BIODEGRADATION OF POLLUTANT-RICH SEWAGE WATER EMPLOYING INDIGENOUS BACTERIA IN PURE AND MIXED CULTURE APPROACH

Sapna KALRA*, Anita BHATNAGAR

* *Department of Zoology, Kurukshetra University, Kurukshetra, Haryana India*
Correspondence author: Kurukshetra University, Kurukshetra, Haryana (India) -136119, phone: 09896634580, fax: 01744-238277, e-mail: anitabhatnagar@gmail.com

Abstract. Aquatic environmental contamination owing to the sewage water discharge is one of the accelerating risks associated with urbanization, industrialization, and population expansion. At present, large amount of sewage water is generated and only 50% is treated up to the standards suggested for disposal or using it for agricultural purposes. Sewage water's indigenous bacterial species assist in self-purification ability of the sewage water. The present research work was carried out to isolate, characterize and then analysing the potential biodegrading bacteria from sewage water. Later physiochemical were evaluated to find out the removal efficiency of sewage water. Total 19 bacterial strains (A-S) were isolated from the sewage water but only 3 bacteria *viz. Bacillus cereus* strain D*, Exiguobacterium indicum* strain J and *Bacillus altitudinis* strain Q showed protease producing as well as antipathogenic activity against *E. coli* bacteria. These three bacterial strains were used as pure culture and in consortium (comprising all three bacterial strains in 1:1:1 proportion) to treat the sewage water and were found to reduce pollution indicating parameters like BOD, COD, alkalinity, hardness, TDS, TSS, ammonia, nitrate and orthophosphate content. Percent reduction in BOD and COD followed the order: Consortium (65.81% and 58.02%) > *Exiguobacterium indicum* strain J (53.65% and 47.81%) > *Bacillus altitudinis* strain Q (50.51 and 44.16%) > *Bacillus cereus* strain D (44.38% and 40.15%) > Control (24.49% and 23.72%). Thus this bacterial treatment can be effectively used as eco-friendly efficacious sewage water treatment alternative so that the sewage water can be safely reused.

Key words: antimicrobial; bacterial strains; bioremediation potential; indigenous; sewage water

INTRODUCTION

In response to expanding population, enhanced farming practises, industrialization, urbanisation, demand for safe and hygienic water has been expanded but the availability of fresh/clean water is limited. Inadequacy of sewage treatment plants, their proper distribution and dearth in resources, leads to dumping of all the sewage water into the nearby aquatic ecosystem that has generated in tons from domestic, industrial, and agricultural runoff. The production of sewage water has been tremendously increasing with in the last few decades and give no sign of slowing down. At the global level, only 52% of the sewage water is treated up to desirable limits, rest of the water is dumped into water bodies or used for agricultural purposes.

Physiochemically, Sewage water has low DO (Dissolved Oxygen), high BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), organic compounds, inorganic compound, xenobiotics, heavy metals. Sewage water contains a wide range of bacteria that may be pathogenic including Proteobacteria, particularly Gammaproteobacteria, being the most common taxon, followed by Actinobacteria, Bacteriodetes, Chloroflexi, Firmicutes, and Planctomycetes. Archaea primarily consisted of two types of methanogens: acetoclastic (*Methanosarcina, Methanosaeta*) and hydrogenotrophic (*Methano-spirillium, Methanoculleus, Methanobrevibacter*) [27].

Thus, inappropriate disposal of sewage water in aquatic bodies or using it for irrigation purposes, without being treated may produces foul odours, pollution, biomagnification, and the mortality of aquatic organisms mainly of the fishes. Epidermal carcinoma, fin/tail rotting, gill illness etc. are some of the most common ill effects in fishes caused by sewage water. Therefore, sewage water must be treated to limit environmental damage.

Conventionally, sewage water is treated in three phases including primary, secondary, and tertiary treatment in sewage treatment plants. Primary treatment involves filtration and sedimentation; secondary treatment uses microbes to treat the sewage; and tertiary treatment employs chemicals to decontaminate the sewage wastewater before it is being utilized for agriculture or dumped into water bodies. But treated sewage water often produce poisonous gases and retain harmful chemicals that were used in treatment hence can cause the severe impact on the ecosystem. Also, conventional sewage treatment processes are expensive to operate and maintain [9] because it requires ample space, large infrastructure and should be in reach to all sources of wastewater. Along with that there is insufficient biodegrading bacterial population present in activated sludge that is used to degrade varied number of pollutants and most of time, physiochemical parameters of treated water do not fall within the standards suggested for irrigation and discharging in rivers/aquatic bodies. Thus, there is need to develop an alternate solution to treat the sewage water without sacrificing the quality of water bodies.

Sewage water has inherent potential to get decontaminated with course of time. The possible reason could be the presence of some autochthonous biodegrading microorganisms in sewage water itself that can utilize pollutants and break them into water and carbon dioxide. A chief role during the self-biodegradation of sewage water is played by bacteria because they are equipped with varied enzymes that can degrade even a complex chemical compound from sewage water. The possible mechanism may be the production of biosurfactant by these bacteria which is

amphiphilic compounds used for solubilizing and desorbing pollutants so that it can be used by microbes as energy source. These bacteria form the biofilm which provides them with a favourable environment for nutrient availability and multiplication. Some other mechanisms could be the biotransformation, bioaccumulation, and enzymatic degradation of chemical compounds via variety of enzymes such as oxygenase, carboxygenase and laccases etc. [17]. These bacteria can be isolated, screened for their bioremediation potential for sewage water and then mass cultured to degrade the toxic compounds of the sewage water. This could be the Eco-friendly, cheaper, and feasible technique to treat all the sewage water generated before being discharged in aquatic bodies.

