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Original Article

Isolation and Polyphasic Identification of Hydrolytic Bacterial Communities as a Prospective Bio-remediation Agents for the Waters of River Yamuna

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Abstract

Nine isolates of indigenous hydrolytic bacteria with potential as bioremediation agents for the polluted water of river Yamuna, namely *Klebsiella pneumoniae, Enterococcus faecium, Aeromonas veronii, Providencia stuartii, Aeromonas veronii, Bacillus sp., E. coli, Bacillus altitudinis, Pseudomonas azotoformans*, were obtained by using procedures of isolation, selection and molecular identification by polymerase chain reaction targeting 16s rRNA gene. These bacteria were initially isolated on Nutrient Agar (NA) media and further selection of bacteria was done through hydrolysis tests on Skimmed Milk Agar (SMA), Starch Agar, Tween Agar, CMC and cellulose media as well as hemolysis test for pathogenicity on Blood Agar Plate (BAP). Finally, the isolates were molecularly identified. Bioremediation prospects for river Yamuna have been analyzed.

Keywords: *Bioremediation; Hydrolytic Bacteria; Pollution; River Yamuna*

Introduction

Hydrolytic bacteria are a group of microorganisms that can perform the process of hydrolysis which includes breaking down composite organic polymers like carbohydrates, proteins and fats into monomeric soluble molecules such as simple sugars, amino acids and fatty acids by producing extracellular hydrolytic enzymes (Ethica *et al.,* 2018). The broken-down organic matter becomes more digestible for other microbes present in polluted water, making hydrolytic bacteria an excellent choice as bioremediation agent. These bacteria have the ability to survive in an extreme environment by utilizing quantum of pollutants for their metabolic processes (Hussain *et al*., 2022).

River Yamuna is relatively the most polluted river in the world (Gupta *et al.,* 2013; Joshi *et al*., 2022). Domestic sewage and industrial effluents are the major contributors of Yamuna River pollution (Shrivastava *et al*., 2001; Prakash *et al.,* 2024). The wastewater discharged from industries and households into river remains untreated and degrade its water quality (Mahajan & Prakash, 2025). About 57 million people are dependent on Yamuna water to meet their daily needs as the river flows through several cities from different states like Uttaranchal, Uttar Pradesh, Himachal Pradesh, Haryana, Rajasthan, Madhya Pradesh and the union territory Delhi (Malik *et al.,* 2014; Kumar *et al*., 2023).

Today, bioremediation is a leading sustainable biological approach to treat water pollution. The process of bioremediation is inexpensive, eco-friendly and efficient as compared to the physical and chemical methods of water treatment (Watanabe, 2001). It is a process that harnesses the metabolic and enzymatic processes of microbes to break down harmful pollutants to eliminate or reduce their levels in the environment (Mckew *et al.,* 2007). In comparison to other bioremediating agents like algae, fungi and plants, bacteria are easy to grow, manipulate and multiply rapidly. Bacteria have the ability to survive in harsh stressful environments and use a large number of pollutants as their carbon source for their different metabolic processes (Hussain *et al.,* 2022).

Studies have been conducted to employ bacteria like *Pseudomonas* sp. ZC1, *Vibrio* sp. ZL2,

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Acinetobacter sp. ZY3, *Citrobacter* sp. GO, and *Enterobacter Cloacae* GM6 for bioremediation of oil contaminated wastewater (Zhang *et al.,* 2025). Ansari *et al.* (2025) developed a bacterial consortium consisting of two bacteria namely *Bacillus licheniformis* KIBGE IB21 and *Glutamicibacter uratoxydans* KIBGE IB41 for treatment of synthetic textile sewage with toxic dyes. This consortium can decolorize 76-88% of synthetic textile sewage and showed 95-100% detoxification on MTS assay. Hashem *et al.* (2025) developed IF consortium which included bacteria belonging to the genera *Hydrogenophaga, Microbacterium* and *Gordonia* for removal of steroid estrogens which act as endocrine-disrupting chemicals commonly found raw domestic sewage. Moreover, alignment of microbial bioremediation strategies with controlled bioreactor systems have shown remarkable results with removal of 90-95% pharmaceutical residues, PFAS analogs and 70-80% reduction in microplastic in wastewater (Mishra *et al.,* 2025). Zhu *et al.* (2025) developed two indigenous *Bacillus* consortia that can effectively remove 34.5% of heavy oil from oilfield wastewater.

The objective of the present study was to initially isolate the indigenous bacteria from the Yamuna, identify and characterize by determining their ability to produce various hydrolytic enzymes (cellulase, lipase, protease, amylase) to be used as a bio-remediating agents for cleaning the Yamuna River (Fig. 1).

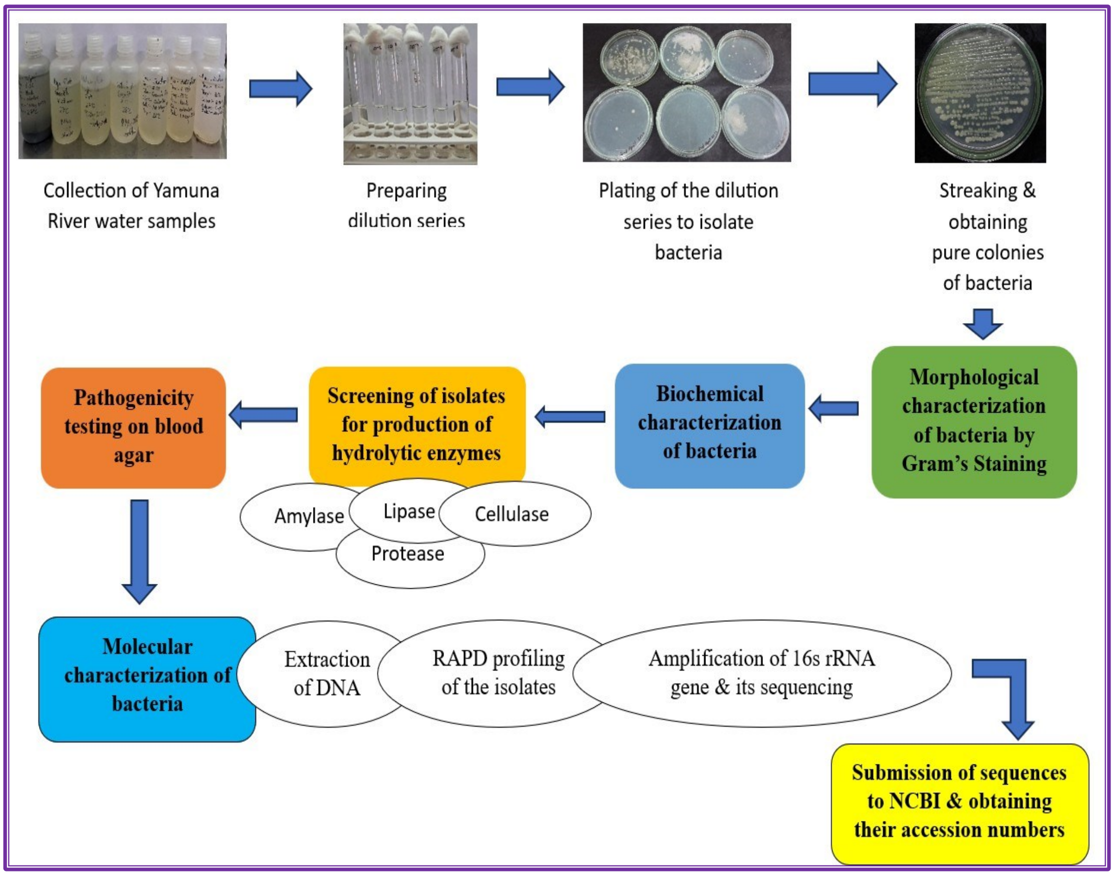


Figure 1: *Research Designed to Achieve the Objectives*

Materials and Methods

River water samples were collected from seven different sites located along the course of the River Yamuna in Agra as depicted in the Fig. 2 and Fig. 3. These sites were: -

