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A Study on the Bioremediation Ability of the Common Microflora Isolated From Tannery Effluents

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Abstract: *Industrial evolution has generated unprecedented disturbances in the environment due to the introduction of anthropogenic pollutants such as organic, inorganic and xenobiotic chemicals in the form of untreated industrial waste water. The tannery effluent wastes are ranked as high pollutants among all other industrial wastes. Tannery industrial wastewater is a serious consequence from the pollution point of view of streams, freshwater and land used for agriculture. The lack of awareness in the modern industrial practice has resulted in the discharge of tannery effluents which exhibit very high value for chromium, sulphide and Chloride. Chromium is known to be one of the most common inorganic contaminants of groundwater at pollutant hazardous sites. This investigation focuses on the ability of microorganisms isolated as Isolate 1 (Bacillus cereus F4810/72), Isolate 2, Isolate 3, Isolate 4 (Brevibacillus brevis US575) to reduce Chromium and degrade leather followed by enzymatic assays of the isolates. It also reveals that the ability to reduce Cr^{6+} is maximum for Bacillus cereus F4810/72 compared to others. It was observed that Brevibacillus brevis US575 has the highest Lipase and Protease activity as well as the same has the maximum ability for leather degradation making it an effective strain for leather degradation. Our study reveals that all the four strains isolated from tannery effluents were found to produce keratinase out of which Bacillus cereus F4810/72 was able to produce highest 26U/ml keratinase. Over a wider range of pH (6-10) and temperature (37ºC - 45ºC) it was found that pH 8 and 40°C was most suitable for keratinase production. So these isolates seemed to have potential for bioremediation of leather effluent pollution and thus, can serve as a potential tool to degrade leather waste.*

Keywords: *Bacillus cereus F4810/72, Bioreduction, Brevibacillus brevis US575, Chromium, Keratinolytic, Xenobiotic, Tannery*

I. Introduction

Bioremediation is a waste management technique that uses naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances. Some examples of bioremediation are bioleaching, rhizofiltration, composting, biostimulation. The most significant advantages of bioremediation over conventional treatment methods are low cost, minimization of chemical usage, reduced amount of secondary sludge production, high efficiency, regeneration of cell biomass, and the possibility of recovering pollutant metals. Leather industries, which employ chromium compounds in the tanning step of leather processing pathway, discharge spent-chromium-burdened effluent into nearby water bodies. Worldwide, chromium is considered one of the most common inorganic contaminants of groundwater at industrial belts. Hexavalent chromium poses a health risk to all forms of life. In bioremediation strategy microorganisms alter the oxidation/reduction state of toxic metals directly or indirectly using various biological and chemical processes. Bioreduction of Cr^{6+} to Cr^{3+} not only decreases the chromium toxicity to living organisms, but also helps precipitate chromium at a neutral pH [1]. Plants that are exposed to environmental chromium contamination are seriously affected and suffers from metabolic stress. The stress imposed by Cr toxicity induces oxidative metabolic stress in plants that leads to the generation of active free radicals. Such active free radicals degrade essential biomolecules and distort plants cellular and organellar membranes [2]. Thus, microbial bioremediation of chromium can be a beneficial and cost competitive strategy for removing many hazardous contaminants from tannery and other industrial wastes.

This study was done to investigate the bioremediation of leather effluent by the microorganisms isolated from industrial leather effluent near Bantala leather complex in Kolkata. In the experiment after characterizing the physical and chemical properties of the sample the morphological and gram characters of the isolated organisms were determined. Then chromate reductase assay was performed by DPC method. The experiment was further progressed to find whether the microorganisms can degrade leather particles and then the leather degradation assay was validated by carrying out protease assay, lipase assay and keratinase assay and finally the most suitable temperature and pH was optimized for keratinase production and the most potent leather degraders were phylogenetically characterized by performing 16s rDNA sequencing.

II. Materials and Methods

2.1. Collection of the soil sample

Water samples were collected from Bantala Leather Industry. The samples were collected in sterile containers at a 5 cm depth from the surface of water to avoid surface contamination. Collected samples were transferred to the laboratory under sterile condition for analysis.

2.2. Physical and chemical characterization of the sample

The sample collected was analyzed and its physical (colour, texture & odour) and chemical properties (pH & electrical conductivity) were noted.

2.3. Isolation & characterization of the microorganisms

Serially diluted (10^{-1}, 10^{-2} upto 10^{-6}) 0.1ml of sample water was inoculated by pour plate method on the N.A plates. The plates were incubated overnight at 37^0 c. After incubation, from 10^{-3} marked plate 4 distinct colonies were selected and pure culture was made using quadrant streaking. From the pure cultures, their Gram character was observed.

2.4. Estimation of chromium (VI) reduction by the tannery effluent isolated bacteria by DPC method

Heavy metal pollution of ground and surface waters by industrial effluents has become a serious threat to the environment especially in developing countries. One example of heavy metal pollution is the high chromium containing liquid effluents discharged from tanneries. In this case, the strains tolerant to Cr (VI) were isolated and their ability to reduce Cr (VI) was estimated by the use of the indicator DPC (Diphenylcarbazide). Chromate solution was made by dissolving $K_2Cr_2O_7$ salt in NB at different concentrations of 0.01ppm, 0.1ppm, 1ppm, 10ppm and 100ppm and the pH was adjusted to 1 by adding 2M H_2SO_4.1-2 drops of DPC was added in the medium and color change was noticed. The intensities of the colors of different concentrations were measured at 590 nm by spectrophotometer and a respective graph was plotted.

