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Effect of cyclodextrin glucanotransferase enzyme in biodegradation of diesel oil

Sivaraman C, Anasuya Ganguly and Srikanth Mutnuri*

Applied and Environmental Biotechnology Laboratory, Department of Biological Sciences, BITS Pilani - K.K. Birla Goa Campus, Zuari Nagar, Goa 403726, India

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Abstract. Microbial degradation of hydrocarbons is found to be an attractive process for remediation of contaminated habitats. However the poor bioavailability of hydrocarbons results in low biodegradation rates. Cyclodextrins are known to increase the bioavailability of variety of hydrophobic compounds. In the present work we purified the Cyclodextrin Glucanotransferase (CGTase) enzyme which is responsible for converting starch into cyclodextrins and studied its role on biodegradation of diesel oil contaminated soil. Purification of CGTase from *Enterobacter cloacae* was done which resulted in 6 fold increase in enzyme activity. The enzyme showed maximum activity at pH 7, temperature 60ºC with a molecular weight of 66 kDa. Addition of purified CGTase to the treatment setup with *Pseudomonas mendocina* showed enhanced biodegradation of diesel oil (57 ± 1.37%) which was similar to the treatment setup when added with *Pseudomonas mendocina* and *Enterobacter cloacae* (52.7 ± 6.51%). The residual diesel oil found in treatment setup added with *Pseudomonas mendocina* at end of the study was found to be 73 ± 0.21%. Immobilization of *Pseudomonas mendocina* on alginate containing starch also led to enhanced biodegradation of hydrocarbons in diesel oil at 336 hours.

Keywords: diesel oil; bioavailability; cyclodextrin; cylodextrin glucanotransferase

1. Introduction

Hydrocarbons are organic compounds that are released in the environment due to natural and anthropogenic activities. Due to their mutagenic and carcinogenic properties their cleanup from the polluted habitats is of principal concern (Arulazhagan *et al.* 2010). Diesel oil is derived from crude oil refining and is complex mixture of saturated and aromatic hydrocarbons (Zanaroli 2010). Diesel fuels have been observed to cause skin and tumorogenic responses in mice (Nessel 1999). Diesel is considered to be harmful and possibly carcinogenic to humans since it contains PAHs those posses' carcinogenic, teratogenic and mutagenic properties (Grant *et al.* 2007). Bioremediation processes are significantly affected by number of biotic and abiotic factors: one principal factor is bioavailability i.e., availability of hydrocarbons towards the degrading microorganisms (Haritash and Kaushik 2009). The poor bioavailability is due to low aqueous solubility of the hydrocarbons in the polluted

*Corresponding author, Professor, E-mail: srikanth.mutnuri@gmail.com

sites (Mukherji and Vijay 2002). The practical application of a bioremediation process can be justified only when the biodegradation rates are higher than the natural attenuation. One of the difficulties of developing bioremediation strategies lies in achieving as good or better results in the field as in the laboratory (Juhasz *et al*. 2000). The strong sorptive capacity of hydrocarbons to organic matter present in the polluted habitats result in low biodegradation rates during natural attenuation of pollutants or in engineered remediation process (Sabate *et al*. 2006). This makes the stakeholders to lose their credibility over usage of biological systems to cleanup these toxic sites. The use of chemical surfactants to enhance the mobility of pollutants in polluted sites results in secondary pollution (Franzetti *et al*. 2006). The composition of the diesel wastes is usually very complicated especially at sites with aged contamination where linear long chain alkanes and cyclic alkanes are the main components. Hydrocarbon bioavailability is increased by cyclodextrins that form water soluble inclusion complexes. Bardi *et al.* (2000) conducted a laboratory experiment to show how β-cyclodextrin increased the degradation of four hydrocarbons, a medium chain (dodecane) and a long chain (tetracosane) aliphatic hydrocarbon and two polyaromatic hydrocarbons (naphthalene and anthracene). Usage of cyclodextrin as bioavailability enhancing agents for degradation of hydrocarbons seems to be promising (Sivaraman *et al*. 2010). But the high cost of cyclodextrins makes its usage economically non-viable in the field scale remediation of polluted sites. Cyclodextrins can be produced by the action of cyclodextrin glucanotransferase (CGTase) enzyme on starch due to intermolecular transglycosylation reactions. The CGTases are known to be produced by various genera of bacterial kingdom such as *Bacillus, Klebsiella, Pseudomonas, Brevibacterium, Thermoanaerobacterium, Corynebacterium, Micrococcus, Clostridium* etc. All known CGTases produce α- β-, and γ-CDs from starch in different ratios (Gawande *et al.* 1999). In the present study an attempt has been made to purify the CGTase enzyme and study its role in biodegradation of petroleum hydrocarbons present in diesel oil in the presence of starch.