Bioremediating potential of these bacteria is evaluated by their ability to cleave the proteinaceous compound by secreting the protease enzyme. These enzymes either help with dissolving varied organic and inorganic contaminants present in wastewater. Now a days, Bioaugmentation i.e., employment of bacterial culture to degrade the pollutants, is frequently used to improve the performance of biological processes [28] to treat the wastewater. It has been demonstrated in various studies that adding local bacterial strains can enhance the breakdown of specific organic pollutants in wastewaters. Qu and team found that *Pseudomonas sp*. (isolated from activated sludge) was particularly efficient in phenol removal when compared to non-augmented industrial wastewater [18]. Zhang and team isolated *Rhodococcus sp*. D32 that can aerobically degrade the organic pollutants such as dexamethasone (widely used endocrine disruptors) from wastewater [28]. Similarly bacterial strains such as *Pseudomonas stutzeri* CSW02 and *Pseudomonas extremaustralis* CSW01, were able to degrade very high concentrations of paracetamol in solution as a sole carbon and energy source [29]. According to Tuo and coworker, *Bacillus sp.* can effectively eliminate quinoline from the environment [25]. The removal of TOC (Total organic carbon) was found to be more effective with bioaugmentation_utilizing three strains of *E. cloacae, Gordonia*, and *P. putida* than single strain treatments. Along with these, attempts have been made to isolate anoxygenic bactria like *Rhodopseudomonas palustris* that have reduced the pollutant parameters at the rate of 80% [24]. Although there has been a constant effort in isolating novel biodegrading bacteria from sewage water but still there is big lacuna between diverse bacteria available and the number of bacteria isolated till date and studies describing their specific pollutant removal efficiency in terms of percent reduction in physiochemical and bacteriological parameters. Also, there is no such data available concerning the dose and minimum days required for the biodegradation of sewage water pollutants via bacterial culture. Hence the present study was conducted to investigate the autochthonous biodegrading bacterial culture that possess protease producing as well as antipathogenic activity from sewage treatment plant located in Kurukshetra city (India) and later evaluating its pollutant removal efficiency, dose and minimum time required in the form of pure culture and mixed culture to bioremediate sewage water.

MATERIALS AND METHODS

Collection of sewage water sample and its physiochemical analysis

Samples of sewage water were collected in triplicates in plastic bottles that had already been sterilized and were tightly sealed. Sample collection site was sewage treatment plant (STP) located in Mirzapur i.e., University STP (29.9540599°N, 76.812977331 °E) in Kurukshetra city. Prior to treatment, the sewage water's quality was assessed. According to the standard methods given by APHA [2], water quality parameters like pH, BOD, COD, DO, alkalinity, hardness, calcium, magnesium, chloride content, ammonia, nitrate, and orthophosphate were measured in triplicates.

Isolation and screening of bacteria

Serial dilution and spread plate method were performed to isolate the pure bacterial culture from the sewage water [11]. For this, the 1 mL of sewage water sample in 9 mL of sterilized distilled water was vortexed to form the suspension of each sample, which was then aseptically transferred on a nutrient agar medium (NAM) plate. The Petri plates were incubated for 24 hours at 37 °C. After that, the bacterial colonies were isolated by streaking them onto the fresh plates with nutrient agar media and again kept for incubation. The purified bacterial isolates were preserved on nutrient agar slants at 4°C. All bacterial isolates (A-S) were subcultured after a time interval of 30 days.

The isolated bacterial strains were screened for protease production on skim milk agar medium (pH: 7.2) comprising skim milk powder 28 g, tryptone 5 g, yeast extract 2.5 g, dextrose 1 g and agar 15 g in 1 liter of distilled water. Plates containing SM agar medium were inoculated by bacterial strains and observed for the formation of zone of proteolysis as protease enzyme produced by these bacteria hydrolyze the casein protein present in skim milk powder [16]. The bacterial strains forming zone of proteolysis were purified and preserved on alkaline nutrient broth.

Antibacterial activity of isolated protease producing bacterial strains were performed according to Scillato and coworker in which Cell-Suspension of the isolated bacterial strains (1.5×10^7 CFU mL^{-1}) were used [20]. For the agar well diffusion assay [6, 12], 9 cm Petri plates were filled with 20 mL of molten nutrient agar and allowed to solidify. Pathogenic *E. coli* was evenly spread on the NAM plates before a well was made in the center of the plate using micropipette tips. The cut wells were filled with 100 µL of the cell suspension of the bacterial strains and labelled accordingly. With the aid of a ruler, the test strain's zones of inhibition were quantified in terms of diameter. For the isolated

bacteria, the zones of inhibition (calculated from the colony's edge to the edge of the clear zone) were recorded. Bacterial strains possessing zone of inhibition with a minimum 20 mm were selected for further studies.

Identification of isolated bacteria using biochemical assay and 16S rDNA based molecular characterization

Pure cultures of isolated bacteria with high biodegradation potential and antipathogenic activity (Strain D, J and Q) were first identified using biochemical characterization (Gram staining, methyl red test, Vogues Parker test, starch, and urea hydrolysis) by using standards biochemical assays in accordance with the Bergey's manual scheme [4].

For 16 S rDNA molecular analysis, Genomic DNA was isolated from the pure bacterial culture using inhouse Bacterial DNA isolation kit and Denovix DS-11 spectrophotometer was used to analyze the purity and concentration of the isolated DNA. Further the amplification of 16S rDNA region of isolated DNA was performed using Bacterial 16S rDNA PCR kit Fast (800) (Make, EmeraldAmp® GT PCR Master Mix). The amplification of 800bp amplicon was obtained using 16S rDNA Primer Mix and TaKaRa Taq™ HS Fast Detect Premix in the sample provided and in the positive control used *E. coli*. No amplicon was obtained in the non-template control (NTC). The test amplicon of 800bp was purified via column purification and bi-directional cycle sequencing was carried out with forward primer and reverse primers using BDT Cycle sequencing kit on ABI 3730 Genetic Analyzer. GeneTool software was used to generate a consensus sequence of 16S rRNA gene obtained by primers were assembled using and BLAST analysis was carried out to compare with the sequences available in the NCBI genebank database. The first ten sequences in the database that showed highest similarity were selected based on maximum identity score and phylogenic tree result.

***In vitro* biodegradation potential of isolated bacteria**

The pollutant removal efficiency of bacterial culture based on per cent reduction of different physico-chemical parameters of sewage water was evaluated in two different experiments.

Experiment 1: This experiment was performed for five days under the controlled conditions in which sewage water was inoculated with isolated bacterial cultures singly and as consortium and kept in incubator to find out the desirable dose at which maximum pollutant removal efficiency (considering BOD, COD, Nitrate, orthophosphate, and ammonia) was obtained. All the treatments were kept in replicate of three.

