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CLONING AND EXPRESSION OF CATECHOL 2, 3 DIOXYGENASE AND CYCLODEXTRIN GLUCANOTRANSFERASE GENE IN *E.COLI* AND ITS ROLE IN BIODEGRADATION

SIVARAMAN C, ANASUYA GANGULY AND SRIKANTH MUTNURI*

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

ABSTRACT

Hydrocarbons are the organic contaminants that enter the environment through natural and anthropogenic processes. Microbial degradation of hydrocarbons is considered as cost effective treatment option. The mass transfer between hydrocarbons to catabolic microflora is highly influenced by its lower aqueous solubility that limits the biodegradation rates. Cyclodextrins with its interior hydrophobic cavity can increase the bioavailability of the hydrophobic compounds. To enhance the biodegradation of hydrocarbons, a catabolic gene (catechol 2, 3 dioxygenase) that is responsible for initial attack on hydrocarbons and cyclodextrin glucanotransferase gene that is responsible for converting starch into cyclodextrins were expressed together in *E.coli*. The recombinant *E.coli* was tested for its enhanced degradation in the presence of naphthalene in the presence of starch. At 96 hours enhanced degradation of naphthalene was found in the samples with recombinant *E.coli* and starch (35.42% $\pm$ 3.42) when compared with biotic control i.e. samples with recombinant *E.coli* (62.08% $\pm$ 1.56).Thus the strategy used in the present study enhances the bioavailability of naphthalene which in turn results in enhanced biodegradation.

Keywords: bioavailability, Naphthalene, Cyclodextrin glucanotransferase gene, bioremediation.

INTRODUCTION

Oils are mixtures of both aliphatic and aromatic hydrocarbons, and the level of hydrocarbon contaminants in the environment is a cause for concern, as they are recalcitrant and exerts toxic and carcinogenic effects on biological receptors (Cerniglia, 1992). They enter into the environment as a result of natural and anthropogenic processes. The fate of these contaminants is associated with both abiotic and biotic processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and microbial transformation (Danne *et al*., 2001). Among these processes, microbial transformation is nature's way of cleanup that results in less or no harm to the environment. The use of microbes to clean up polluted sites is termed as bioremediation which is conceived as an attractive alternative to conventional treatment methods. Microbial diversity has been exploited in the recent decades for its ability to degrade hydrocarbons. However, the low water solubility of hydrocarbons makes its availability less in the aqueous phase where the degrading microbes are present. This lowers the efficiency of bioremediation. Hydrocarbon degrading bacteria use different strategies to cope with this limitation (Wick *et al*., 2002). One strategy of bacteria to

enhance the mass transfer is to produce biosurfactants (Ron & Rosenburg 2002). Another strategy is to grow attached to non-aqueous phase liquids or solid hydrophobic surfaces as substrates with increased mass transfer driven by steep concentration gradient (Mutnuri *et al*., 2005). This strategy requires certain adaptations such as hydrophobic cell surfaces or enhanced fluidity of the lipid membrane as shown recently for *Mycobacteria* (Wick *et al*., 2002; Wick *et al*., 2003). The third strategy is to develop specific high affinity uptake systems. Specific uptake systems may target either the compound of interest itself or may be mediated by carrier molecules such as sorptive or complexing agents such as cyclodextrins (Bardi *et al,* 2000; Sivaraman *et al,* 2010). The high cost of cyclodextrins makes its usage economically non-viable in field scale remediation. Cyclodextrins are produced by the action of cyclodextrin glucanotransferase (CGTase) enzyme on starch. In the present study an attempt has been made to express the CGTase gene and a catabolic gene in *E.coli* as a strategy to enhance the bioavailability of hydrocarbons. The increased bioavailability will in turn help in increasing the biodegradation of hydrocarbons and thus resulting in enhanced bioremediation. In the present study we isolated CGTase producing strain and expressed the CGTase gene in *E.coli* along with catechol 2, 3 dioxygenase, a catabolic gene from a hydrocarbon degrading *Pseudomonas mendocina*. Isolation and characterization of *Pseudomonas mendocina* was shown in our earlier work (Sivaraman *et al*., 2010). This was followed by studying enhanced biodegradation of hydrocarbons in the presence of recombinant *E.coli* and a cheaper substrate i.e. starch.

