Chapter 6
Summary
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Characterization of a methylotrophic bacterial consortium and its potential in treatment of industrial effluents

1. Construction of a bacterial consortium for COD reduction of industrial effluents and characterization of its individual isolates

❖ 118 aerobic, heterotrophic, mesophilic bacterial isolates were obtained from the samples collected from a fertilizer company, domestic sewage treatment plant (STP) and common effluent treatment plant (CETP).

❖ The isolates were screened on the basis of fusel oil (mainly containing methanol) utilization. 24 isolates showing higher growth on fusel oil were selected for the further studies.

❖ The 24 selected isolates were further screened on the basis of their growth in DNR effluent containing fusel oil (1 %, v/v). The isolates showing higher growth were selected to prepare 2 mixed bacterial consortia, viz. FW consortium consisting of the isolates FTE3, FTE8, FTE15, FTE22 and WAS2, and AC consortium consisting of the isolates AC1, AC4, AC5 and AC8.

❖ AC consortium, FW consortium, DNR A sludge, DNR B sludge and Wadi activated sludge were screened for their biotreatment potential. The AC consortium showed the maximum biotreatment potential and, hence, was selected for the further studies.

❖ The growth curves and specific growth rates of all the 4 isolates of AC consortium were found to be comparable, implying that they grew together in the form of AC consortium.

❖ The isolates of AC consortium were able to grow on methanol as a sole carbon source and they showed higher methanol utilization ability when present together as compared to their individual activities.

❖ The ability of the isolates of AC consortium to use methanol was also determined during their growth in DNR effluent by gas chromatography. More than 95 % methanol biodegradation was obtained using isolates AC1 (99 %), AC4 (99 %), AC5 (99 %) and AC8 (100 %), confirming their methylotrophic nature.

❖ PCR amplification of the partial mxaF gene, encoding the methanol dehydrogenase enzyme in methylotrophs, confirmed the presence of about 550 bp sized partial mxaF gene in all the isolates of AC consortium.
The isolates of AC consortium were identified on the basis of their biochemical characters and 16S rRNA gene sequencing. Thus, isolate AC1 was identified as *Bordetella petrii* AC1, isolate AC4 as *Bacillus licheniformis* AC4, isolate AC5 as *Salmonella subterranea* AC5 and isolate AC8 as *Pseudomonas stutzeri* AC8.

Phylogenetic analysis of the isolates of AC consortium indicated that *B. petrii* AC1 was phylogenetically most closely related to *B. petrii* AJ870969, *B. licheniformis* AC4 to *B. licheniformis* EU256500, *S. subterranea* AC5 to *S. subterranea* AY373829 and *P. stutzeri* AC8 to *P. stutzeri* GU339239.

Differential carbon substrate utilization pattern of the isolates of AC consortium confirmed their facultative methylotrophic nature. Among the 10 single carbon substrates provided as sole carbon source, *B. petrii* AC1 was the best of all the 4 isolates in that it showed growth on 8 out of 10 substrates, while *B. licheniformis* AC4, *S. subterranea* AC5 and *P. stutzeri* AC8 could grow on 7 out of 10 substrates. All the isolates showed growth on methanol and formaldehyde. Methyl amine, methyl bromide, methyl chloride and methyl fluoride were utilized by at least 3 of the 4 isolates. Among the multi carbon substrates tested for sole carbon sources, ethanol, N-propanol, isopropanol, N-butanol and fusel oil were growth substrates for all the isolates of AC consortium; while 2-butanol, glucose, fructose and L-glutamate served as substrates for at least 3 out of the 4 members of AC consortium.

The metabolic diversity of the isolates of AC consortium, further studied in terms of their ability to utilize a variety of industrially important alcohols, demonstrated that while the AC consortium and its individual members could grow on all the alcohols tested, *B. petrii* AC1, *P. stutzeri* AC8 and AC consortium showed higher utilization of methanol, ethanol, 1-propanol and 2-propanol; *B. licheniformis* AC4 showed higher utilization of methanol and ethanol; and *S. subterranea* AC5 showed higher utilization of methanol, ethanol and 1-propanol as compared to other alcohols tested.