2. Materials and methods

2.1 Chemicals

All the chemicals (Bushnell Hass Broth, Starch agar Broth, Ammonium sulphate, Sodium hydroxide, Ethanolamine, sodium chloride, sodium acetate, Tris-Hydrochloride, glycine, potassium phosphate, SDS, Acrylamide, Bis-acrylamide, Ammonium persulphate, TEMED, Silver nitrate, Sodium alginate, Calcium chloride and Ethanol) used in this study were of analytical grade purchased from Himedia, Mumbai, India. Diesel oil was purchased from a local fuel re-filling station and used in the experiments as received. Epoxy activated Sepharose 6B was purchased from Sigma-Aldrich, Mumbai, India. Hexane and Ethyl acetate were of analytical reagent grade purchased from SD fine chemicals, Mumbai, India

2.2 Microbial cultures

Pseudomonas mendocina, a hydrocarbonoclastic bacteria used in this study was isolated from bilge oil contaminated waters. This strain is known to degrade aliphatic hydrocarbons (Sivaraman *et al*. 2011). *Enterobacter cloacae* that was isolated from garden soil produces cyclodextrins from starch due to the action of cyclodextrin glucanotransferase (Sivaraman *et al*. 2012).

2.3 Purification of CGTase enzyme

Enterobacter cloacae were grown in starch broth till its late log phase. The supernatant separated by centrifuging the culture at 4000 r/min for 10 minutes at 4ºC served as a source of crude enzyme. All the subsequent purification steps were carried out at 4ºC. CGTase enzyme present in the supernatant was precipitated overnight using ammonium sulphate to a saturation of 70%. The resultant precipitate was separated by centrifugation at 4000 xg for 20 minutes at 4ºC. The precipitate was resuspended in 800 ml of acetate buffer pH 5.5. α-cyclodextrin bound epoxy activated sepharose 6B column was prepared according to the protocol described previously (Sian *et al*. 2005). Briefly, Epoxy activated sepharose (5 g) was washed with about 1 litre of distilled water on a sintered glass filter. 20 ml of coupling solution (350 mg α-cyclodextrin in 20 ml of 0.1 M NaOH) was reacted with the sepharose 6B gel for 16 hours at 45ºC. Following incubation, the gel was washed again with distilled water for 1 hour. Then the washed gel was transferred to 1 M ethanolamine solution (pH 8.0) to block any unreacted epoxy groups and was incubated for 24 hours at 50ºC. The gel was then washed with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris–HCl buffer pH 8.0 containing 0.5 M NaCl. This cycle was repeated three times. Finally, the gel was packed into a 15 mm × 100 mm column and equilibrated with two bed volumes of 0.01 M sodium acetate buffer pH 5.5. The mixture was filtered through Whatmann filter paper to remove any insoluble material before loading onto the column. The column was equilibrated with acetate buffer at a flow rate of 21 ml/hr before loading the mixture containing 0.08 mg/mL of protein. After loading the mixture, the column was washed with acetate buffer pH 5.5 to remove the unbound proteins. The desired CGTase enzyme was eluted by loading 1% α-cyclodextrin in acetate buffer pH 5.5 onto the column. The eluents were collected as 3 ml fractions from the column assayed for CGTase activity. The fractions showing CGTase activity were pooled up and dialyzed against acetate buffer pH 5.5 in a regenerated cellulose tubing (PIERCE 10,000 MWCO). Change of buffer was done three times during the course of the dialysis.

