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SCREENING OF HEAVY METAL TOLERANT AND DIESEL DEGRADING INDIGENOUS BACTERIA FROM PICHAVARAM MANGROVE - ESTUARINE

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ABSTRACT

Mangroves are salt tolerant plants found in tropical and subtropical coastal areas that are impacted by high anthropogenic pollution. Hydrocarbons are the world's most widely used energy and fuel resource. The inevitable spillages have become a global problem in industrialized and developing countries, generated continuous research interest in this field. Heavy metals are liberated by a variety of industrial processes are present in the industrial effluents pollute the atmosphere, soils and aquatic ecosystems due to their mobility in natural ecosystem. These hydrocarbon and metal ion contaminations pose severe risks to human health and the environment. Biological approaches are being used for remediation of hydrocarbon pollution and heavy metals. The study is designed to screen the heavy metal tolerant and diesel degrading bacteria from Pichavaram Mangrove – Estuarine. The tidal water samples were collected, serially diluted and plated on to Nutrient agar plates to isolate indigenous bacteria. Screening for heavy metal tolerance bacteria was done by agar well diffusion method using varying concentrations of heavy metal salt solutions such as Cadmium sulphat**e,** Copper sulphate, Zinc sulphate, Mercuric chloride, Lead acetate, Potassium dichromate, Manganese sulphate and Magnesium sulphate. Atomic absorption spectroscopy was used for the quantification of lead accumulation. Testing the diesel degradation ability of the heavy metal tolerant bacteria was performed using 1% of diesel in Bushnell Haas broth with DCPIP as an indicator. The biomass produced and CO_2 evolved was estimated in the test and control. The functional groups in the test and control were determined by doing FT-IR and the potent isolate was identified to be *Bacillus subtilis* by phenotypic, biochemical and 16s rRNA analysis.

KEYWORDS: Hydrocarbons, Diesel, Heavy Metals, *Bacillus subtilis*, Bioremediation.

INTRODUCTION

Mangroves are salt tolerant plants found in tropical and subtropical coastal areas (Lugo and Snedaker, 1974). Pichavaram mangroves are located between the Vellar and Coleroon estuaries (latitude 11°23’ to 11°30’ N, longitude 79°45’ to 79°50' E) in Tamil Nadu; southeast India is impacted by anthropogenic pollution.[1] Hydrocarbons are the world’s most widely used primary energy and fuel resource. Although diesel is a commonly used fuel for vehicles and machines, it is recognized as a serious threat to ecosystems.[2] Industrialization of coastal regions has increased drastically over the last three decades.[3] The industrial effluents are being introduced into the nearby water bodies there by contaminating the water bodies.

Yim and Tam (1999)[4] indicated the negative effects various heavy metals (e.g., Pb, Cu, Zn, Cd, Cr, and Ni) to both mangrove vegetation and soil microbial activities. These metals can accumulate in mangrove plant tissues, and pose damaging effects in the food chain through biomagnifications.[5] The metal ions and hydrocarbon contaminations pollute environment and cause severe risks to human health by their mutagenic, carcinogenic or toxic effects.

The conventional physicochemical techniques may not be very effective.[6] Bioremediation based on microorganisms, plants or other biological systems is a cost-effective and eco friendly method for hydrocarbon and metal clean-up.[6,7] Many species of microorganisms including bacteria, yeast and fungi obtain energy from hydrocarbons. The indigenous organisms that have adapted to the new environments[8] to withstand high concentrations of heavy metals by a variety of mechanisms through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration.[9] The aim of the study is to isolate the heavy metal tolerant and diesel degrading bacteria from Pichavaram Mangrove – Estuarine.

MATERIALS AND METHODS

Collection of sample: Tidal water samples were collected from the Pichavaram Mangrove using a sterile container and transported to the laboratory in an ice box.

