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RESEARCH ARTICLE OPEN ACCESS

Isolation, Characterization of *B. subtilis* from Song River Shore and their Application to Wastewater Treatment

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Abstract

Treatment of wastewater has been a hotspot of research since ages. Emerging technologies and methodologies have been postulated to resolve the issue worldwide. Almost 97.2% of our earth is covered with water bodies, out of which 12,500 water bodies are situated in India. As per the statistics, nearly 70% water bodies are polluted in Southern Asia. Presence of emerging pollutants exacerbate the quality of flowing water. Amongst all possible ways, microbial bioremediation has been considered one of the most thriving methods to treat wastewater. This research will manifest about the isolation of *B. subtilis* from soil followed by its characterisation and action in treatment of wastewater which was collected from industry. From the study, it was concluded that *B. subtilis* holds the potential of degradation. Significant decrease in values of BOD and COD were achieved.

Keywords: Song River, Microbial Bioremediation, Emerging Pollutants, Soil, *B. subtilis*

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INTRODUCTION

Water is an asset of ecosystem and necessity of industries. In India, almost 60% of the commercial market direct their waste generated into water bodies. Industrial effluents form the majority of contaminants in water bodies. The unregulated disposal of toxins in water severely affects the water quality and the ecosystem. Since past three decades, it has been reported that about 40 million litres of water are polluted and has to be treated. Liberation of heavy metals into water bodies increases the amount of toxicity, thus lowering the pH of water.[1] This iniquitous act sometimes also leads to the acidification of water bodies known as 'ocean acidification'. severely affects the marine life and the livelihood of population surviving on it.[2] In the past various physical and chemical ways were adopted either to regulate the run offs or treat water. The success rate of these methods was moderate as they too generate gaugeable amount of waste such as releasing chemicals and antiquated filters. Scientists performed groundwork to formulate such method of wastewater treatment which aims towards 'zero waste' generation. Bioremediation is a novel technique of wastewater treatment in which bacteria breakdown waste into water bodies. Bio-remediation was discovered in 1972 first to treat the spilled oil in oceans.[3] It was observed that certain group of microorganisms degrade the oil with their catabolic activities.[4] Microbial bioremediation is preferred means of removing impurities of heavy metals and lowering the toxicity of water bodies. The degradation of water bodies brought on by organic pollution was not a significant issue until the last 220 years or so since a comparatively smaller homo sapiens populace inhabited dispersed villages and garbage discharge to streams could be handled by authentic self-characteristics.[5-6] The fate of contemporary world has shifted towards the poles of degradation in water availability and quality of drinking water.[7] The reason behind this situation is increase in population, industrialization and dwelling of modern lifestyle. Disposal of effluents by industries and mismanagement of sewage waste are the two major issues that hamper the quality of drinking water.[8,9] All effluents can either be released into a stream without any treatment or only after the necessary treatment, regulations regarding sanitary conditions and pollution levels may differ.[10-11] Industries such as textile, fertilizer, chemical, agricultural etc. release harmful synthetic dyes and residues into the flowing bodies which severely affect the quality of drinking water.[12-13] Prior to disposal, the sewage needs get specific treatment otherwise, this contaminated water may also leach into the ground thus contaminating underground reservoirs of freshwater. These pollutants when consumed by organisms may biomagnify and cause diseases. Lead, mercury, palladium, manganese is some of the heavy metals that not only accumulate into the environment best has long lasting side effects on the population residing nearby. Organic persistent particles are contaminants that are soluble in water and has a marked effect on animals, plants and people thriving on it.[14-15] Organizations such as Environment Protection Committee has formulated the guidelines for industries dealing with carcinogenic and toxic effluents for safe disposal of their effluents.[16-17]

Other major issue on the cards is management of sewage water in India. Sewage, is a form of wastewater produced by a group of people. It is distinguished by its volume or flow rate, physical state, chemical and poisonous contents, and bacteriological status. Assessment of sewage generation volumes is required to guarantee proper collection, transportation, treatment, disposal, and reuse. With assistance from SPCBs, PCCs, and District bodies the CPCBs periodically assess the amounts of sewage generated and its treatment. Over the past few years India is enthusiastically working towards the management of wastewater by postulating more efficient techniques.[17,18]