2.5. Estimation of chromium (VI) reduction by the Chromium tolerant strains

The Chromium tolerant strains isolated from the tannery effluent sample and were grown for 4 days in NB with 50 ppm Cr (VI) concentration. The cultures were centrifuged at 5000 rpm for 15 mins and the supernatant was collected in centrifuge tubes and marked. The cell pellets were resuspended in sterile water and again these solutions were centrifuged at 5000 rpm for 15mins. The supernatant was collected in centrifuge tubes and marked. All the supernatants were adjusted to pH 1 by adding 2M H_2SO_4.1-2 drops of DPC was added in each centrifuge tube. Colour change was noted and O.D. was taken. Reduction of the Cr was calculated from the standard curve.

2.6. Estimation of the amount of leather degradation by the isolated strains

The percentage of leather degradation will indicate production of certain degrading enzymes which is helping the bacteria to reduce the weight of the leather sample. 1sq.mm pieces of leather were taken and dried in the hot air oven. Initial weights of individual pieces of leather were taken in a digital balance. Then 4 pieces of it are UV- sterilized. 4 Sterile NB mediums were taken and inoculated with the isolated strains and 1 piece of UV-sterilized leather followed by incubation for 1 week at 37^0C. After 1 week the leather pieces were taken out and again dried in the hot air oven. The final weight was noted with the help of digital balance.

2.7. Validation of leather degradation by Enzymatic assays

The percentage of leather degradation will indicate production of certain degrading enzymes which is helping the bacteria to reduce the weight of the leather sample. Some enzymes that are responsible for degradation of the leather are protease, lipase, keratinase etc. Further experiments were carried out using standardized protocol to validate the observations.

2.8. Estimation of the amount of lipid degradation by the leather degrading microorganisms

The isolates were grown in sterile N.B medium for 2 days at 37^0c. After incubation cultures were centrifuged at 5000 rpm for 15 minutes. The centrifuged suspension was used as crude enzyme. 0.08M vanillin was prepared by dissolving 600 mg Vanillin in 50 ml carbinol and is used as substrate. A control tube was made by adding 1ml vanillin, 1 ml water and 0.1 ml concentrated sulphuric acid but no enzyme was added. A blank tube was also made by adding 2 ml water and 0.1ml concentrated sulphuric acid but no vanillin and enzyme was added. 4 test tubes were taken each containing 1 ml different crude enzymes, 1ml vanillin, and 0.1 ml concentrated sulphuric acid. After few minutes optical density at 490 nm was measured spectrophotometrically for each tube.

2.9. Estimation of the amount of protein degradation by the leather degrading microorganisms

The isolates were grown in sterile N.B medium for 2 days at 37^0c. After incubation cultures were centrifuged at 5000 rpm for 15 minutes. The centrifuged suspension was used as crude enzyme. Bovine Serum Albumin (BSA) (stock protein solution)- 1mg/ml was prepared and is used as substrate. 4 tubes were made by adding 1ml BSA, 500µl of NaOH and then 1 ml distinct crude enzymes to stop the reaction in time zero. A blank tube was also made by adding 1ml BSA, 1 ml water and 500 µl of NaOH. To measure the amount of protein in blank and test samples. In the test set 4 test tubes were taken each containing 1 ml BSA, 500 µl of NaOH, and 1 ml distinct crude enzymes. But here NaOH was added after 15 minutes incubation to stop the reaction. Lowry test was then performed for each 9 tubes by Folin-ciocalteau method for protein content measurement and then absorbance was measured at 660 nm accordingly.

2.10. Preparation of feather meal for screening of keratinase producers from the effluent

The feathers (native chicken feathers) were cut with scissors in to small pieces of 3 to 4 cm long and washed several times with tap water. Defatting of feather pieces was done by soaking a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for 2 days. The solvent was replaced every day. The feathers were finally washed several times with tap water to eliminate the solvent residual. Dried at 60°C for 24 hours and then grinded [3].

2.11. Submerged fermentation

Submerged fermentation was performed by inoculating pure culture of the isolates into the production medium containing feather meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO4 (0.03%), K_2HPO_4 (0.04%) and MgCl2(0.01%) of pH 8 which is the optimum pH for the growth of the isolate. The incubation was carried out at 37º C for 3 days at 180 rpm [3].

2.12. Keratinase assay

The broth was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used as crude enzyme. Keratin from feather meal was used as a substrate. The 5 mg keratin was suspended in 1 ml 50 mmol/L Tris-HCl buffer (pH 8.0). The reaction mixture contained 1ml keratin suspension and 1 ml appropriately diluted supernatant. The reactions were carried out at 50°C with constant for 1 hr. After incubation, the reactions were stopped by adding 2 ml 1 [M] NaOH and followed by filtration to remove the substrate. The filtrate was spectrophotometrically measured at 595 nm. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 increases in absorbance between sample and control at 595 nm after one hour under the given conditions [3].