Isolation of Bacteria

For the isolation of Bacteria, the samples were serially diluted and plated on nutrient agar medium and incubated at 37^{o} C for 24 hours

Screening for Heavy metal resistance

Heavy Metals

Screening for resistance to heavy metal salt solutions were carried out using Cadmium sulphat**e,** Copper sulphate, Zinc sulphate, Mercuric chloride, Lead acetate, Potassium dichromate, Manganese sulphate and Magnesium sulphate. The concentration of the heavy metal salt solutions was ranged from 10 mM, 50 mM, 100 mM, 500 mM and 1000 mM. The salt solutions were prepared with phosphate buffer saline, PBS (pH 6.8). The salt solutions were sterilized separately at 110^{o} C for 15 mins.

Agar well diffusion method

The isolates were grown for 24 hours in nutrient broth. Using a sterile wells borer, wells were made on the surface of Nutrient agar seeded with the isolates. To each well 100µl of the metal salt solutions were added and incubated at 37^{o}C for 24 hours. The area of inhibition (nm) was measured from the edge of growing colonies to the edge of the well.[10]

Heavy metal analysis in the sample by Atomic absorption spectrophotometer

The experimental analysis of heavy metals concentration in the water sample before and after treatment, were analyzed by using atomic absorption spectrophotometer (Perkin Elmer Spectra AA 3100).

Sample preparation: Sample was filtered by using filter paper (Whatmann No.5) to remove any suspended materials in the sample. Supernatant was refiltered by using membrane filters (Millipore 0.45 µm). Hydrogen ions concentration was modified by using (1 M NaOH) to get an ideal pH for bacterial cultivation. Samples were stored at 4^{o} C until needed.

Biological treatment of water

The experiments were carried out to test the bioaccumulation of heavy metals by living cell of bacteria in batch systems accordingly.

1. 50 ml of sample was dispensed in to 250 ml Erlenmeyer flasks, the flasks were then inoculated in triplicate with 1 ml of the isolate; the flasks were incubated at 37^{o} C, 250 rev/ min on shaking incubator for 2 weeks.

2. The flasks were examined at regular intervals for microbial growth, after incubation time, bacterial biomass from each flask was collected by filtration through Whatmann No. 1filter paper and washed with generous amount of deionized water. After that the filter paper was oven dried at 50^{o} C for 24 h to estimate the dry weight of the biomass.

3. The remaining filtrates in the flasks were analyzed by atomic absorption spectrophotometer (Perkin Elmer Spectra AA 3100) to determine the remaining heavy metal concentration.[11]

Diesel degradation

Diesel sample used in this experiment was purchased from a local oil filling station and stored in dark at ambient temperature throughout the study. The diesel was sterilized using 0.2µm membrane filter, stored at 4^{o} C in a pre- sterilized sample bottle following aseptic conditions.

Bushnell-Hass (BH) medium was used as the enrichment media with 1% (V/V) diesel as the sole carbon source. The culture was used as inoculums for the hydrocarbon degradation. DCPIP at a concentration of 20 mg/l of the medium was used. The medium was sterilized at 121^{o} C for 15 minutes. 1 ml of the inoculum was added and incubated at 150 rpm for 15 days. Control without the inoculum was also maintained.[12]

Diesel degradation was determined by the change in colour of DCPIP from blue to colourless. The indicator, when oxidized was blue and reduced was transparent. The reaction was observed visually till 15 days of incubation and also spectrophotometrically (600nm) at an interval of 4 days using Shimadzu model UV-Visible spectrophotometer.[13]

Study of diesel degradation

Volumetric and gravimetric estimation of CO_2 evolved during 4-week incubation was performed by doing Sturm test.[14]

Evolution of CO_2

Volumetric analysis

The dissolved carbon dioxide present in the medium was estimated by titration for volumetric analysis.[14] The broth was filtered to remove bacterial mass and the diesel fuel, and then 25 ml of filtrate was taken in a conical flask to which 0.05 ml of 0.1 N thiosulphate solution was added. After the addition of 2 drops of methyl orange indicator, solution was titrated against

0.02 M sodium hydroxide solution. End point appeared as a change in color from orange red to yellow. After this, two drops of phenolphthalein indicator were added and titration was continued until pink color was observed. Volumes of the titrant used were noted and the amount of CO_2 evolved was calculated using the formula:

$$CO_2 = \frac{A \times B \times 50 \times 1000}{V}$$

Where A = volume of NaOH titrant in ml, B = normality of NaOH, and V = volume of sample in ml.