The choice of a certain sewage treatment technique depends on the characteristics and features of the sewage as well as the nature and ability of the water body. Water must be treated biologically to eliminate the organic matter. However, compared to chemical and physical therapy options, biological treatment is more affordable. Treatment of wastewater via sewage treatment plant using chemical and physical methods is not that efficient method of treatment. Construction and design of sewage treatment plants require large amount of capital

as well as land. The maintenance cost of STPs is the following issue. Degradation of wastewater using this technique releases foul smell in the neighboring site. Various types of chemicals are used to disinfect the treated water. Such chemicals also act as secondary pollutants and dissolution of organic compounds is persistent in treated water.[19]

In order to overcome these problems, de novo technique of microbial degradation was introduced. Microbial degradation of wastewater refers to the degradation of organic and inorganic constituents of water. It is regarded as one of the most coherent methods for treatment of sewage water. Microbial degradation is a type of bioremediation in which complex substances are converted into simpler ones with the help of potent microorganisms. It can be carried out by different types of bacteria, fungi, algae etc. It encompasses the strategy of 'zero waste'. It is sought as an eco-friendly method of treatment. Bacterial degradation of wastewater accelerates the rate of decomposition. Effective biodegradation depends on the organic compounds in sewage being susceptible to biochemical oxidation in combination with the presence of certain microbes known as bio-oxidation agents. The organic pollutants found in sewage may require a certain type of microbe to be biodegraded. Effective sewage treatment helps prevent disease by preventing a number of conditions that may be contracted by exposure to microbes that may be existing in unprocessed garbage. Untreated sewage discharges have the potential to pollute drinking water supplies, recreational areas, and fish and shellfish fisheries in surface and ground waterways.[19,20]

Groundwater can get contaminated with germs by untreated sewage from traditional septic systems that fail or sewage that is dumped directly into the environment. Untreated sewage discharge into streams poses a health risk due to the potential for direct disease transmission, making such waters unsuitable for activities like swimming and boating that require direct contact with the water. Incidental contact, like coming into touch by mice, bugs which have been exposed first and are now harboring the pathogens, can also spread disease. Untreated sewage discharges have the potential to poison fisheries as well as harm the ability of receiving streams to maintain thriving aquatic life.[21,22]

Therefore, proper decomposition of wastewater in sewage is essential to inhibit the breeding of disease-causing agents such as mosquitoes and to prohibit leaching into groundwater.[23,24]

Microbial bioremediation deals with the efficiency of microorganism to break down complex water to simpler substances.[25,26] These microbes may or may not be already present at the site. Therefore, these can be added manually at the site to initiate biodegradation. The time taken by such bioremediation is more. So, to enhance the rate of degradation and save time engineered microbes are too being used. Engineered microbes are those microorganisms whose genome has been modified artificially in the laboratory to increase the efficiency of this process.[27,28] B. *subtilis*, also known as grass or hay bacillus is a rod shaped, heat-resistant bacteria found in soil and gastrointestinal tract of humans or ruminants.[29,30] It is one of the most efficient bacteria in treatment of wastewater.[31,32] *B. subtilis* is an aerobic decomposer which is known to degrade heavy metals and reduce toxicity of water.[33,34] In this article we shall witness the effect of *B. subtilis* on wastewater which was taken as a sample from an industry.[24-25]

MATERIALS AND METHODS

Collection of samples

Soil sample was collected in a UV sterilized poly bag with proper labelling from bank of the Song River which is situated in Dehradun, Uttarakhand in India. The eastern and central portions of Doon Valley are drained by it. River Song flows through the Ladwakot forest of Dehradun, which springs from a natural spring on the southern slopes of the Mussoorie Range. Both banks of the Song River are fertile since it runs mostly through central and eastern Dehradun. It is a branch of the revered Ganga.