2.13. Optimization of culture conditions for keratinase production

The effect of pH and temperature on keratinase production was individually tested by carrying out the submerged fermentation at different pH and temperature. The fermentation media were assayed after 3 days for analyzing the keratinase enzyme activity. For analysis of the effect of pH, pH is varied from 6 to 10 and for temperature it was 37°C, 40°C, and 45ºC.

2.14. Identification of Isolate 1 and Isolate 4

As it is found that Isolate 1 has highest Chromium reduction and keratinase enzyme activity, and Isolate 4 has highest leather degrading and Protease, lipase producing abilty hence they were Identified by 16S rDNA analysis. For 16s rDNA sequencing, both of these 2 isolates were grown in Sterile Nutrient agar plate. Then genomic DNA of the isolates was extracted and amplified via Polymerase chain reaction using forward primer, F-73, 5'-AGAGTTTGATCCTGGCTCAG-3', and reverse primer, R-74, 5'-AAGGAGGTGATCCAAGCC-3' (For Isolate 4). The PCR fragments were then analyzed by 1.5% agarose gel electrophoresis using 100 bp DNA ladder and then visualized in gel Documentary system. After this amplification, the 16s rDNA were sequenced and these were compared to 16S rDNA sequences available in the nucleotide databases of the GenBank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), to determine the approximate phylogenetic position and percentages for homology scores to identify the isolates.

III. Results

3.1. Physical and chemical characteristics of the sample

The physical and chemical characteristics of the sample are given below (Table 1 & Table 2)

Table 1: Physical characteristics of the collected sample

Colour	Pale brown colour.
Texture	Coarse texture with 5% detoriated leather particles present
Odour	Extremely foul smell.

Table 2: Chemical characteristics of the collected sample

pH	8
Electrical conductivity	15.79 siemens

3.2. Isolation, identification and characterization of the microorganisms

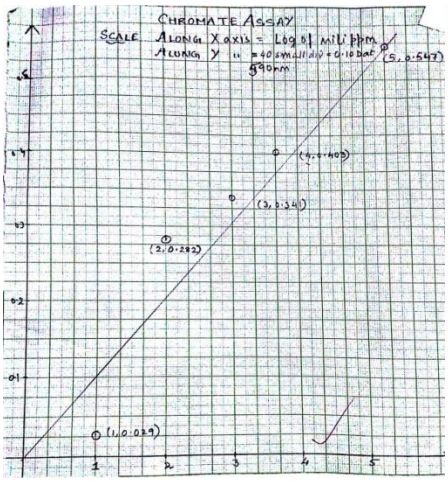
4 distinct colonies were selected from 10^{-3} marked plate and from the pure cultures their Gram characteristics were observed.

Table 3: Morphological and biochemical characterization of the isolates

Designation of the isolates	Gram character & Morphology
Isolate 1 (IS1)	Gram positive , short rods
Isolate 2 (IS2)	Gram positive , short rods
Isolate 3 (IS3)	Gram negative , short rods
Isolate 4 (IS4)	Gram positive , short rods

3.3. Chromium (VI) reduction was observed by the tannery effluent isolated bacteria by DPC method

The observations from the "**Chromium Reduction Test**" indicate the ability of the strains to reduce Cr^{6+}. The amount of Cr salt present in the medium (50ppm) was reduced respectively by the strains which are shown in the graph. The observations indicate that isolate 1(IS1) has the highest ability to reduce Cr^{6+}.



1(A) 1(B)

Fig 1: Observation of the amount of Chromium reduced by the isolates by DPC method. (A)Standard curve for determining the amount of Cr^{6+} reduced by the isolates. (B) Tubes showing standard concentration of chromium for the standard curve preparation. A- 0.01ppm, B - 100ppm, C – Blank, D - 0.1ppm, E – 10ppm, F – 1ppm.

Table 4: O.D readings of different concentration chromium solution prepared for the determination of Standard curve

Concentration of Cr (VI) (ppm)	0.D. at 590nm
0.01	0.029
0.1	0.282
1	0.341
10	0.403
100	0.547

Table 5: Amount of Cr (VI) in the supernatant 1 which determines remaining chromium present in the medium

Isolate no.	O.D. at 590nm	Amount of remaining Cr(vi) (ppm)
Isolate 1 (IS1)	0.475	12
Isolate 2 (IS2)	0.455	27
Isolate 3 (IS3)	0.437	15
Isolate 4 (IS4)	0.495	37

Table 6: Amount of Cr (VI) in the supernatant 2 which determines the amount of Cr^{6+} bound with the cell surface

Isolate no.	O.D. at 590nm	Amount of remaining Cr^{6+}(ppm)
Isolate 1 (IS1)	0.445	22
Isolate 2 (IS2)	0.450	15
Isolate 3 (IS3)	0.497	33
Isolate 4 (IS4)	0.455	10

Table 7: Amount of Cr^{6+} reduced by isolated organisms

Strains	Amount of Cr^{6+} reduced by isolated organisms (ppm)
Isolate 1 (IS1)	16
Isolate 2 (IS2)	8
Isolate 3 (IS3)	2
Isolate 4 (IS4)	3