MATERIALS AND METHODS

1. CHEMICALS

Deoxynucloeotides and *Taq* polymerase with necessary buffers were purchased from Sigma Aldrich Mumbai India. p-GEM Easy Vector System kit was obtained from Promega USA and Synthesized Oligonucleotides were purchased from IDT USA through Bioresource Pvt. Ltd., Pune India. All the other reagents used in the study were purchased from Himedia India at highest grade of purity available.

2. ISOLATION AND CHARACTERIZATION OF STARCH DEGRADERS FOR β-CYCLODEXTRIN PRODUCTION

1g of subsurface garden soil was sampled in sterile sampling bags from ten different places within our institute campus and was inoculated into ten different 250ml conical flasks containing 100 ml of sterile starch broth (peptone 5g/L; beef extract 3g/L; starch 2g/L) each. The conical flasks were then incubated in a rotary shaker (at 110 rpm, 37°C). At late log phase, 1ml of the cultures were sub-cultured into starch broth and incubated at conditions as described above. Streak plating was done to isolate the starch degraders. Around 40 individual distinct colonies were isolated and were stored at 4°C. CGTase activity of the starch degrading isolates was measured as β-CD forming activity by phenolphthalein method (Rahman *et al*., 2006). Briefly the protocol is as follows- The starch degrading isolates were grown in sterile starch broth. The cells were harvested at late log phase by centrifuging the culture at 11180 x g for 10 mins in a refrigerated centrifuge (Eppendorf, Hamburg, Germany). The supernatant was separated by centrifugation from starch degrading isolates and was used as source of crude enzyme for detecting CGTase activity The reaction mixture containing 0.04g starch in 0.1 M phosphate buffer (pH 6.0) and 1 ml enzyme solution were mixed. The mixture was incubated at 60°C for 10 min in a water bath. The reaction was stopped by adding 3.5 ml of 0.03 M NaOH solution. 0.5 ml of 0.02% (w/v) phenolphthalein in 0.005 M sodium carbonate was then added to the reaction mixture. After 15 min, the decrease in colour intensity was measured at 550 nm. The percentage of reduction in the original colour intensity was interpreted with a standard curve (% OD reduction versus β-CD in mg produced) for the calculation of CGTase activity. One unit of enzyme activity was defined as the amount of enzyme that forms l μmol of β-CD from soluble starch in 1 min. A standard graph was prepared by measuring the optical density with increasing concentrations of β-CD complexed to

definite amount of phenolphthalein. Among the 40 starch degrading isolates, 11 strains showed β-CGTase enzymatic activity. These cyclodextrin producing isolates were used in further experiments.

3. CONFIRMATION OF CGTase GENE IN β-CYCLODEXTRIN PRODUCING ISOLATES

The cyclodextrin producing isolates were grown in sterile starch broth. The cells were harvested at late log phase by centrifuging the culture at 11180 x g for 10 mins in a refrigerated centrifuge (Eppendorf, Hamburg, Germany). The supernatant was discarded and the cell pellet was resuspended in 100µL of sterile distilled water. DNA was extracted from the suspension according to Marmur 1961 with minor modifications. The purity of the extracted DNA was tested by running 1% agarose gel electrophoresis with 1X TAE as running buffer. PCR was performed with primers coding for CGTase gene as per literature survey. The primer set (256f and 570r) was chosen because this region encodes for partial sequence of CGTase gene. The reaction conditions were followed as per the literature. For the positively amplified strains, a PCR was performed using primer sets coding for the complete sequence of CGTase gene (256f and 2078r) (Vollu *et al*., 2008). The amplicons were visualized by running 2% agarose gel along with a 100bp ladder.

