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Short Communication

Treatment of Saline Pharmaceutical Wastewater by a Moderately Halophilic Bacterial Consortium

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Abstract

Saline wastewater containing high salt concentrations and high organic and chemical demand remains a challenge for the microbial strains used for cleanup of pollutants. In this present study, an attempt has been made to investigate the treatment of saline pharmaceutical wastewater by the isolated moderately halophilic bacterial consortium under salt conditions. The results showed that the bacterial consortium was able to utilize the total phenols and reduce the BOD and COD under high salt conditions. Batch studies conducted with the diluted saline pharmaceutical wastewater showed a COD removal of 92%, BOD removal of 90% and total phenols were degraded up to 92% in the presence of the bacterial consortium.

Lab-scale reactor study for the treatment of raw saline pharmaceutical wastewater using the bacterial consortium was conducted. The study revealed that the bacterial consortium potentially reduced 89% of COD, 84% of BOD and degraded total phenols almost completely up to 97% in 5 days.

INTRODUCTION

Saline and hypersaline environments are frequently contaminated with organic compounds as a result of industrial activities [1,2]. These hypersaline wastewaters are generated during manufacture of chemicals such as pesticides, herbicides and pharmaceuticals and during oil and gas recovery processes [3]. Major organic contaminants of the saline environment are the xenobiotics that include mainly polyaromatic hydrocarbons and phenol and its substituents. The main concern with xenobiotic compounds is the toxicity threat they pose to public health. The xenobiotic compounds like phenols, biphenyl compounds, phthalates, etc. act as endocrine disruptors [4,5].

Phenolic compounds are released into the saline environments from industrial discharges from coal refining, petroleum refining, phenol manufacturing, pharmaceuticals etc and oil-spills [6,7]. According to Prasad and Ellis (1978), phenol and its derivatives are among the most frequently found pollutants in sea shores, rivers, industrial effluents and landfill run-off waters. Phenol and its substituents are among the major hazardous compounds in industrial wastewaters [9,10]. Phenolic compounds produced from waste materials due to the use of petroleum based products, the decomposition of algae and discharge of chemicals from various industries [11]. Also, as they are relatively stable (Half-lives for biodegradation range from <1 to 9 days) and soluble in water/organic solvents, their degradation to reach safety levels in the range of 0.1–1.0 mg /L is not an easy task.

There are much literature information is available on the biodegradation of phenolic compounds under non-saline conditions; however, very few reports have been documented, especially on biodegradation of phenol by moderately halophilic bacteria [12-15]. Biological treatment of industrial effluents have proved to be advantages as they are environment friendly and cost effective way when compared with physico-chemical methods [16] and offer efficient removal of wide range of pollutants in wastewater treatment. In addition, biological treatment of saline wastewater usually results in low removal efficiencies because of the adverse effects of salt on microbial flora [17], but by a proper adaptation of the biomass to a desired salt concentration or use of halophilic microorganisms, the detrimental effects of salinity on the overall bioprocess performance can be also mitigated [18,19].

Many industries such as agro-food, petroleum and leather industries are likely to generate highly saline wastewater. The discharge of such wastewater containing at the same time high salinity and high organic content without prior treatment is known to adversely affect the aquatic life, water portability and

agriculture. Saline effluents are conventionally treated through physico-chemical means, as biological treatment is strongly inhibited by salts (mainly NaCl). However, the costs of physico-chemical treatments are very high, alternative methods for the treatment of organic matter are nowadays increasingly used is aerobic or anaerobic biological treatment. Salt-tolerant halophilic organisms may be used singly or in activated sludge culture for effective biological treatment of saline wastewater. Biological treatment of hypersaline wastewater by pure halophilic bacteria has been studied in biofilms and in a sequencing batch reactor [20]. Inclusion of halophilic bacteria in activated sludge culture was shown to improve COD removal efficiency especially at high salt contents in a rotating biological contactor [21].

There have been several reports on the removal of phenolic compounds from synthetic saline wastewater [22-25]. Hence, the present study was chosen to investigate the degradation of different phenolic compounds under saline conditions by a moderately halophilic bacterial consortium. The second importance application of the study was to treat the real time phenol- contaminated saline pharmaceutical wastewater.

MATERIALS AND METHODS

Chemicals

The Phenolic compounds (Phenol, Catechol, o-cresol, p-cresol, m-cresol and chlorophenol substituents (2-Chlorophenol, 4-Chlorophenol, 2,4- Dichlorophenol, 2,4,6- Trichlorophenol and Pentachlorophenol) were purchased from Sigma Aldrich and all other chemicals (analar grade) were purchased from Merck, India.

Samples

Soil and water samples were collected from different habitats in Chennai such as salt pan, Puliket marine back water lake; Sea harbour (Chennai), phenol containing wastewater, tannery affected soils and soil from sea food industries. Directly, after collection, soils were kept at 4°C until used. Later, soil samples were prepared for culturing by mixing 50 g moist weight with agitation in sterile distilled water (1:1 w/v) for 1 h at room temperature.