Gravimetric method

For the gravimetric analysis, the flasks were taken out and the bacterial activities were stopped by adding 1% 1N-HCL. For extraction of diesel oil, 50ml of culture broth was mixed with 50ml Petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layers. Acetone was then added to it and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, diesel oil and acetone; clumping cells make a middle layer and the bottom aqueous layer contains acetone, water and biosurfactant in soluble form. The lower two layers were spread out while top layer containing petroleum ether mixed with diesel oil and acetone was taken in a preweighed clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tared beaker.[15,16]

The percentage of diesel oil degraded was calculated as follows

% diesel degraded = (weight of diesel oil degraded/ original weight of diesel introduced) X 100

where the weight of diesel oil degraded was determined as the weight of diesel oil plus flask minus the original weight of the flask.

Biomass determination on diesel fuel hydrocarbons: Bacterial biomass was determined by filtering the culture broth through Whatmann No. 1 filter paper. Recovered biomass samples were weighed and dried in pre-weighed aluminum foil at 60^{o} C to a constant weight and the dry mass was obtained by subtraction. Three replicate flasks were maintained for the bacterial isolate. The flask with inoculum but without diesel fuel substrate was maintained as control.

Gain in biomass under treatment and the control was recorded; and difference between gain in treatment and control was considered to be due to biodegradation activity of the bacteria.

Analysis of the degraded diesel by Fourier transmission infra-red spectroscopy (FTIR)

FTIR was used to detect the functional group of a compound. Perkin Elmer, UK make, paragon 500 model FTIR was used for the analysis. The spectrum was taken in the mid IR region of 400-4000 cm^{-1} with 16-scan speed. The samples were mixed with spectroscopically pure chloroform in the ratio of 1:100 and pellets were fixed in the sample holder and the analyses were carried out.

Identification of heavy metal tolerant and diesel degrading bacterial isolate

The most potent heavy metal tolerant and diesel degrading bacterial isolate was identified by observing morphological characters, by doing several basic biochemical tests and by 16s rRNA analysis.[17] Different types of biochemical tests were done such as Gram's staining, Motility, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Catalase test, Oxidase test and H_2S production test (HIMEDIA). The bacteria isolated were identified based on physical characterization and the biochemical test outlined in Bergey's manual of determination bacteriology.[18]

RESULTS

The isolates were screened for the heavy metal tolerance against heavy metal salts by agar well diffusion method. Out of four different isolates screened, the orange colour pigmented isolate showed resistance to lead acetate, magnesium sulphate and manganese sulphate and sensitive to zinc sulphate, mercuric chloride and potassium dichromate (Figure 1).

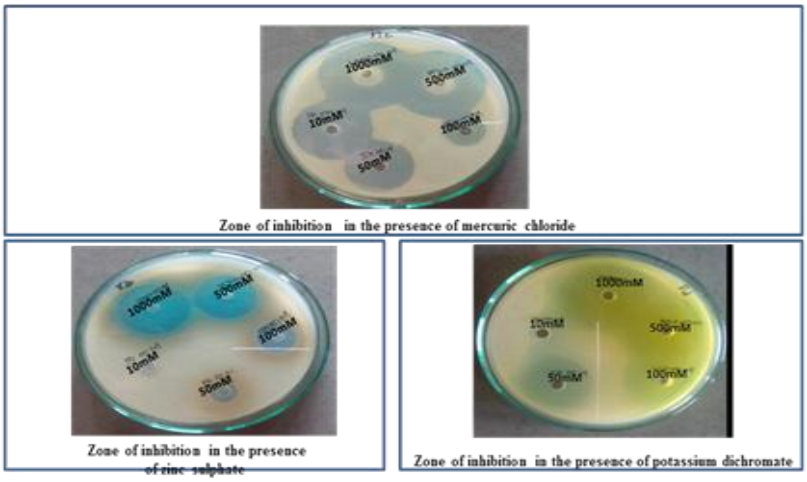


Figure. 1: Screening for heavy metal resistance by agar diffusion method.