The biodegradation ability of the AC consortium was further checked on different carbon substrates and xenobiotics in terms of their growth and COD reduction. It was observed that out of the 15 substrates used for this study, the AC consortium showed 100 % degradation of acetate, succinate and pyruvate; methanol, ethanol, propanol, 2-propanol, 2-butanol and acetone were maximally utilized at > 50 %,
whereas butanol, formaldehyde, \textit{tert}-amyl alcohol, benzene, xylene and toluene were utilized at < 50%.

- The methanol dehydrogenase present in the isolates of AC consortium was in the range of 0.16 - 0.56 units/ml with the specific activity in the range of 0.48 - 0.87 units/mg.

- Methanol tolerance of the isolates of AC consortium, when analyzed, showed that the isolates of AC consortium could tolerate methanol up to 1.2 g/l, which is a highly toxic concentration of methanol, thus, implying that the AC consortium could be used for treatment of industrial effluents containing high concentrations of methanol.

2. Biodegradation of xenobiotics by the AC consortium

2A. Methyl \textit{tert}-Butyl Ether (MTBE) biodegradation by the AC consortium

- The isolates of AC consortium could grow on all the soluble xenobiotics selected.
  - Among the soluble xenobiotics tested, \textit{B. petrii} AC1 showed higher utilization of 2-chloroethanol (CE), MTBE, trimethylamine hydrochloride (TMAH) and allyl chloride; \textit{B. licheniformis} AC4 of \textit{tert}-amyl methyl ether (TAME), MTBE and allyl chloride; \textit{S. subterranea} AC5 of MTBE; \textit{P. stutzeri} AC8 of MTBE and allyl chloride, and AC consortium of CE, TAME, MTBE, TMAH and allyl chloride.

- MTBE was maximally utilized by AC consortium and its individual isolates. Hence, MTBE was selected for the further xenobiotic biodegradation studies.

- MTBE biodegradation ability of the AC consortium and its individual isolates, when checked, indicated that the AC consortium was more effective than its individual members in reducing the COD of MTBE containing medium from 700 mg/l to below detection limit in 120 h, indicating its higher potential to biodegrade MTBE.

- The effect of different cations of MM2 medium, viz., \text{Mg}^{2+}, \text{Ca}^{2+}, \text{Mn}^{2+}, \text{Na}^{+} \text{and Fe}^{2+} on MTBE utilization by the isolates of AC consortium showed that \text{Mg}^{2+} at 0.3 g/l was statistically significant for maximum utilization of MTBE by AC consortium and its individual isolates, except \textit{S. subterranea} AC5 that showed maximum growth on MTBE at the \text{Mg}^{2+} concentration of 0.2 g/l.
❖ All the AC isolates showed maximum growth at the MTBE concentration of 4.5 g/l, whereas the AC consortium showed maximum growth at higher MTBE concentration of 7.0 g/l.

❖ The AC consortium and *P. stutzeri* AC8 showed similar and maximum growth on MTBE as compared to the other isolates, viz. *B. petrii* AC1, *B. licheniformis* AC4 and *S. subterranea* AC5 individually. Elimination of *P. stutzeri* AC8 from the AC consortium drastically affected the growth of AC consortium and thereby MTBE utilization.

❖ The growth and COD reduction ability of the AC consortium and its individual isolates in the optimized MM2 medium increased as compared to that obtained in the original medium. The GC analysis showed that the initial MTBE concentration of 7.4 g/l was reduced to 0.13 g/l by *B. petrii* AC1, 0.14 g/l by *B. licheniformis* AC4, 0.15 g/l by *S. subterranea* AC5, 0.12 g/l by *P. stutzeri* AC8 and 0.12 g/l by AC consortium.

❖ The AC consortium could also grow effectively on TBA, the first metabolic intermediate of MTBE, and reduce its COD to below detection limit, indicating its potential to biodegrade TBA.

❖ The MM2 medium supplemented with the cations, Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\) and Na\(^{+}\), at 4 % concentration significantly enhanced the growth of AC consortium and its individual members on TBA.

❖ *B. petrii* AC1 and *B. licheniformis* AC4 showed maximum growth on TBA at the concentration of 7.8 g/l and 5.4 g/l respectively, while *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth on TBA at the concentration of 3.9 g/l.