Composition of culture medium

The mineral salts medium contained 50.0 g/L NaCl, 0.25 g/L KH_2PO_4, 1.0 g/L NH_4Cl, 2.0 g/L Na_2BO_7, 0.0125 g/L $FeCl_3$, 0.06 g/L $CaCl_2$ and 0.05 $MgCl_2$. The medium was supplemented with a specified amount of added NaCl, adjusted to pH 7.4 ± 0.2, and distilled water 1 L [15]. The final pH of the medium was adjusted to 7.4 with 0.1 N NaOH, and the medium was sterilized in an autoclave (121°C for 15 min) prior to the addition of the phenolic compounds.

Enrichment of the bacterial consortium

The mineral salts medium (100 mL) was added, with phenolic compound as the sole carbon source. Five millilitres of 10^4–10^5 cfu/mL bacterial consortium was added. The conical flask was shaken at 160 rpm at 37 °C for 48 h. After growth, 5 mL of enriched culture was then transferred to fresh medium and incubated under the same conditions. The culture was serially transferred in the same phenol-containing medium to enrich the bacterial consortium.

Biodegradation on phenolic compounds

Phenolic compounds were added to the mineral salts medium. The bacterial consortium isolated from the saline environment was grown and the plate count (cfu/mL) was checked daily. Cell morphology and the motility of cells in exponentially-growing liquid cultures were examined on freshly-prepared wet mounts by light microscopy. Plate counting (cfu/mL) was done on nutrient agar medium. The bacterial consortium was studied for its growth on phenolic compounds with substituted phenols/ chlorophenols as the sole carbon source.

For the degradation study, mineral salts medium containing phenolic compounds were inoculated with the bacterial consortium. Different conditions used for the degradation of phenol were (i) medium + Phenolic compound + bacterial consortium; (ii) medium + Phenolic compound and (iii) medium + bacterial consortium, with (ii) and (iii) serving as controls. The bacterial consortium was added to the medium at concentrations of 10^4– 10^5 cfu/mL. The culture, in duplicate, was incubated at 37°C with shaking at 150 rpm and extracted every 24 h interval for 5 days. Each culture was acidified to pH 2.5 with 1N HCl and extracted twice with dichloromethane (v/v). The extracts were filtered through anhydrous sodium sulphate and condensed to 1 mL in a rotavapour (Buchi, Germany) for further gas chromatographic analysis.

Analytical method

The measured parameters in the wastewater samples were Biomass, phenol, COD, chloride phosphate and pH; whereas phenol, COD and chloride were measured in outlet samples. The parameters of pH, DO and temperature of the wastewater were daily measured in order to control the optimum condition for bacterial growth in the reactor. For evaluating biomass characterization the parameters of mixed liquor suspended solid (MLSS), were measured in the wastewater samples routinely. In order to measure phenol and COD, the samples were filtered through a filter with 0.45 mm pore size before analysis. Phenol concentrations were measured spectrophotometrically, using a UV Spectrophotometer by the colorimetric 4-aminoantipyrine according to the procedure given in the Standard Methods [26]. The pH, DO and temperature were measured using specific electrodes.

Gas chromatographic analysis of the degradation of products

The ability of the consortium to utilize the phenolic compounds as sole carbon source was determined by growing it in the mineral salts medium containing Phenolic compounds. The cell suspensions were clarified by centrifugation at 10,000 rpm for 15 min, at 6°C. The culture supernatant was extracted with dichloromethane, condensed and filtered through a 0.2mm Gelman filter acro disc, prior to analysis in gas chromatograph (Chemito GC Model No 1000) equipped with FID detector and capillary column (Varian Chromopak capillary column CP SIL 8 CB, 30 m X 0.32 mm, 1.00 µm film thickness with detection limit of 10 ppt of the compound. Nitrogen was used as a carrier gas,

with injector temperature 220°C, detector temperature 250°C and the oven temperature of the column at 150°C. The standard solutions of different phenolics were as reference to evaluate the concentration of phenolic compounds. Analytical data on degradation of phenolic compound species were interpreted based on the peak area percent and retention time by mass spectra library.

Analysis of phenolic compounds by GC-MS

GC-MS analysis was performed with GC-MS-QP2010 [SHIMADZU] with an inert mass selective detector and a computer workstation was used for the phenolic compounds analysis. The samples were silylated before analysis, as it is the most widely used technique for the derivatization of functional groups present in the compounds. The silylating reagent, N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was used in identifying compounds that would otherwise go undetected in the GC analysis and do not produce by-products that can damage the capillary GC column. The GC–MS was equipped with: an Agilent DB-5 capillary column (30m x 0.25mm id x 0.25 µm); with an injection volume of 1 µL, split ratio of 20 injection at 280ºC and an ion source temperature at 200ºC. Oven operating temperature was 80ºC with the holding time of 1 min, finally reaching 300ºC for 2 mins with the total time of 41.67 mins. The masses of primary and secondary phenolic compound ions were determined by using the scan mode with impact ionization (70 eV, 200ºC) for pure phenolic compound standards (Merck). Qualitative analysis of phenolic compounds was performed by using the selected ion monitoring (SIM) mode. Fragmented products were identified using computer station library search. Retention time of the fragmented products are further compared and confirmed by analysing authentic standards. Helium was used as the carrier gas. A GC–MS library search was used to confirm the metabolites without standards.

