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Bacterial assisted treatment of anaerobically digested distillery wastewaterSankaran K^a, Lakshmi Pisharody^a, Suriya Narayanan G^a and Premalatha M^{a*}*^aAlgal Biotechnology Laboratory, Dept. of Energy & Environment, National Institute of Technology, Tiruchirappalli 620 015, Tamil Nadu, India*

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Abstract

The present study focused on the treatment of anaerobically digested distillery wastewater (ADSW) by bacterial method as the high COD (30-35,000 ppm), BOD (8-10,000 ppm), total solids (60-65,000 ppm) and the presence of organic compounds even after anaerobic treatment of the wastewater indicated that the conventional treatment methods were far from reaching the safe limits of disposal. ADSW has organic and inorganic compounds which need to be degraded. This study was done with culture rich in *Pseudomonas sp.* which was more effective and would bring further treatment process easier. The consortia of bacterial culture was taken from DEE soil and ADSW contaminated soil and the bacteria were grown in Kings B media to enrich *Pseudomonas sp.* The group of bacterial system was resulted in the reduction of organic pollution load (COD) of ADSW up to 26.05% for ADSW contaminated soil bacteria and 18.15% for DEE soil bacteria. Conventional immobilization of bacteria in sodium alginate also practiced to find the reduction level of pollution in ADSW and compared with free bacterial treatment. This bacterial system can further be combined with other aerobic treatment for enhanced COD reduction.

Keywords

Bioremediation; Microbial growth; Culture development; Batch Processing; Wastewater Treatment; Improved ADSW characteristics.

1. Introduction

Central Pollution Control Board (CPCB) is listed distilleries among top 17 most polluting industries of India (www.cpcb.nic.in). According to the Environment (Protection) rules 1986, CPCB prescribed safe disposal limits for distilleries (Table 1). Distillery wastewater (Spent wash) treatment is being carried out generally by three routes in industry. (a) concentration followed by incineration (b) direct oxidation by air at high temperature followed by aerobic treatment (c) anaerobic digestion with biogas recovery followed by aerobic polishing (www.environmental-expert.com). Out of three routes, anaerobic digestion is mostly followed in South Indian Distilleries because of economy and environmental factors. However, the characteristics of treated wastewater through the route require further treatment to reach safe disposal limit (Table 2). Physical/chemical methods are costlier and are less employed in industries. In recent years, wastewater treatment using biological systems have attracted the attention of researchers everywhere and have helped in the development of efficient low cost wastewater treatment system¹. Many have been utilizing biological processes for removing organic compounds from effluent. Microorganisms such as fungi^{2,3}, yeast^{4,5}, algae⁶ and bacteria have been tried for degradation and decolorization of distillery effluent. Various bacterial groups such as *Lactobacillus plantarum*⁷; *Bacillus licheniformis*, *Bacillus sp.* and *Alcaligenes sp.*⁸, *Klebsiella oxytoca*, *Serratia mercerscens* and *Citrobacter sp.*⁹, *Pseudomonas aeruginosa PAOI*, *Stenotrophomonas maltophila*, and *Proteus mirabilis*¹⁰, *Pseudomonas putida*, *Aeromonas sp.* are reported as potential organism to treat distillery wastewater¹¹. This approach can remove most of the biologically removable organics, CODs, and color^{12,13,14,15}.

Identification and optimization of biotechnological treatment methods is a necessity of the present time¹⁶.

Many articles deal only with treatment of distillery wastewater with dilution or artificial creation of wastewater for obtaining better treatment efficiency or success of the experiments. However, real wastewater characteristics largely differ from the experimental one. In our study, real anaerobically digested distillery wastewater (ADSW) directly taken for the experiments with very minimum deviation from original characteristics. The results showed in this work are very close to real time treatment of distillery wastewater and help as input for scaling up of operation. We are experimented and compared ADSW treatment with culture reach in *Pseudomonas sp.* isolated from ADSW contaminated soil and from DEE soil. Conventional immobilization also performed for finding closeness/deviation of results with free bacterial treatment.

2. Materials & Methods

2.1. Soil Sample Collection

Soil from two different locations were collected for understanding the change in the potentiality of the bacteria to degrade the pollutants in the effluent as the source changes. The sources were: Soil near to Department of Energy & Environment and soil contaminated with ADSW around Trichy Distilleries & Chemicals Ltd., Tiruchirappalli.