4. EXPRESSION OF CATABOLIC GENE AND CGTase GENE IN E.coli

E.coli were used as competent cells. Log phase culture of *E.coli* was aliquoted into eight 50-ml prechilled, sterile polypropylene tubes and the tubes were left on ice for 5 to 10 min. The cells were then centrifuged for 7 min at 1600 × g, 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution. The cells were centrifuged for 5 min at 1100 × g, 4°C. The supernatant was decanted and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution and the cells were kept on ice for 30 min. The cells were then centrifuged for 5 min at 1100 × g, 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml ice-cold $CaCl_2$ solution. The cells were then dispensed into prechilled, sterile polypropylene tubes and were freezed immediately at −70°C.

The PCR product of C23DO gene amplified from *P. mendocina* (Sivaraman *et al*., 2011) and CGTase gene were purified to remove the excess PCR components and non-specific bands which may interfere with the efficiency of ligation reactions. The insert was purified from 1% agarose gel using gel extraction kit (Axygen Biosciences, India). The insert was quantified using Agarose gel electrophoresis with lambda-DNA as standard.

Ligation and Transformation reactions were performed according to p-GEM easy vector system II kit. Ligation reaction was performed at 1:3 ratio of vector to insert. After a complete mixing of the reaction mixture with a pipette, the reaction tubes were incubated overnight at 4°C for enhancing ligation. The constructs containing CGTase gene and C23DO gene were transformed into *E.coli* cells using Heat-Shock method (Ausubel *et al*. 2003). To confirm the transformation of the constructs, LB/Ampicillin/X-gal/IPTG plates were used. The plates were incubated at 37˚C overnight. White colonies were picked up and inoculated in starch modified media which contain 0.2% hydrocarbon and ampicillin (0.1mg/mL). At late log phase the cells were harvested and inoculated in mineral medium which contains starch and hydrocarbon as the sole carbon source. 2 mL of the log phase culture was used in plasmid isolation (Birnboim and Dolly 1979) to confirm the presence of C23DO and CGTase genes in the recombinant *E.coli* using PCR as described before.

5. CONFIRMATION OF β-CYCLODEXTRIN PRODUCTION BY THE RECOMBINANT E. coli

The recombinant bacterium was checked for the β-Cyclodextrin production using optimised medium (Ibrahim *et al*., 2005). The starch concentrations used in the experiments were 2% and 4% keeping all other constituents as constant as per literature. The β-CD producing activity of the recombinant strain was compared with the native isolate.

6. NUCLEOTIDE SEQUENCING

The plasmid from the rec. *E.coli* was isolated and amplification of C23DO gene was done using PCR. Following this the C23DO PCR product was purified to remove unbound labeled and unlabeled nucleotides and salts. The purified reaction was loaded onto the 96 capillary ABI 3700 automated DNA analyzer, and electrophoresis was carried out for 4 h. The nucleotide sequences obtained from the ABI DNA analyzer were studied using the BLAST software available on the NCBI website (www.ncbi.nlm.nih.gov). After editing the sequence, the BLAST software was used to identify the nucleotide sequence.