Total viable count

Collected soil sample was weighed 10 gram and suspended in 90 ml of distilled water. Four soil suspensions were prepared as sample

Table 1. Morphology Characteristics of *B. subtilis*

Isolate No.	Colony Characteristics colour	Cell Features Gram's Staining	Test Organism Identification
SRB1	*White, Flat & Tender opaque*	*+Ve, rod*	*Bacillus Spies*

for the research. First sample was autoclaved for 15 mins at 121 psi. The second sample was heat shocked and placed in water bath at 60°C for one hour. The process of autoclaving and heat shock was followed to supress the growth of non-spore forming organisms, indirectly allowing only bacillus to grow. Out of that each sample seven serial dilutions (namely: 10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8},10^{-9} X2) were prepared. 100 microlitre of dilution was spread efficiently on nutrient agar plates followed by an incubation of 24-48 hours at 37°C.post incubation time.[8,9]

Characterization of B*. subtilis*

The colony morphology (Colour, texture and from) done by the microscopy observation and IMVIC and Triple Sugar Iron Agar tests and Motility test were used to further biochemically characterize bacterial isolates. To distinguish between positive and "false positive" responses, appropriate positive and negative controls were utilized.[11,12]

Molecular Screening 16S rRNA

To determine the proportion pattern resemblances, DNA isolated from the Song River isolates and16S rRNA-F and 16S rRNA primer used for the 16S rRNA gene sequence analysis. This Screening was done in the outside Lab. After receiving the Sequence that sequence compared by the NCBI GenBank data base and use BLAST local searching alignment tool.[1-10] The Tamura-Nei model and the Maximum Likelihood approach were used to infer the evolutionary history. The tree (-2103.06) with the highest log probability is displayed. By automatically applying the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Tamura-Nei model, and then choosing the topology with the highest log likelihood value, the initial tree(s) for the heuristic search were created. There were 11 nucleotide sequences in this investigation. Codon positions 1st+2nd+3rd+Noncoding were included. The final dataset had 1519 locations altogether.

Potential Isolate Searching

Determination of COD, BOD and pH was performed on wastewater sample and after mixing the bacterial culture and wastewater in different dilution (900:100,700:300,500:500) and all diluted sample incubated in different intervals (24, 48, 72 Hour). Measured the BOD using three days by

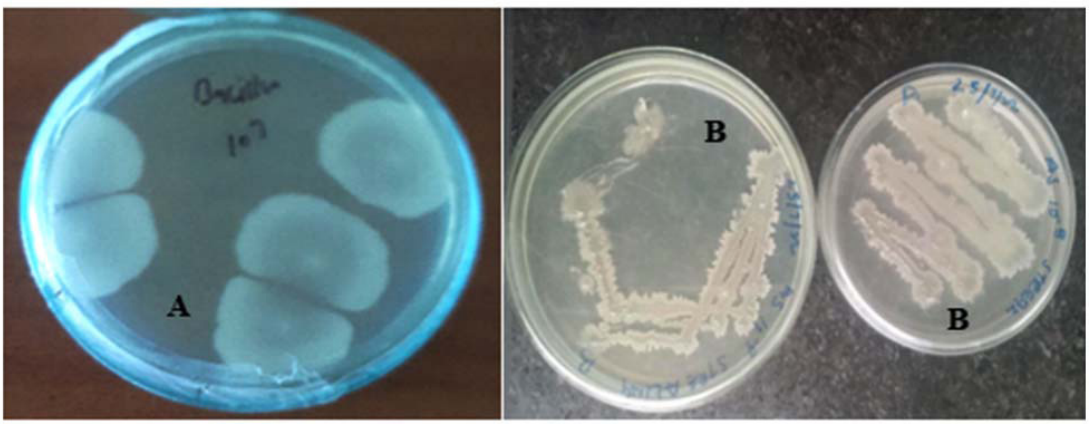

Figure 1. (a) Colonies of B. *subtilis* (b) Streak Plate of B. *subtilis*

Table 2. Biochemical test of *B. subtilis*

Test Isolates	Indole Test	Methyl Red Test	Voges Test	Citrate Proskaur	Catalase Test	TSI Test Test
1. Bacillus Spies.	-ve	-ve	+ve	+ve	+ve	A/A With Gas

the BOD incubator, COD measured by the COD digestion and dichromate titration method and measuring pH value with a pH meter.[6-7]

RESULTS

Total viable count

After the 72 hr of incubation of plate we obtain 5 x 10^7 colonies on the nutrient agar plate. And the NA plate shows White to creamy, Flat Soft and tender Opaque colonies. (Figure 1 and Table 1)

Characterization of B. *subtilis*

As per the microscopy (Gram staining) observation bacterial isolate was purple in colour (Figure 2) and bacterial isolates shows negative test for indole and in the MR, Test indicates negative test while presence of red coloration shows positive test for VP. In the citrate utilization the bacteria show positive result and changing the colour of media. The catalase test is showing bubbles on the slide means this test is positive and Urease, TSI test are negative for *B. subtilis* (Table 2).