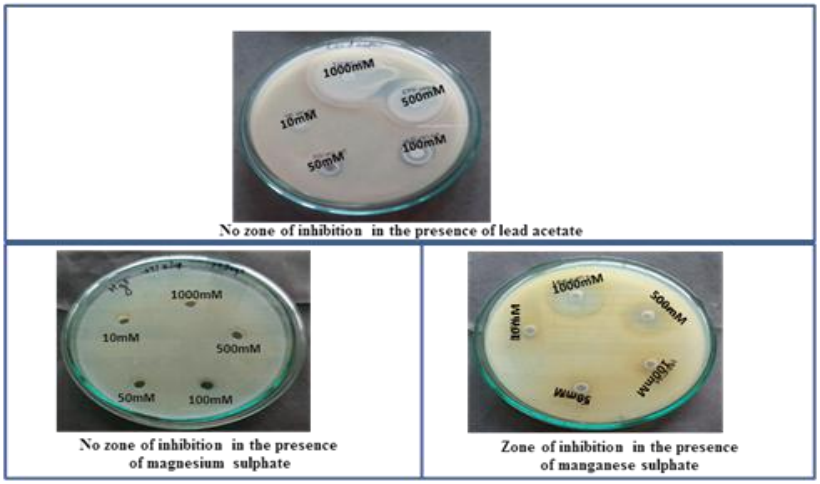


Figure. 2: Atomic Absorption Spectroscopy analysis of water sample.

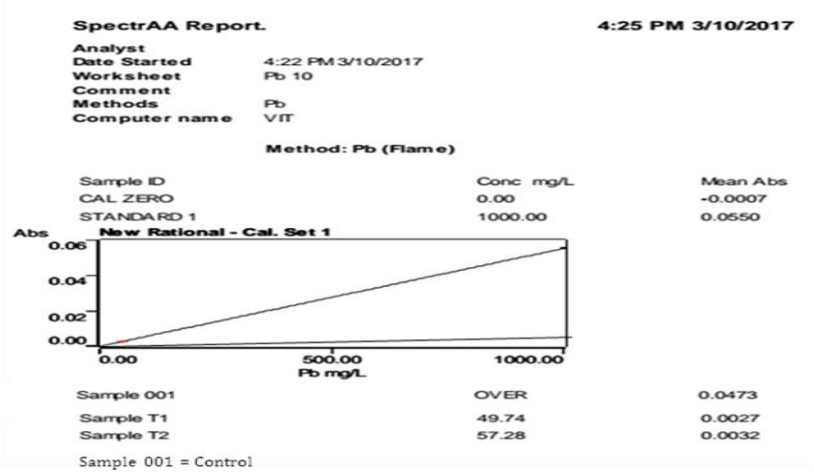


Table. 1: Dry Weight accumulation by bacterial isolates during the water analysis.