Experiment 2: Second experiment was performed for 21 days under the normal atmospheric conditions in which sewage water was inoculated with same bacterial cultures and consortia (in replicate of three) after the interval of 7 days at the desirable dose to degrade most of the pollutants. Various physiochemical parameters such as BOD, COD, DO, alkalinity, hardness, calcium content, magnesium content, ammonia, nitrate, and orthophosphate content were observed on 7^{th}, 14^{th} and 21^{st} day.

For the assessment of biodegradation potential, inoculum of the selected bacterial strains was prepared by inoculating nutrient broth with bacterial colonies which were later kept in incubator for 24 hrs. for the growth of mother culture. Thereafter the cultures were centrifuged at 7000 rpm for 15 min at 4°C and then supernatant was discarded, and pellets were suspended in 1% normal saline to acquire the desired optical density at 600 nm. For this, concentration of the bacterial inoculum was decided based on optical density of bacterial suspension at 600 nm. Two different doses of bacterial suspension were prepared in which one concentration was 2 x 10^8 CFU/mL having OD_{600} of 0.25 and the other concentration was 4 x 10^8 CFU/mL having OD_{600} of 0.5. Volume of the inoculum and volume of the sewage water sample were in the ratio of 1 :100 (V/V). Thus 3.0 mL of each bacterial suspension was inoculated with two different doses (OD_{600}-0.25 and OD_{600}- 0.5) in separate flask containing the 300 mL of sewage water sample and kept for 5 days at 37°C in incubator [3].

For biodegradation study in both the experiments, Total five treatment groups were formed including one control and four treatments with isolated and identified strains (Table 1). Each treatment was further divided into two doses (for experiment 1) as mentioned above with two different bacterial concentration. In bacterial consortium treatment, 1.0 mL of each bacterial suspension was inoculated into the sewage water sample. After incubation, the samples were analyzed for different physico-chemical parameters and its removal efficiency was determined using the following formula [10]:

Removal Efficiency (R_E %) = $(C_0-R_C)/C_0 \times 100$

where, C_0 = Initial Concentration before Treatment, R_C = Final Concentration after Treatment

Table 1. Treatment Groups

Treatments	Dose A	Dose B
Control	No inoculation	No inoculation
Treatment 1	T1A: Strain D (OD_{600}: 0.25)	T1B: Strain D (OD_{600}: 0.5)
Treatment 2	T2A: Strain J (OD_{600}: 0.25)	T2B: Strain J (OD_{600}: 0.5)
Treatment 3	T3A: Strain Q (OD_{600}: 0.25)	T3B: Strain Q (OD_{600}: 0.5)
Treatment 4	T4A: Consortium (OD_{600}: 0.25)	T4B: Consortium (OD_{600}: 0.5)

Statistical analysis

All the experiments were performed in triplicates therefore all data were subjected to Analysis of variance. A difference among various bacterial treatment' means was determined using Duncan's multiple range test. Statistical significance was set at probability value of $p<0.05$.

RESULTS

Isolation and screening of bacterial strains

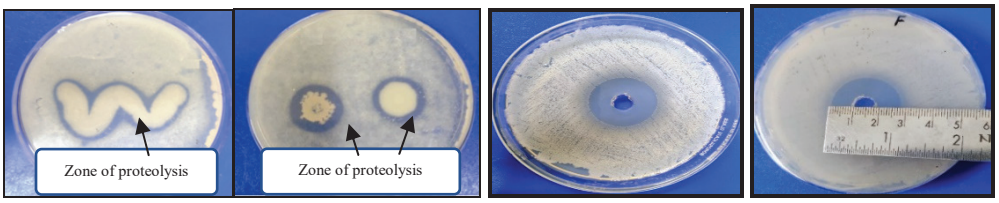
Total 19 (A-S) morphologically different bacterial strains were isolated by repeatedly streaking method and their pure culture was stored in nutrient broth and agar slants. These strains were later examined for protease producing activity on skim milk agar (SMA) plates. Out of 19 bacterial strains, 10 (A, D, F, H, J, M, O, P, Q, R) were able to produce zone of proteolysis (Fig 1a and b) on SMA plates as these bacterial colonies hydrolyze the casein protein of skim milk. Out of 10 bacterial colonies, only five (A, D, H, J, Q) were able to produce zone of inhibition against *E. coli* bacteria as these isolated bacteria restrict the growth of pathogenic bacteria in their vicinity. But out of these 5 bacteria only 3 (D, J, Q) were able to produce accountable zone of inhibition (Fig 1 c and d) of more than 20 mm.

Biochemical characterization of selected strain:

All the three strains (D, J and Q) were found to be gram positive and were of rod shaped as showed in Fig 2. These strains showed positive results for catalase tests and motility. Various other biochemical characteristics possessed by this bacterial strain are described in Table 2.

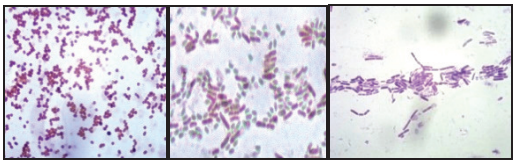
16S rDNA molecular analysis:

These isolated antibacterial protease producing bacteria were later identified by 16 S rDNA molecular technique. The isolated strains D, J and Q showed similarity with *Bacillus cereus* S8 (Accession No: MT611946.1) (here called as *Bacillus cereus* strain D), *Exiguobacterium indicum* DSAM 62 (Accession no: MH819520.1) (called as *Exiguobacterium indicum* strain J) and *Bacillus altitudinis* NPB34b (Accession no: MT634735.1) (called as *Bacillus altitudinis* strain Q) respectively based on nucleotide homology and Phylogenetic tree analysis. Phylogenetic tree of the strains along with the genomic DNA gel electrophoresis image comprising 1kb DNA ladder are shown in Fig. 3a to 3c.