Estimation of phenolic compounds in phenol-contaminated saline wastewater

Phenol-contaminated saline wastewater was collected from a pharmaceutical industry in Chennai. 125 mL of 10^4–10^5 cfu/mL bacterial consortium was added to a 3 L reactor filled with 2.5 L of the saline pharmaceutical wastewater as a seed culture. The inoculated saline crude pharmaceutical–wastewater was aerated and mixed. Every 24 h interval, degradation of phenolic compounds in the saline pharmaceutical wastewater was analysed by GC and GC- MS.

Scanning electron microscopy

The sample preparation for SEM was carried out according to the method of Prior and Perkins (1974) [27]. The isolates were grown on mineral salt medium (MSM) for 24 h. The cells were centrifuged at 8000 x g for 10 min and the pellets were immediately resuspended in 2% glutaraldehyde with 0.05M phosphate buffer and 4% sucrose (pH 7.3). Cells were fixed overnight at 4°C. The specimens were centrifuged at 8000 x g for 10 min, washed four times in distilled water, placed on aluminium foil disks, air dried, platinum coated and examined under SEM (JEOL JSM-6360).

Genomic DNA extraction and amplification of 16s rRNA of the cultured isolates

Extraction of genomic DNA from stable enrichment cultures in mineral salts medium and the isolates was done by the method described by Yates et al. (1997) [28]. DNA was washed twice with 70% ethanol and dissolved in Tris–EDTA buffer (pH 8.0) and analyzed by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining.

Amplification, Cloning, and Sequencing of 16S rRNA

The 16S rRNA of the enrichment cultures and isolates was amplified as described by [28]. The 1.5-kilobase partial sequence of the 16S rRNA gene was amplified from the pooled chromosomal DNA representing the bacterial strains using a polymerase chain reaction (PCR) and universal Eubacteria-specific primers 16F27 (5'-CCA GAGTTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3').

The reaction mixture contained 1.5 mM $MgCl_2$, 0.2 M each dNTP, 25 pmoles each primer, 50 ng template DNA, and 2.5 U Taq DNA polymerase (Bangalore Genei, Bangalore, India) with a reaction buffer supplied by the manufacturer in a total volume of 100 ml. A hot start PCR was performed at 98°C 5 min before the addition of the Taq DNA polymerase. Thirty cycles of 60 sec at 94°C, 60 sec at 55°C, and 90 sec at 72°C, followed by a final extension of 12 min at 72°C was followed. A control PCR reaction containing all reagents was setup with autoclaved water instead of DNA to check for any non-specific contamination.

The PCR products were purified using a QIA Quick gel extraction kit (Qiagen, USA) and then inserted into pGEM-T Easy vector (Promega, USA) following manufacturer's protocols. The ligated vectors were inserted into *Escherichia coli* Bone shot DH5a T1R MAX efficiency competent cells (Invitrogen, USA) by heat shock treatment. White colonies were picked and confirmed for inserts by alkaline-SDS rapid colony lysis method and PCR. The PCR products were grouped according to the DNA patterns obtained by agarose gel electrophoresis after HaeIII digestion. Two representatives from each distinct pattern were selected for sequencing. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1625XP). An internal primer (16F536, 5'-GTG CCA GCA GCC GCG GTR ATA-3') was also used in addition to the other primers. The sequencing of the 16S rRNA gene insert from the clones was done using the 16F27N primer.

Sequencing was performed on 3730 DNA analyzer (Applied Biosystems, USA) using ABI Big-Dye Version 3.1 sequencing kit, as per manufacturer's instructions. In the case of isolates, 30 colonies were selected randomly for each isolate and checked for inserts. Five positive clones from each isolate were sequenced. The partial 16S rRNA sequences of the enrichment culture clones and the nucleotide sequences obtained from the 3730 DNA analyser were studied by BLAST software available at the NCBI website (www.ncbi.nlm.nlm.gov). After editing, the sequence was analysed with BLAST software to identify the specific type of bacteria corresponding to the nucleotide sequence. The isolates

were deposited to Gen Bank and the accession numbers are EU780459, EU780460, EU780461, EU780462, EU780463, and EU780464.

RESULTS AND DISCUSSION

Growth of the bacterial consortium on the phenolic compounds

The isolated bacterial consortium was studied for utilization of different phenolic compounds to be used as a sole source of carbon and energy in the presence of 50 g/L NaCl concentration. As the consortium was isolated from phenol contaminated sites and acclimatized to grow on phenol during the enrichment period, the consortium was able to grow on all the selected phenolic compounds which are indicated by increase in cfu/ml on different phenolic compounds (Figure 1). The isolated bacterial consortium was able to grow on phenols (Phenol, Catechol, *o*-Cresol, *m*-Cresol, *p*-Cresol) and on substituted chlorophenols (2-CP, 4-CP, 2,4-DCP and 2,4,6-TCP) but the growth was very less on PCP, this may be due to the toxicity of the compound. When compared to other phenolic compounds pentachlorophenol has a complex structure because of the chlorine molecules attached to the aromatic ring structure.