2.4 Effect of pH and temperature on purified CGTase enzyme

The optimum pH of the purified enzyme was found by reacting the enzyme with soluble starch in buffers at various pH. The buffers used were 0.1 M sodium acetate buffer for pH 4-5, 0.1 M potassium phosphate buffer for pH 6-8 and glycine-NaOH buffer for pH 9. Similarly the optimum temperature was found by incubating the enzyme with soluble starch in phosphate buffer pH 6.0 for 10 minutes at various temperatures ranging from 30ºC-80ºC. CGTase assay was done using phenolphthalein method (Rahman *et al*. 2006).

2.5 Determination of molecular weight of the purified CGTase enzyme

The molecular weight of the purified enzyme was checked in 10% SDS-Polyacrylamide Gel with 97 kDa ladder at a constant voltage of 40 V for 3 hours (Laemmli 1970). The protein bands were visualized by silver staining.

2.6 Immobilisation of P. mendocina on alginate containing starch

Cells of *P.mendocina* were immobilized on sodium alginate and its viability was checked by the

method of Karmalidis *et al*. (2010). *P. mendocina* was grown in Bushnell Hass medium with diesel oil as sole carbon source till its log phase. The culture was centrifuged at 4000 xg for 10 minutes. The pellet was washed with sterile distilled water and centrifuged again. A mixture containing 2% alginate and 2% soluble starch were mixed with 10% of the concentrated culture. Immobilised beads were obtained by adding culture mixture as droplets to 0.1 M calcium chloride using 1 ml pipette. The beads were hardened by incubating the mixture at room temperature for 1 hour. The formed beads were collected by gentle centrifugation (1000 xg) and washed once with 0.9% NaCl containing 5% glycerol and stored at 4ºC. To determine the viable counts, one gram of beads was resuspended in 9 mL of phosphate buffer (0.1 M, pH 7) followed by vigorous vortexing until gel beads were completely dissolved. The colony forming units (*cfu*) were determined by plating on LB agar plates.

2.7 Biodegradation experiments

Garden soil was collected from our institute campus, sieved to remove plant debris and stones. Soil was characterized for its pH, Moisture content, Organic Carbon, Nitrogen and Phosphorous. pH was determined at 1:10 suspensions according to Sakata (1987). Moisture content was determined using oven drying method. Organic carbon and nitrogen were analysed using Walkley-Black method and Kjeldahl method respectively (APHA 1998). Before starting the experiments the soil was sterilized at 121ºC for 1 hour to kill the indigenous microbes present. The biodegradation experiments were carried out in duplicates in borosilicate bottles covered with Teflon cap. Sterile soil was taken in glass trough and diesel oil was added to a concentration of 20% and mixed well with sterile spatula. The experimental setup were as follows: Treatment A: Sterile Garden soil (C: N: P -100:10:1) + 20% Diesel Oil. Treatment B: Sterile Garden soil (C: N: P -100:10:1) + 20% Diesel Oil + *E. cloacae* + 2% Starch. Treatment C: Sterile Garden soil (C: N: P -100:10:1) + 20% Diesel Oil + *P.mendocina* + 2% Starch. Treatment D: Sterile Garden soil (C: N: P -100:10:1) + 20% Diesel Oil + *P. mendocina* + *E. cloacae* + 2% Starch. Treatment E: Sterile Garden soil (C: N: P-100:10:1) + 20% Diesel Oil + immobilised *P. mendocina* containing 2% Starch + Purified CGTase enzyme (65 U/mL). Moisture content of the soil was maintained at 35% in all the setups during the course of the experiment. The parameters analyzed during the course of experiment were residual hydrocarbons, CGTase activity and microbial colony forming units (*cfu*). 2 g of soil was taken in a conical flask and extracted with 20 ml of hexane. The contents were stirred in a magnetic stirrer for 20 minutes and centrifuged to obtain a clear organic phase. Following this, the organic phase was condensed to 1 ml using Rota-evaporator. The samples were analyzed in a gas chromatograph with conditions described previously (Sivaraman *et al*. 2010). 0.5 g of soil was resuspended in saline solution and plated onto Nutrient agar and starch agar to enumerate total and *E. cloacae* as microbial colony counts. 0.1 g of soil was resuspended in 1 ml of phosphate buffer, vortexed for 1 min and centrifuged at 4000 r/min for 5 minutes. The supernatant was used to assay the CGTase activity.