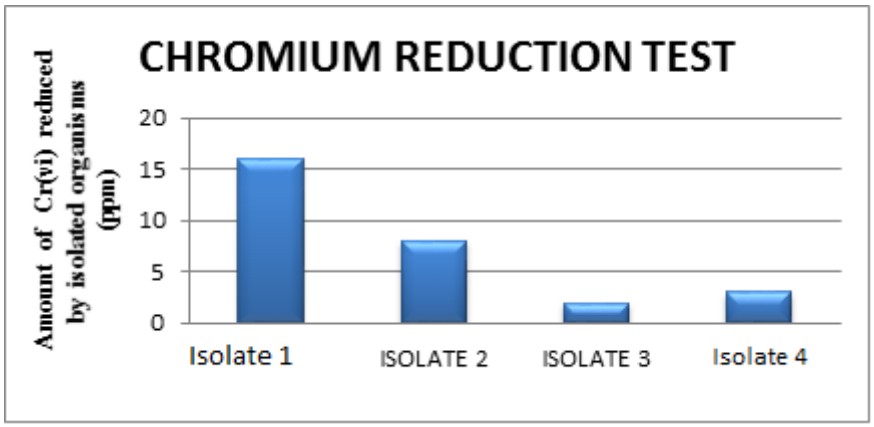


Fig 2: Graphical representation to show the amount of Cr^{6+} reduced by isolated organisms. Among the isolates, Isolate 1(IS1) shows the highest chromate reducing activity.

3.4. The isolates have immense leather degrading activity

In the estimation of "Leather Degradation from the isolated strains" it was noted that the Isolate 4(IS4) has the maximum ability to degrade leather making it an efficient strain for leather degradation.

Table 8:- Amount of leather degraded by isolated microorganisms

Isolate no.	Initial weight (gm)	final weight (gm)	% of reduction (gm)
Isolate 1 (IS1)	0.102	0.099	2.94
Isolate 2 (IS2)	0.123	0.119	3.25
Isolate 3 (IS3)	0.106	0.104	1.88
Isolate 4 (IS4)	0.134	0.127	5.22

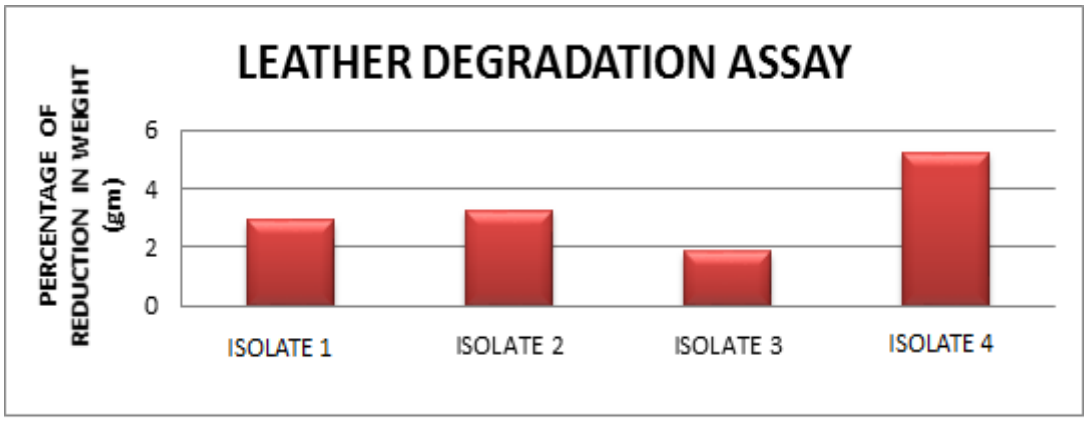


Fig 3: Graphical representation demonstrating the amount of leather degradation by the isolates. It was observed that Isolate 4 (IS4) has the highest leather degrading activity.

3.5. Lipolytic activity was shown by the isolates

The "Lipase Assay" was performed to determine the degree of lipid degradation by the enzyme Lipase isolated from extracellular broth medium of Leather degrading microorganisms. In this assay it was found that Isolate 4(IS4) has the highest lipase activity for the degradation of lipids.

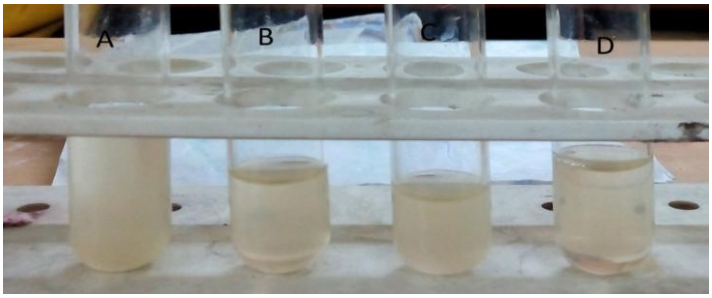


Fig 4: Tubes of test samples showing Vanillin degradation in lipase assay. A- Isolate 1(IS1) B - Isolate 2 (IS2), C - Isolate 3 (IS3), D – Isolate 4 (IS4). Highest lipolytic activity was shown by Isolate 4 (IS4).