2.2. Methodology for Isolation of Bacteria

2.2.1. Serial Dilution Method

Bacteria were isolated from the soil by serial dilution method. The technique used to make a single dilution was repeated sequentially using more and more dilute solutions as the "stock" solution. At each step, 1ml of the previous dilution was added to 9ml of distilled water. Each step results in a further 10-fold change in the concentration from the previous concentration. Serial

dilution was the cheapest and most common method used for isolation of microorganism. The water used for the dilution was autoclaved so as to sterilize the water completely. 1ml of solution from each of the test-tube should aseptically transferred using sterile pipettes into nutrient agar plates, all the processes were performed in a laminar air flow to maintain sterile environment. The Agar plates were made airtight by covering its sides by parafilm and it was incubated for 24hrs at room temperature so as to obtain colonies. Even slants of the same was made for future use which was stored at -20 °C. The colonies from these plates were picked by a sterile loop and were streaked on the plates having Kings B Agar media so as to promote the growth of *pseudomonas species* predominantly. The plates were incubated for 24hrs at 32°C in sterile environment.

2.2.2. Agar Plate preparation

Agar bacterial plates were prepared by pour plate method. Here we prepared 2 sets of Agar plates one having nutrient agar and the other a selective media for *Pseudomonas species* i.e Kings B media. The composition of both the media was given in Table 3 and Table 4 respectively.

The directions followed while preparing the plates for nutrient agar were as follows: Suspended 43.41 grams in 950 ml distilled water; Heated to boiling with frequent agitation to dissolve the medium completely; sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 45-50°C. Mixed well before dispensing. The plates to which the media was poured was completely sterile and the entire transferring activity was carried out in an aseptic environment in a laminar air flow to avoid cross contamination (Himedia technical data).

The procedure for preparation of the plate for Kings B Agar media was: Suspended 42.23 grams of dehydrated medium in 1000 mL distilled water containing 15 mL of glycerol; Heated to

boiling to dissolve the medium completely; Mixed well; Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically poured into sterile Petri plates (Himedia technical data).

2.2.3. Bacterial Cultivation

The colonies from the Agar plate having Kings B Agar media was picked and aseptically transferred to sterile Kings B broth. And the medium was incubated in an incubator at 32°C in order to grow the bacteria. The bacterial growth analysis was done using a spectrophotometer (Merck Spectroquant Pharo 300) at 600nm.

2.3. ADSW Treatment

The bacterial culture when at exponential phase was inoculated into the effluent and the treatment studies were done for 72hrs. The COD analysis was carried out for the initial and final treatment time. The COD was measured at 605 nm by dichromate method using Thermal digester (TR 420) and spectrophotometer (Merck Spectroquant Pharo 300) instruments¹⁷

$$\% \text{ Reduction in COD} = \frac{\text{COD of untreated effluent} - \text{COD of treated effluent}}{\text{COD of untreated effluent}} \times 100 \dots\dots\dots (1)$$

2.4. Bacterial Immobilization

Bacteria were immobilized using sodium alginate. 4mL of bacterial culture in Kings B media was dispersed into sterile autoclaved sodium alginate solution and thoroughly mixed using a magnetic stirrer at 600rpm. After homogenizing the solution, this sodium alginate solution was added in a drop wise manner into 0.1M CaCl₂ solution. The beads were kept suspended in CaCl₂ solution for 3hrs; after which the CaCl₂ solution was decanted and the beads were washed with distilled water. These beads further inoculated into the effluent for treatment of the effluent (ADSW)¹⁸.

3. Results & Discussion

3.1. Bacterial growth study

Bacteria were isolated from soil contaminated with ADSW. After serial dilution, the bacteria was allowed to grow on a common media (Nutrient Agar) for a period of 24hrs at room temperature as per microbiological methods^{19,20}. Grant & Holt²¹ picked the colonies from the common media Agar plates and streaked into a selective media Agar plates (Kings B) and was incubated for 24hrs at 32°C. Similar way the colonies were transferred to Kings B plate in our experiments. The colonies from the plate was picked further and inoculated into Kings B broth. 500mL of Kings B broth was prepared and autoclaved to make it completely sterile and the colonies were picked and inoculated into the media aseptically in a laminar air flow to carry out the bacterial growth analysis as done by Johnsen & Nielsen²². The analysis was done by measuring OD (600nm), bacteria wet weight & dry weight, substrate concentration at a regular interval of hour for 24hrs until it reached stationary phase. Fig. 1 (a, b &c) showed the bacterial growth. Fig. 1 (a) indicated that the increase in the absorbance value because of increase in cell density and reduction in the absorbance of substrate due to the substrate utilization by bacteria. Increase in bacterial wet, dry weight and reduction in substrate concentration showed in Fig. 1 (b) & (c) also the indication for bacterial growth.