3. Results

3.1 Purification of CGTase enzyme

The cell free supernatant separated from *E. cloacae* was precipitated with ammonium sulphate and

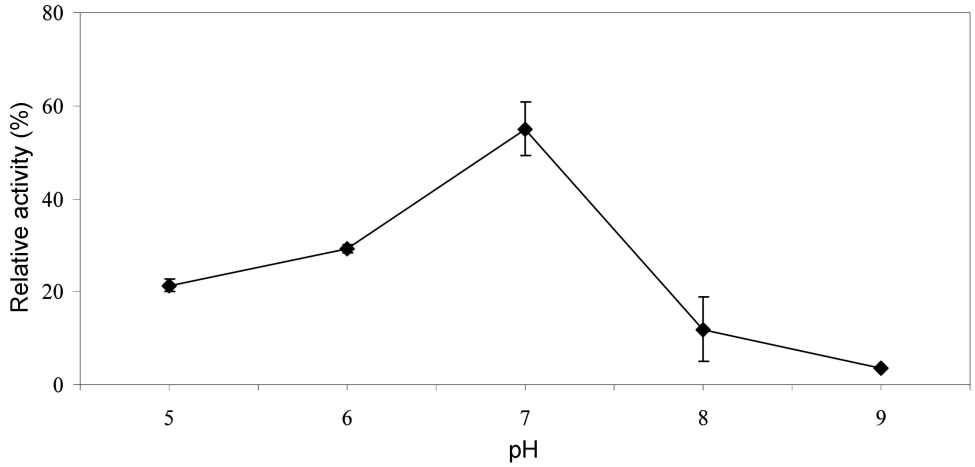

Fig. 1 CGTase activity at different pH

purified using α-cyclodextrin bound epoxy activated sepharose 6B column. The specific activity of the purified CGTase increased to 65 U/ml against the crude CGTase i.e., 11.3 U/ml.

3.2 Effect of pH and temperature on enzyme activity

The purified enzyme was found to be active at a pH range of 5-8 with maximum activity found at pH 7 (Fig. 1). The CGTase was most active in a 35-70^{o}C with maximum activity at 60^{o}C (Fig. 2).

3.3 Molecular weight

The molecular weight of the purified denatured protein was determined to be 66 kDa by SDS-