7. BIODEGRADATION OF HYDROCARBONS IN THE PRESENCE OF RECOMBINANT E.coli

Biodegradation experiments were performed in the presence of naphthalene to investigate the enhanced biodegradation of the recombinant bacterium. The recombinant *E.coli* harboring C23DO gene and CGTase gene was tested for its ability to enhance and degrade the hydrocarbon was studied. The experimental design was as follows: abiotic control - BH medium + 0.2 % starch + 0.1 mg/mL Ampicillin + 100mg of naphthalene. Biotic control- BH medium + 0.1 mg/mL Ampicillin + 100mg of naphthalene + 1 ml of rec. *E.coli* cell suspension. Test sample - BH medium + 0.2 % starch + 0.1mg/mL ampicillin + 100mg of naphthalene + 1 ml of rec. *E.coli* cell suspension. The following parameters were analyzed every 24 hours for 120 hours. Estimation of Residual naphthalene, protein analysis in the biomass and CGTase activity in the bioremediation experiments were carried out. The amount of residual naphthalene and proteins in the biomass were quantified as explained previously (Sivaraman *et al*., 2010). 3µL of organic phase containing naphthalene was injected into fused silica capillary column in Chemito GC Chromatograph equipped with flame ionisation detector. The GC conditions were: 50°C for 2 min. 5°C rise upto 250°C and hold for 2 min. The injector and detector temperature were maintained at 250°C. The carrier gas used was nitrogen at a flow rate of 25mL/min. One ml of the medium is sampled out from all the conical flasks and centrifuged in a refrigerated centrifuge at 12000rpm for 10 mins. The supernatant obtained was used to assay the CGTase activity. 0.5 mL of the supernatant was taken as a source of crude enzyme and the experimental protocol was followed as explained before to quantify the enzymatic activity.

RESULTS AND DISCUSSION

The primary challenges for designing effective bioremediation methodologies are: complexity of petroleum hydrocarbons combined with their low bioavailability, hydrophobic nature, strong sorption phenomena and high persistence in the contaminated site. The efficiency of bioremediation will depend on the extent of the bioavailability of the contaminant, its mass transfer rate and subsequent metabolism by the microflora (Guerin 1999; Mohan *et al*., 2006). Bioavailability of hydrocarbons is influenced by many factors including chemical structure, molecular weight and toxicity (Doyle *et al*., 2008). Synthetic surfactants had been used to enhance the bioavailability but their release in environment leads to possible risks of contamination (Wang *et al*., 1998). Hence use of surfactants can be matter of debate in the remediation of hydrocarbons became a successful remediation should not culminate with secondary pollution. Cyclodextrins are cyclic molecules with hydrophobic interior cavity and hydrophilic shell towards the exterior. The structural configuration enables cyclodextrin to bind with variety of molecules and can change the physicochemical properties of the guest molecules (Valle 2004). Recalcitrance of hydrocarbons after its release in the environment is due to its poor water solubility. In our previous study, we concluded that addition of cyclodextrins enhanced the bioavailability of hydrocarbons which in turn increased their degradation (Sivaraman *et al*., 2010). However high cost of cyclodextrins makes their usability economically non-viable in field scale remediation. In the present study a new strategy was adopted wherein a catabolic gene responsible for hydrocarbon degradation and CGTase gene responsible for producing cyclodextrins were expressed together in *E.coli* and its efficiency in degrading naphthalene was studied.

1. CHARACTERISATION OF STARCH DEGRADERS FOR CYCLODEXTRIN PRODUCTION

There are enzymes similar to amylases that can convert starch to various products. One such enzyme is Cyclodextrin glucanotransferase (CGTase) which can hydrolyse starch (Biwer *et al* 2002). Cyclodextrin glucanotransferase (CGTase) catalyses the conversion of starch into cyclodextrins which are capable of forming inclusion complexes with many hydrophobic compounds including hydrocarbons (Jeang *et al* 2005; Rahman *et al* 2006). As an initial step, forty starch degrading strains were isolated from garden soil using standard microbiological procedures. The isolates were maintained in slants and stored at 4 ºC. Cyclodextrin glucanotransferse is an extracellular enzyme and hydrolyses starch to cyclodextrins (Rahman *et al*., 2006). The supernatant obtained from the late log phase cultures of the individual strains were used to analyse the production of β-CGTase. The optical density values were compared with the standard values obtained from the aliquots of increased concentrations of β-cyclodextrin with definite volumes of phenolphthalein. The supernatant of 11 strains showed decrease in phenolphthalein's colour indicating production of cyclodextrins. Cyclodextrins forms inclusion complexes with phenolphthalein and results in decrease in intensity of colour of phenolphthalein (Qi & Zimmerman 2005).