3.2. Bacterial treatment of ADSW with ADSW contaminated soil born bacteria

3.2.1. Bacterial Inoculum Optimization

As the bacteria showed potential to degrade the pollutants in the effluent in the preliminary studies there is a need to optimize the amount of bacterial inoculum; which are both economical and give maximum degradation.

Gay *et al.*, (1996)²³ found the significance of inoculum concentration and pre-incubation temperature on *Listeria monocytogenes*. Through their approach we too took 2 sets of 6 conical flasks each containing same amount of ADSW (1000 mL) and the conical flasks were inoculated with 100, 120, 140, 160, 180, 200 mL of bacterial culture respectively. All the inoculated cultures were maintained at room temperature and 180 rpm in an incubator cum shaker. Measurements such as biomass (wet), chemical oxygen demand (COD) and total dissolved solids (TDS) were taken for every 3 hours. Measurements showed that the conical flask inoculated with 180 mL of bacterial culture was given maximum degradation in 72 hrs. (3 days) (Fig. 2, 3 & 4). (Average value was plotted in figure). There after again ADSW reduction was reduced a little and so no further concentration of bacterial inoculum was added. Fig.2 showed the increase in wet biomass value (g) with respect to time which indicated the bacterial growth on ADSW in the similar way experimented by Eroglu *et al.*, 2010²⁴ for measuring biomass accumulation as a function of growth time of *R. sphaeroides* under continuous illumination. In our study, inoculum concentration of 180 ml and 200 ml resulted maximum biomass value (grams) however, for 200 ml concentration the stationary phase was started at 69 hours whereas for 180 ml concentration it was started only in 72 hours. COD & TDS reduction were also maximum i.e. 21.29% & 26.00% respectively for the 72 hours treatment study with 180 ml concentration of bacterial inoculum (Fig. 3 & 4).

3.2.2. ADSW treatment study

The soil from the sight Trichy Distilleries & Chemicals Ltd. which was contaminated with the ADSW was collected and the bacteria were isolated by the same method of serial dilution followed by growing it in a common media then Kings B media. The bacterial colonies obtained from the plates were further picked and allowed to grow in Kings B broth. Optimized 180mL of bacterial culture of OD 0.69 was inoculated into the effluent (1 litre ADSW) and the treatment

study was carried out for 72 hours and the results were shown in Fig. 5 (a, b, c & d). Similarly one more trail was performed and the average value was plotted in the Fig. 5. Increase in biomass (wet & dry) with respect to treatment time were shown in Fig. 5a, indicating the growth of bacteria rich in *Pseudomonas sp.* on ADSW²⁴. Fig 5 b, c & d showing the reduction of COD, total carbon (TC) and TDS values before and after 3 days of treatment respectively. The treatment efficiency were COD, TC & TDS up to 26.05%, 21.50% and 26.18% respectively.

3.3. Bacterial treatment of ADSW with DEE soil born bacteria

The soil from Dept. of Energy & Environment was collected and serially diluted to isolate bacterial colonies. The sample was poured into agar plates and further transferred to Kings B agar media to get culture rich in *Pseudomonas sp.* in the same way performed for ADSW contaminated soil bacteria. To carry out treatment study, the colonies were transferred to Kings B broth. Optimized 180 ml culture was used as inoculum for the treatment of 1 litre of ADSW. The results were reported in Fig. 6 (a, b, c & d). Increase in biomass (wet & dry) with respect to treatment time were shown in Fig. 6a, indicating the growth of bacteria rich in *Pseudomonas sp.* isolated from DEE soil on ADSW²⁴. Fig 6 b, c & d showing the reduction of COD, total carbon (TC) and TDS values before and after 3 days of treatment respectively. The treatment efficiency were COD, TC & TDS up to 18.15%, 13.94% and 14.50% respectively.

