ISOLATION, SCREENING, AND CHARACTERIZATION OF BACTERIA FROM KORANGI CREEK RIVER WITH ASSESSMENT OF THEIR BIOREMEDIATION POTENTIAL

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خلاصہ

ماحول نامیاتی آلودگیوں کی ایک سے آلودہ ہے، جن میں سے اکثر گندے پانی میں داخل ہو سکتے ہیں۔ میونسپل اور صنعتی گندے پانی آبی ماحول میں ان آلودگیوں کا بنیادی حصہ دار ہیں اور عام طور پر مختلف نامیاتی مرکبات کی کافی مقدار پر مشتمل ہوتے ہیں۔ لہذا، گندے پانی اور دیگر آلودہ پانی کو صافرنے کے موثر طریقے تلاش کرنا ماحول کی حفاظت کا ایک اہم حصہ ہے۔ بائیو میڈیشن کے مختلف طریقوں میں سے، ان سیٹو بائیوڈیگریڈیشن سب سے زیادہ طاقتور اور ماحولیاتی شعور کے طریقوں میں سے ایک کے طور پر ابھرتا ہے۔ یہ قدرتی طور پر پائے جانے والے بیکٹیریا کو نقصان دہ آلودگیوں کو جو انسانی صحت اور ماحول دونوں کے لیے نقصان دہ ہے مکمل طور پر توڑنے یا ختم کرنے کے لیے استعمال کرتا ہے۔ اس مطالعے کا مقصد کاربوہائیڈریٹس، پروٹینز، صنعتی رنگوں اور ہائیڈروکاربن سمیت نامیاتی آلودگیوں کے سپیکٹرم کے خلاف بائیوڈی گریڈیشن کی صلاحیتوں کو ظاہر کرنے والے بیکٹیریا کو isolate ور شناخت کرنا ہے۔ مختلف مورفولوجیکل اور بائیو کیمیکل طریقوں کے ذریعے۔ کراچی میں دریائے کورنگی کریک سے میونسپل گندے پانی کو جمع کیا گیا اور اس کی فزیکو کیمیکل خصوصیات جیسے پی ایچ، درجہ حرارت، کل تحلیل شدہ ٹھوس، چالکتا، اور نمکیات کا اندازہ لگایا گیا۔ ڈالو پلیٹ کا طریقہ بعد میں مقامی بیکٹیریل تناؤ کی بایوڈیگریڈیشن صلاحیت کو isolate کرنے اور جانچنے کے لیے استعمال کیا گیا۔ الگ تھلگ تناؤ کی شناخت مائکروسکوپک اور بائیو کیمیکل تجزیہ کے ذریعے کی گئی۔ نامیاتی آلودہ گندے پانی سے تین بیکٹیریل strain کا انتخاب کیا گیا جو ایک خاص نامیاتی مرکب کے وجود میں بڑھنے کی صلاحیت کے لحاظ سے تھے۔ تینوں بایوڈیگریڈنگ بیکٹیریل تناؤ کے لیے مختلف خصوصیات کا مطالعہ کیا گیا اور ان کی شناخت کلیبسیلا نمونیا سب ایس پی کے طور پر کی گئی۔ Ozaenae، گروپ A Streptococcus pyogenes اور Nonenterococcus Streptococcus bovis ۔ یہ مطالعہ اہمیت کا حامل ہے کیونکہ یہ انتہائی لاگت سے موثر اور ماحول دوست طریقے سے نامیاتی آلودگیوں کو مؤثر طریقے سے کم کرنے اور ختم کرنے کے لیے فطرت کے microbes کی صلاحیت کو استعمال کرتا ہے۔