S. No	Sample	Dry weight of culture (g/100ml)
1.	Water with culture	1.007
2.	Only water (control)	0.104

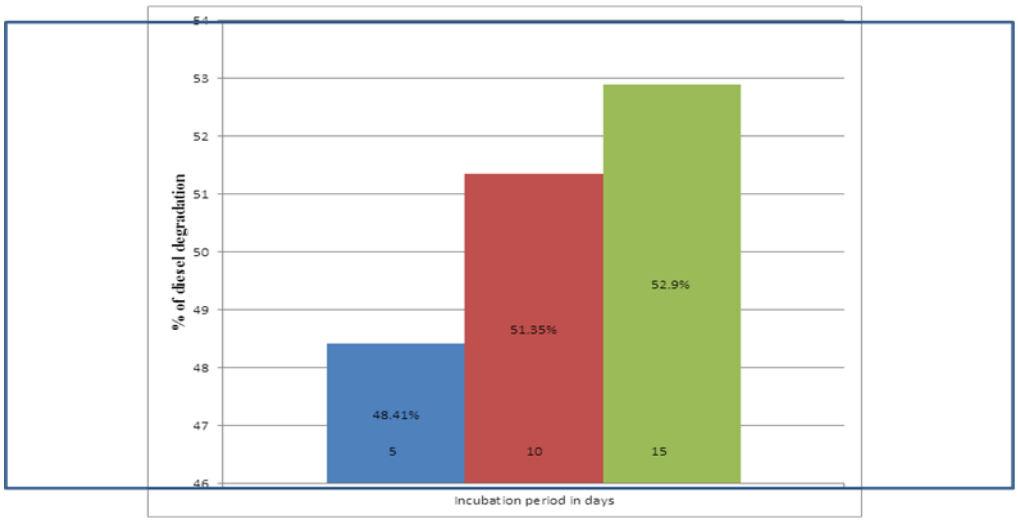
The dry weight accumulation of bacterial isolate during the water analysis was found to be increased in the water inoculated with the culture (Table 1).

Table. 2: Dry weight accumulation by the bacterial isolate during the cultivation with diesel.

Controls (g/l)	Treatments (g/l)	Gain via Biodegradation Weight (g/l)
0.107	0.665	0.561

During the degradation of diesel, the bacterial inocula grew within a day and showed greater accumulation of biomass. The gain of dry weight of the isolate was found to be 0.561g/l (Table 2).

Figure. 3: Degradation of diesel by heavy metal resistant isolate.



Graph. 1: Percentage of diesel degradation at different days of incubation against 1% diesel.

Table. 3: Volumetric analysis of CO_2 evolution.

Controls (g/l)	Treatments (g/l)	Gain via Biodegradation Weight (g/l)	% of CO_2 evolution
0.447	0.770	0.323	72

The diesel biodegradability of the isolate was noticed at the fourth day itself by the change in the colour of the indicator, from blue to colourless. This is due to the reduction of the indicator by the oxidized product of the hydrocarbon degradation, which supports the facts

that the isolate is potential hydrocarbon oxidizers (Figure 3). The percentage of diesel degradation of 15 days was calculated to be 52.9% (Graph 1).

The CO_2 evolution test are used to study the assimilation of polymeric carbon;[19, 20, 21, 22] and liberation of carbon dioxide during the degradation of petroleum hydrocarbons can be used as a reliable indication of the microbial activity in the medium.[23] The evolution of CO_2 taken by the volumetric method showed 72% of CO_2 emission in the biodegraded diesel sample after 15 days of incubation (Table 3).

In fig. 4, the IR spectroscopy of the control (diesel in Bushnell Haas media) showed the characteristics bands at **543** cm-1, **993** cm^{-1} and **1390** cm^{-1} (C-O stretch); **1463** cm^{-1} (C=C aromatic nuclei), **1639** cm-1 (C=C stretch), **2092** cm^{-1} (disubtituted benzene). **2355** cm^{-1} , **3431** cm^{-1}, **3466** cm^{-1}, **3504** cm^{-1} and **3566** cm^{-1} (N-H stretch). The spectrum has the various CH stretch bands that all hydrocarbons show near 3000 cm^{-1}.

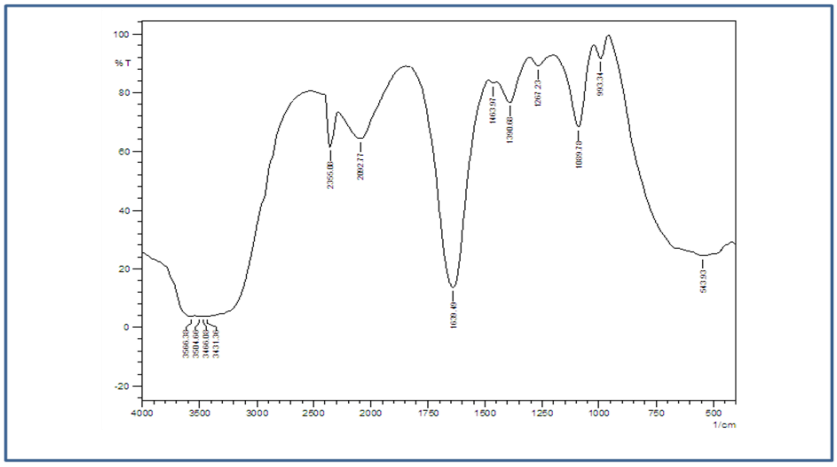


Figure. 4: FTIR analysis of culture media containing 1% diesel (Control).