3.4. ADSW treatment by Bacterial Immobilization

Conventional method of immobilization¹⁸ was also performed with sodium alginate for the purpose of finding deviations of parameters in the bacterial treatment of ADSW. The results were shown in Fig. 7 (a & b). It was observed that the reduction of COD and TDS were less than that of free bacterial treatment isolated from ADSW contaminated soil (19.87% & 23.15%). However, immobilization possesses the advantages of reusability of culture and separation

processes. It could be further improved by having optimum inoculum concentration in the immobilized cell and treatment repetition.

4. Conclusion

Bacterial culture rich in *Pseudomonas sp.* was effective in the treatment of ADSW with the pollution load COD 25-32,000 ppm. Effect of inoculum concentration showed that a minimum of 180 ml of mother culture requires for the treatment of 1 litre of ADSW. It also showed the ability of organism growth on ADSW for 3 days as lag phase is lengthy. The bacteria which are isolated from ADSW contaminated soil shown higher treatment efficiency of the parameters such as COD, TDS, Total carbon and wet and dry weight measurement of biomass while comparing the DEE soil bacteria. Further, if the experiment was repeated continuously, the pollution reduction of ADSW shown in the article could be achievable within a day as the bacteria wholly adapted for the treatment environment and hence the economy associated with the treatment will greatly be improved. Thus, the organic compounds of ADSW are appreciably treated by bacterial culture rich in *Pseudomonas sp.*

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Abbreviations

COD	-	Chemical Oxygen Demand
DEE	-	Department of Energy & Environment
TDS	-	Total Dissolved Solids

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Table 1. Safety disposal limits prescribed by CPCB for distilleries

S.No.	Parameter	Standard
1.	pH	5.5-9
2.	Total Suspended Solids	100 ppm
3.	B.O.D., 27 ⁰ C, 3 days Inland water (river, lakes, streams)	30 ppm
4.	B.O.D., 27 ⁰ C, 3 days Disposal on land for irrigation	100 ppm
5.	COD	250 ppm

Table 2. Characteristics of anaerobically digested distillery wastewater (ADSW)

S.No.	Parameter	Value
1.	pH	7.5-8.0
2.	Total Suspended Solids	1800-3,000 ppm
3.	BOD	8,000-10,000 ppm
4.	COD	25,000–32,000 ppm

Table 3. Composition of Nutrient Agar

Ingredients	g / L
Peptone	5.000
Sodium Chloride	5.000
Beef Extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Table 4. Composition of Kings B Agar media

Ingredients	g/ L
Proteose peptone	20.000
Dipotassium hydrogen phosphate	1.500
Magnesium sulphate. heptahydrate	1.500
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Fig. 1. (a) Graph showing the absorbance of bacterial growth and substrate with regard to time (b) showing the changes in the substrate concentration and bacterial wet weight with regard to time (c) showing the availability of substrate and changes in the dry weight of bacteria with regard to time.

Fig. 2. Bacterial growth with varying inoculum concentration during ADSW treatment

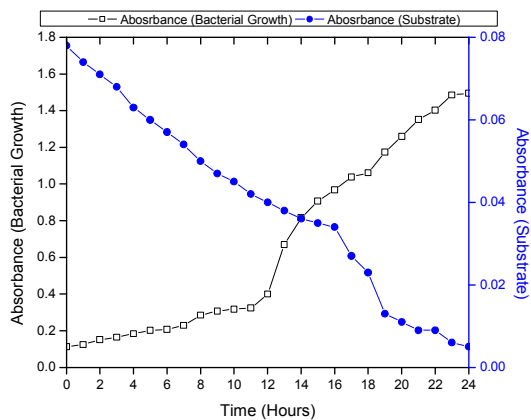
Fig. 3. COD Reduction in ADSW treatment with varying bacterial inoculum concentration

Fig. 4. Total Dissolved Solids (TDS) Reduction in ADSW treatment with varying bacterial inoculum concentration

