CHAPTER 4

SUMMARY AND CONCLUSIONS

Shortage of pure drinking water, environmental pollution and energy crisis are the three equally important critical issues emerged globally as a result of increasing population and industrialization. Microbial desalination cell (MDC) technology is suggested as a new method to solve all these problems simultaneously in an energy efficient way. Lab scale MDC reactors were designed and assembled for simultaneous biodegradation, desalination and power generation.

Various parameters were optimized for obtaining maximum output from MDC. MDC reactor was found to perform better than MFC reactor in terms of power generation, COD removal and desalination efficiency. This was because of the additional desalination activity taking place inside the MDC. During desalination, the anions were transferred from the middle chamber to the anolyte solution which increased the conductivity of the anolyte and stabilized anolyte pH. This inturn increased the microbial growth and metabolism, which resulted in increased COD removal efficiency of MDC. As the microbial metabolism accelerated, more and more electrons were transferred across the circuit resulting in better power generation. More ionic gradients were created which led to increased desalination activity.

When both MFC and MDC were fed with lactate medium and inoculated with electrochemically active Shewanella putrefaciens, MDC could achieve a COD removal of 85±3% where as only 79±6% COD removal
efficiency was achieved by MFC. The maximum power density of 7.01±0.46mW/m$^2$ was achieved in MDC when compared to 6.86±0.62mW/m$^2$ in MFC. 72.34±0.35% desalination was also observed in MDC with 3.5% salt water. This clearly indicated the application potential of MDC over MFC technology.

Two standard cultures, *S.putrefaciens* and *A. hydrophila* obtained from MTCC, were initially used in MDC. It was observed that *S.putrefaciens* generated more power, desalination and higher COD removal. This was attributed to the electron transfer mechanisms involved in these anodophilic bacterial species. *Shewanella* was found to produce redox mediators such as riboflavin and FMN that acted as electron shuttles to transfer electrons from the cells to the anode. *Shewanella* also possessed nanowires and outermost cytochrome C type protein that would also have contributed in the electron transfer processes under anaerobic conditions.

*B.subtilis moh3*, isolated from oil refinery sludge, was used as anodic catalyst in MDC. The performance of the reactor was almost similar to *Shewanella* catalysed MDC, highlighting the electrochemical activity of the strain. Maximum power density of 6.81±0.36mW/m$^2$, 70% desalination and 81.2% COD removal were observed. The biofilm formed by the bacteria on anode was observed by scanning electron microscope. Cyclic voltammetric analysis revealed the formation of redox peaks when *B.subtilis* was used as anodic cultures in MDC. It was supposed that the excreted redox compounds (mediator) in the broth solution were mainly responsible for the electrochemical activity of *B.subtilis*. The degradation potential and electrochemical activity of *B.subtilis moh3* was found promising to be used for bioremediation of toxic contaminants in MDC with simultaneous power generation and desalination.
The influence of pure cultures and mixed cultures on MDC performance was assessed by observing the performance of a *Shewanella* mediated MDC and a waste water fed MDC. It was found that the waste water-MDC produced more power, desalination as well as COD removal. This can be attributed to the synergistic effect of different electrochemically active bacterial species present in the waste water sample which employ different electron transfer mechanisms.

The effect of cathode-catholyte combinations on MDC operation was studied using three different combinations of cathode and catholytes. It was observed that potassium ferricyanide with carbon cloth was found to be the best combination for producing maximum power (7.56mW/m²). Using ferricyanide as the electron acceptor in the cathode chamber increased the power density due to the availability of a good electron acceptor at high concentrations. Eventhough air cathode was the cheap and easily available one, it required the use of expensive catalysts on the cathode to catalyse the reduction reaction to form water.

The configuration of MDC including the surface area of the anode and the volume of the chambers also influenced the performance of MDC. The influence of reactor configuration is mainly by the effect of internal resistance of the reactor. When the size and complexity of the reactor increase, the internal resistance also increases which limits the performance of MDC. MDC-1 possessed total internal resistance of 2108.9 Ω and MDC-2 had total internal resistance of 8519.3 Ω. Due to this, MDC1 performed better than MDC1. Hence it was suggested to employ a higher surface area to volume ratio, lower diffusion lengths or inter membrane space and a closer spacing between the electrodes to minimize the internal resistance of the system.
Hydrocarbon degrading bacteria were isolated from oil contaminated sludge samples by enrichment method using automobile waste oil as the carbon source. Two best degrading bacteria were identified as novel strains *Bacillus subtilis moh3* (KF021537) and *Pseudomonas stutzeri* by morphological, biochemical and molecular techniques. Since the isolates were novel strains, growth were optimized for both the cultures and found that temperatures of 30°C and 35°C were the optimum for growth of *B. subtilis* and *P. stutzeri* respectively. pH 7 was found to be the optimum for both bacteria. Both the bacteria showed maximum growth with glucose as the carbon source. Organic nitrogen sources yielded maximum growth than inorganic nitrogen sources in both the bacteria. Peptone was the best nitrogen source for *B. subtilis* whereas Yeast extract served as the best nitrogen source for *P. stutzeri*. It was found that the isolated strains were almost similar to the existing strains of the same species in their growth conditions.