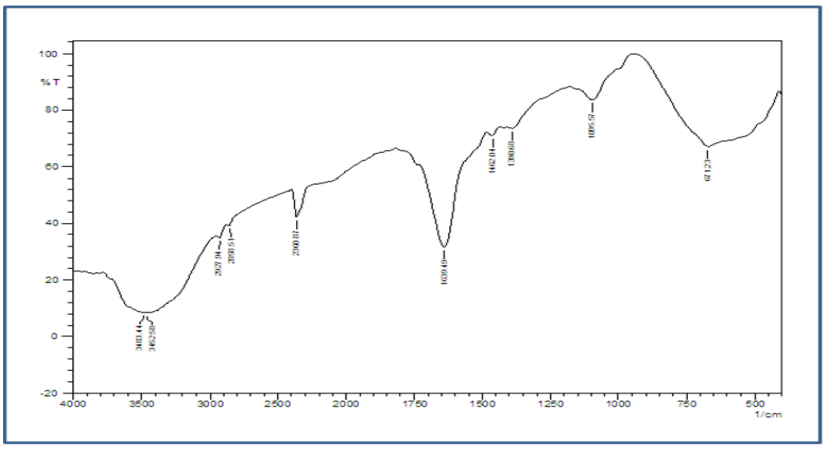


Figure. 5: FTIR analysis of culture media containing 1% diesel and culture (Test).

In fig. 5, the IR spectroscopy of the test (diesel and the isolate in Bushnell Haas media) showed the characteristics bands at **671** cm-1 **(=C – H bending; cis - disubstituted alkenes and aromatic)**, **1095** cm^{-1} (C-H aliphatic stretch); **1390** cm^{-1} (C-O stretch); **1462** cm^{-1} (C=C aromatic nuclei), **2360** cm^{-1} (meta disubtituted benzene) , **2858** cm^{-1} (C-H stretch), **2927** cm^{-1} (C-H aliphatic stretch), **3452** cm^{-1} and **3483** cm^{-1} (N-H stretch). Besides, it was noticed that the transmittance at 1463 **cm^{-1}** and 1639 cm^{-1} **(C=C aromatic nuclei)** were higher in control.

Figure. 6: Morphological and biochemical test results of the isolate.

Alignments

Bacillus subtilis strain 30C1-2 16S ribosomal RNA gene, partial sequence
Sequence ID: **JN366786.1** Length: 1457 Number of Matches: 1
Range 1: 33 to 738

Score	Expect	Identities	Gaps	Strand	Frame
1110 bits(601)	0.0()	689/727(95%)	24/727(3%)	Plus/Plus	

Features:

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Sbjct  33   GCTTGCTCCCT-GTAT-GTTA-GCGGCGGAC-GGGTGAG-TA-ACACGTGGGTAACCTGC  86
Query  62   CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGC  121

Sbjct  87   CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGC  146
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Sbjct  207  GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA  266
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Query  721  CCGTGGG  727
Sbjct  733  C-GTGGG  738
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Figure. 7: 16s rRNA analysis of the isolate.

The diesel degrading bacteria was identified to be *Bacillus subtilis* based on the morphological, biochemical characteristics and 16s rRNA analysis (Figure 6 & 7).

CONCLUSION

The present study is an evident that the *Bacillus subtilis* showed tolerance to the lead also degrades diesel. The heavy metal tolerance is due to the high peptidoglycan and teichoic acid in their cell walls. This isolate is a promising candidate for biosorption which can be used for bioremediation and detoxification of lead and diesel. Genetic improvement of the isolate may help to develop the field of existing methodologies to decontamination processes.

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