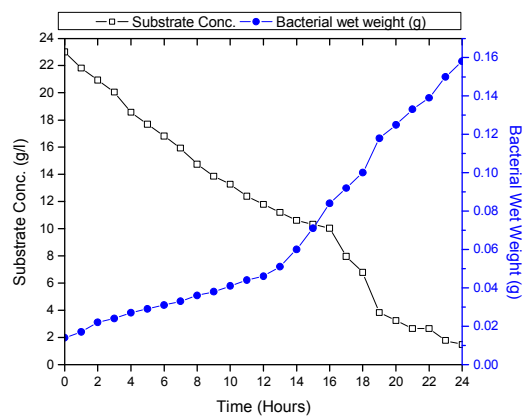
Fig. 5. (a) Bacterial biomass growth (Wet & dry weight) with regard to time during the treatment of ADSW (b) COD reduction due to reduce in organic load (reduction percentage – 26.05%) (c) Reduction in the total carbon (reduction percentage – 21.50%) (d) Reduction in total dissolved solids concentration during bacterial treatment (reduction percentage – 26.18%) – Source: ADSW contaminated soil

Fig. 6. (a) Bacterial biomass growth (Wet & dry weight) with regard to time during the treatment of ADSW (b) COD reduction due to reduce in organic load (reduction percentage – 18.15%) (c) Reduction in the total carbon (reduction percentage – 13.94%) (d) Reduction in total dissolved solids concentration during bacterial treatment (reduction percentage – 14.50%) – Source: DEE soil

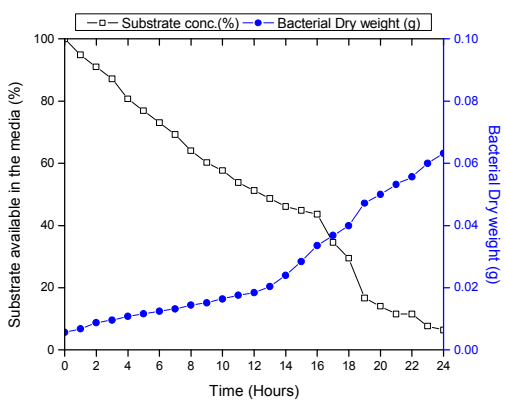
Fig. 7. Treatment of ADSW with bacterial culture rich in *Pseudomonas sp.* in immobilized form (a) COD reduction with respect to days (reduction percentage – 19.87%) (b) Total dissolved solids reduction with respect to days (reduction percentage – 23.15%)



(a)



(b)



(c)