❖ TBA utilization by the individual isolates and AC consortium showed considerable increase in terms of their growth and COD reduction on optimization of MM2 medium as compared to that without optimization. The GC analysis showed that the initial TBA concentration of 7.75 g/l was reduced to 0.47 g/l by *B. petrii* AC1, 0.53 g/l by *B. licheniformis* AC4, 0.54 g/l by *S. subterranea* AC5, 0.49 g/l by *P. stutzeri* AC8 and 0.47 g/l by AC consortium in the optimized medium.

❖ Detection of metabolic intermediates of MTBE on its biodegradation by the AC consortium, analyzed using GC-MS, indicated that MTBE was completely
utilized by AC consortium and neither TBA nor TBF were accumulated during its biodegradation.

❖ The AC consortium reduced the initial COD of the MTBE containing synthetic effluent from 650 mg/l to below detection limit in 5 h at flask level, from 950 mg/l to below detection limit in 78 h at batch reactor level and from 1000 mg/l to below detection limit in 10 d at continuous reactor level. Hence, the AC consortium has a strong potential for treatment of MTBE containing effluents.

2B. 1,2-Dichloroethane (DCE) biodegradation by the AC consortium

❖ The AC consortium and its individual members could utilize all the insoluble xenobiotics tested in their vapor phase, except 4-chloroaniline where growth of all the isolates and AC consortium was lowest. However, the AC consortium and its individual isolates showed maximum utilization of DCE and, hence, DCE was selected for the further xenobiotic degradation studies.

❖ The AC consortium could grow on DCE as well as reduce the COD of DCE containing medium from 750 mg/l to below detection limit in 120 h.

❖ Out of all the cations, viz. Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Na$^{+}$ and Fe$^{2+}$, tested, Mg$^{2+}$ at the concentration of 0.5 g/l was statistically significant for the maximum utilization of DCE by the individual isolates and AC consortium.

❖ Out of the different concentrations of DCE tested, DCE at 12.5 g/l was statistically significant for maximum growth of the individual isolates and AC consortium.

❖ As compared to the AC consortium, the combinations in which one of its members was eliminated showed a sizeable decrease in their growth on DCE.

❖ Enhancement in DCE utilization by the individual isolates and AC consortium was obtained in terms of their growth and COD reduction on media optimization. The GC analysis showed that the initial DCE concentration of 12.56 g/l was reduced to 6.06 g/l by *B. petrii* AC1, 6.97 g/l by *B. licheniformis* AC4, 5.58 g/l by *S. subterranea* AC5, 5.48 g/l by *P. stutzeri* AC8 and 4.97 g/l by AC consortium in the optimized medium.

❖ All the isolates and AC consortium could also grow on and biodegrade CE, the first metabolic intermediate of DCE.
Out of all the nutrient conditions tested, it was shown that the addition of Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$ and Na$^+$ together at 4% concentration in MM2 medium had a statistically significant effect on the growth of AC consortium and its individual members on CE.

*B. petrii* AC1, *S. subterranea* AC5, *P. stutzeri* AC8 and AC consortium showed maximum growth on CE at 6.0 g/l, while *B. licheniformis* AC4 showed maximum growth on CE at 8.4 g/l.

CE utilization by the individual isolates and AC consortium was enhanced in terms of their growth and COD reduction on media optimization. The GC analysis showed that the initial CE concentration of 8.4 g/l was reduced to 0.44 g/l by *B. petrii* AC1, 0.35 g/l by *B. licheniformis* AC4, 0.35 g/l by *S. subterranea* AC5, 0.46 g/l by *P. stutzeri* AC8 and 0.34 g/l by AC consortium in the optimized medium.

The detection of metabolic intermediates of DCE on its biodegradation by the AC consortium, analyzed using GC-MS, indicated that DCE was completely utilized by AC consortium and CE was not accumulated during its biodegradation.

### 3. Biotreatment of different industrial effluents using AC consortium

The biodegradability indices of all the 4 effluents selected for the biotreatment studies, viz. DNR effluent obtained from a fertilizer company, ECO effluent from common effluent treatment plant, COR effluent from a pesticide company and UPL effluent from a chemical company, were in the range of 0.3 – 0.6.

The AC consortium, used as a special microbial seed for biotreatment of these 4 effluents, showed statistically significant COD reduction of DNR effluent from 770 mg/l to below detection limit in 8 h, of ECO effluent from 1000 mg/l to below detection limit in 9 h, of COR effluent from 1600 mg/l to below detection limit in 12 h and of UPL effluent from 1600 mg/l to below detection limit in 8 h in the flask level treatability studies.