Abstract

The environment is contaminated by an array of organic pollutants, many of which can permeate into wastewater. Municipal and industrial wastewater are the primary contributors of these pollutants in the aquatic environment and typically comprise substantial amounts of different organic compounds. Therefore, finding efficient ways to manage wastewater and other contaminated water is a crucial part of protecting the environment. Among the various methods of bioremediation, in-situ biodegradation emerges as one of the most potent and eco-conscious approaches. It harnesses naturally occurring bacteria to entirely break down or detoxify harmful pollutants, detrimental to both human health and the environment. This study aims to isolate and identify bacteria exhibiting biodegradation capabilities against a spectrum of organic pollutants, including carbohydrates, proteins, industrial dyes, and hydrocarbons from Korangi river via different morphological and biochemical methods. Municipal wastewater was collected from Korangi Creek River in Karachi and its physicochemical characteristics such as pH, temperature, total dissolved solids, conductivity, and salinity were assessed. The pour plate method was subsequently employed to isolate and assess the biodegradation potential of native bacterial strains. Isolated strains were identified by using microscopic and biochemical analysis. Three bacterial strains were selected from organic-contaminated wastewater based on their amylase activity, azoreductase activity, and kerosene-degrading activity. Different features were studied for all three biodegrading bacterial strains and were identified as *Klebsiella pneumoniae* subsp. *Ozaenae*, Group A *Streptococcus pyogenes* and *Nonenterococcus Streptococcus bovis* by microbiological culture techniques and biochemical identification tests. This study assumes significance as it utilizes the ability of nature's microorganisms to efficiently degrade and eliminate organic contaminants in a cost-effective and eco-friendly way.

Keywords: Bioremediation; waste water; azo dyes; plastic

Introduction

The contamination of water bodies caused due to a combination of chemical discharges and domestic waste poses a significant global concern (Kebede *et al*., 2018; Sohail *et al*., 2019). Industrial effluents introduce a complex mixture of substances, including hydrocarbons, dyes, proteinaceous compounds, and suspended solid particles (SS), into the environment. The uncontrolled and untreated release of industrial effluents, often enhanced

with various metals, stands as the primary culprit behind the deteriorating quality of surface waters (Saha *et al*., 2018; Benabdelkader *et al*., 2018; Odukoya *et al*., 2017). To mitigate the environmental impact of industrial effluents and household waste disposal, effective measures are imperative. The total volume of wastewater discharged into major rivers and water bodies amounts to 392,511 million gallons per day (MGD) (Soomro *et al*., 2011).

The rapid industrialization and urbanization witnessed globally have led to the release of substantial volumes of waste pollutants, including toxins, into the environment, resulting in increased levels of pollution (Hoskeri *et al*., 2014; Mulla *et al*., 2017; Edalli *et al*., 2018). Most notably, the textile dyestuff and dyeing industries release a significant portion of colored effluents, containing dyes, into the environment (Bharagava *et al*., 2018). Azo dyes, constituting more than half of the colorants used, are the predominant choice in the textile sector (Haq and Raj, 2018). These effluents containing azo dyes are not only recalcitrant but also phytotoxic, carcinogenic, and mutagenic (Hussain, 2006; Singh and Arora, 2011). Given the environmental implications, treatment of dye-containing wastewater before disposal is imperative.

Within municipal wastewater treatment facilities, the breakdown of proteins and polysaccharides into smaller molecules through extracellular enzymes is essential (Sheng and Yu, 2006). These enzymes find applications in waste treatment across various food-processing industries and domestic activities (Gupta *et al*., 2002; Ichida *et al*., 2001). Sectors like confectionery, ice cream, dairy, and meat processing show promise for the use of hydrolytic enzymes like lipases, amylases, and proteases in wastewater treatment. The advantage of not requiring extensive purification of enzymes makes their production cost-effective (Rigo *et al*., 2008). These characteristics have spurred interest in enzyme production techniques and the search for novel microorganisms with diverse enzyme-producing capabilities (Cavalcanti *et al*., 2005; Leal *et al*., 2006).

The proliferation of the oil industry and the subsequent increase in oil and petroleum refinery operations have led to a substantial rise in oils, particularly kerosene, present in wastewater from these facilities (Mohsen and Amin, 2010). Various methods have been widely employed to remove kerosene from oil refinery effluents, including solvent extraction (Ahmad *et al*., 2003), photocatalytic processes, sedimentation, flocculation, and coagulation (Masschelein *et al*., 2002). However, researchers around the world are actively seeking more cost-effective alternatives to address this challenge.

Bioremediation can be defined as a process that aims to either eliminate or decrease the levels of hazardous waste in polluted areas by harnessing microorganisms and/or their enzymes, all while avoiding any additional harm to the local environment (Rahman *et al*., 2003). The primary goal of bioremediation is to bring down the concentrations of organic pollutants to levels that fall below what regulatory authorities consider safe or acceptable. This method relies on naturally occurring bacteria to completely break down or detoxify pollutants that pose threats to both human health and the environment. What makes this approach particularly attractive is its cost-effectiveness and the ability to convert pollutants into harmless byproducts, namely carbon dioxide (CO_2) and water (H_2O) (Mills *et al*., 2004).