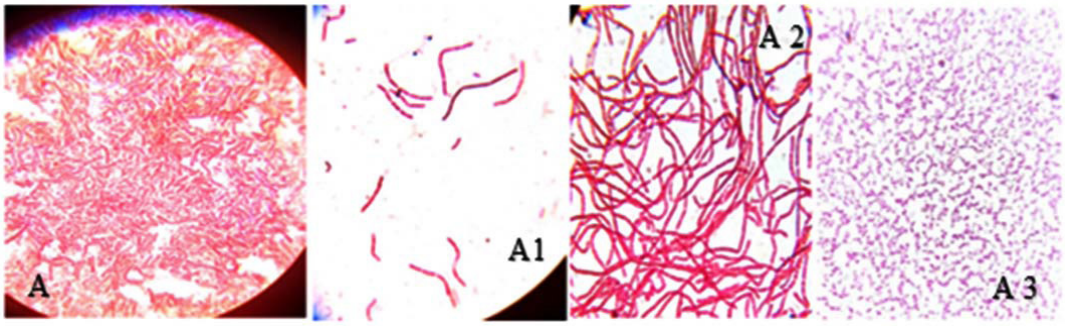

Figure 2. Gram staining to analyze the characteristic of *B. subtilis*

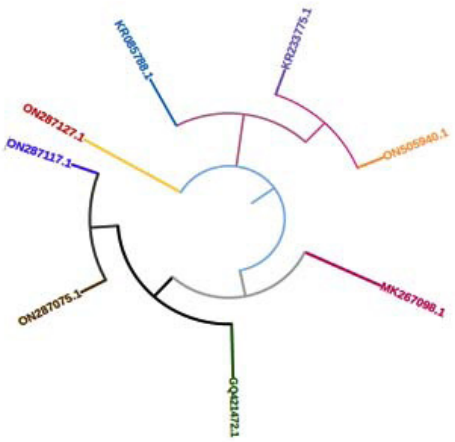

Figure 3. Phylogenetic tree of the strain screened from soil

Molecular Screening 16S rRNA

A nucleotide base sequence analysis using BLAST found that *B. subtilis* was identical to 98.80% to 100% (Table 3). Phylogenetic relationships of 16S rRNA gene sequences and 1500 bp was observed when resolved on agarose gel (Figure 3 and Figure 4). And distance matrix showed the isolated *B. subtilis* and Other *B. subtilis* strain distance (Table 4).

Forward Seq data

TGTTAGGGGGTTTCCGCCCCTTAGT
TGCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGGTCGCAAGACTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACAT

CCTCTGACAATCCTAGAGATAGGACGTCC
CCTTCGGGGGCAGAGTGACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGC
ACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGACAGAA
CAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCC
ACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC
TCGACTGCGTGAAGCTGGAA TCGCTAGTAACGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGTCGGTGAGGTAACCTTTTAGGAG
CCAGCCGCCGAAGGTGGGACAGATGATTGGGG
TGAAGTCGTA ACAAAGGGTAACCGAAGA