(a) (b) (c) (d)

Fig. 1 (a) & (b): Zone of proteolysis formed by the isolated bacteria on skim milk agar plate; **(c) & (d):** Zone of inhibition (more than 20mm) formed by the isolated bacteria on agar plate



(a) (b) (c)

Fig. 2. Gram staining slide of the isolated bacterial strains 2(a) strain D, 2(b) strain J and 2(c) strain Q

Table 2. Biochemical characteristics of isolated bacterial strain

Biochemical characterization	Strain D	Strain J	Strain Q
Gram stain	G+	G+	G+
Shape	Rod shape	Rod shape	Rod shape
Spore formation	+	-	+
Motility	+	+	+
Catalase	+	+	+
Methyl red test	-	-	-
Voges Proskauer test	+	+	+
Starch hydrolysis	+	+	-
Urease production	-	-	-
Mannose fermentation	-	+	+
Lactose fermentation	-	-	-
Glucose fermentation	+	+	+
Maltose fermentation	+	+	+

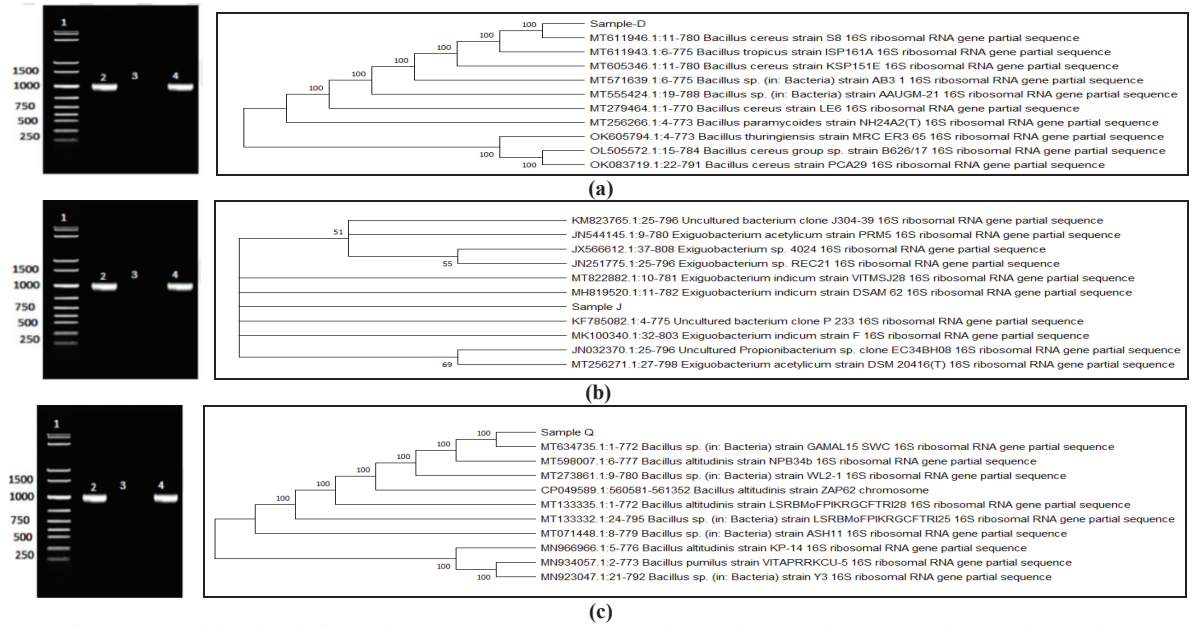


Figure 3. Dendrogram comprising the Phylogenetic tree 3(a) *Bacillus cereus* strain D, 3(b) *Exiguobacterium indicum* strain J and 3(c) *Bacillus altitudinis* strain Q showing relationship with other closely related strains acquired from NCBI GenBank database with their accession number along with the Agarose gel electrophoresis image depicting lane 1: 1Kb DNA ladder, Lane 2: 16S amplicon (Positive Control), Lane 3: Negative Control, Lane 4: Sample.

Physiochemical characteristics

(Pre- Treatment): Physiochemical parameters of the sewage water sample were analyzed before treatment and described in table 3.

(Post treatment): For first experiment, total five experimental groups were formed in which one control and four treatments were included. Table 3 shows the Mean and standard error values of water parameters in which it was observed that initial pH was near to neutral which turned alkaline after the microbial treatment. BOD was found to be reduced up to 30 % and highest reduction was found in the treatment T4 B in which consortium of the bacterial suspension were added with OD_{600}- 0.5.

Pure culture of *Bacillus altitudinis* strain Q was also found to be effective in reducing the BOD upto 27% from the water sample. *Exiguobacterium indicum* strain J was found to most effective in reducing the chemical load of the sewage water. Strain D i.e., *Bacillus cereus* was found to be least effective among all. Similar results were obtained for orthophosphate and ammonia. Content of nitrate is slightly reduced when compared with initial concentration. Overall treatment T4 B consortium of strains at dose B (4 x 10^8 CFU/mL) showed the significant ($p<0.05$) reduction in pollution load from the sewage water sample followed by the pure culture of *Exiguobacterium indicum* strain J that was also observed to be effective on the reduction of pollutants. Overall, the pollutant removal efficiency of treatments followed the trend: T4B>T4A>T2B>T3B>T1B>T3A>T2A>T1A>Control.

Further on the basis of the results obtained from experiment 1, Dose B (OD_{600}: 0.5) was selected as desirable dose and used for experiment 2 because pollutant removal efficiency was highest at this dose. After the treatment for 21 days (Experiment 2), Like the results obtained in the first experiment, among the monoculture bacterial treatment, T3 i.e., Pure culture of *Bacillus altitudinis* strain Q was found to be most effective in reducing the BOD content upto 54%.

Table 3. Mean and standard error of the physiochemical parameters of pre-treated and post-treated sewage water sample for different experimental groups with two different doses.