In spite of notable progress in bioremediation research over recent decades, the challenge of effectively eliminating organic pollutants from the environment remains a difficult task in practical applications. The objectives of this study encompass an assessment of the water quality in the Korangi Creek River, which has been polluted by both domestic waste and industrial discharge. Additionally, the study seeks to identify strains within the collected samples that exhibit bioremediation capabilities specifically against carbohydrates, proteins, plastics, azo-dyes, and kerosene. Hence, the significance of the present research depends on the utilization of nature's microorganisms to efficiently break down and eliminate these hazardous organic contaminants in a manner that is both cost-effective and environmentally friendly.

Materials and Methods

Selection of the Study Area

On Karachi's southeast shore, Korangi Creek represents a habitat that has experienced some anthropogenic stress. It gets industrial effluents from the industrial states of Korangi and Landhi. Domestic effluents from smaller coastal towns as well as untreated wastewater from Karachi city are also discharged into the creek. The mangrove habitats are exposed to the hazardous pollutants from Korangi Creek, which could pose major risks to the health of the local fisheries and other wildlife (IUCN 2005; Shahzad *et al*. 2009).

Sample Collection

Three samples of 1 liter of municipal wastewater were collected from Korangi Creek River, Karachi (**Figure 1**), in pre-sterilized bottles. The wastewater sample was transferred to the laboratory immediately where its physical properties such as pH, temperature, TDS (Total Dissolved Solids), conductivity, and salinity were measured. For further analysis, the rest of the sample was stored at 4 ˚C to avoid any chemical or physical changes in the wastewater.

Physicochemical Analysis

Equipment for on-site measurements was calibrated and checked according to the instruction manual. Temperature was measured using an alcohol thermometer, while conductivity and total dissolved solids (TDS) were measured using the PASCO Wireless Conductivity Sensor PS-3210. pH was measured using the PASCO Wireless pH Sensor PS-3204. The results were recorded via the SPARKvue application. The salinity of the sample was found by raising the conductivity to the power of 1.0878 and then multiplying the result by 0.4665 (Bennet, 1976).

Isolation and Identification of Indigenous Bacteria

The collected municipal wastewater sample was serially diluted in 10^{-2} –10^{-5} concentrations. The pour plate method was followed to assess the protease, amylase synthesis and, kerosene, azodye, and polythene degradation potential of indigenous bacteria by using minimal media as detailed below. Isolated bacterial strains were characterized and identified via colonial, microscopic, morphological, and biochemical assays as per Bergey's Manual of Determinative Bacteriology, 2018.

Amylase Activity Assay

1 mL from each of the 4 dilutions was dispensed in an empty plate, after which molten M9 Minimal Media (with starch as the carbon source) [(v/v) M9 Salts; 1M Magnesium Sulfate; 20% Starch; 1M Calcium Chloride; Agar; Distilled water, pH 7.2 ± 0.1] was poured on top of it. The sample was thoroughly mixed with the medium by gentle shaking of the plate after which the plates were incubated at 37 ◦C for 24 hours. After incubation, the plates were inverted on top of iodine crystals for 5-10 minutes. The clear zone of hydrolysis around the colony indicated a positive result (Saleem and Ebrahim 2014).

Protease Activity Assay

1 mL from each of the 4 dilutions was dispensed in an empty plate, after which molten Skim Milk Modified Agar Medium [(w/v) Tryptone; Yeast extract; Glucose monohydrate; SM powder; Agar; Distilled water, pH 7.5 ± 0.2] was poured on top of it. The sample was thoroughly mixed with the medium by gentle shaking of the plate after which the plates were incubated at 37 ◦C for 24 hours. Any zone of clearance observed around the colonies was indicative of the proteolytic activity (Masi *et al*., 2014).

Azoreductase Activity Assay

1 mL from each of the 4 dilutions was dispensed in an empty plate, after which molten M9 Minimal Media (with Fast Red E dye as the carbon source) [(v/v) M9 Salts; 1M Magnesium Sulfate; 20% Starch; 1M Calcium Chloride; Agar; Distilled water, pH 7.2 ± 0.1] was poured on top of it. The sample was thoroughly mixed with the medium by gentle shaking of the plate after which the plates were incubated at 37◦C for 24 hours. Any zone of clearance observed around the colonies was indicative of the azoreductase activity (Khehra *et al*., 2005).