Reverse Seq Data

GCTTAGTGCGTTAGCTGCAGCACTA
AGGGGCGGAAACCCCCTAACACTTAGCACTC
ATCGTTTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCA
GTTACAGACCAGAGAGTCGCCTTCGCCACT
GGTGTTCCTCCACATCTCTACGCATTTCACCG
CTACACGTGGAATTCCACTCTCCTCTTCTG
CACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCG
GTTGAGCCGGGGGCTTTCACATCAGACTTAAGA
AACCGCCTGCGAGCCCTTTACGCCCAATAATTC
CGGACAACGCTTGCCACCTACGTATTACCGCGGC
TGCTGGCACGTAGTTAGCCGTGGCTTTCTGGT
TAGGTACCGTCAAGGTACCGCCCTATTCGAACG
GTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCC
GAAAACCTTCATCACTCACGCGGCGTTGCTCCGT
CAGACTTTCGTCCATTGCGGAAGATTCCCTA
CTGCTGCCTCCCGTAGGAGTCTGGGCCGTG
TCTCAGTCCCAGTGTGGCCGATCACCCTCTCA
GGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTT
ACCTCACCAACTAGCTAATGCGCCGCGGGTCCATC
TGTAAGTGGTAGCCGAAGCCACCTTTTATGTT
TGAACCATGCGGTTCAAACAACCATCCGGTATT

Table 3. Sequences producing significant alignments of *B. subtilis*

Description	Max Score	Total Score	Query Cover	E value	Per. Identity	Accession
Bacillus subtilis strain IAM 12118	2802	2802	100 %	0.0	100.00 %	MK267098.1
Bacillus subtilis strain NOK4	2798	2798	99 %	0.0	100.00 %	ON287117.1
Bacillus subtilis strain NOK42	2798	2798	99 %	0.0	100.00 %	ON287075.1
Bacillus subtilis strain BA20	2789	2789	100 %	0.0	99.87 %	OP547499.1
Bacillus subtilis strain L4	2785	2785	99 %	0.0	99.87 %	GQ421472.1
Bacillus subtilis strain IHB B 10201	2784	2784	99 %	0.0	99.87 %	KR233775.1
Bacillus tequilensis strain IHBB 9348	2784	2784	99 %	0.0	99.87 %	KR085788.1
Bacillus subtilis strain BAO8	2784	2784	100 %	0.0	99.80 %	OP547498.1
Bacillus subtilis strain ATULGPJ_O1E	2784	2784	99 %	0.0	99.80 %	ON505940.1
Bacillus subtilis strain NOK33	2784	2784	99 %	0.0	100.00 %	ON287127.1

Table 4. Distance Matrix of *B. subtilis*

Bacillus_subtilis		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000
MK267098.1	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000
ON287117.1	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000
ON287075.1	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.001	0.001	0.000
OP547499.1	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.001	0.001	0.000
GQ421472.1	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.001	0.001	0.000
KR233775.1	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.001	0.001	0.000
KR085788.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.001	0.001	0.000
OP547498.1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		0.001	0.001
ON505940.1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		0.001
ON287127.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	

AGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGG
CAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAA
CATCAGGGAGCAAGCTCCCATCTGTCCGCT
CGACTTGCATGTATTAGGCACGCCGCCAGCGT
TCGTCCTGAGCCAGGATCAAACTC TAAAG

Reverse complement

CTTTAGAGTTTGATCCTGGCTCAGGACG
AACGCTGGCGGCGTGCCTAATACATGCAAGT
CGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCTGTAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACCGGATGGTTGTTTGAACCGCA
TGGTTCAAACATAAAAGGTGGCTTCGGCTACC
ACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAAC GGCTCACCAAGGCAACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAG
GGAAGAACAAGTACCGTTCGAATAGGGCGGTAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
GCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG
GGAACTTGAGTGCAGAAGAGGAGAGTGGA