Degradation of selected phenolic compounds by the bacterial consortium

The bacterial consortium was studied for degradation of phenolic compounds (100 mg/L) at 50 g/L NaCl. The bacterial consortium was able to utilize all the selected phenolic compounds and the percentage of degradation is represented in the Figure (2). Phenol (100 mg/L) was almost completely degraded (99%) by the bacterial consortium in 5 days. Degradation efficiency of *o*-cresol was (93%), *m*-cresol (82%), *p*-cresol (79%) respectively. In the case of substituted chlorophenols the degradation efficiency reduced, for 4- chlorophenol (84%) , 2,4-DCP (82%) and 2,4,6- TCP (74%) respectively. The degradation percentage of the phenolic compounds reduced based on the structure of the substrate, where the degradation decreased with substituted phenols. The isolated bacterial consortium was able to grow on all the selected phenols. The growth of bacterial consortium on PCP was very slow, probably because of its recalcitrant structure with five chlorine atom substitutes attached to the aromatic ring structure. The toxicity of phenolic compounds tends to

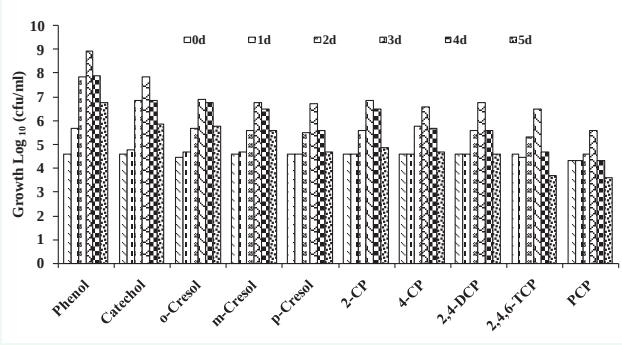

Figure 1 Growth of the bacterial consortium on different phenolic compounds.

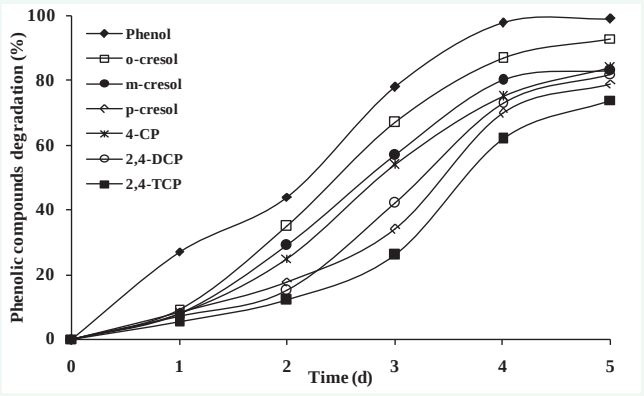

Figure 2 Degradation of selected phenolic compounds by the bacterial consortium.

increase with relative degree of chlorination [29,30]. This view was also supported by Saber and Crawford (1985) [31], where they reported that PCP was resistant to degradation because of its stable aromatic ring system and high number of chlorine substitution. In present study, it was found that the growth of the consortium on PCP was inhibited thereby a reduction in the viable cell count was observed.

Garcia et al. (2005), studied the degradation of low-molecular- weight aromatic compounds (benzoic acid, *p*-hydroxy benzoic acid, cinnamic acid, phenylacetic acid, *p*-coumaric acid, ferulic acid, salicylic acid) by a group of halophilic bacteria. When the isolates were enriched on phenol, they were able to utilize a greater number of aromatic compounds than the rest of the isolates enriched on other aromatic compounds other than phenol, showing their wider substrate specificity. But the mixed isolates were unable to utilize *p*-Cresol. In the present study it could be noted that the isolated bacterial consortium which was enriched with phenol as the carbon source was able to utilize all the phenolic compounds in the mineral salts medium. Salinity studies showed that the isolated bacterial strains were able to grow at a range of NaCl concentrations from (5% to 10%) [32].

Treatment of diluted saline pharmaceutical wastewater

The saline pharmaceutical wastewater was analysed for the presence of phenolic compounds. The batch study was performed in shake flasks with combined pharmaceutical wastewater supplemented with mineral medium (80:20 v/v) along with the bacterial consortium. The results of the batch experiments in shake flasks are shown in Figure (3).

The initial COD and BOD concentration were 16,180 mg/L and 1961 mg/L, respectively after dilution with the mineral salts medium (80:20 v/v). The maximum COD and BOD removal efficiencies were 92% and 90% respectively with an effluent COD and BOD concentrations of 1,452 mg/L and 212 mg/L, at the end of 5 days. In the batch study the bacterial consortium removed about 92% of total phenols with a maximum dry cell weight of 655 mg/L in the log phase on the 4th day. There was no significant increase in the biomass of the consortium after 5 days; this might be due to the depletion of nutrients and toxic intermediates produced during the degradation.