Kerosene-Degrading Activity Assay

M9 minimal media (with kerosene as the carbon source) [(v/v) M9 Salts; 1M Magnesium Sulfate; 1M Calcium Chloride; Distilled water, pH 7.2 ± 0.1] was prepared to check for the kerosene-degrading activity of the microbes present in the sample; however, agar was not used for this purpose. Three different concentrations of kerosene (0.5%, 1%, and 1.5%) were incorporated into different flasks containing minimal media. 1 mL of water sample was then dispensed into each flask and the flasks were kept inside the shaking incubator at 37 ◦C for 7 days. The turbidity of the samples was indicative of the kerosene-degrading activity by the bacterium, whose enrichment was then performed on nutrient agar using the pour plate method (Ekram *et al*., 2020).

Polyethylene Degrading Assay

All samples were serially diluted in sterile PBS diluent and then alternate dilutions were plated onto the enrichment media containing 0.1 g/L Yeast extract, 0.25 g/L MgSO4.H2O, 5.8 g/L KH2PO4, 3.7 g/L K2HPO4, 2.0 g/L KNO3, and 0.25 % Polyethylene powder. 45 mL of this enrichment media was inoculated with 5 ml of undiluted sample. Upon autoclaving, the powder melted to form a solid piece and each piece was placed in a flask. A total of three flasks were prepared, one for each sample. Flasks were placed in a shaking incubator at 30°C and 170 rpm for 1 month (Usha *et al*., 2011).

Results and Discussion

Korangi is Karachi's major industrial hub and home to a diversity of industries, mainly petroleum and textile. The effluent from such industries predominantly contains heavy toxic metals like Hg, As, Fe, Zn, and Cd. It is also rich in hydrocarbons; both biodegradable and non-biodegradable, such as oils, greases, wax, and volatile

compounds like butane, pentane, and hexane. Wastewater is a massive source of multiple species of bacteria and other microorganisms that can be used for bioremediation purposes.

The physicochemical parameters of collected samples were recorded to assess the level of pollution that has occurred in the Korangi Creek River due to various industrial and anthropogenic activities, as depicted in Table 1.

Indigenous bacterial colonies were isolated from the samples, and their morphological characteristics are exhibited in Table 2. Three different bacterial species with bioremediation capabilities were selected Amylase-producing and kerosene-degrading strains were isolated from the contaminated site, as depicted in Figure 3. Microbial proteases play a crucial role in solubilizing proteinaceous waste, thereby reducing the biological oxygen demand in aquatic systems. However, no growth with a clear zone was observed in this study when checking for protease activity.

While several physicochemical decolorization methods have been employed over the past two decades, only a handful have gained acceptance within the textile industry. However, ongoing use of these techniques has drawbacks, including high costs, sludge production, and the release of harmful compounds (Senan and Abraham, 2004). Furthermore, some of these methods fail to completely eliminate the organic compounds responsible for secondary contamination (Thangaraj and Senthil, 2020). In this study, textile dye-reducing strains were isolated from the contaminated site, as shown in Figure 3**.** Microbial decolorization of dyes has emerged as an economically viable and environmentally compatible solution, given its affordability and ecological compatibility (Kalyani *et al*., 2009). Under specific environmental conditions, microorganisms can fully mineralize synthetic dyes or break them down into non-colored compounds (Singh, 2017).

In the polyethylene assay, we found out that indigenous bacterial species present in sample 1 degraded the smallest percentage of plastic, 0.64%, while sample 2 degraded a much larger amount, 5.24%, and sample 3 successfully degraded the largest percentage of plastic, 8.56%, within 30 days of incubation (Table 3). The volume in each flask was greatly reduced by almost 50% after one month of incubation. The enrichment media in a flask for sample 1 contained minuscule pieces, which could be of plastic and hence may be termed 'micro plastics'. The media clarity for polyethylene assay in flask 2 was highest as compared to the other two i (Figure 2). Our findings, particularly samples 2 and 3 showed confident results where a good quantity of plastic was degraded, comparatively. The raw sample contained multiple species of bacteria and other microorganisms.