Hydrocarbon degradation ability of the isolates was further confirmed by using two different assays. DCPIP assay exploited the ability of the dye to accept electrons easily and to be reduced if the bacteria degraded the hydrocarbons. Dioxygenase assay was done to check for the presence of dehydrogenase enzyme which is important in hydrocarbon degradation. Both the cultures were found positive for these two assays, confirming their hydrocarbon degradability.

Biodegradation was carried out in batch scale using MSM media and 1% automobile waste oil as the sole carbon source. It was observed that the growth of bacteria was directly proportional to waste oil degradation as waste oil was the only carbon source available. Degradation percentages of 63.17 ± 0.61 and 59.86± 0.21 were observed respectively for *B. subtilis* and *P. stutzeri* that was estimated gravimetrically. The effect of various factors such as temperature, pH, initial oil concentration, initial inoculum...
concentration and incubation period on waste oil biodegradation were studied. It was observed that the preference of both temperature and pH was found to be similar to that seen during growth optimization studies. The degradation percentage was found to be decreasing with increasing initial oil concentrations. This could be due to the toxicity of engine oil on a fixed number of bacterial cells when the oil concentration was increased. The inoculums concentrations of up to 1.5% was found to be good for degradation, after that the degradation rate decreased. This was because of the fact that, more the number of cells in media, the more were the competition for substrates. Incubation period of 21 days was found to be the optimum for both the bacteria, by the time 67.9% and 63.75% degradation were observed in the culture flasks of *B. subtilis* and *P. stutzeri* respectively.

Biodegradation of waste oil was conducted in MDC by *B. subtilis* cultures and 74.77 ± 0.69% biodegradation was observed within 14 days. Desalination of 68.36 ± 0.6%, Maximum power density of 3.1 ±0.3 mW/m² and current density of 142.9 ± 0.6 mA/m² were observed. The FT-IR and GC-MS analyses of both untreated waste engine oil sample and the waste engine oil treated in MDC had clearly indicated the biodegradation of long chain, branched and cyclic aliphatic hydrocarbons into simpler and harmless by-products.

In the FT-IR spectrum, two most intense peaks at 2924 and 2854 cm⁻¹ in untreated oil, corresponding to C–H stretching in aliphatic compounds and C–N (nitriles), respectively, got their intensity reduced in treated sample. A new band was formed at 1739 cm⁻¹ in treated oil which is associated with carbonyl groups in ketones, aldehydes or acids due to microbial degradation of certain compounds in used engine oil. In the untreated sample, there was a strong band at 1459 cm⁻¹ corresponding to C–C stretch in aromatic nuclei and a less intense band at 1376 cm⁻¹ produced by a mixture of compounds with
small chain length and branching vibration from C–H of the methylene(–CH2–)chain in waste oil sample. Both the peaks disappeared in the treated oil and some weak peaks formed at 1462, 1456 and 1379 cm$^{-1}$ due to the presence of a mixture of hydrocarbon compounds with small chain lengths and C–H branching vibrations within the –CH– groups. This confirmed the degradation of compounds including aromatic nuclei into simpler products.

The most abundant peaks in GC-MS spectra were located between 16 and 18 min. The most likely compounds were identified as 5,8-diethyldodecane, 9-hexylheptadecane, 9-octylheptadecane, 9-(2-cyclohexyl ethyl) heptadecane, 1(1,5-dimethyl hexyl)- 4-(4-methylpentyl) cyclohexane, 1-(1,5-dimethylhexyl)-4- (4- methyl pentyl) cyclohexane, etc. These include mostly long chain, branched and cyclic alkanes. Most of the peaks present in untreated oil got reduced to a great extent in treated oil. A few number of peaks present in untreated oil were absent in treated oil and some new peaks were visible in the treated oil chromatogram. Alkanes are usually the easiest hydrocarbons to be degraded by their conversion to alcohol via a mixed function enzymatic activity. The detection of alcohol production would indicate the potential of microorganisms to transform hydrocarbons. Peaks corresponding to alcohols, ketones, some short chain aliphatic hydrocarbons and organic acids were detected in the GC-MS spectrum of degraded engine oil which represented the degradation products.

Physical characterization of fresh, used and MDC treated engine oil samples were done and found that most of the physical properties of the treated oil were significantly different from that of fresh oil and hence was not able to be reused as engine oil. As the toxicants got biodegraded, it was safe to be discarded or could be used for some other purposes such as lighting industrial burners, as additive in manufactured products, in concretes, greasing nuts and bolts, destroying wasp and mosquito larvae etc.
Biosurfactant activity assays were done to check the ability of the oil degrading bacterial isolates to produce biosurfactants. It was observed that *B. subtilis* and *P. stutzeri* produced biosurfactants, better than other isolates. Surface tension value of 32.28±0.42 mN/M and 36.44±0.26 mN/M were observed for the crude biosurfactants of *B. subtilis* and *P. stutzeri* respectively.