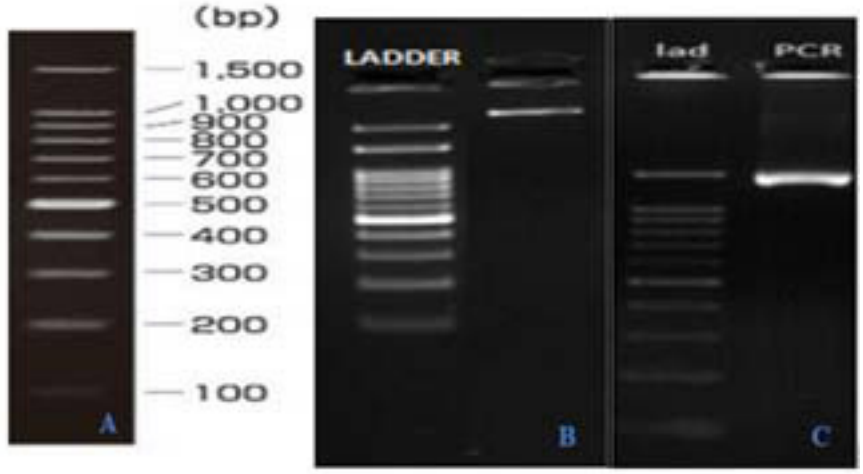

Figure 4. (a) Marker (b) gDNA (C) 16SrRNA amplicon of *B. subtilis*

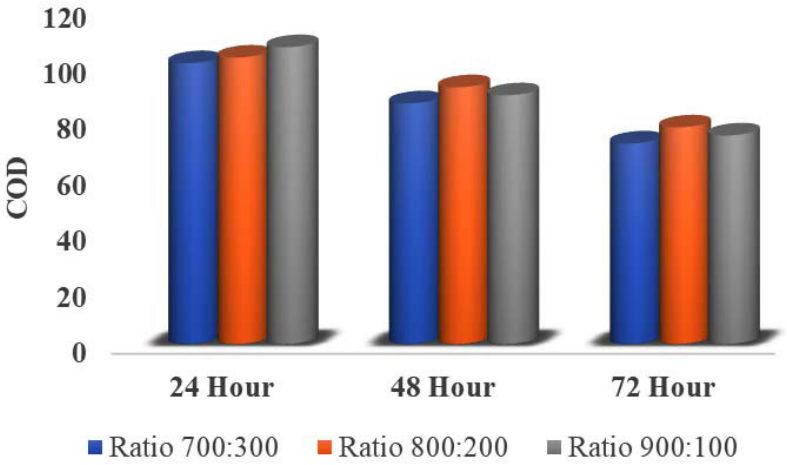

Figure 5. Using *B. subtilis* in a 700:300, 800:200, 900:100 concentration reduces COD of wastewater

ATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACT\GACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAG
TGCTGCAGCTAACGCACTAAGC

Consensus data

TTAGAGTTTGATCCTGGCTCAGGACGAAC
GCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
GGACAGATGGGAGCTTGCTCCCTGATGT
TAGCGGCGGACGG GTGAGTAACACGTGGGTAA
CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACCGGATGGTTGTTTGAACCGC
ATGGTTCAAACATAAAAGGTGGCTTCGGCTAC
CACTTACAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCAACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGG CAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGTTTTCGGATCGTAAAGCTC
TGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG

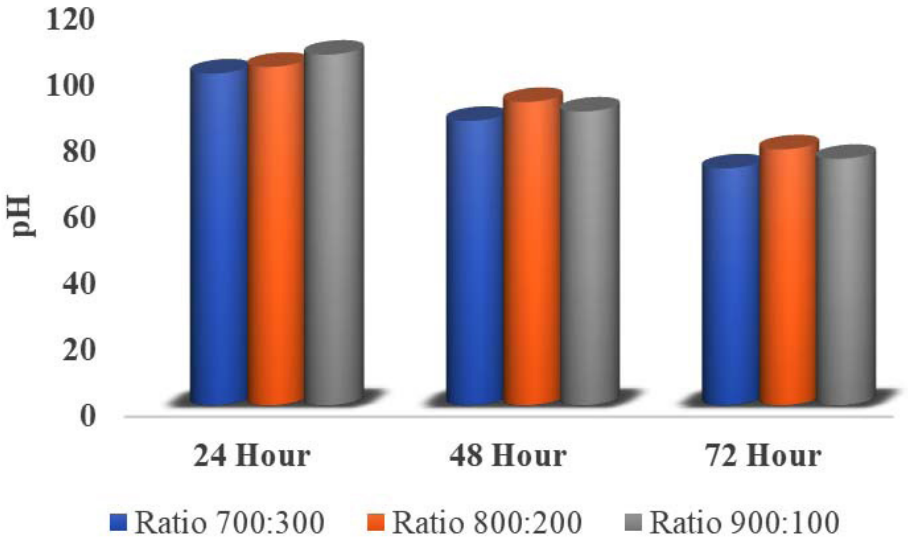

Figure 6. Using *B. subtilis* in a 700:300, 800:200, 900:100 concentration reduces pH