I. Tajganj Cremation Ground (27º9'37.2852" N and 78º3'14.6232" E)
II. Hathi Ghat (27º11'26.8" N and 78º01'33.7" E)
III. Jawahar Bridge (27º10' N and 78º2' E)
IV. Poiya Ghat (27º13'51.67" N and 78º0'51.51" E)
V. Parvati Ghat (Balkeshwar) (27º12' 37.6" N and 78º1'38.7" E)
VI. Kailash ghat (27º10'34.5" N and 78º02'38.9" E)
VII. Dussehra Ghat (27º07'23" N and 78º02'22" E)

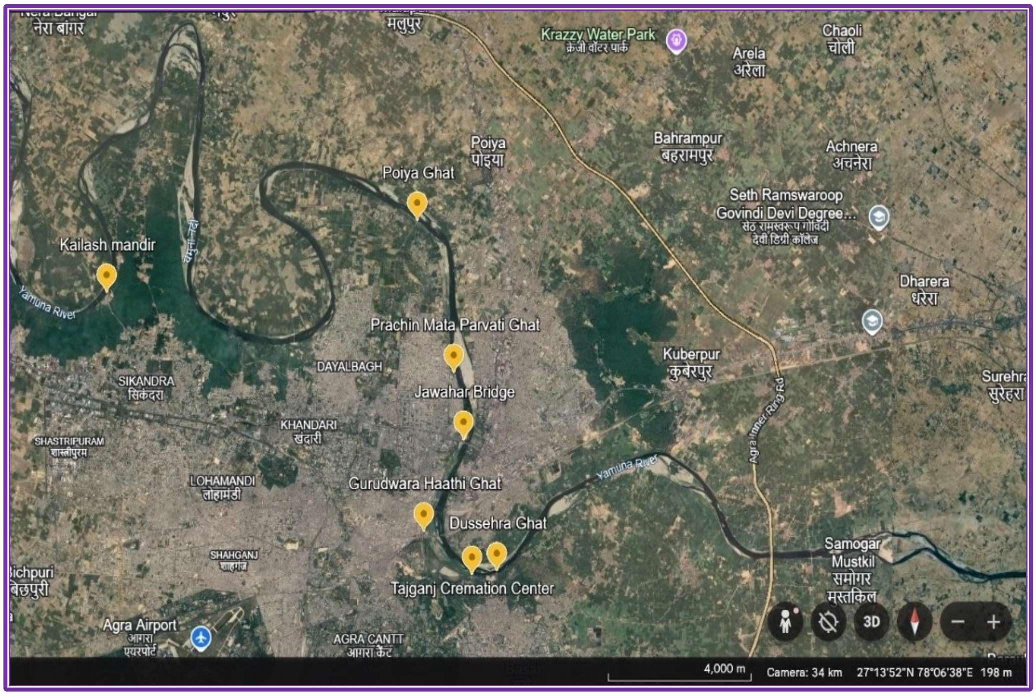


Figure 2: *Sampling Sites on the River Yamuna*


Tajganj
Hathi Ghat
Jawahar Bridge


Parvati Ghat
Kailash Ghat
Dussehra Ghat


Poiya Ghat

Figure 3: *Shows All the Sampling Sites from Where Yamuna Water Was Collected in Agra Region*

Grab samples were collected in the months of April and May, 2024 in sterile 140ml polyethylene bottles with screw caps following the triple rinse method from a depth 10cm. It was made sure that the bottles were not completely filled and about 1/3rd of the space in the bottle was left empty for the microbes to breathe. Location, time of collection, color, odor and date were noted on the bottles. These samples were transferred to the Lab, kept in an ice box to maintain the temperature of 4℃ till processing. These samples were processed within 5 hours of collection.

Isolation

Bacteria from the river water samples were isolated using serial dilution technique reported by Purwaningrum *et al*., 2021. 1ml of Yamuna River water sample was mixed with 9ml of 0.85% of saline solution and it was serially diluted up to 10^6 fold dilution. 100µl of each dilution was inoculated on the nutrient agar plates by spreading. These plates were incubated at 37℃ for 24 hours. Isolated and unique bacterial colonies were picked from the different dilution plates using sterile inoculating loop and incubated in nutrient broth tubes at 37℃ for 24 hours. After incubation, these bacterial cultures were further purified at least three times by streaking on nutrient agar plates. Isolated and purified colonies then obtained were sub cultured and maintained for further use. These sub- cultures were revived after every 30 days by inoculating 100µl of the pure bacterial culture into 3ml of fresh nutrient broth.

Morphological Characterization

Gram's Staining (Willey et al., 2008)

Thin smear of bacterial culture was made with loop full of distilled water on a sterile glass slide and was air dried and then heat fixed. The fixed smear was flooded with crystal violet stain for 1 minute, then rinsed with distilled water. Next, the smear was flooded with a mordant, Gram's iodine for 1 minute and again rinsed with water. The smear was then treated with 95% ethyl alcohol for a short period of 30 seconds and rinsed immediately with water. Finally, the smear was counter stained with safranin stain for 1 minute and rinsed with distilled water. The prepared slides were air dried and then observed under a light microscope at a magnification of 40x and 100x of objective lens. Shape, color, edge, elevation, consistency and arrangement were studied by observing a well grown colony of bacteria under the microscope.

Biochemical Characterization

Biochemical tests performed were namely catalase test, methyl red test, Voges Proskauer test, motility test, nitrate reduction test, oxidase test, citrate test, urease test and sugar fermentation tests (a set of five different sugars were used that is glucose, lactose, sucrose, raffinose and arabinose) were conducted as per the standard protocols. Positive and negative reactions were observed for the given tests. This biochemical profiling reflects the different families to which a microbe may probably belong.

Screening of lipase producing bacteria (Sierra, 1957)

The enzymatic characterization was performed by streaking the microorganisms on tween agar plates. The tween agar plates were prepared according to the protocol described by Kumar *et al.* (2012) and were incubated at 37℃ for 24 hours. After incubation, the formation of opaque zones around the bacterial colonies indicated hydrolysis of tween to fatty acids by microbes. The fatty acids bind with calcium present in the medium to form insoluble crystals, thus showing lipase production.

Screening of protease producing bacteria (Ramadhan et al., 2021)

Protease activity of microbes was qualitatively determined by streaking bacteria on skimmed milk plates. A positive protease activity was shown by formation of clear zone around the bacterial colony.

Screening of amylase producing bacteria (Sharma et al., 2015)

The ability to produce amylase was screened by streaking the isolated bacteria on starch agar plates. Starch agar plates were formed by dissolving 5g of peptone, 3g of beef extract, 2g of soluble starch, 22 g of agar in 1L of water. After incubating the streaked plates at 37℃ for 24 hours, the plates were flooded with Gram's iodine. On adding iodine, the plates turned bluish black in color and clear zones appeared around bacterial colonies with a positive amylase activity.

Screening of cellulase producing bacteria (Gupta et al., 2012)

Bacterial isolates were screened for the production of cellulase by streaking them on cellulose and CMC (carboxy methyl-cellulose) plates. After incubating the streaked plates at 37℃ for 24 hours, halo formation around the bacterial colonies shows positive cellulase activity.