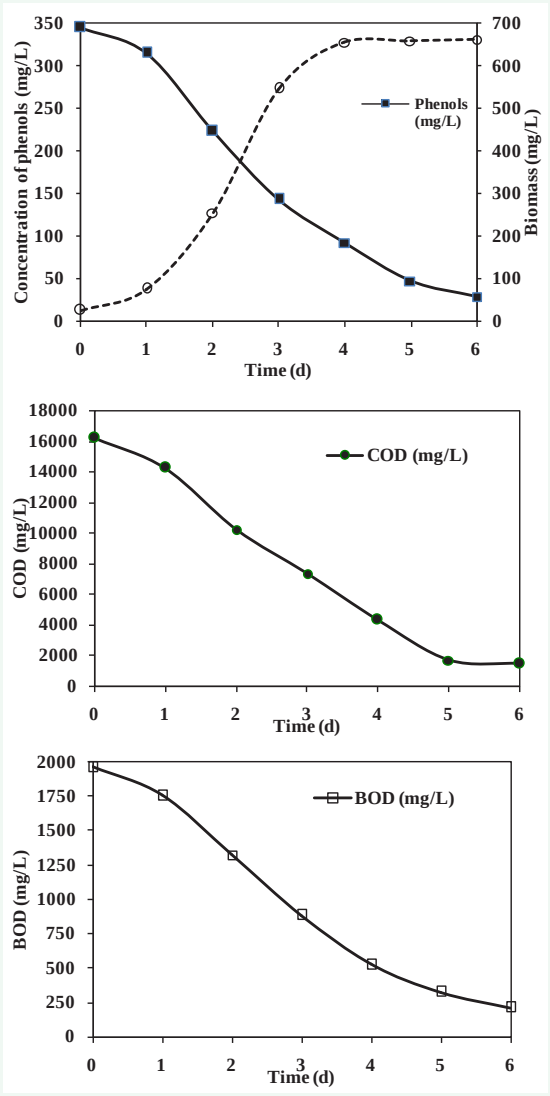

Figure 3 Reduction of Total Phenols, COD and BOD during the treatment of diluted saline pharmaceutical wastewater.

COD reduction was only 55% on the 3 rd day and the COD removal gradually increased to 84% on the 5th day which reached a maximum of 92% at the end of 6 days. It is clear from the figure that the COD, BOD reduction starts during the logarithmic growth phase and the COD, BOD reduction reached maximum during the stationary phase. Phenolic compounds in the wastewater were rapidly degraded or transformed into their metabolites, which is also indicated by the COD removal. Because phenol was used as the sole substrate the difference between COD equivalent of measured phenol and COD measured in the effluent could be explained by accumulation of organic intermediates (metabolites) that were generated during the partially phenol biodegradation caused by inhibitory effect of phenol concentration in synthetic saline wastewater on microbial activities [30].

Afzal et al. (2007), reported the degradation of phenol with monocultures of *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei*, where both the strains were able to degrade phenol (1500 mg/L) in 7 days. As individual cultures of *Pseudomonas aeruginosa* showed 96% removal of both COD and BOD, while *Pseudomonas pseudomallei* showed 86% and 80% of COD and BOD removal respectively.

In present study, the time taken by the bacterial consortium for the treatment of the saline wastewater was only 6 days and there was no lag period during the degradation of phenols and the consortium could effectively degrade the organics under saline conditions. The results obtained in the batch study on the treatment of phenol containing saline pharmaceutical wastewater by the bacterial consortium have shown the feasibility for high COD and BOD removal along with total phenols removal in the saline pharmaceutical wastewater.

Treatment of raw saline pharmaceutical wastewater

The characteristics of saline pharmaceutical wastewater used were: pH -7.8, colour- brown, salinity 3.5-3.6%, a high COD 20,198-20,226 mg/L, BOD 3,822-3,952 mg/L, Total Nitrogen - 980- 995 mg/L, phosphate was 182-198 mg/L, and total phenols of 565- 595 mg/L. During the treatment process the pH in the bioreactor was 7.8 in the raw effluent and at the end of 5 days, the pH was 7.4.

Initial COD and BOD were 20,226 mg/L and 3,952 mg/L, which started to decrease from the second day of treatment and finally attained 2,225 mg/L and 625 mg/L, respectively on the 5th day of the treatment. The BOD and COD removal efficiencies were 84% and 89% respectively. In this study, when the BOD of the saline wastewater decreased, the MLVSS increased from 585 mg/L to 1492 mg/L in 5 days. The MLSS concentration during the treatment study was between 585 mg/L to 1568 mg/L which is represented in the Figure (4). The removal of nitrogen and phosphate during the treatment of saline pharmaceutical wastewater is shown in the Figure (5). Microscopic examinations of the isolated bacterial consortium existing in the bioreactor during the experiments is demonstrated in Figure (6).

Efficiency of bacterial consortium in treating SPW

During the treatment process the pH in the bioreactor was between 7.4 and 7.8. Initially the pH was 7.8 in the raw effluent and at the end of 5 days, the pH was 7.4. This trend can be possibly attributed to the conversion of the recalcitrant compounds in the wastewater, and production of acidic intermediates.