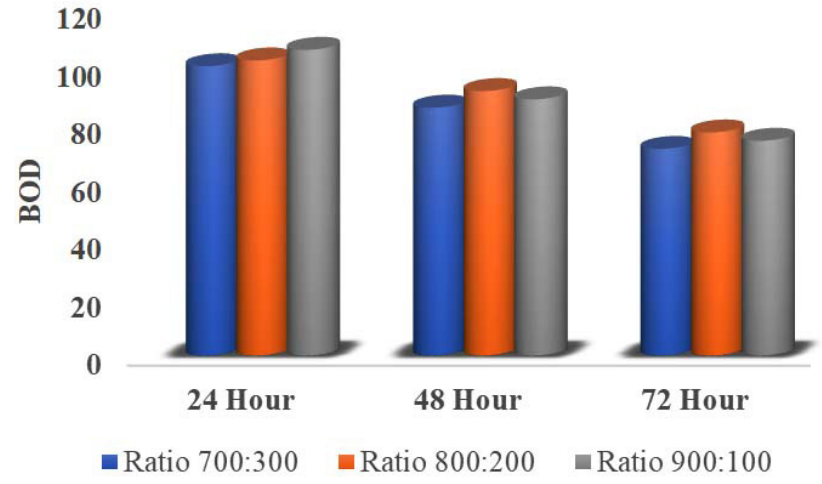

Figure 7. Using *B. subtilis* in a 700:300, 800:200, 900:100 concentration reduces BOD

GCGGTACCTTGACGGTACCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGC
GTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGAT GTGGAGGAACACCAGTGGCGAAGGCGA
CTCTCTGGTCTGTAACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCG GTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACAATCCTAGAGAT
AGGACGTCCCCTTCGGGGGCAGAGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGATCTTAGTTGCCAG
CATTCAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACAGAACAA
AGGGCAGCGAAACCGCGAGGTTAAGCC
AATCCCACAAATCTGTTCTCAGTTCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGCTGGAAC
GCTAGTAATCGCGGATCAGCATGC
CGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTT
TGTAACACCCGAAGTCGGTGAGGTAACCTTTTA
GGAGCCAGCCGCCGAAGGTGGGACAGA
TGATTGGGGTGAAGTCGTAACAAGGGTAA CCGA

Potential Isolate Searching

The COD, BOD, and pH values of wastewater are 352.18 mg/l, 125.12 mg/l and 8.96. Then microbes degrade the values of different concentrations in the ratio of 700:300 in 24hr is 40.29, 48hr is 35.26, and 72hr is 29.56, in 800:200 ratio in 24hr is 55.54, 48hr is 42.12, and 72hr 27.25, in 900:100 ratio in 24hr is 60.23, 48hr is 52.12, and 72hr is 32.56 in BOD. Concentrations in the ratio of 700:300 in 24hr is 100, 48hr is 85.71, and 72hr is 71.42, in 800:200 ratio in 24hr is 102, 48hr is 91.42, and 72hr 77.14, in 900:100 ratio in 24hr is 105.7, 48hr is 88.75, and 72hr is 74.28 in COD. Concentrations in the ratio of 700:300 in 24hr is 7.5, 48hr is 7.12, and 72hr is 6.9, in 800:200 ratio in 24hr is 7.46, 48hr is 7.2, and 72hr 6.88, in 900:100 ratio in 24hr is 7.98, 48hr is 7.39, and 72hr is 7.12 in pH (Figure 5, Figure 6 and Figure 7).

DISCUSSION

The paramount objective behind the experiment was to treat sewage generated wastewater via microbial degradation. Since, B. *subtilis* is one the most abundantly found microbe, it was decided to introspect into its ability to degrade wastewater. For the purpose of isolation, soil sample from the bank of Song River situated in Dehradun was collected. Bacteria was isolated using basic techniques of microbiology such as dilution, pouring, spreading, streaking etc. In order to gauge the action potential for degradation by the bacteria.