2. CONFIRMATION OF CGTase GENE IN CYCLODEXTRIN PRODUCING ISOLATES

The DNA samples obtained by extraction from the 11 probable strains for cyclodextrin production were used as template for detecting Cyclodextrin glucanotransferase gene by polymerase chain reaction. The primers for this study were selected from the work of Vollu *et al* 2008. Out of 11 strains, positive amplifications were obtained for 3 strains using the primers (256F and 570R) that code for partial sequence of CGTase gene. The DNA from those 3 strains was further used as template to amplify for the complete sequence of CGTase gene using the primers 256F and 2078R. Initially, multiple bands were obtained while attempting to amplify the complete sequence using CGTase gene specific primers for all the three strains. The band corresponding to 1,822 bp was excised from the agarose gel and purified and a second round amplification was done using the purified template. A single band was obtained corresponding to 1822 bp in all the 3 strains confirming the presence of CGTase gene. Randomly, one strain was selected to express the CGTase gene in the cloning experiments.

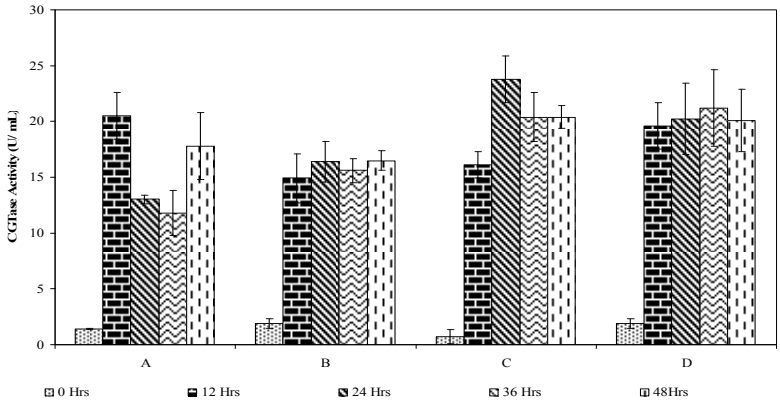
3. EXPRESSION OF CATABOLIC GENE AND CGTase GENE IN *E.coli*

The PCR product of C23DO and CGTase gene were ligated with p-GEM vector and transformed into *E.coli*. Positive clones were picked up and grown in expression medium containing 0.2% peptone, 0.2% starch, and 0.1mg/mL of ampicillin and 0.5% of hydrocarbon. Initially the growth of the recombinant *E.coli* was very slow (Growth rate at 48 Hrs = 0.0372). After two successive transfers, the growth of *E.coli* became stabilized gradually (Growth rate at 48 Hrs = 0.0163).

4. PRODUCTION OF CYCLODEXTRINS BY RECOMBINANT *E.coli*

The β-CGTase activity of the recombinant *E.coli* was compared with the activity of the native isolate. The enzymatic activity of the recombinant *E.coli* was less when compared with the native isolate (Figure 1). Cyclodextrins are produced by action of cyclodextrin glucanotransferase enzyme on starch (Valle *et al*., 2004). Since starch is the main substrate for biosynthesis of cyclodextrins, two different concentrations of starch were added to the medium and cyclodextrin production was studied (Ibrahim *et al*., 2005). However no significant difference in cyclodextrin yield was observed on increasing the concentration of starch. This pattern was found both in recombinant *E.coli* and native isolate. The maximum enzyme activity was recorded at 12 hrs for both the recombinant *E.coli* and native isolate with 2% starch, whereas the activity was comparably less with 4% starch. Reduction in the phenolphthalein intensity was recorded through the entire duration of study, indicating the sustained production of cyclodextrins. This implies that produced β-cyclodextrin accumulates in the medium. In our previous studies,

2.5mM of cyclodextrin resulted in enhanced biodegradation of hydrocarbons, when compared to 5mM of cyclodextrin (Sivaraman *et al*., 2010). In the present work, the amount of cyclodextrin produced by the rec. *E.coli* is nearly 140 folds less than the concentration previously used. However since cyclodextrin was being produced by the recombinant isolate, a biodegradation experiment was attempted using the recombinant isolate.