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In present study, when the BOD of the saline wastewater decreased, the MLVSS increased from 585 mg/L to 1492 mg/L in 5 days. The MLSS concentration during the treatment study was between 585 mg/L to 1568 mg/L which is represented in the Figure (4). Woolard and Irvine (1995) reported the degradation of phenol with 99.5% removal by moderately halophilic microorganisms during the treatment of synthetic hypersaline wastewater by sequential batch biofilm reactor, where the increase in MLVSS increased from 50 mg/L to 1020 mg/L in 180 h.

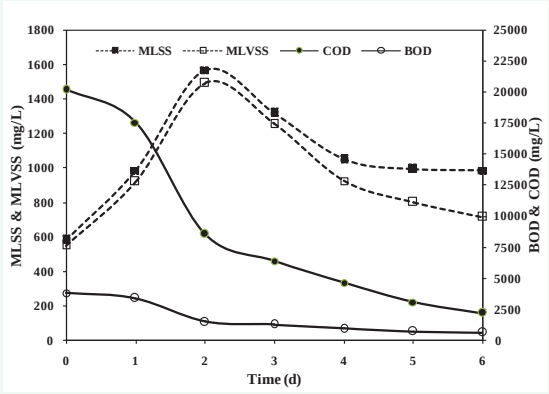

Figure 4 Reduction of COD and BOD during the treatment raw SPW with concentrations of MLSS and MLVSS.

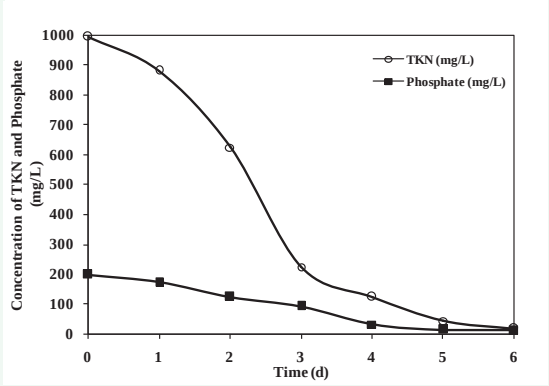

Figure 5 Removal of TKN and Phosphate during the treatment of raw SPW.

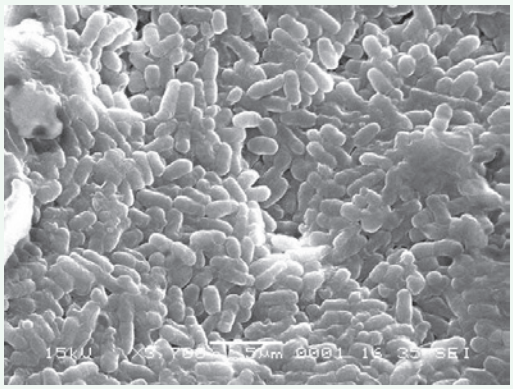

Figure 6 Scanning Microscopic Examination of the Bacterial consortium in the Bioreactor.

In the present work, when the MLVSS increased, the concentrations of nitrogen in the saline pharmaceutical wastewater decreased from 995 mg/L to 18 mg/L and phosphate decreased from 198 mg/L to 12 mg/L, which showed that they were utilized by the bacterial consortium for MLVSS production. The increase in MLSS and MLVSS during the treatment process is represented in the Figure (4).

Degradation of phenolic compounds in saline pharmaceutical wastewater

In the raw wastewater the total phenols contributed to 595 mg/L, which was utilized by the bacterial consortium. At the end of 5 days the total phenols concentration was 15 mg/L which amounted to degradation of 97% as represented in Figure (7). The growth of the bacterial consortium was shown as biomass (dry cell weight), which increased from 24.6 mg/L at the start to a maximum of 652 mg/L on 4th day of treatment process. The phenol containing saline pharmaceutical wastewater was analysed for the presence of phenolic compounds by GC-MS which is represented in the Figure (8).

The GC-MS chromatogram showed five major peaks, the first peak representing 2,4-DCP with m/z (41,49,63,73,97,98,126,162), the 2nd peak at retention time of 9.52 represented Phenol with m/z (25,39,55,66,74,94) followed by the 3rd peak 2,4,6-TCP at retention time of 10.615 with masses m/z (40,48,62,73,97,99,125,132,143,160,169,196,198). At the retention time of 14.099, 4th

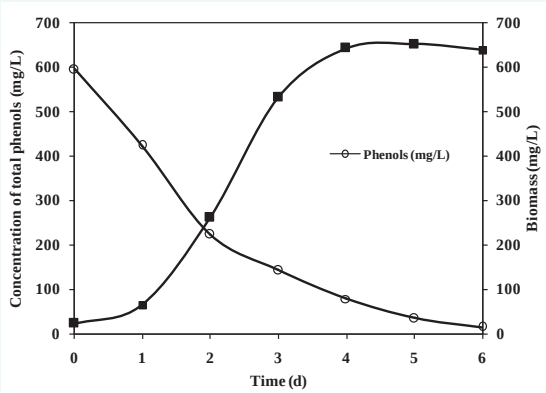

Figure 7 Removal of Total Phenols during the treatment of SPW.