Table 9: O.D values indicating Vanillin degradation by extracellular lipase enzyme of the isolates

Designation of the isolates	O.D value at 490 nm	
	Control	Sample
Isolate 1 (IS1)	0.259	0.211
Isolate 2 (IS2)	0.259	0.219
Isolate 3 (IS3)	0.259	0.193
Isolate 4 (IS4)	0.259	0.190

Table 10: Table showing lipolytic activity of the isolates

Designation of the isolates	Concentration of degraded vanillin/ ml of crude enzyme
Isolate 1 (IS1)	0.015 M
Isolate 2 (IS2)	0.013 M
Isolate 3 (IS3)	0.021 M
Isolate 4 (IS4)	0.022 M

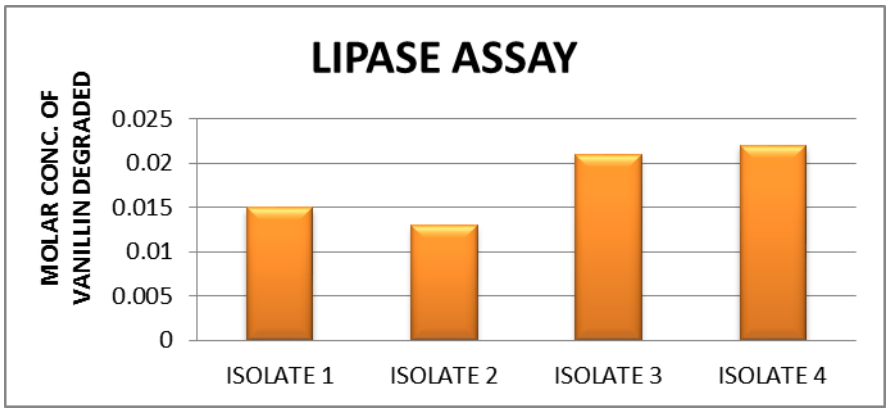


Fig 5: Graphical representation to show the amount of lipid degradation by the strains. Highest lipase activity was found in Isolate 4 (IS4).

3.6. The isolates has the ability to degrade protein

The "Protease Assay" was performed to determine the degree of protein degradation by the enzyme Protease isolated from leather degrading microorganisms. In this assay it was found that Isolate 4 (IS4) shows highest Protease activity i.e. the amount of BSA degraded per minute.

Table 11: O.D values indicating BSA degradation by extracellular protease enzyme of the isolates

Isolate no.	O.D value at 750 nm	
	Control	sample
Isolate 1 (IS1)	0.150	0.204
Isolate 2 (IS2)	0.185	0.249
Isolate 3 (IS3)	0.184	0.322
Isolate 4 (IS4)	0.149	0.275

Table 12: Table showing proteolytic activity of the isolates

Isolate no.	Amount of BSA degraded /min
Isolate 1 (IS1)	0.024mg
Isolate 2 (IS2)	0.022 mg
Isolate 3 (IS3)	0.050 mg
Isolate 4 (IS4)	0.056mg

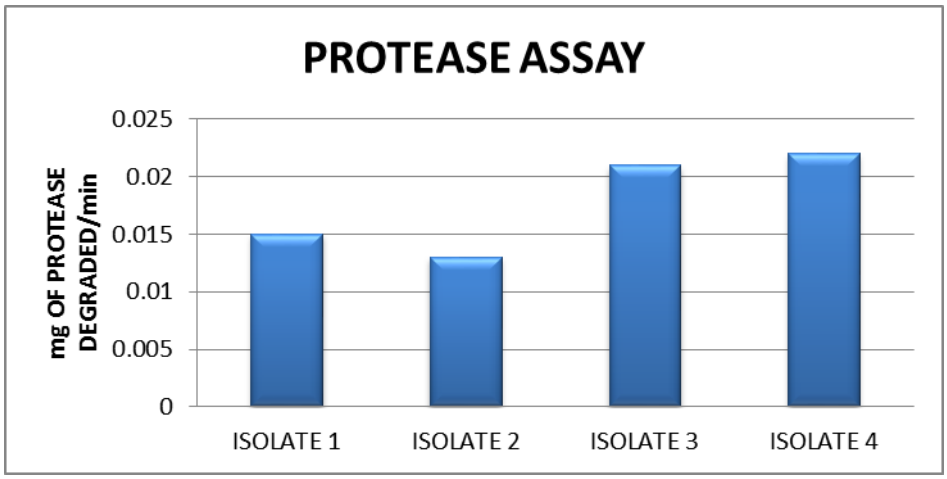


Fig 6: - Graphical representation demonstrating the amount of protein degradation by the isolates. *Isolate 4 (IS4)* has the highest ability to degrade protein.

3.7. The leather degrading microorganisms showed Keratinase production

In the "Keratinase Assay" it was observed that all the four strains isolated from leather industrial polluted water samples were found to produce keratinase. These bacteria were grown on basal medium with feather as its primary source of carbon and nitrogen. The maximum enzyme activity at optimum temperature, pH and substrate concentration and incubation time was 26 U/ml by Isolate 1(IS1). Hence, it can be concluded that this keratinolytic isolate can be utilized for recycling keratin-rich agro industrial byproducts.