Fig.1

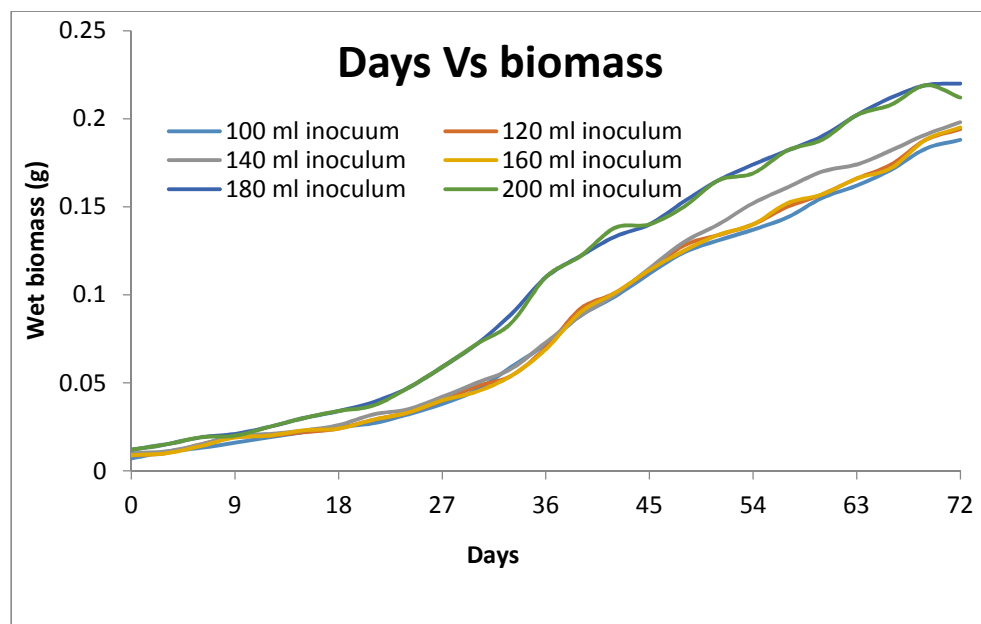


Fig.2

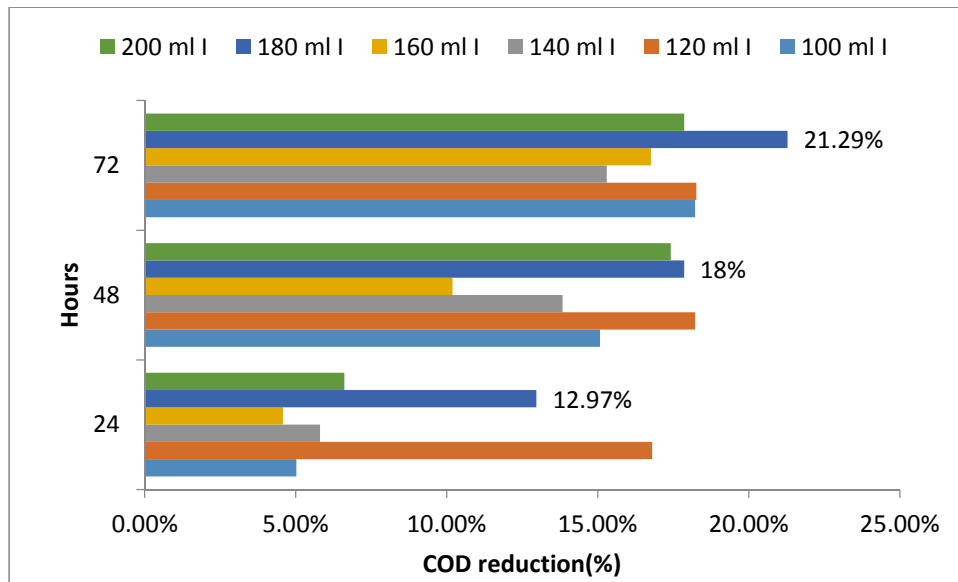


Fig. 3

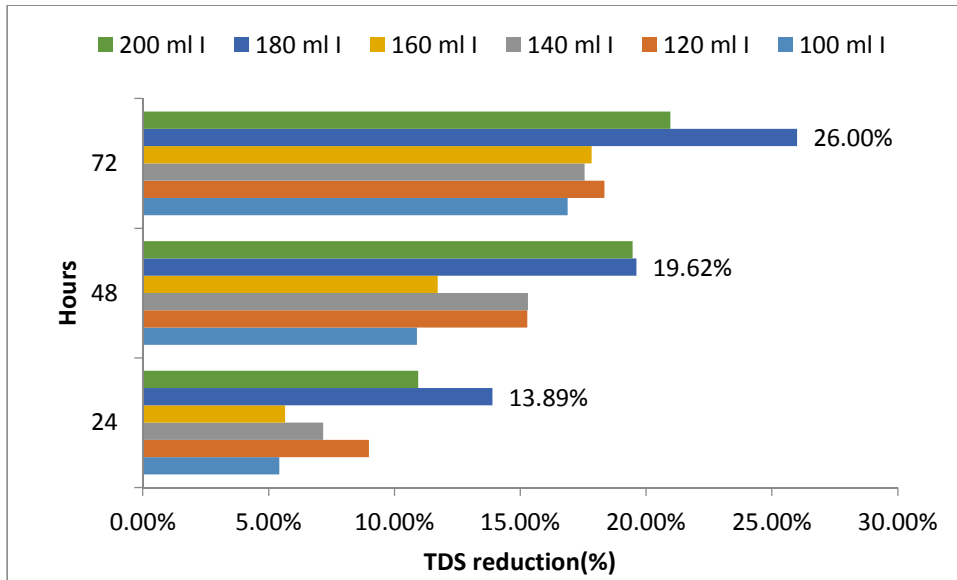
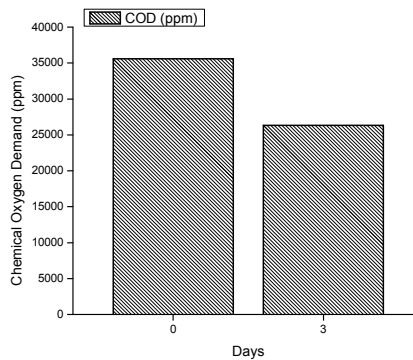
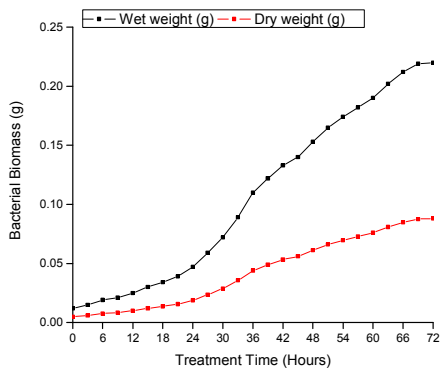