Biosurfactant production experiments were conducted and biomass and biosurfactant yield were monitored throughout the experimental period of one week. It was observed that *B. subtilis* produced maximum biosurfactants during late log phase of growth and production was completed within 72 hours. In *P. stutzeri*, the maximum biosurfactant production was observed during the stationary phase of growth and the production was completed within 96 hours of growth. It was observed that both biomass and biosurfactant yield were higher in *P. stutzeri* than *B. subtilis*, but the surface tension values were lower in *B. subtilis* supernatants indicating its potential as an efficient biosurfactant producer.

The biosurfactants were extracted from the production broth by acid precipitation, cold acetone precipitation and foam fractionation. Foam fractionation method recovered more biosurfactants with a surface tension value of 30.86±0.34 mN/M for *B. subtilis* and 34.84±0.36 mN/M for *P. stutzeri*. This reduction in surface tension values was due to the increased purity or enrichment of the extracted biosurfactant.

Biochemical characterization revealed the chemical nature of extracted biosurfactants tentatively as lipopeptides from *B. subtilis* and rhamnolipids from *P. stutzeri*. This was further confirmed by FTIR analysis after purification by TLC. The single bands formed on TLC plates were scratched out and extracted with solvents to get purified biosurfactants. These purified biosurfactants exhibited even lower surface tension values as 26.28 mN/M for lipopeptide biosurfactant and 31.6 mN/M for rhamnolipid
biosurfactant. FTIR spectrum of purified biosurfactant from *Bacillus* exhibited characteristic peaks for lipopeptides and that of *Pseudomonas* biosurfactant showed characteristic bands for rhamnolipids, which confirmed the tentative identification of biosurfactants.

Critical Micelle Concentration of the biosurfactant was determined which highlighted the effectiveness of their surface active property. A lower CMC of 60mg/L was observed for *Bacillus* biosurfactant when compared to the *Pseudomonas* biosurfactant whose CMC value was 80mg/L. Stability of biosurfactant was checked under wide ranges of temperature, pH and salinity conditions. Both the biosurfactants showed significant stability over the range of conditions where lipopetide biosurfactant was found to be highly stable than the rhamnolipid biosurfactant. The low CMC value and good stability of biosurfactants make it suitable for wide range of industrial applications.

Both the biosurfactants were found to have antimicrobial activity against a number of pathogenic bacteria. This property could aid in the pharmaceutical and cosmetic applications of biosurfactants. Interestingly rhamnolpid biosurfactant exhibited antibacterial activity against the isolated *B.subtilis moh 3* strain which accounted for the inhibition of growth of *B.subtilis* cultures when both *Bacillus* and *pseudomonas* cultures were used as consortium for waste oil biodegradation. The role of extracted biosurfactants in oil bioremediation was observed by conducting waste oil biodegradation experiment using *B.subtilis* cultures with biosurfactant amended to the medium. An enhanced degradation percentage (78.96%) was observed when compared to previous degradation experiments without biosurfactant amendments.

The application of MDC for dye degradation and decolourization was observed using *B.subtilis* and *Aeromonas hydrophila* cultures. Physical parameters such as temperature, pH, initial dye concentration, initial
inoculums size and incubation period were optimized for growth and degradation. *B. subtilis* decolourised MG with an efficiency of $56\pm 5\%$ and $92\pm 4\%$ for SY within 72 hours.

Both the dyes were decolourized completely by *B. subtilis* when the degradation was conducted in MDC. Higher power production and desalination were observed for MG-MDC with maximum power densities of $3.01\pm 0.04 \text{mW/m}^2$ for MG-MDC and $2.86\pm 0.25 \text{mW/m}^2$ for SY-MDC. The decreased power production in SY-MDC may be due to the fact that Sunset Yellow is an azo dye which requires electrons during the reduction of its azo bonds. The glucose present in M9 medium acted as an electron donor for bacterial growth and a part of these electrons were utilized by the azoreductase enzyme for decolourising the azodye by reduction of azo bond. Hence remaining electrons were only getting transferred to the anode for bioelectricity production leading to decreased power generation.

The kinetics of biodegradation during waste oil degradation with different initial substrate concentrations were explored using second order kinetic models. The experimental data for biodegradation in both batch experiment as well as MDC, fitted well to Quiroga’s second order kinetics with $R^2$ values $>0.91$ with the maximum of 0.99 for MDC data. In MDC biodegradation, the model could predict the maximum growth rate of bacteria (0.451g/day) and, maximum (8.623g/L) and minimum (2.074g/L) concentrations of substrate which were very close to their experimental values. Also the growth kinetics of bacteria during batch degradation at each initial oil concentration was explored and the kinetic parameters obtained experimentally were not significantly different from the values predicted by the model. There was a good agreement between the theoretical and experimental biodegradation pattern especially for MDC assisted biodegradation (experimental-74.77% and predicted-75.94%), signifying the validity of the model.