TREATMENTS		BOD (mg/L)	COD (mg/L)	pH	Nitrate (mg/L)	Orthophos-phate (mg/L)	Ammonia (mg/L)
PRE-TREATMENT		104± 2.31^{A}	156± 2.3^{A}	7.52 ±0.11^{E}	1.12 ± 0.01^{AB}	1.41± 0.015^{A}	3.74± 0.03^{A}
POST TREATMENT	**CONTROL**	94.67± 1.3^{B}	146± 3.46^{B}	8.11 ± 0.005^{D}	1.11 ± 0.013^{ABC}	1.34± 0.006^{B}	3.26± 0.27^{B}
	T1 A	90± 2.3^{C}	129.33 ± 1.33^{C}	8.14 ±0.011^{CD}	1.1 ±0.008^{ABCD}	1.27± 0.014^{D}	3.17± 0.003^{C}
	T1 B	85.3± 0.67^{C}	114.67± 1.33^{EF}	8.186 ±0.008^{B}	1.08± 0.008^{DE}	1.29± 0.003^{CD}	3.14± 0.008^{CD}
	T2 A	88± 1.54^{C}	116.67 ±0.67^{DEF}	8.18± 0.11^{B}	1.11± 0.013^{ABC}	1.31± 0.006^{C}	3.14± 0.017^{CD}
	T2 B	78.67± 0.66^{D}	108.67± 0.67^{G}	8.14± 0.145^{C}	1.1± 0.008^{ABCD}	1.19± 0.003^{F}	3.12± 0.05^{CD}
	T3 A	86.67± 1.33^{C}	120± 2.3^{DE}	8.26± 0.014^{A}	1.1± 0.008^{ABCD}	1.25± 0.008^{E}	3.13± 0.014^{CD}
	T3 B	76.67± 1.76^{DE}	120.67± 1.76^{D}	8.21±0.003^{B}	1.09±0.008^{BCDE}	1.18± 0.008^{F}	3.09± 0.008^{D}
	T4 A	74.66± 1.33^{DE}	117.23± 1.33^{DEF}	8.21±0.008^{B}	1.08± 0.008^{DE}	1.16± 0.01^{F}	3.015± 0.003^{E}
	T4 B	72.67± 0.66^{E}	112.67± 0.67^{FG}	8.26± 0.15^{A}	1.07± 0.003^{E}	1.127± 0.008^{G}	2.96±0.008^{E}

All values are mean ± S.E. of mean
Mean with different letters in the same column are significantly ($p<0.05$) different (Duncan's multiple range test)

Table 4. Various physiochemical properties of sewage water observed on 7th, 14th and 21st day of bacterial treatment under the normal lab conditions

PHYSIO-CHEMICAL PARAMETERS	7TH DAY					14TH DAY					21ST DAYS				
	CONTROL	T1	T2	T3	T4	CONTROL	T1	T2	T3	T4	CONTROL	T1	T2	T3	T4
TEMPERATURE (˚C)	29.53±0.03^{B}	29.1±0.057^{C}	29.85±0.057^{A}	29.58±0.033^{B}	29.58±0.33^{B}	28.35±0.12^{B}	28.56±0.03^{B}	28.59±0.02^{B}	28.36±0.12^{B}	29.73±0.06^{A}	29.57±0.03^{B}	29.43±0.08^{B}	29.75±0.02^{A}	29.86±0.01^{A}	29.76±0.017^{A}
pH	8.13±0.013^{C}	8.45±0.003^{B}	8.49±0.005^{B}	8.65±0.012^{A}	8.47±0.014^{B}	8.25±0.005^{E}	8.52±0.006^{D}	8.66±0.015^{B}	8.72±0.01^{A}	8.62±0.01^{C}	8.33±0.008^{B}	8.58±0.02^{AB}	8.73±0.006^{AB}	8.58±0.29^{AB}	8.91±0.02^{A}
BOD (mg/L)	121.33±1.33^{A}	101.33±1.33^{B}	104±2.31^{B}	98.6±1.33^{B}	89.33±2.67^{A}	108.67±2.4^{A}	82.67±0.66^{B}	78±2^{BC}	76±1.15^{C}	54.66±1.76^{D}	98.66±1.76^{A}	72.66±1.33^{B}	61.33±1.3^{C}	64.66±1.36^{C}	44.66±1.76^{D}
COD(mg/L)	164.67±2.66^{A}	140.67±2.4^{B}	132.67±1.76^{B}	139.33±0.66^{C}	116±2.3^{D}	152±2^{A}	123.33±1.76^{B}	112.67±1.76^{C}	118±1.15^{BC}	93.33±1.76^{D}	139.33±1.76^{A}	109.33±1.33^{B}	95.33±2.40^{C}	102±1.15^{D}	76.66±1.76^{E}
ALKALINITY (mg/L)	388.67±5.69^{A}	358±5.03^{B}	328±2.3^{C}	325.33±3.71^{C}	300±1.15^{D}	348±3.46^{A}	306.67±5.6^{B}	295.33±4.37^{B}	278±4.16^{C}	244.67±4.37^{D}	324.67±2.4^{A}	294±2.3^{B}	269.33±2.91^{C}	258±4.16^{D}	207.33±2.4^{E}
CHLORIDE (mg/L)	101.4±4.33^{A}	63.53±2.45^{B}	49.14±1.66^{C}	39.65±1.73^{D}	28.51±2.18^{E}	74.55±3.09^{A}	46.32±2.2^{B}	35.27±2.3^{C}	36.52±2.82^{C}	22.74±1.15^{D}	70.66±0.66^{A}	38.37±1.13^{B}	30.61±1.28^{C}	30.3±2.283^{C}	16.45±2.38^{D}
HARDNESS (mg/L)	232.67±1.76^{A}	215.33±3.71^{B}	200.67±2.9^{C}	204±4^{C}	185.33±3.52^{D}	193.33±2.9^{A}	163.33±2.4^{B}	160.67±1.76^{B}	145.33±2.9^{C}	114±3.05^{D}	177.33±2.4^{A}	151.33±1.76^{B}	146.67±2.4^{B}	138±1.15^{C}	110±1.15^{D}
CALCIUM (mg/L)	61.63±0.31^{A}	48.96±1.79^{B}	42.06±0.97^{C}	46.27±1.16^{BC}	35.89±2.39^{D}	53.76±1.71^{A}	40.63±1.28^{B}	33.81±1.72^{C}	40.64±1.27^{B}	26.5±1.19^{A}	49.24±0.64^{A}	30.67±1.09^{B}	24.60±2.29^{C}	29±0.76^{B}	20.56±1.16^{C}
MAGNESIUM (mg/L)	30.78±0.24^{A}	24.89±0.79^{B}	21.45±0.68^{BC}	23.56±0.78^{C}	18.23±1.07^{D}	27.28±0.86^{A}	20.65±0.5^{B}	17.34±0.74^{C}	20.42±0.72^{B}	13.45±0.41^{A}	24.84±0.24^{A}	15.51±0.42^{B}	12.67±1.25^{C}	15.14±0.24^{B}	10.64±0.50^{C}
AMMONIA (mg/L)	3.62±0.034^{A}	3.33±0.017^{B}	3.29±0.018^{B}	3.32±0.017^{B}	3.08±0.038^{C}	3.47±0.03^{A}	3.10±0.01^{B}	2.94±0.02^{C}	2.96±0.02^{C}	2.81±0.017^{A}	3.34±0.02^{A}	3.08±0.05^{B}	2.78±0.04^{C}	2.82±0.02^{C}	2.18±0.03^{D}
NITRATE (mg/L)	2.48±0.02^{A}	2.41±0.04^{AB}	2.34±0.011^{BC}	2.37±0.017^{BC}	2.29±0.036^{C}	2.43±0.02^{A}	2.35±0.017^{B}	2.31±0.017^{BC}	2.26±0.006^{D}	2.26±0.02^{D}	2.37±0.02^{A}	2.36±0.01^{A}	2.3±0.03^{AB}	2.26±0.01^{BC}	2.21±0.024^{C}
ORTHO -PHOSPHATE (mg/L)	2.77±0.055^{A}	2.51±0.024^{B}	2.35±0.017^{C}	2.38±0.023^{C}	2.15±0.017^{D}	2.56±0.013^{A}	2.28±0.04^{B}	2.14±0.017^{C}	2.13±0.024^{C}	1.87±0.02^{D}	2.49±0.03^{A}	2.16±0.006^{B}	1.96±0.02^{C}	1.89±0.02^{C}	1.72±0.01^{D}