Table 13: Table showing the keratinolytic activity of the isolates observed after Keratinase assay

Isolate no.	O.D observed at 595 nm	Enzyme activity (U/ml)
Isolate 1 (IS1)	0.260	0.260/0.01 = 26
Isolate 2 (IS2)	0.217	0.217/0.01 = 21.7
Isolate 3 (IS3)	0.191	0.191/0.01 = 19.1
Isolate 4 (IS4)	0.178	0.178/0.01 = 17.8

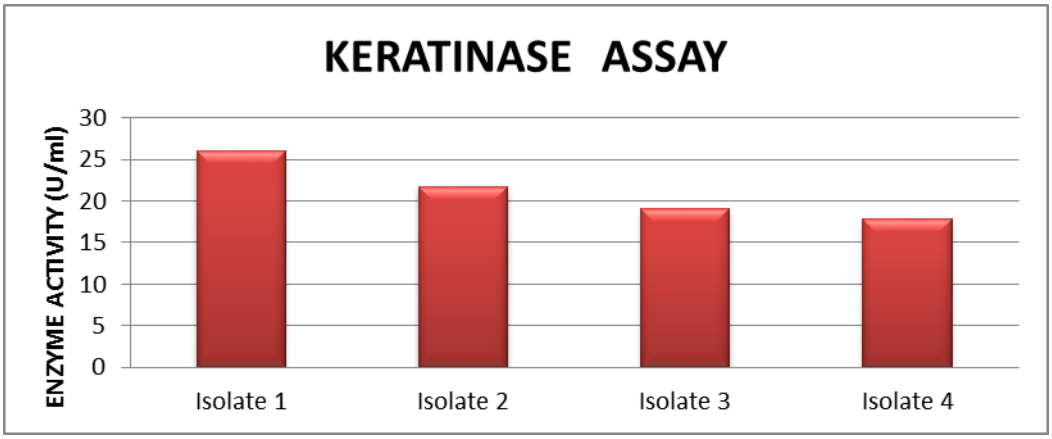


Fig 9:- Graphical representation showing keratinase activity of all 4 isolates. Of which Isolate 1(IS1) shows the maximum keratinase activity.

3.8. Optimization of culture conditions for keratinase production

The effect of pH and temperature on keratinase production was individually tested by carrying out the submerged fermentation at different pH and temperature.

3.8.1. Effect of pH

The isolate showed keratinase production from pH 7 to 10, and maximum activity was shown by Isolate 4 (IS4) at pH 8 giving 26 U/ml after 3 days. This indicates that pH 8 is the optimum temperature for keratinase production.

Table 14: Keratinase produced by the isolate at varying pH

Isolate no.	Enzyme activity (U/ml)				
	pH 6	pH 7	pH 8	pH 9	pH 10
Isolate 1 (IS1)	6	12	26	15	15
Isolate 2 (IS2)	2	6.5	12	7	7.5
Isolate 3 (IS3)	4	9	20	10	13
Isolate 4 (IS4)	1.5	5	8	4.5	5

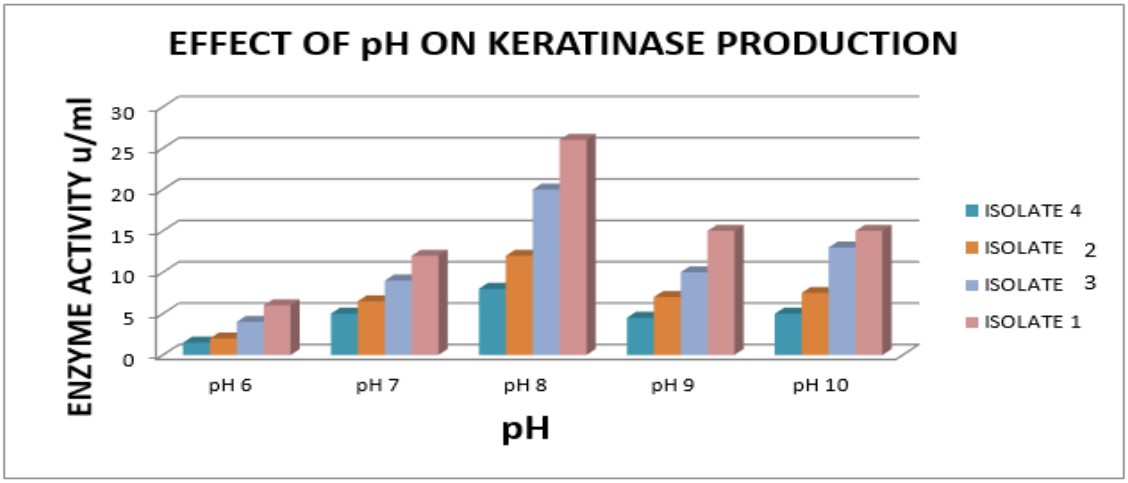


Fig 10: Graphical representation showing the effect of pH on Keratinase production. It has been found that the Isolate 1(IS1) has the highest ability of producing 26U/ml at a pH of 8 which is the optimum pH.

3.8.2. Effect of temperature

The isolate showed keratinase production at a temperature range of 37- 45°C after 72h. Maximum production of 30 U/ml at 40°C was observed which indicates that 40°C is the optimum temperature for keratinase production.