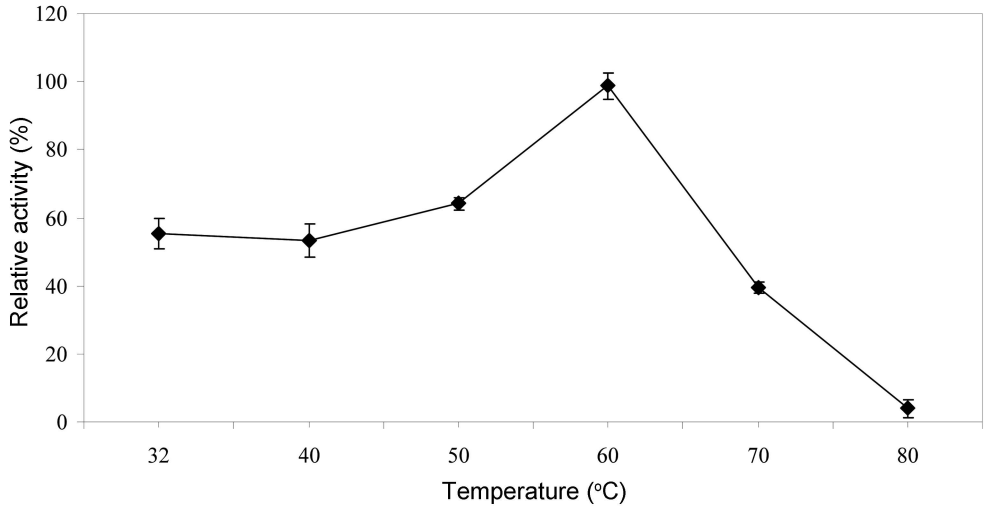

Fig. 2 CGTase activity at different temperature

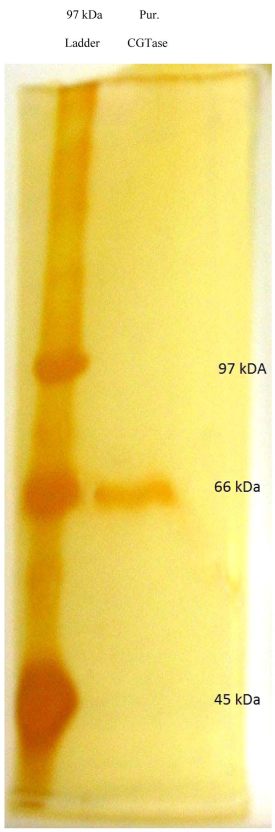

Fig. 3 Determination of molecular weight of CGTase on SDS-PAGE

Polyacrylamide gel (Fig. 3).

3.4 Biodegradation of diesel oil contaminated soil

The effect of purified CGTase enzyme on the biodegradation of diesel oil contaminated soil in the presence of hydrocarbonoclastic *P. mendocina* was studied. A parallel setup was made to compare the effect of *E. cloacae* which produces CGTase enzyme on biodegradation of diesel oil. The pH of the soil was found to be 6.92 at 1:10 suspensions. The residual diesel oil present in treatment *A* and *B* were 94 ± 2.92% and 83 ± 0.19% respectively (Fig. 4). The intrinsic bioremediation activity of a microbe is due to its ability to degrade the pollutants without any additional carbon source. Treatment *C* added with *P. mendocina* left a residual diesel oil of 70 ± 2.49% in 96 hours. At 96 hours enhanced degradation of diesel oil was seen in treatment *E* (63 ± 3.62%) but this was insignificant when compared to treatment *D* (52 ± 1.2%) (Fig. 4). Production of cyclodextrin was initially higher in treatment *E* but over time the activity of the enzyme was decreased (Fig. 5). The enhanced degradation in treatment *D* was due to increase in production of cyclodextrin which was quantified by decrease in optical density of phenolphthalein during CGTase assay. In treatment *D* the enzyme activity increased from 5.7 ± 0.1 to 7.6 ± 0.3 (Fig. 5). *E. cloacae* and *P. mendocina* were visualized as white and orange colored colonies on the nutrient agar plates which enabled their differentiation.