Fig.4



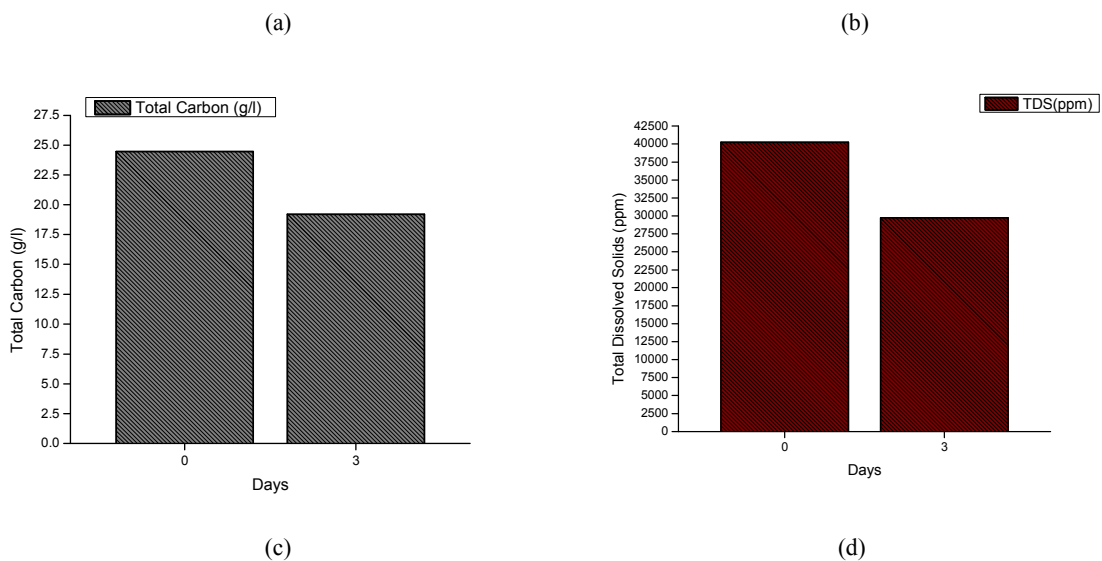
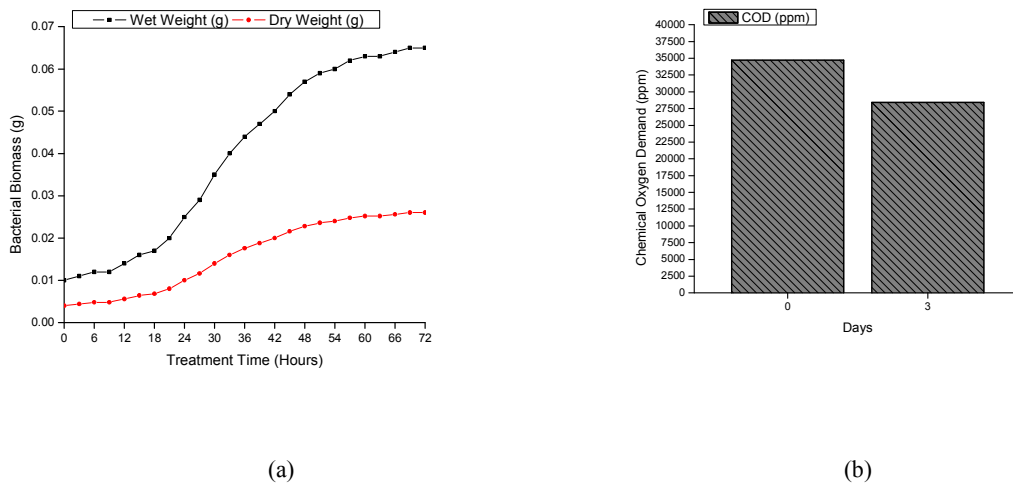
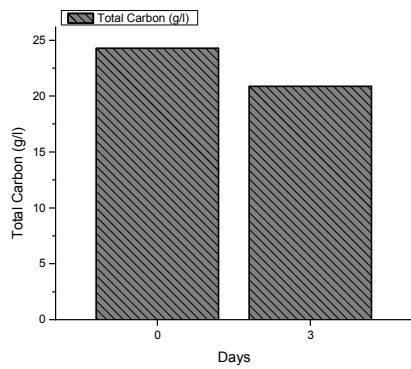
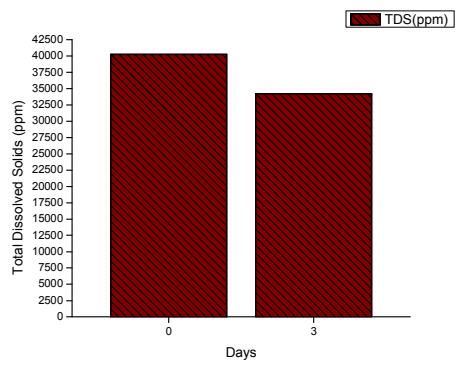