All values are mean ± S.E. of mean

Mean with different letters in the same row are significantly ($p<0.05$) different among the particular day (Duncan's multiple range test)

Table 5. Removal efficiency of pollutants in terms of percent reduction in physiochemical parameters

Parameters	7 DAYS					14 DAYS					21 DAYS				
	Control	T1	T2	T3	T4	Control	T1	T2	T3	T4	Control	T1	T2	T3	T4
BOD	7.14	22.45	20.41	24.4	31.63	16.83	36.73	40.3	41.83	58.16	24.49	44.38	53.06	50.51	65.81
COD	9.85	22.9	27.37	23.72	36.49	16.78	32.48	38.32	35.4	48.9	23.72	40.148	47.81	44.16	58.02
ALKALINITY	13.11	19.96	26.67	27.27	32.93	22.2	31.44	33.97	37.85	45.3	27.42	34.27	39.79	42.32	53.65
CHLORIDE	28.069	54.93	65.14	71.87	79.77	47.11	67.14	74.97	74.08	83.86	49.87	72.77	78.28	78.49	88.32
HARDNESS	8.87	15.6	21.4	20.1	27.41	24.28	36.03	37.07	43.08	55.35	30.54	40.73	42.55	45.95	56.91
CALCIUM	14.79	32.3	41.8	36.06	50.37	25.67	43.82	53.26	43.81	63.36	31.92	57.59	65.97	59.9	71.56
MAGNESIUM	17.91	37.05	48.24	41.36	58.56	24.84	43.1	52.21	43.72	62.93	31.57	57.27	65.09	58.29	70.68
AMMONIA	6.21	13.81	14.59	13.98	20.12	9.93	19.6	23.66	23.14	27.2	13.29	20.03	27.97	26.94	43.34
NITRATE	4.72	7.28	10.1	8.82	12.02	6.53	9.4	11.28	12.81	13.07	7.92	8.15	10.48	11.88	13.52
ORTHOPH -OSPHATE	4.15	13.26	18.57	17.64	25.49	11.18	20.87	25.71	26.18	35.17	13.72	25.02	32.17	34.48	40.37

While the consortium shows the removal efficiency upto 65%. COD reduction was upto 50% via pure culture of *Exiguobacterium indicum* strain J and consortium shows the maximum reduction upto 58% as shown in table 5. Similar results were obtained for the calcium, magnesium, and ammonia content. While pure culture of *Bacillus altitudinis* strain Q was effective reducing alkalinity, chloride and orthophosphate content. On an average removal efficiency follows the order: T4>T2>T3>T1>Control. Most of the biodegradation occurs during first 14 days later the speed of biodegradation slows down due to the limited availability of organic matter.

DISCUSSION

Discharge of untreated sewage due to the lack of sewage treatment infrastructure may lead to detrimental effects to the aquatic ecosystem. Sewage water often has a high content of BOD, COD, and other pollutants that marks the presence of pathogenic microorganism [7]. In addition to these, sewage water harbor numerous bacteria that has inherent capacity to degrade the pollutants [5]. Thus, in the above context, present study was performed to isolate bioremediating bacteria from sewage water. Initially total 19 bacterial strains were isolated and out of these 19 strains, three novel bacteria (*Bacillus cereus* strain D*, Exiguobacterium indicum* strain J and *Bacillus altitudinis* strain Q) were isolated from the sewage water as they possess the protease producing capacity and show antibacterial activity against the pathogenic *E. coli.* Hence these were considered as autochthonous beneficial bacteria which can lower the number of pathogenic bacteria present in sewage water. Moreover when the data was compared with findings of Xiu and co worker [5,27], Sewage water comprises common pathogenic organisms belonging to genus *Acinetobacter, Aeromonas*, *Pseudomonas, Vibrio*, *Staphylococcus, Mycobacterium* and *Helicobacter* while the bacteria isolated in our study that showed antibacterial activity, were not under that list thus these finding further supports the beneficial character of isolated bacteria.These bacteria were used as inoculum in the form of pure culture and consortium to treat the sewage water in two different doses. Later the removal efficiency was calculated by comparing the physiochemical parameters before treatment and after the treatment. Along with that optimum time required for degradation was also observed on basis of the days required for maximum pollutant reduction.