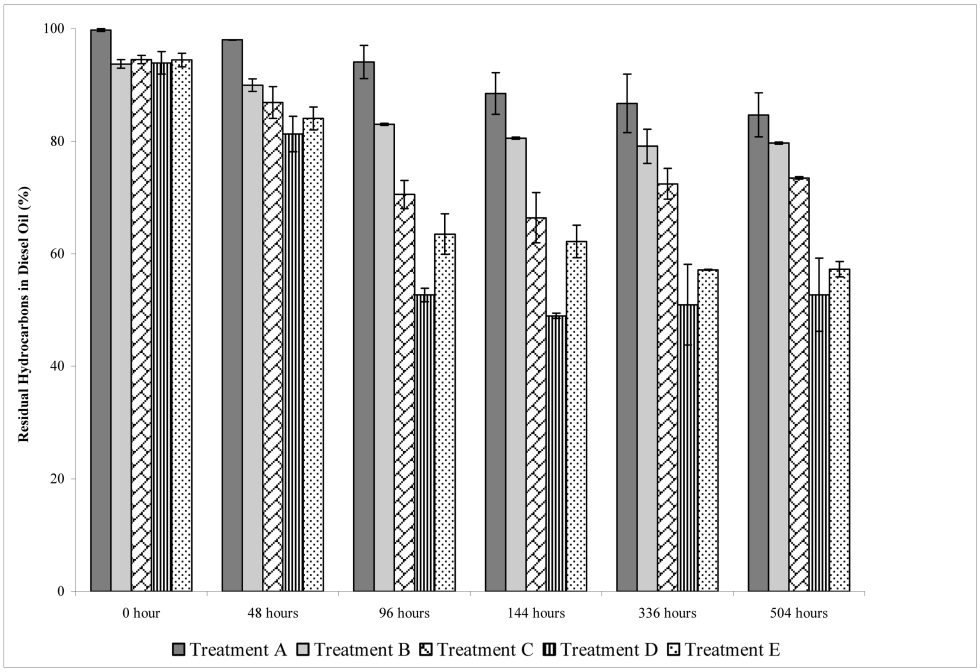

Fig. 4 Residual hydrocarbons in diesel oil during biodegradation experiments

At 96 hours there was increase in *cfu* of *P. mendocina* in treatment *C* and treatment *D* i.e., 66×10^7 and 74×10^7*cfu* respectively (Fig. 6). Immobilised cells of *P. mendocina* survived till 336 hours of the study with a *cfu* of 24×10^7 (Fig. 6). A decrease in population of *E. cloacae* which was observed throughout the study. At 96 hours in Treatment *C* 47×10^7 *cfu* of *E. cloacae* was found which decreased to 0.21×10^7 at 504 hours whereas in treatment *D* the decrease was from 43×10^7 to 0.19×10^7. The residual diesel oil in Treatment *A* (control microcosms) decreased with time, indicating a linear trend in the abiotic loss due to volatilization.

4. Discussion

Bioremediation is a more eco-friendly, cost-effective and efficient environmental clean-up technique when compared to chemical treatment or physical removal of hazardous compounds from contaminated soil or water (Tyagi *et al*. 2011). Micro-organisms utilizing hydrocarbons as energy and carbon source are ubiquitous in nature (Ron and Rosenberg 2001). They are capable of degrading various types of hydrocarbons like short chain, long-chain and aromatic compounds, including polycyclic aromatic hydrocarbons. Biodegradation of hydrocarbons in the polluted environments is affected by number of abiotic and biotic factors (Doyle *et al*. 2008). One of the main abiotic factors which affect the overall bioremediation process is the poor aqueous solubility of hydrocarbons. This results in low bioavailability of hydrocarbons towards the indigenous catabolic microflora. Surfactants can be used to increase the solubility of hydrocarbons but the results obtained while using surfactant as solubilising agent is either stimulatory or inhibitory. There was a