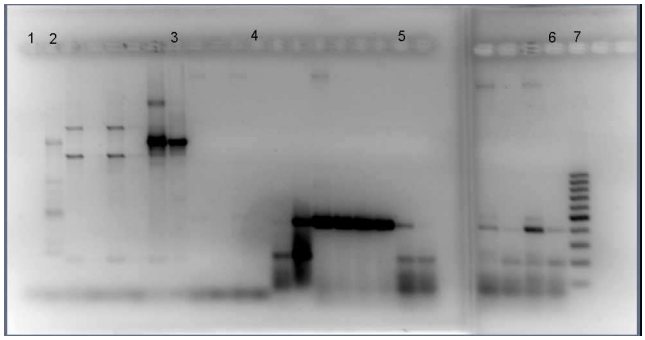


A: E.cloacae + 2% Starch B: E.cloacae + 4% Starch C: Rec. E.coli + 2% Starch D: Rec. E.coli + 4% Starch

Figure 1: ***Comparative analysis of β-CGTase activity by recombinant E.coli and E.cloacae with varied concentrations of starch***

5. BIODEGRADATION OF NAPHTHALENE IN THE PRESENCE OF RECOMBINANT *E.coli*

Naphthalene was selected as the model compound because meta-cleavage of catechol like metabolites from a wide range of aromatic compounds was mediated by C23DO enzymes (Junca *et al*., 2004; Mesarch *et al*., 2004). C23DO genes are known to occur in bacteria that catabolise a wide range of pollutants like benzene, toluene, xylenes, phenol, naphthalene and other aromatic compounds. Further, it is the simplest and most soluble PAH being a frequent constituent of PAH polluted environments (Pumphrey & Madsen 2007). Therefore a study was carried out to check for enhanced biodegradation of naphthalene in the presence of rec. *E.coli*. Before starting the biodegradation experiments, the presence of CGTase and C23DO gene in rec. *E.coli* was confirmed using PCR (Figure 2). The PCR product of C23DO was sequenced and it was matched with the sequences available in the NCBI website using BLAST. The sequence showed 100 % similarity with the Uncultured bacterium C23O gene for catechol 2,3-dioxygenase (Kasuga *et al*., 2007). The amount of residual naphthalene was quantified every 24 hours for a span of six days. Estimation of proteins in the biomass was performed to correlate amount of degradation with increase in biomass. Further, CGTase assay was performed with the supernatant to know the extent of the enzyme activity during the degradation experiments.



1-CGTase negative control; 2-CGTase positive control; 3-Recombinant CGTase; 4-C23DO negative control; 5-Recombinant C23DO; 6-C23DO positive control; 7-100bp ladder.