Pathogenicity Testing (Purwaningrum et al., 2021; Darmawati et al., 2021)

In this test, the bacterial isolates were streaked on three different plates namely MacConkey Agar Plate (MAP), Blood Agar Plate (BAP) and Chocolate Agar plate (CAP).

Pathogenicity test on MAP

The isolates were streaked on MAP plates which was prepared by dissolving 51.55g of MacConkey Agar in 1000ml of water. The solution was heated till boiling and then autoclaved at 121℃ for 15 minutes.

Pathogenicity Test on BAP

BAP plates were prepared by dissolving 40.5g of blood agar media in 1000ml of water. This media was sterilized by autoclaving at 121℃ for 15 minutes. The sterilized blood agar media was cooled down to 50℃ and 7% (v/v) sheep blood was added (prepared as per manufacturer's instructions). The isolated bacteria were streaked on these blood agar plates and were incubated at 37℃ for 24 hours.

Pathogenicity Test on CAP

CAP media was prepared in a similar way as blood agar media with an additional step that was followed after adding blood to agar media. The flask in which media was prepared was plugged once again and placed in a water bath maintained at 80℃ for approximately 10 minutes till the blood turned chocolate brown in color. The flask was then swirled to homogenize the media and poured into sterilized plates. These plates were streaked and observed after 24 hours.

DNase Test

The bacteria were streaked on DNase agar plate to determine the pathogenic character on the basis of hydrolysis of DNA by the enzymes produced by isolates. After incubation the plates were flooded with 1N HCl solution. If clear zones develop around the bacterial growth, it shows the bacteria are pathogenic.

Molecular characterization of isolated bacteria

Extraction of genomic DNA (Ekhalil et al., 2015)

The genomic DNA was extracted by snap chill method. In this method, 1ml of freshly revived culture was transferred to a microfuge tube and spun for 3 minutes to allow the cells to separate from the broth in the form of a pellet at the bottom of the tube. The supernatant so produced was discarded and the pellet was resuspended in 500µl of distilled water followed by spinning for 3 minutes. The supernatant was again discarded and 200µl of distilled water was added to the pellet. The cells of the pellet were uniformly distributed in the water by vortexing. Then a hole was created in the cap of the microfuge tubes and placed in boiling water bath for 10 minutes. After the lapse of time, the tubes were immediately placed in ice for 15 minutes. The microfuge tubes were again spun for 2 minutes to sediment the cell debris and allow the DNA to aggregate in the supernatant. 200µl of DNA was carefully collected in a fresh microfuge tube and stored at 4℃ till further use.

Qualitative and quantitative analysis of extracted DNA (Green & Sambrook, 2012)

The quantitative and qualitative analysis of DNA was done by both ways electrophoretically and spectrophotometrically. Spectrophotometrically, DNA samples having an absorbance ratio between 1.8 and 2.0 were considered pure. The DNA samples having a concentration less than 100µl/ml were immediately rejected.

Random Amplified Polymorphic DNA (RAPD) fingerprinting (Baker et al., 2002; Kumari & Thakur, 2014)

RAPD PCR analysis of isolated bacteria was done using the universal primer OPA-11 having a sequence 5' CAATCGCCGT 3'. A 50µl master mix consisted of 5µl buffer F, 3µl Mg$Cl2$, 2µl dNTP mix, 5µl primer OPA-11, 0.85µl Taq DNA polymerase, 5µl DNA and 29.15µl of DNase RNase free water. A RAPD PCR program given in Table 1 was run to obtain the desired amplification. The amplified products were examined using 1.5% agarose gel electrophoresis. The PCR products and the 100bp ladder ran on agarose gel at 75V for 55 minutes. Later the amplicons stained with ethidium bromide were visualized through a gel documentation unit under ultra violet light.

Table 1: *RAPD PCR Run Program for Amplification*

Stage	Temperature	Time	Cycles
Initial Denaturation	94℃	5 minutes	1 cycle
Denaturation	94℃	1 minute	45 cycles
Annealing	32℃	1 minute	
Elongation	72℃	2 minutes	
Final Extension	72℃	8 minutes	1 cycle
Final Hold	4℃	∞	1 cycle

Amplification of 16s rRNA gene by Polymerase Chain Reaction (Galkiewicz & Kellogg, 2008)

To amplify the conserved 16s rRNA gene in the selected bacteria the universal primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'TACGGCTACCTTGTTACGACTT3') having Tm 51.78℃ and 52.97℃ respectively were used. Table 2 presents the constituents required to prepare the PCR mix. The PCR cycling program used with the above-mentioned primers was 1 cycle of initial denaturation at 94℃ for 5 minutes, 30 cycles of denaturation, annealing and extension altogether. Denaturation took place at 94℃ for 30 seconds followed by annealing at 48℃ for 30 seconds and extension at 70℃ for 1 min. The final elongation took place for 10 minutes at 72℃ and final hold at 4℃. The PCR products were analyzed on 1.5% agarose gel at a potential difference of 75V for 55minutes. 1kb ladder was used as the size marker and the gel was photographed under UV light by a gel documentation unit. The EtBr trapped by the amplicons cause them to shine.

Table 2: *Preparation of Master Mix for Amplification of 16s rRNA Gene*

Constituents	Stock Conc.	Quantity (µl)	Final Conc.
DNase RNase free water		29.15	
Buffer F	10X	5	1X
$MgCl_2$	25mM	4.0	2.0 mM
dNTP mix	10mM	2.0	0.4 mM
Forward Primer (8F/ 27F)	10µM	2.0	1µM
Reverse Primer (1492R)	10µM	2.0	1µM
Taq DNA polymerase	3 units/µl	0.85	0.05U/µl
Genomic DNA Template	100ng/µl	5	10ng/µl
Total		**50**	

16s rRNA gene Sequence Analysis

The amplified products of 16s rRNA gene obtained after PCR were sent for sequencing where the samples were purified and sequenced by Sanger sequencing using automated Applied Biosystems Sequencer. Once the sequence was obtained the samples were identified using BLAST (Basic Local Alignment Search Tool) tool and submitted to NCBI (National Center for Biotechnology Information) in order to obtain their accession numbers.