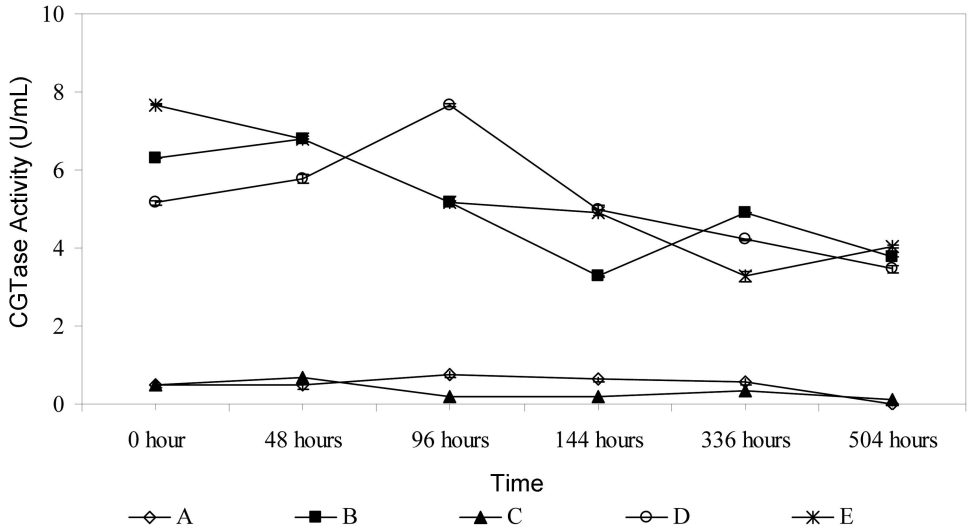

Fig. 5 CGTase assay in biodegradation experiments

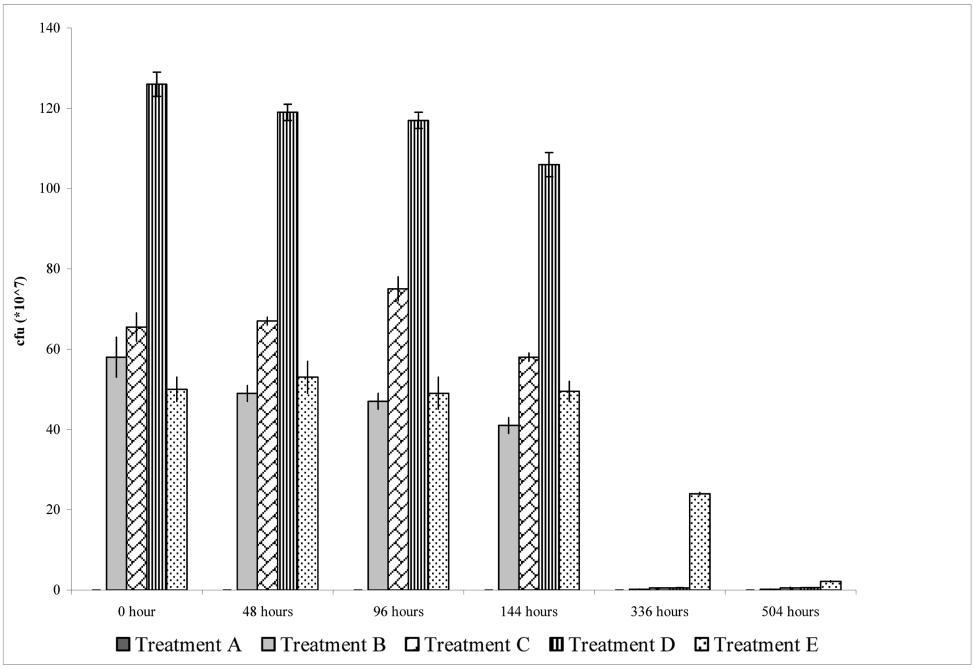

Fig. 6 Microbial counts in biodegradation experiments

debate in the last decade whether the surfactants-solubilised hydrocarbons are bioavailable (Volkering *et al*. 1995, Willumsen and Arvin 1999, Willumsen *et al*. 2001). It is also reported that negative effects of surfactants which are due to the toxicity of surfactants over the microorganisms. There was growing interest on usage of biosurfactants in remediation process but microorganisms

produce biosurfactants for many other purposes apart from increasing the bioavailability of the hydrophobic substrates (Johnsen and Karlson 2004).

Cyclodextrins could be a better alternative. They have a torus-shaped structure with hydrophobic interior cavity and hydrophilic exterior surface and therefore can form inclusion complexes with a variety of hydrophobic guest compounds (Valle 2004). Cyclodextrins are shown to increase the bioavailability of variety of hydrophobic pollutants which results in enhanced biodegradation rates (Bardi *et al*. 2000, Fava *et al*. 2003, Reid *et al*. 2004). In our previous work, we found that biodegradation of hydrocarbons like hexadecane, tetradecane and octadecane was enhanced by β-cyclodextrin (Sivaraman *et al*. 2010). Diesel oil consists mostly of linear and branched alkanes with different chain lengths and contains a variety of aromatic compounds. Many of these compounds, especially linear alkanes, are known to be easily biodegradable (Sticher *et al*. 1997). However, due to their low water solubility, the biodegradation of these compounds is often limited by slow rates of dissolution, desorption, or transport. In general, the bioavailability of hydrophobic compounds is determined by their sorption characteristics and dissolution or partitioning rates and by transport process to microbial cells (Rocha *et al*. 2001). In the present work we studied the biodegradation of diesel oil contaminated soil in the presence of *P. mendocina* (hydrocarbonoclastic bacteria) together with *E. cloacae* (cyclodextrin producing bacteria) so as to enhance the bioavailability of hydrocarbons. Further we studied the effect of purified CGTase on the biodegradation of diesel oil contaminated soil. Cyclodextrins are produced by the action of Cyclodextrin glucanotransferase (CGTase) on starch. CGTase is an extracellular enzyme that produces cyclodextrin from starch using intermolecular transglycosylation reactions.