During experimentation it was observed that the bacteria is an extremophile and could successfully withstand high temperature and pressure inside an autoclave. Isolation of this bacteria was performed several times with distinct techniques. Initially, sample was prepared by dissolving soil into distilled water using magnetic stirrer. In this method, the probability of isolation of B. subtilis was extremely low as the culture was highly mixed. For the next time, the soil solution was heated in a water bath for 60 mins at 60°C, but no significant result was obtained. For the final time, the sample was autoclaved for 15 mins at 15psi and hence nearly pure culture of bacteria was obtained. For further identification and screening, biochemical tests and 16S rRNA molecular sequencing confirmed the presence of B. *subtilis*.

Microbial degradation is a part of bioremediation which is a modern-day technique for remediation and treatment. It has been practiced since a decade in ocean and seas to treat spilled oil. The idea of application of this method in treatment for wastewater generated by sewages was inspired from there. In the research, the bacteria were inoculated and incubated for 72 hours in a specific media called BS media. The composition of this media was prepared in the form of broth for 1L. Thereafter, bacterial broth and water sample were into different

ratios of 700:300; 800:200; 900:100. These ratios were prepared to study the effect of microbial degradation. To measure the degradation potential of B. *subtilis*, parameters such as BOD and COD were performed.

BOD stands for the demand of oxygen made by the microbe to break down complex organic compounds into simpler ones. This process is temperature dependent i.e., degradation of organic waste only occurs in temperature range of operation of that bacterium. Aquatic life depends on a particular amount of dissolved oxygen in water bodies for its respiratory requirements. Aquatic life need oxygen to survive, but when organic matter is present in water body, aerobic microorganisms use the oxygen from the water to break down the organic material. Water contains molecular oxygen that is either a result of photosynthesis carried out by aquatic plants or air oxygen that has been dissolved. A body of water or water sample is more contaminated the higher the BOD is. The consequences of rising BOD are analogous to those of dissolved oxygen depletion. Aquatic life suffers when the BOD of a body of water considerably rises. The bacteria that break down organic waste considerably reduce the amount of oxygen that aquatic species need for respiration and metabolism. Fish and aquatic vegetation die as a result, completely upsetting the aquatic ecology. Even low oxygen creatures like shellfishes and carps are at risk when the oxygen content falls below 6 ppm. In this study, volumetric titration method was used to calculate the biochemical oxygen demand. Titrations were performed at regular time interval of 24 hours for three days. It was observed that for the first ratio, the slope of BOD decreased in a steady manner and rate of degradation was constant throughout. Hence a descent rate of degradation was achieved. For the second ratio, the rate of degradation increased post first 24 hours. The degradation potential of B. *subtilis* was better in the second concentration. In the third ratio, the initial rate of degradation for first 24 hours was slow. Post 72 hours a significant decrease in BOD was observed.

COD testing is used to estimate the amount of oxidation that will occur and the concentration of organic compounds in a water sample. COD testing is another method for figuring out how many inorganic compounds are present in a sample. For COD testing, a strong oxidizing agent is frequently employed. Organic material oxidizes in an acidic environment to produce CO2 and water. The amount of an oxidizing agent used during the test must be known so as to calculate Organic matter content or demand for oxygen. A larger proportion of oxidizable organic matter and, consequently, a lower concentration of DO is indicated by an elevated COD in water. Critical DO depletion brought on by organic pollution can result in the extinction of aquatic life. The volumetric titration technique was employed in this experiment to determine the chemical oxygen requirement. Titrations were performed at regular time interval of 24 hours for three days. It was observed that for the first ratio, the slope of COD decreased in a steady manner and rate of degradation was constant throughout. Hence a descent rate of degradation was achieved. For the second ratio, the rate of degradation was less as compared but post first 24 hours the value for COD was decreased. The degradation potential of B. *subtilis* was better in the second concentration. In the third ratio, the initial rate of degradation for first 24 hours was a bit slower than the prior ratios but post 72 hours a significant decrease in COD was observed.

CONCLUSION

The results achieved in this research emerged as a ray of hope to adopt microbial degradation of any wastewater. The significant decrease in the values of COD and BOD proved the strong ability of novel B. *subtilis* for the purpose of microbial degradation. This experiment can successfully be adopted to various sectors dealing in generation of wastewater. For the future perspective, it is an environment friendly approach of treatment with zero waste generation. Since the bacteria is available abundantly in the nature, the cost of this application will be comparatively less than conventional methods.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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