In our investigation of bacterial treatment for 5 days and 21 days, it was observed that pH of the sewage water sample that undergone bacterial treatment was raised up to 8.26 this may be due to the biodegradation of nitrogen compounds which solubilize into ammonia. BOD reduction followed the trend: Consortium > T3 ≥ T2 > T1 > Control and *Bacillus altitudinis* strain Q was observed as most efficient one while *Exiguobacterium indicum* strain J proved to be potential COD reducer. *Bacillus altitudinis* has also been studied for degradation of butachlor upto 80% (a herbicide) [13]. Some other strains of *Bacillus* such as *Bacillus flexus* have reduced the COD upto 81% from alkaline wastewater [8]. Reduction in BOD and COD content was also observed in control group, this may be due to the presence of biodegrading bacteria in sewage water. Likewise eco-friendly bioremediation of sewage water was also performed by Anusha and coworkers using *Bacillus cereus* strain SDN1 that have significantly reduced the calcium content, hardness, and nitrate content [1].

Bacillus sp. solubilize phosphate and utilize phosphate containing compounds as carbon source in synthetic phosphate containing medium [10] likewise Sunar and coworkers isolated *Bacillus altitudinis* from the soil which is potent phosphate biodegrading bacteria [23]. Similarly in our treatments, *B. altitudinis* strain Q utilized the phosphate content and reduced it upto 40%. Ammonia reduction was found to be highest (43%) in consortium treatment while the control group also shows the reduction up to 13%, this may be due to the presence of denitrifying bacteria in sewage water such as *Pseudomonas sp*. and *Bacillus sp.* in sewage water. Nitrate content reduction was very less in our treatments, this may be due to the presence of nitrifying bacteria in sewage water that oxidized ammonia into nitrate however *Bacillus cereus* is considered as heterotrophic denitrifying bacteria that degrade the nitrate from the water thus treatment T1B shows the decrease in nitrate content up to 8.5%. These findings were supported by Siripong and Rittmann who demonstrated that the Ammonia-oxidizing bacteria (AOB) belonging to the *Nitrosomonas europaea/eutropha*, *Nitrosomonas oligotropha*, *Nitrosomonas communis*, and *Nitrosospira* lineages from the wastewater plant often convert the ammonia into nitrate thus there may be rise in the nitrate content after treatment or there may be no reduction at all [21]. Mixed culture bacterial treatment often observed to be more effective in wastewater where diverse pollutants are present [19]. Larcher and Yageau reported the major reduction in sulfamethoxazole present in wastewater by the consortium of bacteria comprising the "*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Rhodococcus equi*, *Rhodococcus erythropolis*, *Rhodococcus rhodocrous*, and *Rhodococcus zopfii"* rather than pure culture [14]. Similarly in our study, among all the treatments significant reduction in pollution indicating parameters was shown by the consortium of the bacteria. As consortium comprises of the three different bacteria which works in synergistic approach and reduced the BOD, COD upto 65% and 58% respectively at OD_{600} of 0.5. The presented study is supported by the results of Sonune and Garode [22]. They isolated bacterial isolates *Bacillus licheniformis NW16, Pseudomonas sp. NW9, Paenibacillus borealis NS3 and Aeromonas hydrophilia NS17* from municipal wastewater and

observed a reduction in BOD, COD, and phosphate content after treatment.

Acc to Li and coworker, the possible mechanism of biodegradation involves the four main process. First one is the production of biosurfactant by bacteria that emulsify the pollutants. These emulsified pollutants get absorbed on the cell surface of microorganism in second step [15]. Later this absorbed complex is internalized and finally undergo enzymatic degradation involving the various enzymes. Ligninolytic laccase enzymes were isolated from the bacteria that catalyzed the mono-electronic oxidation of a substrate and involved the degradation of wide range industrial pollutants [7].

The current study revealed that the indigenous sewage water bacteria have effective pollutant removal efficiency from sewage water at bacterial dose comprising 4 x 10^8 CFU/mL (OD_{600} of 0.5) and this may be the highest by the consortium of bacteria. Further the pace of biodegradation slows down after the 14 days of inoculation. This may be due to the less amount of organic material left in the sewage water. These findings were also supported by experiments of Anusha and colleague in which show significant reduction using *B. cereus* after 15 days of treatment [1].

The findings reported in this study are the results of laboratory scale experiments, as the use of bacterial strains on a commercial scale necessitates further large-scale experiments. There is also a need to further analyze the favourable conditions in order to extract their maximum potential to overcome the shortcomings. Further experiments are needed to identify a potential carrier/medium for these bacterial strains to transport in order to sustain their survival.

Acknowledgement: SK is thankful to the Council of Scientific and Industrial Research, New Delhi, India for support in the form of Junior Research Fellowship.

Conflict of interest: There is no actual or potential conflict of interest in relation to this article.

Funding: The necessary funding was in the form of a Junior Research Fellowship (JRF) to one of the authors (Sapna Kalra) provided by the Council of Science and Industrial Research (CSIR)

REFERENCES

[1] Anusha, P., Ragavendran, C., Kamaraj, C., Sangeetha, K., Thesai, A.S., Natarajan, D., Malafaia, G., (2023): Eco-friendly bioremediation of pollutants from contaminated sewage wastewater using special reference bacterial strain of *Bacillus cereus* SDN1 and their genotoxicological assessment in *Allium cepa.* Science of The Total Environment, 863: 160935.

[2] APHA, (2017): Standard methods for the examination of water and wastewater, 23rd edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington

[3] Ayyasamy, P.M., Banuregha, R., Vivekanandhan, G., Lakshmanaperumalsamy, P., (2002): Treatment of sago factory effluent by aerobic microbial consortium, 22(5): 554-558.

[4] Bergey, D.H., (1994): Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins, 1071 p.

[5] Bhatnagar, A., Kalra, S., (2022): Autochthonous antimicrobial microorganisms: application in wastewater treatment. Journal of Applied and Natural Science, 14(2): 660-676.

[6] Cavalini, L., Jankoski, P., Correa, A.P.F., Motta, A.S., (2021): Characterization of the antimicrobial activity produced by *Bacillus sp.* isolated from wetland sediment. Anais da Academia Brasileira de Ciências, 93: 1-13.