In the flask level treatability studies, the AC consortium showed 100% COD reduction of DNR effluent in 8 h, ECO effluent in 9 h, COR effluent in 12 h and UPL effluent in 8 h, as compared to 48, 50, 50 and 56% COD reduction of DNR, ECO, COR and UPL effluents respectively in 12 h by the indigenous microflora of the respective industrial effluents.
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- The AC consortium showed statistically significant COD reduction of DNR effluent from 1600 mg/l to below detection limit in 60 h, of ECO effluent from 950 mg/l to below detection limit in 72 h, of COR effluent from 1850 mg/l to below detection limit in 96 h and of UPL effluent from 1900 mg/l to below detection limit in 72 h at batch reactor level.

- In the batch reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 60 h, ECO effluent in 72 h, and COR and UPL effluents in 96 h, as compared to 56, 68, 57 and 57 % COD reduction of DNR, ECO, COR and UPL effluents respectively by their indigenous microflora.

- The environmentally important parameters like BOD, pH, MLSS, MLVSS, SVI and F/M ratio of all the 4 effluents were also estimated for their efficient biotreatment by the AC consortium. BOD of the effluents was reduced in the range of 20 – 27 mg/l, pH in the range of 7.0 – 7.6, MLSS and MLVSS in the range of 103 – 132 mg/l, SVI in the range of 198 - 208 ml/g and F/M ratio in the range of 0.03 – 0.05.

- The AC consortium showed statistically significant COD reduction of DNR effluent from 1300 mg/l to below detection limit in 15 d, of ECO effluent from 1100 mg/l to below detection limit in 9 d, of COR effluent from 2000 mg/l to below detection limit in 10 d and of UPL effluent from 1800 mg/l to below detection limit in 10 d at continuous reactor level.

- In the continuous reactor, the AC consortium showed 100 % COD reduction of DNR effluent in 15 d, ECO effluent in 9 d, and COR and UPL effluents in 10 d, as compared to 62, 73, 65 and 67 % COD reduction of DNR, ECO, COR and UPL effluents respectively by their indigenous microflora.

- The desirable BOD value of 30 mg/l for discharge of effluents was obtained at the end of each reactor study; the pH obtained was in the fixed range of 5.5 – 9.0; the MLSS and MLVSS values were reduced approximately to 100 mg/l; the SVI obtained was approximately 100 ml/g, and the F/M ratio was lower than the stipulated value of 0.04.

- One-way ANOVA performed for the comparison of difference in % COD reduction obtained in control and AC consortium seeded flask, batch and continuous reactors showed a p-value of 0.005, indicating that there was a significant difference in the observed means of % COD reduction in all the 3 reactors at 95 % level of significance.
The reactor studies were further scaled up to a pilot scale 2000 l continuous reactor level with DNR effluent. Here, the AC consortium reduced the COD of DNR effluent from about 1300 mg/l to below permissible limit and maintained it over a period of 120 d.

The desirable MLSS and MLVSS values of 100 mg/l were obtained by the AC consortium for most of the reactor run. Hence, the AC consortium is an efficient microbial seed for biotreatment of DNR effluent at a larger scale.

4. Development of a bench scale Moving Bed Biofilm Reactor (MBBR) for COD reduction of industrial effluents by AC consortium

Biofilm forming ability of the isolates of AC consortium was checked on a microscopic glass slide. All the isolates showed different stages of biofilm formation on microscopic examination of the glass slides. It was observed that the AC isolates formed a complete biofilm when present as a consortium.

*B. licheniformis* AC4 and *P. stutzeri* AC8 showed highest biofilm formation, while *B. petrii* AC1 showed least in AMS medium using microtiter plate biofilm assay. The AC consortium showed a comparable biofilm formation to *B. licheniformis* AC4 and *P. stutzeri* AC8.

Biofilm forming ability of the AC consortium, when checked on commercially available Kaldnes type K1 biofilm carriers in DNR effluent, showed that the AC consortium formed a strong biofilm on these carriers. Biofilm formation was higher on the inner surfaces of the biofilm carriers as compared to their outer surface.

The methanol concentration of 0.5 % along with 0.01 % yeast extract added as a growth factor showed better growth and biofilm formation of the individual isolates and AC consortium.