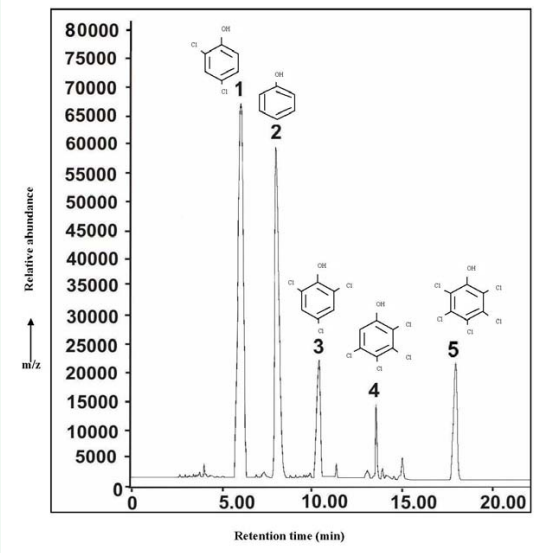

Figure 8 GC-MS Chromatogram of the raw Saline pharmaceutical wastewater.

peak was observed which represented 2,3,4,5-Tetrachlorophenol with masses m/z (40,44,61,83,97,98,131,168,196,232) and the 5th peak represented pentachlorophenol at the retention time 18.514 showing masses m/z (40,47,60,83,95,101,115,130,141,165,169,196,202,214, 230,239,266,268).

The individual phenols and the intermediates at the end of 4 days were shown by GC-MS analysis as shown in the Figure (9). It was found that phenolic compounds were metabolized almost completely, with formation of their intermediates at 9.717, peak 2 representing 4-Chlorophenol m/z (26,39,50,65 ,73,92,99.8,100,128), peak 3- Catechol at 9.863 min with the masses m/z (40,53,64,81,92,110,112,136, 151,166,207), peak 4- Hydroquinone retention time of 11.525 min with the m/z (25,27,39,55,63,81,92,110,112), and final intermediate at peak 5 being dichlorocatechol 14.131 with masses (40,51,63,83,87,98,115,126,144,170,185,200,259,274). It was also found that few compounds were available in traces at peak 1- 2,4-DCP, peak 6 and peak 7 represented tetra-chlorophenol and pentachlorophenol.

Woolard and Irvine (1995) used a sequencing batch biofilm reactor (SBBR) with moderately halophilic bacteria isolated from the Great Salt Lake, in order to treat a synthetic effluent containing 150 g/L of salt and here they showed a degradation efficiency of 99.5% of phenol (100 mg/L) concentration in 150 h. Panswad and Anan (1999) showed 71% COD removal using an anaerobic/aerobic process and with a synthetic wastewater containing 3% salt, where the seeding material was acclimated to high salinity conditions.

In the present study the isolated bacterial consortium was able to degrade 97% of the total phenol present in the raw saline pharmaceutical wastewater with initial concentration of phenol 595 mg/L within 6 days. The bacterial consortium has the ability to degrade higher concentration of phenols as well as different phenolic compounds which proved the feasibility in the treatment of raw saline wastewater.

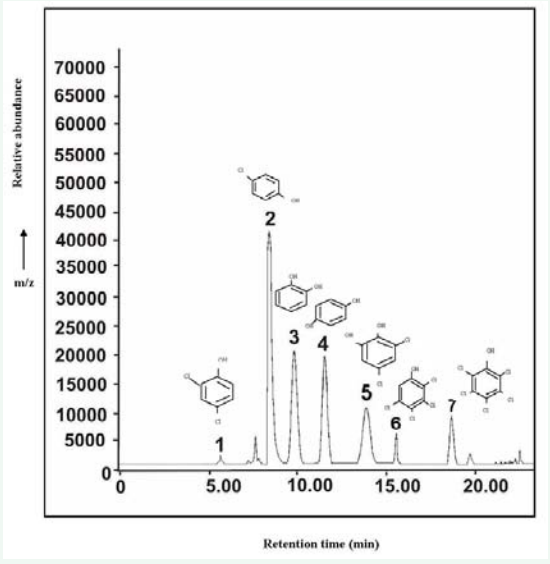

Figure 9 GC-MS Chromatogram of treated Saline pharmaceutical wastewater.

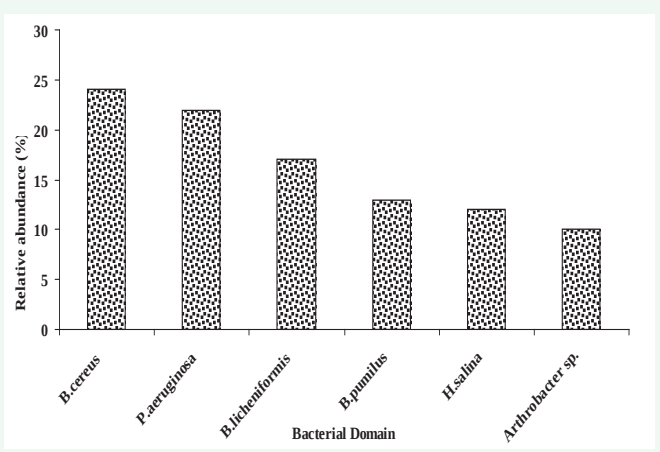

Figure 10 Comparison of relative abundance of bacteria identified in clone library.