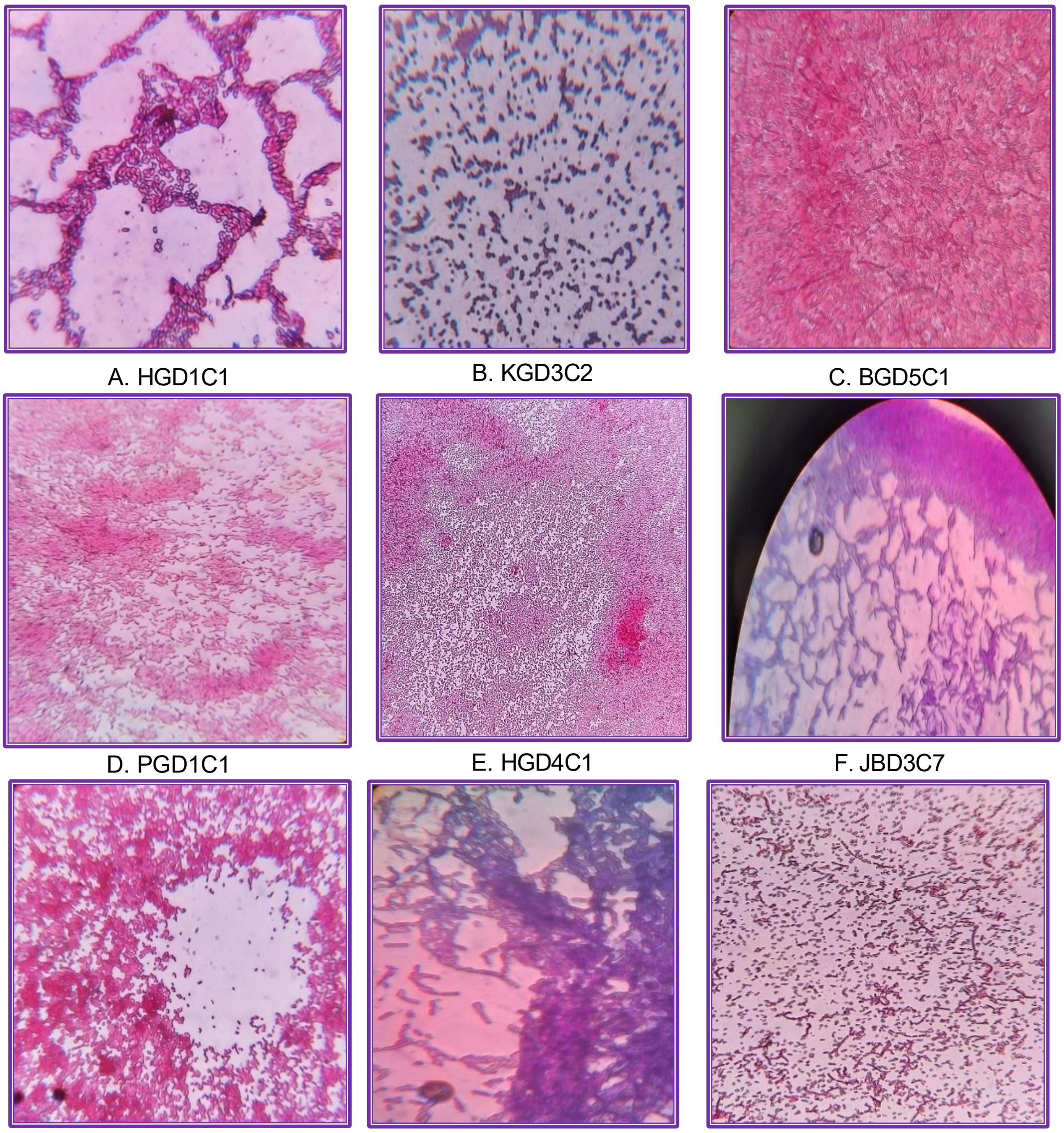
Results

Morphological Characterization of Isolates

Gram's staining helps to determine the shape of the bacterial cells as well as the composition of their cell walls. Gram positive bacteria appear purple on Gram's staining as they have thicker peptidoglycan cell walls that retain the violet-iodine complex on staining with crystal violet followed by the use of mordant Gram's iodine. Gram negative bacteria appear pink on Grams's staining as they have an additional outer membrane that contains lipopolysaccharides and inner thin peptidoglycan layer that loses its violet colour on the use of a decolourizer. Table 3. summarize the macroscopic observation of colony morphology on Nutrient Agar. Table 4 and Figure 4 show the results of Gram are staining on bacterial cells. Table 4 shows that only one of the selected isolates was coccus shaped while the others were rod shaped. The observations also showed that three selected isolates were Gram positive and the others were Gram negative.

Table 3: *Colony Morphology of Selected Bacteria on Nutrient Agar After 24 Hours of Incubation*

Isolate	Shape	Color	Edge	Elevation	Consistency
A1(HGD1C1)	round	white	even	convex	moist
B2 (KGD3C2)	round	white	even	convex	moist
C3 (BGD5C1)	punctiform	cream	even	convex	moist
D4 (PGD1C1)	round	white	even	slightly convex	moist
E5 (HGD4C1)	round	cream	even	convex	moist
F6 (JBD3C7)	irregular	white	serrate	flat	dry
G7 (DGD2C1)	round	white	even	convex	moist
H8 (KGD4C3)	punctiform	greyish white	even	flat	moist
I9 (BGD5C2)	round	slightly yellow	even	flat	moist



A. HGD1C1
B. KGD3C2
C. BGD5C1
D. PGD1C1
E. HGD4C1
F. JBD3C7
G. DGD2C1
H. KGD4C3
I. BGD5C2

Figure 4: *Gram Staining of Selected Isolates and Their Observation Under Optic Microscope at 100X Magnification*

Table 4: *Observation of Bacterial Cell Morphology by Gram-Staining*

Name	Shape	Arrangement	Gram-Staining Result
A1 (HGD1C1)	Rod	Cluster	Gram-Negative
B2 (KGD3C2)	Coccus	Cluster	Gram-Positive
C3 (BGD5C1)	Rod	Chain	Gram-Negative
D4 (PGD1C1)	Rod	Cluster	Gram-Negative
E5 (HGD4C1)	Rod	Cluster	Gram-Negative
F6 (JBD3C7)	Rod	Chain	Gram-Positive
G7 (DGD2C1)	Rod	Cluster	Gram-Negative
H8 (KGD4C3)	Rod	Chain	Gram-Positive
I9 (BGD5C2	Rod	Chain	Gram-Negative

Biochemical Characterization

Catalase test

Active bubbling on addition of H_2O_2 to well grown colonies of selected bacterial isolates on agar plate shows a positive catalase test while no bubbling indicates negative catalase test.

Methyl Red test

Colonies of selected bacterial isolates were inoculated in the glucose phosphate broth, when a drop of methyl red was added to freshly revived culture, a positive test was indicated by red color, a negative test was indicated by yellow color and cultures showing orange color were incubated for longer durations.

Voges-Proskauer test

The selected bacterial colonies inoculated in VP broth that show a color change to red on addition of α naphthol and KOH are considered to give a positive test while no color change shows negative test.

Motility test

The bacterial colonies that show turbidity after incubation of 24 hours show a positive test while no turbidity indicates negative motility test.

Nitrate Reduction

A positive test is indicated by the production of pink red compound on the addition of sulphanilic acid and α naphthylamine reagent and a negative test is indicated by no color production or producing red on further addition Zn.

Oxidase

A positive test is indicated by the production of a deep blue color on addition of tetramethyl-p-phenylenediamine (TMPD) while no change in color occurs in case of the negative test.

Citrate Test

Positive test is indicated by the growth of bacteria in citrate media giving it a Prussian blue color while negative test is indicated by no color change and no growth of bacteria on citrate media. Sometimes the growth of bacteria on citrate can turn media color from green to yellow instead of blue. In this case if there is an evident growth of bacteria on the media, it is considered positive otherwise negative.