Purification of CGTase is a critical step which mainly involves use of gel filtration, column chromatograph or combination of both the techniques. CGTase were purified upto 9.5 fold when using a combination of DEAE cellulose and Sepharose CL-6B gel filtration setup (Cao *et al*. 2004). Gawande *et al.* (1999) purified CGTase using series of steps such as Ultra filtration, Starch affinity chromatography and gel filtration and obtained 400 fold purification yield. There are also reports found on low recovery of CGTase when using three to five steps of purification and hence more attention has to be paid in CGTase purification (Lee *et al*. 2006). In our work we used affinity column to purify the CGTase separated from *E. cloacae*. A six-fold purification of CGTase was obtained when using α-cyclodextrin bound epoxy activated sepharose 6B column. As a next step to purification we studied the effect of pH and temperature on the purified enzyme. The purified enzyme was found to be active at a pH range of 5-8 with maximum activity at pH 7. CGTase are active to a maximum in near- neutral pH. Most of the reported CGTase exhibited optimum pH ranging from 5.0 to 8.0 (Nakamura and Horikoshi 1976, Lee *et al*. 1992, Penninga *et al*. 1995). The CGTase was most active from 35-70^{o}C with maximum activity at 60^{o}C. This result is in agreement with the results obtained by the previous researchers (Rahman *et al*. 2006, Sian *et al*. 2005). The molecular weight of the purified protein on 10% SDS-Polyacrylamide gel was found to be 66 kDa which is very close to the reported molecular weight of CGTase. Purified CGTase are monomeric and had a molecular weight range of 68-88 kDa. Next to purification and characterization, the effect of purified CGTase on biodegradation of diesel oil contaminated soil was studied. To account for intrinsic bioremediation activity of *P. mendocina* an additional setup was made which resulted in $73 \pm 0.21\%$ residual diesel oil at the end of the study. This strain harbors catabolic genes such as *C23DO, nid* and *ndo* and is capable of degrading aliphatic hydrocarbons which was evident from our previous study (Sivaraman *et al*. 2011).

Addition of Purified CGTase showed enhanced bioavailability of diesel oil which resulted in

increased biodegradation in the presence of *P. mendocina* (57 ± 1.37%) but no significant activity when compared to the treatment setup added with *P. mendocina* and *E. cloacae* (52.7 ± 6.51%). The difference in extent of could be possibly explained by the fact that the activity of purified CGTase enzyme could be decreasing when added to the natural environments where the optimum conditions for increased enzyme activity are not maintained. Immobilization of *P. mendocina* was done in alginate along with starch (Karmalidis *et al*. 2010) so that action of CGTase on starch occurs close to *P. mendocina* which might in turn help in enhanced bioavailability of hydrocarbons. Immobilization of *P. mendocina* in alginate showed better survival in the treatment setup which formed 24×10^7 *cfu* when compared to free cells in Treatment *B* 0.205×10^7 *cfu* at 336 hours. A dying population of *E. cloacae* was found throughout the course of the study which could be attributed to toxicity of diesel oil and also may be due to addition of starch without any pre-treatment.

Gelatinization or physical treatment opens up the structure of starch granules, which become susceptible to CGTase action, whereas raw starch as such should be relatively inaccessible to the enzyme (Lee and Park 1991). In the present study starch was added without any pretreatment which could be the reason for low cyclodextrin production which was visualized by CGTase assay. Several mechanisms had been suggested to be the main causes for decline of added inoculants in the contaminated soil which includes water tension, pH, temperature, chemicals (toxic waste) organic and inorganic nutrients (Veen *et al*. 1997). We overcame the above causes by sterilizing the soil which was apparent from nil *cfu* formation in Treatment *A* i.e., Abiotic control, adjustement of C:N:P ratio and maintaining the moisture content.

5. Conclusions

In the present work purification of CGTase from *E. cloacae* was done which resulted in 6 fold increase in enzyme activity. The enzyme was active at neutral pH and 60°C. The molecular weight of the purified enzyme was found to be 66 kDa. Addition of purified enzyme in a diesel oil treatment setup greatly enhances the biodegradation of petroleum hydrocarbons present in the diesel but not different from the treatment setup with *P. mendocina* and *E. cloacae* added together (Treatment *D*). Immobilization of *P. mendocina* on alginate and starch resulted in better survival (24×10^7 *cfu* in Treatment *E* when compared to free cells in Treatment *B* 0.205×10^7 *cfu* at 336 hours.) which resulted in enhanced biodegradation of hydrocarbons in diesel oil which might be due to the action of CGTase on starch occurring close to *P. mendocina*. To conclude addition of hydrocarbonoclastic bacteria together with CGTase producing strain will be a good strategy to enhance the biodegradation of hydrocarbons.

Acknowledgements

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