Table 15: Keratinase produced by the isolate at different temperature

Isolate no.	Enzyme activity (U/ml)		
	37°c	**40°c**	**45°c**
Isolate 1 (IS1)	26	30	24.5
Isolate 2 (IS2)	19.1	22.5	18
Isolate 3 (IS3)	21.7	25	21
Isolate 4 (IS4)	17.8	20	16

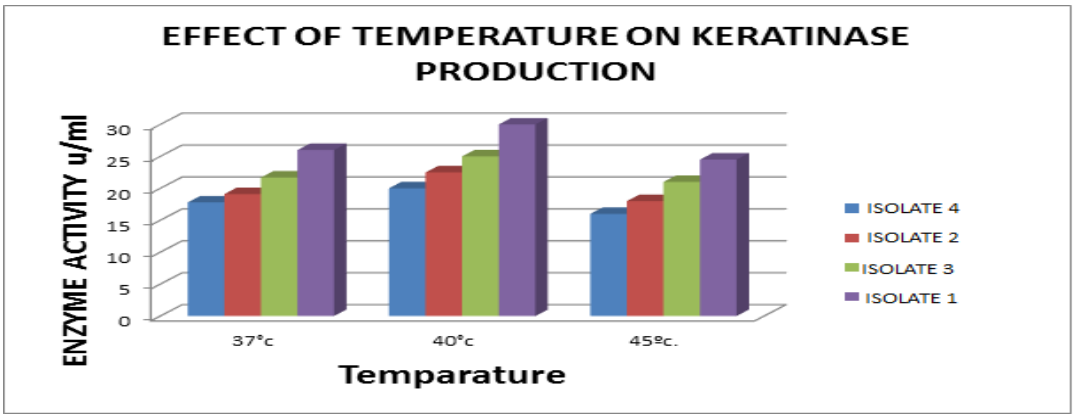
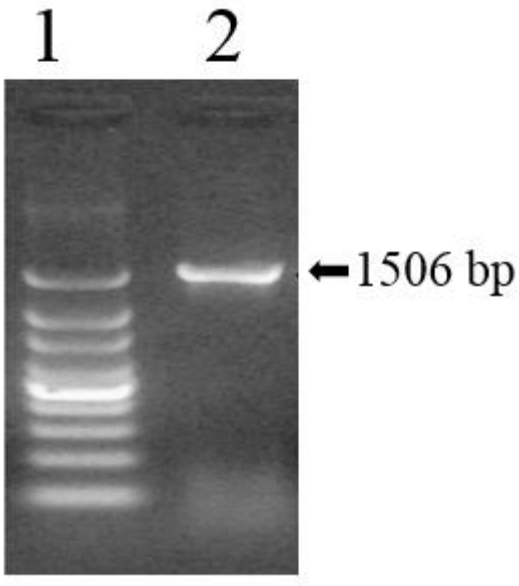


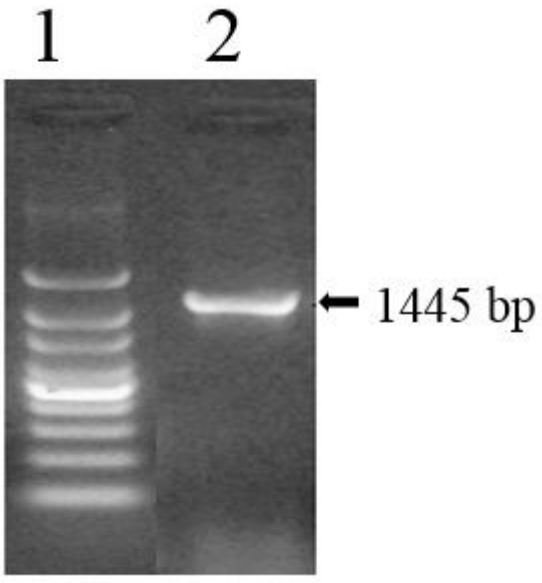
Fig 11: Graphical representation showing the effect of temperature on Keratinase production. It has been found that at 40°c Isolate 1(IS1) produces highest amount of keratinase (26U/ml) so it is the optimum temperature for keratinase production.

3.9. Identification of 'Isolate 1' and 'Isolate 4'

16s rDNA of these 2 isolates after PCR amplification when analysed it was found that the amplicon for isolate 1 gave a band of less than 1500bp DNA fragment whereas for isolate 4 the fragment was more or less similar to the 1500bp DNA fragment. From the sequencing of the rDNA and its alignment in BLAST programme, it was identified that Isolate 1 was ***Bacillus cereus* F4810/72** and Isolate 4 was found to be ***Brevibacillus brevis* US575** and both shows 100% similarity in the alignment.