[7] Chandra, R., Chowdhary, P., (2015): Properties of bacterial laccases and their application in bioremediation of industrial wastes. Environmental Science: Processes & Impacts, 17(2): 326-342.

[8] Chen, K., Shi, W., Yang, J., Zhang, T.C., Zhao, H., (2014): Isolation and characterization of a *Bacillus* strain for alkaline wastewater treatment. In Proceedings of the 2012 International Conference on Applied Biotechnology (ICAB 2012), 2: 1267-1276.

[9] Chew, C.M., Aroua, M.K., Hussain, M.A., Ismail, W.M.Z.W., (2016): Evaluation of ultrafiltration and conventional water treatment systems for sustainable development: an industrial scale case study. Journal of Cleaner Production, 112: 3152-3163.

[10] El Bestawy, E., Ahmed, A.H., Amer, R., Kashmeri, R.A., (2014): Decontamination of domestic wastewater using suspended individual and mixed bacteria in batch system. Journal of Bioremediation & Biodegredation, 5(5): 1-8.

[11] Hesnawi, R., Dahmani, K., Al-Swayah, A., Mohamed, S., Mohammed, S.A., (2014): Biodegradation of municipal wastewater with local and commercial bacteria. Procedia Engineering, 70: 810-814.

[12] Hwang, C.F., Chen, Y.A., Luo, C., Chiang, W.D., (2016): Antioxidant and antibacterial activities of peptide fractions from flaxseed protein hydrolysed by protease from *Bacillus altitudinis* HK02. International Journal of Food Science & Technology, 51(3): 681-689.

[13] Kaur, R., Goyal, D., (2020): Biodegradation of butachlor by *Bacillus altitudinis* and identification of metabolites. Current Microbiology, 77: 2602-2612.

[14] Larcher, S., Yargeau, V., (2011): Biodegradation of sulfamethoxazole by individual and mixed bacteria. Applied Microbiology and Biotechnology, 91(1): 211-218.

[15] Li, X., Li, H., Qu, C., (2019): A review of the mechanism of microbial degradation of petroleum pollution. In IOP conference series: materials science and engineering, 484 (1): 012060.

[16] Masi, C., Gemechu, G., Tafesse, M., (2021): Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. Annals of Microbiology, 71(1): 1-11.

[17] Mishra, S., Lin, Z., Pang, S., Zhang, Y., Bhatt, P., Chen, S., (2021): Biosurfactant is a powerful tool for the bioremediation of heavy metals from contaminated soils. Journal of Hazardous Materials, 418: 126253.

[18] Qu, Y., Zhang, R., Ma, F., Zhou, J., Yan, B., (2011): Bioaugmentation with a novel alkali-tolerant *Pseudomonas* strain for phenol wastewater treatment in sequencing batchreactor. World Journal of Microbiology and Biotechnology, 27(8): 1919-1926.

[19] Safitri, R., Priadie, B., Hawadish, A., (2015): Domestic waste water bioremediation by consortium of bacteria. Lucrări Științifice-Universitatea de Științe Agricole și Medicină Veterinară, Seria Zootehnie, 63: 134-141.

[20] Scillato, M., Spitale, A., Mongelli, G., Privitera, G.F., Mangano, K., Cianci, A., Stefani, S., Santagati, M., (2021): Antimicrobial properties of *Lactobacillus*

cell-free supernatants against multidrug-resistant urogenital pathogens. Microbiologyopen, 10(2): 1173.

[21] Siripong, S., Rittmann, B.E., (2007): Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Research, 41(5): 1110-1120.

[22] Sonune, N.A., Garode, A.M., (2015): Bioremediation potential of bacterial isolates for municipal wastewater treatment. Current world environment, 10(2): 619-615.

[23] Sunar, K., Dey, P., Chakraborty, U., Chakraborty, B., (2015): Biocontrol efficacy and plant growth promoting activity of *Bacillus altitudinis* isolated from Darjeeling hills, India. Journal of Basic Microbiology, 55(1): 91-104.

[24] Sylvia, K., Kumar, A., Lalsiamliani, F., Nagotra, M. and Pandian, M., (2021): Photosynthetic Based Technology for The Bioremediation of Domestic Sewage Water using Anoxygenic Bacteria. Current Trends in Biotechnology and Pharmacy, 15(5): 373-378.

[25] Tuo, B.H., Yan, J.B., Fan, B.A., Yang, Z.H., Liu, J.Z., (2012): Biodegradation characteristics and bioaugmentation potential of quinoline-degrading strain of *Bacillus sp*. isolated from petroleum-contaminated soil. Bioresource Technology, 107: 55-60.

[26] Vargas-Ordóñez, A., Aguilar-Romero, I., Villaverde, J., Madrid, F. and Morillo, E., (2023): Isolation of novel bacterial strains *Pseudomonas extremaustralis* CSW01 and *Stutzerimonas stutzeri* CSW02 from sewage sludge for paracetamol biodegradation. Microorganisms, 11(1): 196.

[27] Xie, Y., Liu, X., Wei, H., Chen, X., Gong, N., Ahmad, S., Lee, T., Ismail, S. and Ni, S.Q., (2022): Insight into impact of sewage discharge on microbial dynamics and pathogenicity in river ecosystem. *Scientific Reports*, 12(1): 6894.

[28] Zhang, J.N., Gong, X.F., Ying, G.G., Wang, A.G., Dong, M.J., Liu, Y.S., Deng, T.C. and Xu, M.Y., (2024): Innovative bioremediation of dexamethasone in aquatic ecosystems using *Rhodococcus sp*. D32: Pathway discovery and reduction of ecotoxicity. Journal of Cleaner Production, p.141319.

[29] Zhang, Q.Q., Yang, G.F., Zhang, L., Zhang, Z.Z., Tian, G.M., Jin, R.C., (2017): Bioaugmentation as a useful strategy for performance enhancement in biological wastewater treatment undergoing different stresses: Application and mechanisms. Critical Reviews in Environmental Science and Technology, 47(19): 1877-1899.

Received: January 13, 2024
Accepted: April 19, 2024
Published Online: April 22, 2024
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House