Bacterial colonies showing amylase activity, azoreductase activity, and kerosene-degrading activity were coded as **AM1, ID2**, and **HC3** respectively. The biochemical identification of isolated strains is depicted in Table 4. Based on biochemical assays isolate AM1 was identified as *Klebsiella pneumoniae* subsp. *Ozaenae,* ID2 as β-hemolytic Group A *Streptococcus pyogenes* and γ-hemolytic isolate HC3 was identified as *Nonenterococcus Streptococcus bovis* (Table 5).

Previous works have also reported the plastic biodegradation potential of the soil bacterium including *Staphylococcus sp, Pseudomonas sp*. (Mohan and Srivastava 2010; Riandi *et al.,* 2017; Singh *et al.,* 2016)

Table 1. Physio-chemical analysis of the waste-water samples

Samples (1ml)	Temperature (ºC)	pH	Electrical Conductivity (µS/cm)	Total dissolved ()solids (mg/L)	Total suspended solids (mg/L)	Total solids (mg/L)
1	33	7.65	8161.0	4715.34	125	4840.34
2	31	7.79	4505.0	3393.57	80	3518.57
3	31	7.79	3832.7	2968.28	110	4068.28

Table 2. Morphological Characterization of bacteria isolated from waste-water samples

Colony No.	Color	Density	Texture	Elevation	Shape	Margin
1	Yellow	Opaque	Smooth	Umbonate	Irregular	Undulate
2	Pink	Translucent	Smooth	Flat	Irregular	Lobate
3	White	Opaque	Smooth	Flat	Irregular	Lobate
4	White	Opaque	Rough	Crateriform	Irregular	Undulate
5	Off-white	Translucent	Smooth	Raised	Circular	Entire

Table 3. Change in weight of plastic incubated with waste-water

Sample no.	Initial weight (W_i) (mg)	Final weight (W_f) (mg)	Difference (W_f- W_i) (mg)	$\%change = \frac{difference\ in\ weight}{initial\ weight}$
1	0.250	0.2484	0.0016	0.64%
2	0.250	0.2369	0.0131	5.24%
3	0.250	0.2286	0.0214	8.56%

Table 4. Tests used for the Biochemical Identification of Isolated Bacterial strains

Strain Code	Strain Identified
AM1	*Klebsiella pneumoniae* subsp. *Ozaenae*
ID2	*Streptococcus pyogenes* (Group A)
HC3	*Nonenterococcus Streptococcus bovis*

Table 5. Identification of isolated strains

Strain Code	Catalase	Oxidase	EMB agar	MacConkey's agar	Indole test	MR test	VP test	Citrate test	H_2S test
AM1	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(-)

Strain Code	Catalase test	Hemolysis test	Bacitracin Sensitivity test
ID2	(-)	β hemolysis	(+)
HC3	(-)	γ hemolysis	(-)