Identification of the bacterial isolates in the bacterial consortium

The bacterial strains present in the consortium were identified using cloning technique followed by 16s RNA sequencing. From the bacterial 16s rRNA clone library, 200 clones were randomly selected and each clone was subjected to colony- PCR. DNA isolated from a single representative clone was PCR- amplified and was analyzed for 16S rRNA sequencing. The sequences (with an average length of 900 base pairs) obtained from the above study were subjected to phylogenetic analysis.

A total of 175 clones were found positive for the insert and were partially sequenced, and 58 of which were found to contain the amplified 16S rRNA gene. On the basis of sequence similarity to the existing Gen Bank database entries, the clones were clustered together to form three major groups: the Firmicutes group, the Gamma proteobacteria group, and the Actinobacteria. Firmicutes contributed the major phyla (32 clones, 55%) γ-Proteobacteria (20 clones, 34%), and Actinobacteria group (6 clones, 10%). Most of the phylotypes were related to pollutant degrading bacteria.

Detailed phylogenetic affiliations of 16S rRNA gene phenotypes of the bacterial strains isolated from the bacterial consortium are presented in Table (1). The relative abundance of bacteria identified in clone libraries is depicted in Figure (10). It could be seen that the predominate bacterial species present in the consortium belongs to *Bacillus cereus* (14 clones, 24%), followed by *Pseudomonas aeruginosa* (13 clones 22%), then *Bacillus licheniformis* (10 clones, 17%) other group of bacteria showed clones less than 10 as *Bacillus pumilus* (8 clones, 13%) *Halomonas salina* (7 clones, 12%) and *Arthrobacter* sp. (6 clones, 10%). The nucleotide sequences of the identified six strains were submitted to GenBank. The Genbank accession numbers EU780459, EU780460, EU780461, EU780462, EU780463, and EU780464 for the six bacterial strains is available in http://www.ncbi.nlm.nih.gov/ Genbank. Figure (11) shows the phylogenetic affiliation of the bacterial strains present in the consortium.

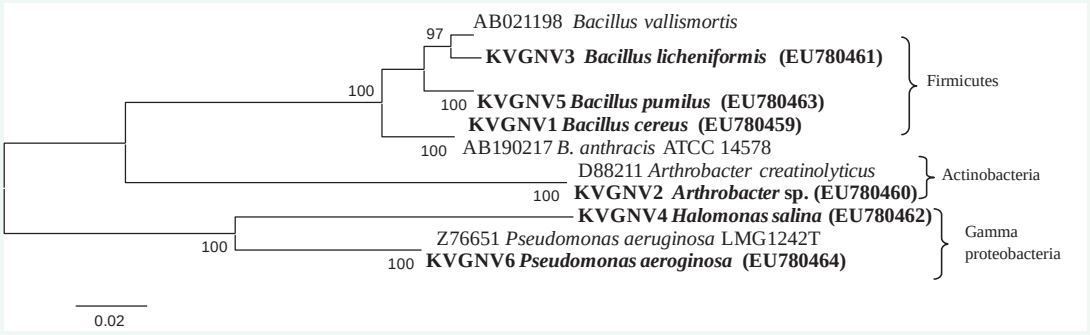

Figure 11 Phylogenetic tree of the evolutionary relationships of the bacterial strains.

Table 1: Bacterial strains identified by 16S rRNA Sequence.

Isolate No. (Strain no. registered in database) (Accession No.)	No. of Clones	Phylum	Nearest Phylogenetic neighbour (Accession No.)	Affiliation	Similarity (%)
KVGNV1 EU780459	14	Firmicutes	*Bacillus cereus* ATCC 10987, complete genome (AE017194.1)	*Bacillus cereus*	99%
KVGNV2 EU780460	6	Actinobacteria	*Arthrobacter* sp. TCCC23001(EU231606.1)	*Arthrobacter* sp.	99%
KVGNV3 EU780461	10	Firmicutes	*Bacillus licheniformis* 16S ribosomal RNA gene, partial sequence (EF059752.1)	*Bacillus licheniformis*	99%
KVGNV4 EU780462	7	Gamma Proteobacteria	*Halomonas salina* 16S rRNA gene, strain F8-11 T (AJ295145.1)	*Halomonas salina*	99%
KVGNV5 EU780463	8	Firmicutes	*Bacillus pumilus* strain BSH-4 16S ribosomal RNA gene, partial sequence (EF488975.1)	*Bacillus pumilus*	98%
KVGNV6 EU780464	13	Gamma Proteobacteria	*Pseudomonas aeruginosa* strain MM1 16S ribosomal RNA gene, partial sequence. (EU583722.1)	*Pseudomonas aeruginosa*	99%

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