Out of potassium acetate, sodium succinate and methanol used as the carbon sources, potassium acetate at the concentration of 8 g/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.

Out of NH₄Cl, NH₄NO₃ and (NH₄)₂SO₄ used as the nitrogen sources, NH₄Cl at the concentration of 1 g/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.
❖ Out of KH$_2$PO$_4$ + Na$_2$HPO$_4$, KH$_2$PO$_4$ + K$_2$HPO$_4$, Na$_2$HPO$_4$ and KH$_2$PO$_4$ used as the phosphorus sources, KH$_2$PO$_4$ + Na$_2$HPO$_4$ at the concentration of 25 ml/l was statistically significant for highest biofilm formation by the AC consortium and its individual isolates.

❖ Out of the different concentrations of macronutrients, CaCl$_2$ at the concentration of 0.4 g/l, MgSO$_4$ at the concentration of 1.5 g/l and FeCl$_3$ at the concentration of 6 mg/l were statistically significant for highest biofilm formation by the AC consortium and its individual isolates.

❖ 3 %, 24 h old inoculum after an incubation period of 120 h supported highest biofilm formation by the AC consortium and its individual isolates.

❖ The optimized AMS medium composed of (per liter): potassium acetate, 8.0 g; NH$_4$Cl, 1.0 g; phosphate buffer (KH$_2$PO$_4$ + Na$_2$HPO$_4$), 25.0 ml; MgSO$_4$, 1.5 g; CaCl$_2$, 0.4 g; FeCl$_3$, 6.0 mg; trace element solution, 0.5 ml, pH, 6.8. The biofilm forming ability of the AC consortium and its individual isolates increased considerably in the optimized AMS medium as compared to the original AMS medium.

❖ The individual isolates as well as the AC consortium formed a strong and dense biofilm in the DNR effluent on a microtiter plate.

❖ The AC consortium reduced the initial COD of DNR effluent from 1600 mg/l to below detection limit in 108 h at flask level MBBR.

❖ 100 % COD reduction by AC consortium was obtained in 7 d in the suspended reactor, while the same was obtained in 6 d in the MBBR filled with 60 % (v/v) biofilm carriers, with the COD removal rate of 316.67 mg/l/d. The biomass obtained on the biofilm carriers was 802 mg/l and the biomass suspended in the reactor was 3721 mg/l.

❖ 100 % COD reduction was achieved by the AC consortium in 6 d in the suspended reactor, while the same was obtained in 5 d in the MBBR filled with 30 % (v/v) biofilm carriers, with a higher COD removal rate of 380 mg/l/d, as compared to that obtained in the MBBR with 60 % (v/v) biofilm carriers. The biomass obtained on the biofilm carriers was 1320 mg/l and the biomass suspended in the reactor was 3217 mg/l.

❖ The COD was maintained at below permissible limit in DNR, ECO, COR and UPL effluents during the course of MBBR run in a continuous mode for 120 d. The initial COD of the DNR effluent of 1700 mg/l was reduced to below
permissible limit when run continuously for 60 d, followed by the COD reduction of the ECO effluent from 1300 mg/l to below permissible limit for the next 20 d, further followed by the COD reduction of the COR effluent from 1100 mg/l to below permissible limit for the next 20 d and lastly the COD reduction of the UPL effluent from 1400 mg/l to below permissible limit for the last 20 d in continuity.

❖ The MBBR was subjected to shock loading of 1700 mg/l DNR effluent, 1300 mg/l ECO effluent, 1100 mg/l COR effluent and 1400 mg/l UPL effluent. The organic loading rates of DNR, ECO, COR and UPL effluents were 0.41, 0.31, 0.26 and 0.34 kg COD/m³/d respectively. The COD removal rates for DNR, ECO, COR and UPL effluents were 113.33, 260, 366.67 and 175 mg/l/d respectively.

❖ ESEM analysis of the biofilm carrier showed that the AC consortium grew and formed a prominent biofilm on the carrier.

❖ The time taken by AC consortium for 100 % COD reduction of DNR effluent was the same in both suspended reactor and MBBR. Thereafter, the COD reduction ability of the biofilm of AC consortium increased gradually and, hence, 100 % COD reduction of ECO, COR and UPL effluents was obtained in very less time in MBBR as compared to the suspended reactor. Hence, different kinds of industrial effluents could be effectively treated by the AC consortium using MBBR.