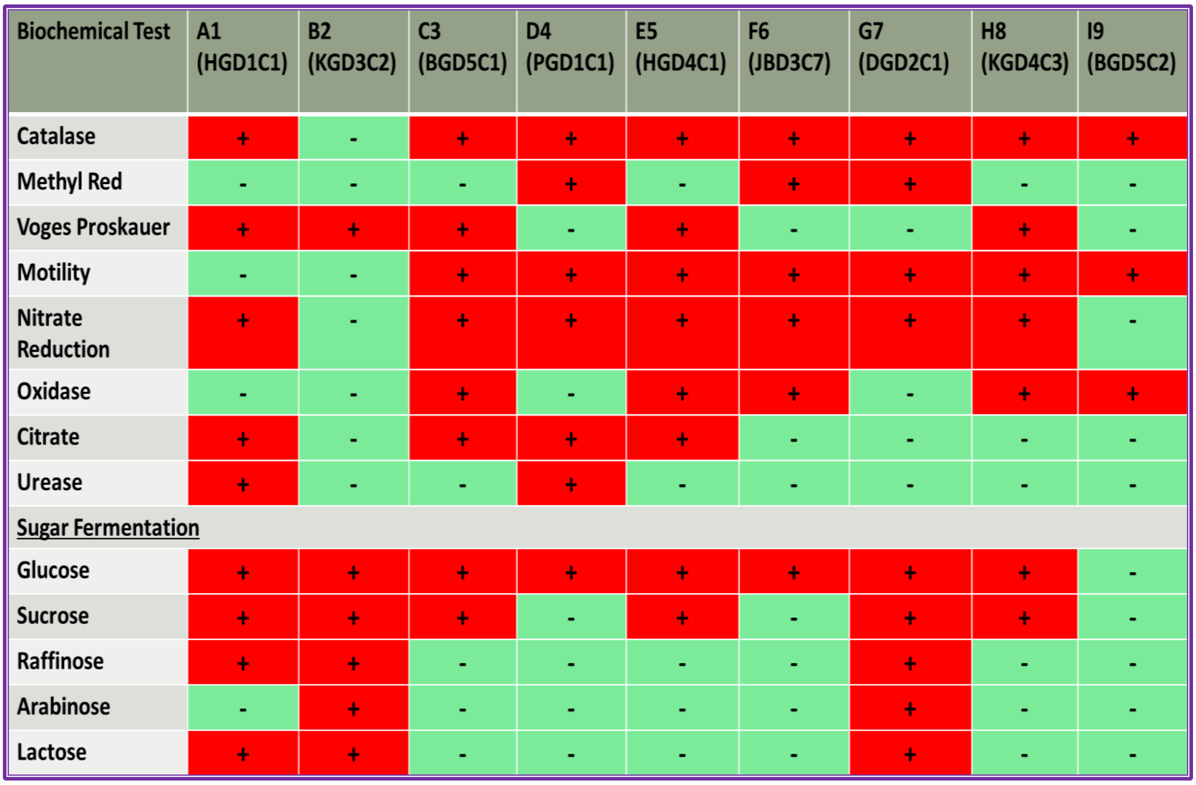
Urease Test

Positive test indicated when media color changes to bright pink while negative test indicated by no color change.

Sugar Fermentation

Bromocresol purple was used as the pH indicator for this test. Positive test was indicated by the yellow color while negative test was indicated purple color. The observations of the different biochemical tests performed on the selected bacteria are summarized in table 5.

Table 5: *Summarizing The Different Biochemical Tests for the Selected Isolates*



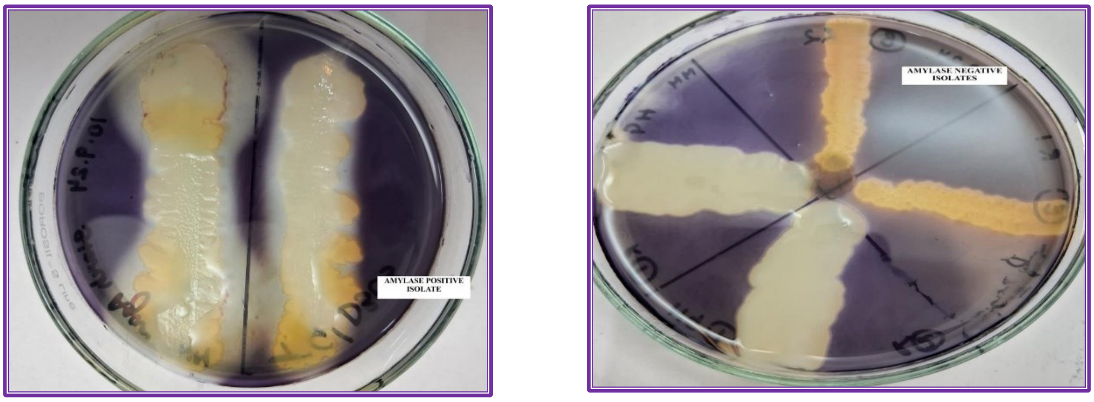
Biochemical Test	A1 (HGD1C1)	B2 (KGD3C2)	C3 (BGD5C1)	D4 (PGD1C1)	E5 (HGD4C1)	F6 (JBD3C7)	G7 (DGD2C1)	H8 (KGD4C3)	I9 (BGD5C2)
Catalase	+	-	+	+	+	+	+	+	+
Methyl Red	-	-	-	+	-	+	+	-	-
Voges Proskauer	+	+	+	-	+	-	-	+	-
Motility	-	-	+	+	+	+	+	+	+
Nitrate Reduction	+	-	+	+	+	+	+	+	-
Oxidase	-	-	+	-	+	+	-	+	+
Citrate	+	-	+	+	+	-	-	-	-
Urease	+	-	-	+	-	-	-	-	-
Sugar Fermentation									
Glucose	+	+	+	+	+	+	+	+	-
Sucrose	+	+	+	-	+	-	+	+	-
Raffinose	+	+	-	-	-	-	+	-	-
Arabinose	-	+	-	-	-	-	+	-	-
Lactose	+	+	-	-	-	-	+	-	-

Enzymatic Characterization

Testing all the isolated bacteria for the production of different hydrolytic enzymes like cellulase, amylase, protease and lipase by inoculating them on CMC plate, starch plate, skim milk agar plate (SMA) and tween 80 agar plate respectively. Formation of a hallow around the bacterial colony after incubation of 24 hours on CMC, starch and SMA media shows the production of cellulase, amylase and protease enzymes respectively while formation of insoluble crystals of calcium and fatty acids in the vicinity of the growing bacterial colony shows the production of lipase enzyme by the bacteria (Figure 5). Table 6 shows the different enzymes produced by the selected bacterial isolates.

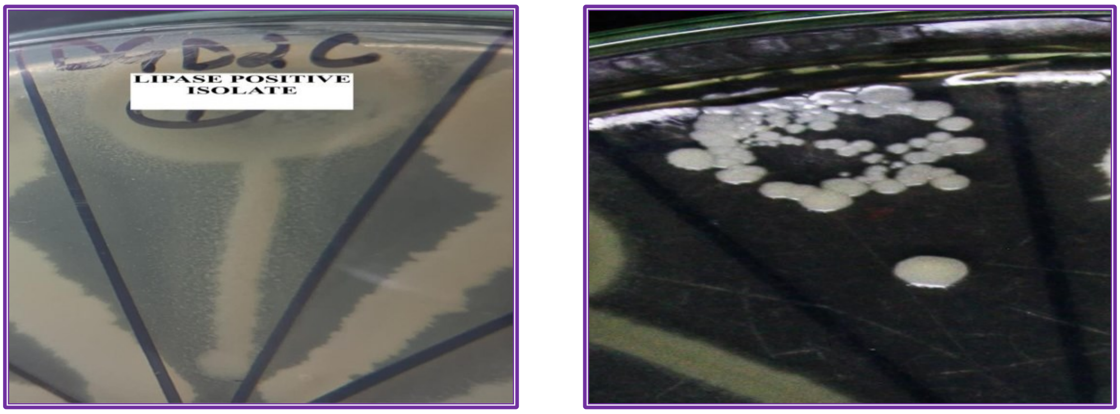
Pathogenicity Testing of Hydrolytic Bacteria

MacConkey is a selective and differential agar which allows the growth of non-fastidious Gram- negative enteric bacteria and selectively inhibits the growth of gram-positive bacteria. It also helps to differentiate between lactose fermenting bacteria and non-lactose fermenting bacteria. Lactose fermenting bacteria produce violet-colored colonies while non lactose fermenting bacteria appear yellow or colorless on MacConkey agar. The hydrolytic bacteria able to grow on MacConkey were marked “+” and those not able to grow on MacConkey were marked “-” (Table 6).