12(A)

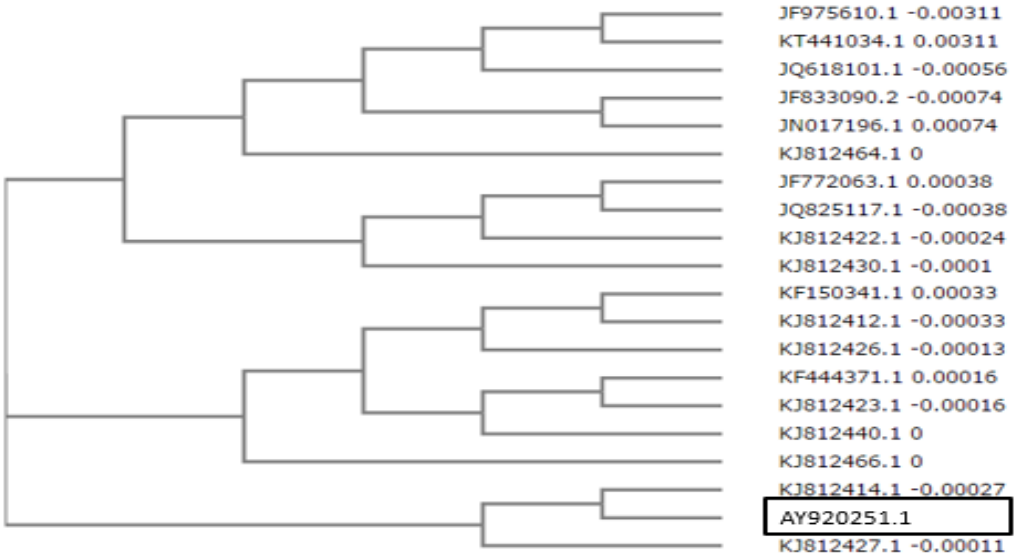


12(B)

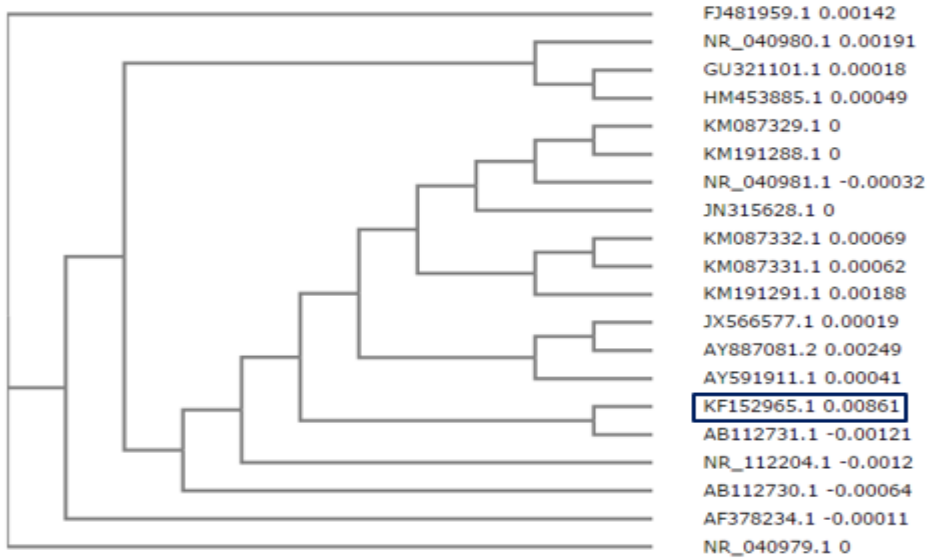
Fig 12: 0.15% Agarose gel showing *16S rDNA* amplicon. Lane 1: 100 bp DNA ladder; Lane 2: *16S rDNA* amplicon. (A) Isolate 1 (B) Isolate 4

Table 16: BLAST result of 16s rDNA gene sequence of Isolate 1 and Isolate 4

Designation of the isolate	BLAST Match	Strain	rDNA	Accession No.	Identity
Isolate 1	*Bacillus cereus*	F4810/72	1445 bp	AY920251.1	100%
Isolate 4	*Brevibacillus brevis*	US575	1506 bp	KF152965.1	100%



13(A)



13(B)

Figure 13: Phylogenetic Tree of Isolate '1' and Isolate '4' obtained after analyzing the BLAST result. (A) Isolate 1 (B) Isolate 4

IV. Conclusion & Discussion

It was reported that Algae, *Chlorella vulgaris*, *Anabena doliolum* and fungal biomass can bioaccumulate chromium [4], [5]. Even activated sludge processes can be applied for efficient removal of chromium [6]. Cyanobacteria and plant biomass have also been found to remove chromium from industrial effluents [7], [8], [9]. Isolation of *Arthrobacter* sp. and *Bacillus* sp. from tannery waste contaminated soil was already reported and their tolerance to hexavalent chromium along with their ability to reduce chromate in cell suspensions and cell extracts were also examined [10]. It was found that isolate B4 is a potent degrader of lipid

by producing lipolytic enzyme where Olive oil (2%) was added to the basal medium and it was used as the production medium for lipase production, the test isolate was identified using morphological and biochemical characteristics according to Bergey's Manual of determinative Bacteriology and was identified as *Bacillus* sp. [11]. It was found that the bacterial isolate which was isolated from deterioted leathers samples produced 38 U/ml keratinase enzymes in optimum pH, temperature and substrate concentration [3].

In our study the organisms were broadly isolated and characterized and finally 16s rRNA was carried out for identification and they were found to be Bacillus cereus F4810/72, Brevibacillus brevis US575. Our Investigation reveals that the organisms has not only reduces Chromium (Cr^{6+} to Cr^{3+}) but also accumulates chromium, thus changing it from cyclical pool to 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 processing of leather. Keratinolytic isolate can be utilized for recycling keratin-rich agro industrial and poultry waste byproduct. 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 (VI) and at the same times producing enzymes like protease, lipase & keratinase.

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