Fig.1. Selection of sites and Collection of samples

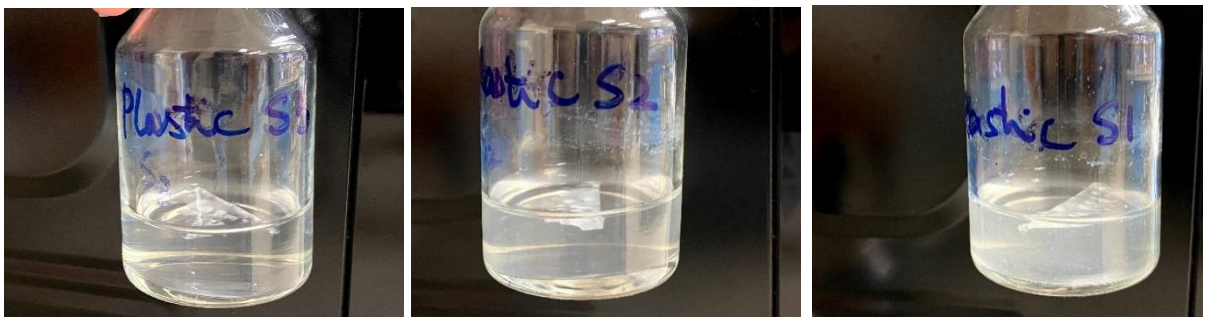

Fig. 2. Enrichment media flasks containing waste-water samples and plastic

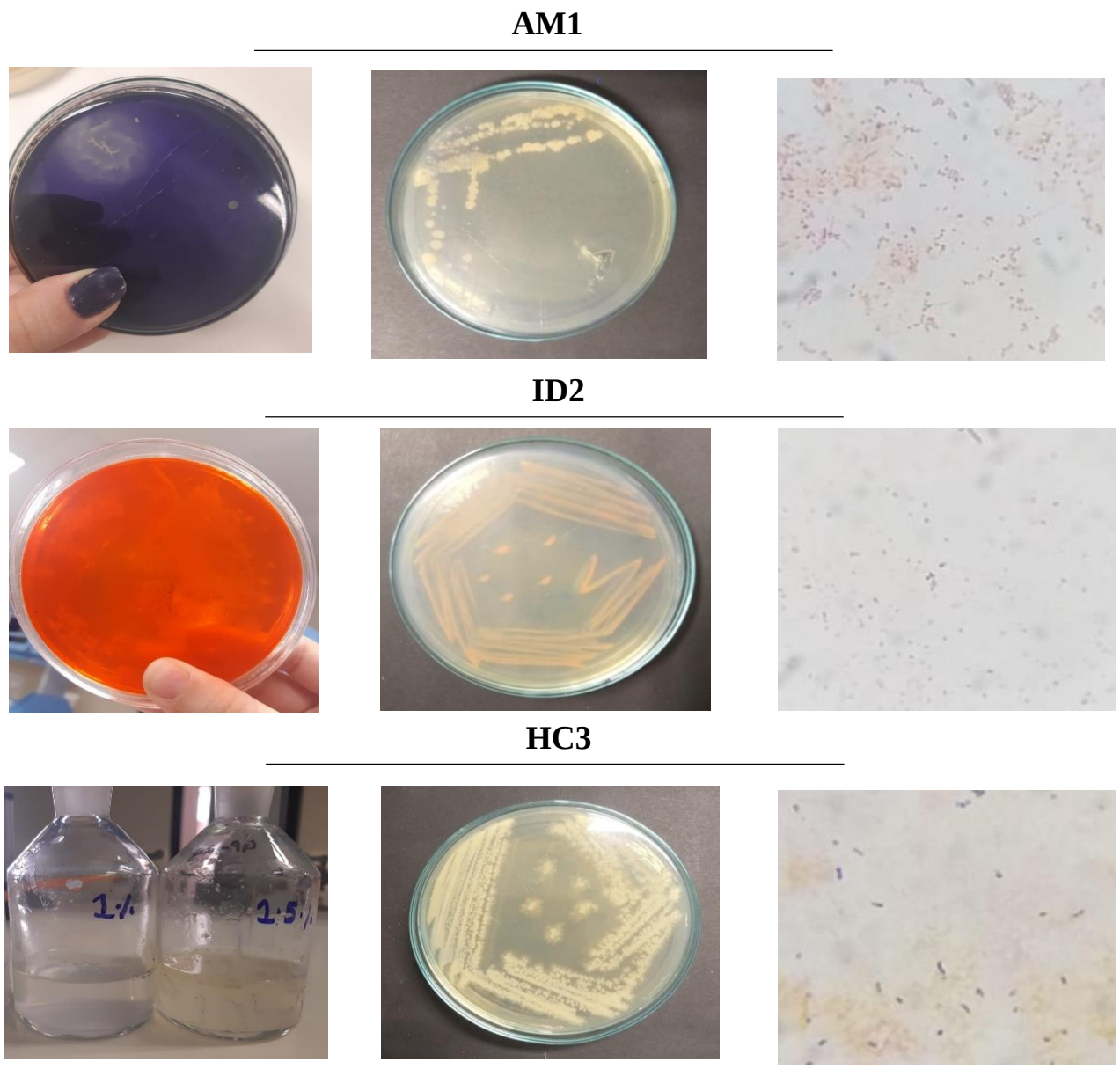

Fig. 3. Morphological Characteristics, isolation of cultures and Gram staining of the three bacterial isolates; AM1, ID2 and HC3 from Korangi Creek River, Karachi using Amylase, Azodye reductase and Kerosene degradation assays respectively

Conclusion

In the present investigation, three bacterial strains from the contaminated wastewater of Korangi Creek River, Karachi, were isolated and characterized for their bioremediation potential. The three isolates showed the ability to utilize different organic materials as nutrients as they showed growth on minimal media, which was devoid of any nutrients other than one specific organic material. However, bench and pilot-scale research is challenging to translate to full-scale field operations. For sites with complex combinations of pollutants that are not evenly diffused in the environment, bioremediation solutions need to be developed and engineered.

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