Fig. 5



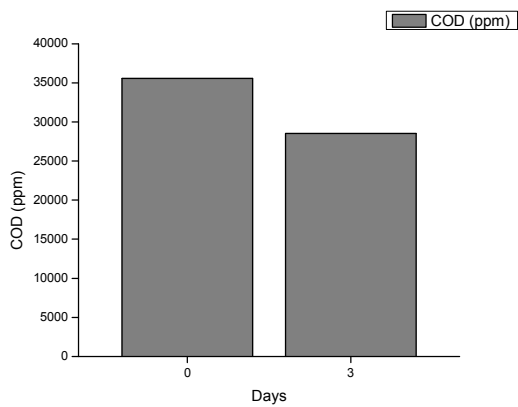


(c)

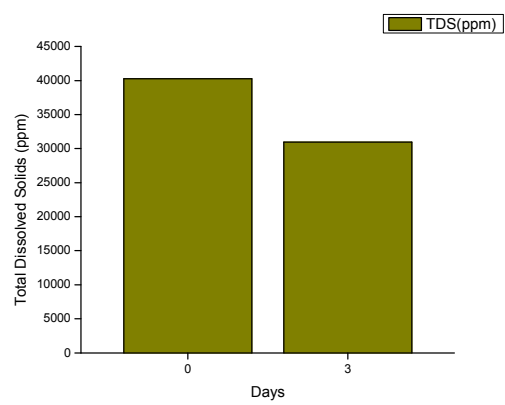


(d)

Fig. 6

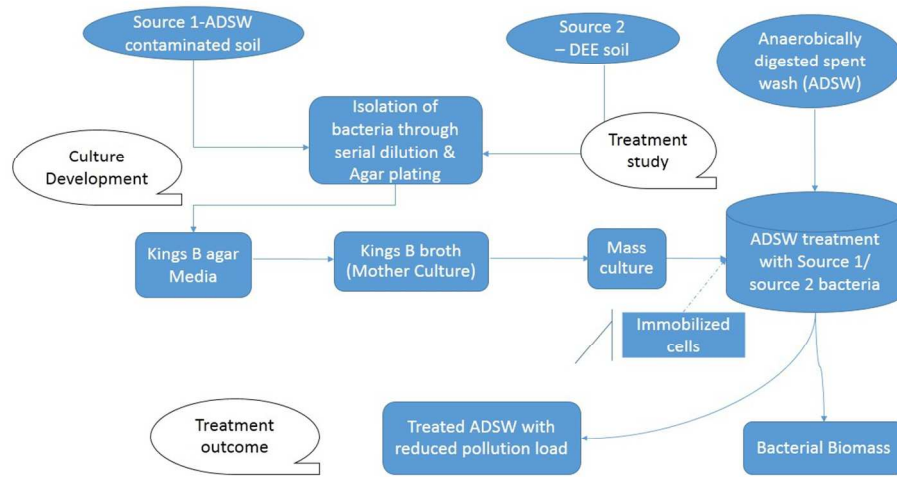


(a)



(b)

Fig. 7



338x190mm (96 x 96 DPI)