A. Bacteria showing amylolytic activity

B. Bacteria showing no amylolytic activity



C. Bacteria showing lipolytic activity

D. Bacteria showing no lipolytic activity

E. Bacteria plated on Skim Milk Plate Agar for testing their protease production

F. Bacteria plated on CMC Plate for testing their cellulase production

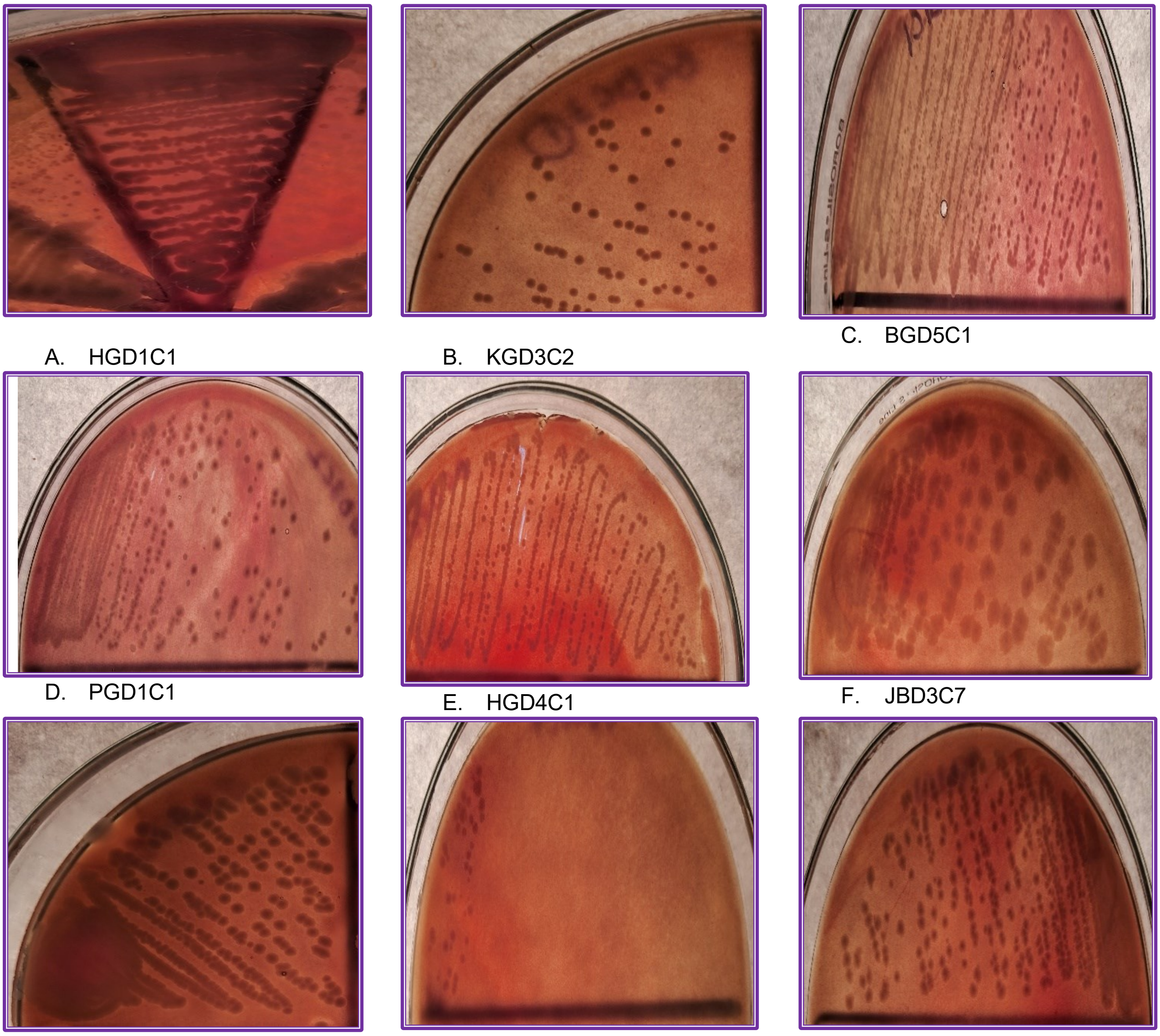
Figure 5: *Screening of Hydrolytic Bacteria*

Table 6: *This Table Presents the Different Enzymes Produced by the Isolates and their Growth on MAP And Pattern of Hemolysis on BAP And CAP*

Sl. No.	Isolate Code	Cellulase Enzyme Production Plate Test		Amylase Enzyme Production Plate Test	Protease Enzyme Production Plate Test	Lipase Enzyme Production Plate Test	Growth On Macconkey Agar Plate (Map)	Pathogenicity Test on Blood Agar Plate (Bap)	Pathogenicity Test on Chocolate Agar Plate (Cap)
		Cellulose	**CMC**						
1.	A1 (HGD1C1)	+	+	--	--	+	+(VIOLET)	γ	-
2.	B2 (KGD3C2)	-	+	++	++	++	-	γ	-
3.	C3 (BGD5C1)	+	-	++	++	++	+ (YELLOW)	γ	-
4.	D4 (PGD1C1)	-	-	++	++	++	+ (YELLOW)	γ	-
5.	E5 (HGD4C1)	+	-	++	--	++	+ (YELLOW)	γ	-
6.	F6 (JBD3C7)	+	+	++	++	--	-	γ	-
7.	G7 (DGD2C1)	-	+	+	++	++	+ (VIOLET)	γ	-
8.	H8 (KGD4C3)	-	0	++	--	++	-	γ	-
9.	I9 (BGD5C2)	+	+	--	--	--	+(YELLOW)	γ	-
10	KGD1C1	0	0	--	++	++	+ (YELLOW)	γ	-
11	BGDC3	-	-	+	--	+	+ (YELLOW)	γ	-
12	PGD3C3	0	-	--	--	++	+ (VIOLET)	γ	-
13	DGD4C1	+	+	--	--	++	+ (VIOLET)	γ	-
14	JBD3C	-	-	++	++	++	+ (VIOLET)	γ	-

Bacterial colonies on blood agar can show three types of hemolysis namely α, β and γ. The ones that showed no hemolysis was labelled "γ", the ones that showed clear zone around the bacterial colonies were labelled "β" and the ones that showed partial hemolysis with brown or green color around the bacterial colonies were labelled "α". Initially 108 bacteria were isolated from Yamuna water. Out of the 108 isolates only 14 did not show hemolysis on 7% blood agar plate after 24 hours of observation. Figure 6 shows the hemolysis pattern of the selected bacterial isolates.

Due to inactivation of certain clotting factors in chocolate agar plate, fastidious bacterial species like *Streptococcus pneumonia, Neisseria meningitides, N. gonorrhea* and *Hemophilus influenza* can grow on this media. After inoculation of bacterial isolates on CAP and its incubation for 24 hours, bacteria showing hemolysis on CAP and colony characteristics similar to highly pathogenic bacterial species a mentioned above were marked "+" otherwise "- ". None of the 14 isolates showed hemolysis on CAP nor were their colony morphology similar to the fastidious bacteria.



A. HGD1C1
B. KGD3C2
C. BGD5C1
D. PGD1C1
E. HGD4C1
F. JBD3C7
G. DGD2C1
H. KGD4C3
I. BGD5C2

Figure 6: *Pathogenicity Testing of the Selected Isolates on Blood Agar*

DNase Test

The bacteria that were able to hydrolyze the DNA present in agar were marked pathogenic otherwise nonpathogenic (Table 7 and figure 7).

Table 7: *Result of Dnase Test*

Isolate	DNase Test	Pathogenicity
A1(HGD1C1)	-	Non pathogenic
B2 (KGD3C2)	-	Non pathogenic
C3 (BGD5C1)	+	pathogenic
D4 (PGD1C1)	-	Non pathogenic
E5 (HGD4C1)	+	pathogenic
F6 (JBD3C7)	+	pathogenic
G7 (DGD2C1)	-	Non pathogenic
H8 (KGD4C3)	-	Non pathogenic
I9 (BGD5C2)	+	pathogenic

Figure 7: *Dnase Agar Plate*

Molecular Characterization

RAPD Fingerprinting

The RAPD fingerprinting of the 14 isolates that showed no hemolysis on blood agar was done. Electrophoresis was performed for the PCR product that was obtained after running the PCR program mentioned in Table 1. Figure 8 shows the different banding patterns of the 14 isolates. The RAPD banding patterns were analyzed with the help of a software Total Lab quant. It was observed that isolates with codes KGD1C1, KGD3C2, PGD3C3 had a similar banding pattern. BGD5C2, DGD4C1, BGDC3 isolates also have same banding pattern. HGD4C1 and JBD3C also had same banding pattern. BGD5C2, KGD3C2 and HGD4C1 i.e. one isolate from each of the above three groups of isolates showing similar banding pattern was chosen for further sequencing.

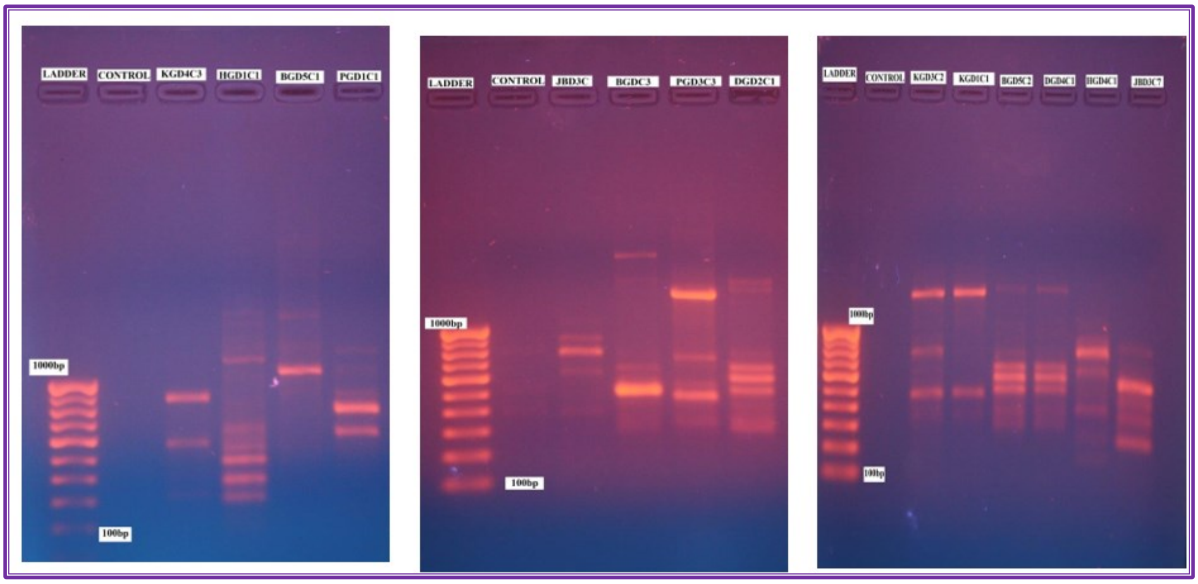


Figure 8: *RAPD Analysis of the Different Bacteria Isolated from Yamuna River Water*

PCR Amplification of the 16s rRNA Gene

Figure 9 shows the 16s rRNA gene amplification of the selected 9 isolates. A single band of size 1500bp for each isolate was observed on the agarose gel visualized under UV transilluminator.

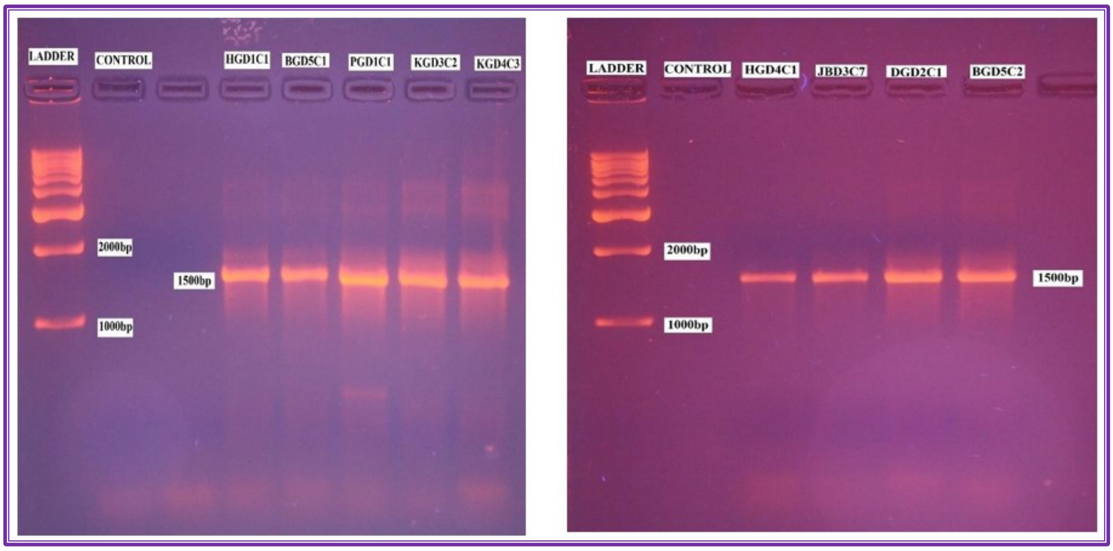


Figure 9: *Visualization of 16s rRNA Gene PCR Product on Agarose Gel*

16s rRNA Gene Sequencing for Bacterial Identification

The isolates were identified by comparing the sequence of each isolate with all the bacterial sequence data in the NCBI database using BLAST tool. Table 8 shows the list of isolates along with their accession numbers.

Table 8: *List of the Selected Bacterial Isolates Along with Their Accession Numbers Obtained From NCBI*

Sl. No.	CODE	NAME	ACCESSION NO.
1.	A1 (HGD1C1)	*Klebsiella pneumoniae*	PX071922
2.	B2 (KGD3C2)	*Enterococcus faecium*	PX103144
3.	C3 (BGD5C1)	*Aeromonas veronii*	PX072486
4.	D4 (PGD1C1)	*Providencia stuartii*	PX088152
5.	E5 (HGD4C1)	*Aeromonas veronii*	PX092361
6.	F6 (JBD3C7)	*Bacillus sp.*	PX125312
7.	G7 (DGD2C1)	*E. coli*	PX092665
8.	H8 (KGD4C3)	*Bacillus altitudinis*	PX106388
9.	I9 (BGD5C2)	*Pseudomonas azotoformans*	PX125316

Discussion

The focus of this study is on the isolation, selection and polyphasic identification of bacteria that can act as a bioremediation agent for the treatment of waste present in the river Yamuna. Initially, 108 isolates of bacteria were obtained from water samples and morphological characteristics of the bacterial isolates were observed as every bacterial colony has its own unique characteristics that can be used in the preliminary identification of bacterial species (table 3) (Tshikhudo *et al.*, 2013). Gram's staining provided the differential staining process that was used to categorize bacteria as Gram- positive or Gram-negative (Table 4 and Figure 4). Possibilities that morphological characteristics of two different bacteria can appear similar, biochemical tests were performed to rule out the overlapping (Table 5). This detection method also has certain limitations like long incubation periods (48 to 78 hours); inability to detect contaminated cultures; slow growing cultures and viable but non culturable bacteria (VNBC) (Reza *et al.*, 2014; Tantawiwat *et al.*, 2005). For the bacterial isolate identified as *Enterococcus faecium* (KGD3C2) in this study, only biochemical test that distinguishes *E. faecium* from *E. durans* is its ability to ferment arabinose (Deibel *et al.*, 1963). Interestingly the isolate BGD5C2 identified as *Pseudomonas azotoformans* in this study also shows a negative nitrate reduction test though it should be positive as the test was performed under aerobic condition that inhibits the process of anerobic denitrification.

It is important that the isolates produce one or more hydrolytic enzymes (amylase, cellulase, protease and lipase) so that they can degrade the organic substances like proteins, carbohydrates and lipids that are the major components of the sewage contaminating river Yamuna in Agra. The bacterial isolates were also tested for their pathogenicity by culturing them on blood agar plates. The isolates showing no hemolysis was only chosen (Table 6 and Figure 6). Report of Nacescu *et al.* (1992) on 2 30 *Aeromonas* strains describes that only 56.51% *Aeromonas* strains were hemolytic on 1% sheep erythrocytes suspension after 48 hours while the percentage dropped to 48.25% when hemolytic activity was observed on 5% sheep blood agar after 48 hours and that strains like *A. hydrophila* and *A. sobria* isolated from meat were more hemolytic than those isolated from freshwater. In our course of investigations, we found similar results where the isolates C3 (BGD5C1) and E5 (HGD4C1) which were later identified as *A. veronii* when cultured on 7% sheep blood agar, showed no hemolysis after 24 hours of incubation. Further, pathogenicity testing with DNase test was conducted and only five isolates out of nine namely *E. faecium, K. pneumoniae, E. coli, B. altitudinis* and *P. stuartii* was found to be non-pathogenic. Similarly, all the selected bacteria possess two main characteristics which are common among different bioremediation agents: -

They are indigenous to Yamuna water

They produce one or more extracellular hydrolytic enzymes that can decompose components of waste. Finally, the bacteria were identified by 16s rRNA sequencing (table 8) and the different bacterial species identified in this study use various metabolic pathways to degrade a wide range of pollutants as per the available literature. The occurrence of opportunistic pathogens like *Klebsiella pneumonia* and *Enterococcus faecium* in Yamuna, coincidently, points to an adaptive response against stressful environmental conditions due to high organic content and antimicrobial substances. The metabolic versatility of these bacteria in degrading urea, nitrate and hydrocarbons cannot be neglected despite their clinical significance (Ozyurek & Bilkay, 2018; He *et al.*, 2023; Cheng *et al.*, 2022). These strains can be used in controlled and monitored bioreactors or to sediment bioaugmentation strategies that are used to clean river water. Although *Providencia stuartii* are studied in clinical settings, Youssef *et al.* (2019) associated it with degradation of aromatic compounds and pollutants like dyes and phenols which are common components of industrial effluents discharged in Yamuna. *E. coli* is an indicator of fecal contamination and can contribute to pollutant degradation especially under facultative anerobic condition. *E. coli* strains were identified that can metabolize nitrogenous compounds and degrade simple hydrocarbons even in low oxygen zone of river and in wetland systems (Humphrey *et al.*, 2024). Metabolic diversity has been observed among the collected bacterial isolates which supports the approach of synergistic bioremediation.

In a study conducted by Ethica and Raharjo (2014), a pathogenic bacterial strain *Alcaligenes sp*. JG3 secreting hydrolytic lipase was characterized, it was an effective biodegradation agent which possesses the ability to degrade fat and glycerol. Rashid *et al.* (2022) isolated 3 bacterial strains identified as *Bacillus paramycoides*, *Alcaligenes faecalis* and *B. paramycoides* from different wastewaters (domestic, hospital, textile and pharmaceutical wastewaters) to be used as a low-cost monoculture system for bioremediation of water. Purwaningrum *et al.* (2021) molecularly identified two hydrolytic bacteria *B. paramycoides* and *B. firmus* that were lactose fermenting and non-hemolytic on blood agar that can be used to remediate liquid biomedical waste. Similarly, a number of different bacteria like *Pseudomonas aeruginosa, Aeromonas* sp., *Enterobacter* sp., *Acinetobacter* sp. was isolated for remediation of synthetic wastewater, tannery wastewater, oil refinery wastewater, chemical industry wastewater respectively (Paisio *et al.*, 2013; Yan *et al.*, 2020; Iqbal *et al.*, 2018; Paisio *et al.*, 2014).

The isolation and identification of *B. altitudinis* from Yamuna River water provides a crucial insight about the river's indigenous competence for self-purification. It has been globally recognized that *Bacillus* species are metabolically versatile and robustly stress tolerant due to their ability to produce endospores. *B. altitudinis* was particularly found to be highly efficient metal tolerant bacteria. Heavy metal contamination of Yamuna from industrial and urban runoff is a critical environmental challenge which acutely amplifies in Agra region as it is a major center for shoe and leather tanning industry. This industry discharges significant quantities of chromium into the river. Active removal of heavy metals by processes of biosorption and bioaccumulation. Goswami *et al.* (2025), places *B. altitudinis* as the most ecologically significant bioremediation agent among the other isolates. Despite extreme pollution, endemic microbial populations *like B. altitudinis* flourish in Yamuna water and can be harnessed for bioremediation strategies.

One of the limitations of this study is that the bacteria that were selected as bioremediation agents were characterized as non-pathogenic bacteria on the basis of in vitro experiments only but in vivo tests should also be conducted to confirm the same before these bacteria are used in the field without standard monitoring systems.

A significant avenue for further study is to develop modular encapsulated bacterial filters that can be retrofitted into existing sewage treatment plants (STPs). Such studies would allow large scale treatment of contaminated water, ensure consistent purification rate and facilitate integration of this biological treatment into existing wastewater treatment infrastructure.

Conclusion

Bacterial species identified as *B. altitudinis, E. coli, K. pneumoniae, E. faecium* and *P. stuartii* in this study can be used as a bioremediating agent to treat Yamuna River water. They can be used individually or assembled into a consortium to improve degradation efficiency. An essential trait for the application of these bacteria in dynamic aquatic environments like Yamuna River water is reinforced by the presence of both aerobic and facultative bacteria that enables the process of degradation at varying redox conditions. These isolated bacteria are of indigenous origin, therefore, it increases their probability of surviving, proliferating and functionally expressing in situ as well and reduces the use of any external inoculum for adaptation with increasing feasibility of extensive remediation. Enforcement of strict strategies and standard operating procedures (SOPs) and regular containment monitoring strategies can resolve any environmental and public issues concerning river waters.

Conflict of Interest

The authors declare they have no conflict of interest.

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