Figure 2: ***Visualization of CGTase and C23DO PCR products in 2% agarose gel***

In the beginning native *E.coli* was supplied with naphthalene as sole carbon source and its growth curve was followed. There was no apparent increase in the optical density at 600 nm. The degradation time of naphthalene was shortened on the addition of starch to the recombinant *E.coli*, possibly due to the production of cyclodextrins. The percentage of residual naphthalene present in the biotic control (samples with recombinant *E.coli*) was less than the test sample (sample with recombinant *E.coli* and starch) at 48 hours. The highest concentration of residual naphthalene in the test sample may be explained by the fact that adequate complexation time to increase the solubility was necessary to enhance the degradation of hydrocarbons (Badr *et al*., 2004; Sivaraman *et al*., 2010). At 96 hours the percentage of residual naphthalene found in the test sample and biotic control were 35.42% $\pm$ 3.42 and 62.08% $\pm$ 1.56 respectively (Figure 3). The enhanced degradation was supported by consistent reduction in phenolphthalein intensity in the test sample indicating production of β-cyclodextrins (Figure 4). The growth of biomass in the biotic control was slightly lower when compared with the test sample at 96 hours (Figure 5). There was a consistent production of cyclodextrins in the test sample during the biodegradation experiments which was confirmed by reduction in phenolphthalein intensity while assaying for the supernatant for β-CGTase activity. This indicates that the production of cyclodextrins possibly led to enhanced availability of naphthalene for the recombinant *E.coli*. It is a well known fact that β-cyclodextrins increase the bioavailability of hydrocarbons and hence increased biodegradation (Schwartz and Bar 1995; Bardi *et al*., 2000).

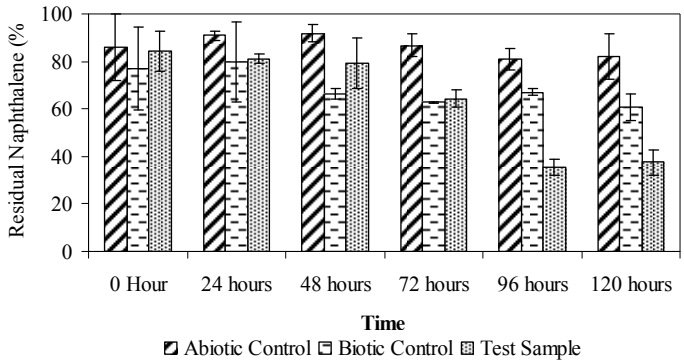


Figure 3: ***Biodegradation of Naphthalene in the presence of recombinant E.coli***

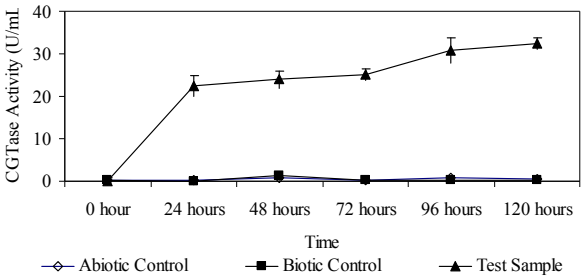


Figure 4: ***CGTase activity in the biodegradation experiments***

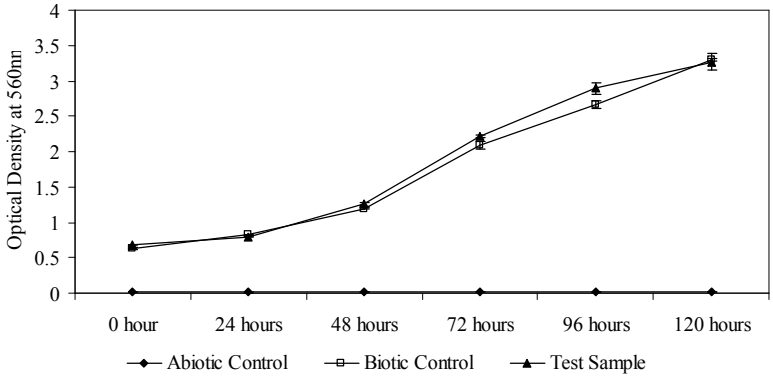


Figure 5*: Biomass assay in the biodegradation experiments*

Catechol 2, 3 dioxygenase is a key enzyme in naphthalene degradation since the pathways converges through catechol. However C23DO is not responsible for the initial attack. Recombinant *E.coli* with C23DO gene and CGTase showing increased degradation of naphthalene may be due to close linkage of genes for naphthalene metabolism and catechol degradation through the meta-pathway (Dunn and Gunsalvs 1973). Also there are several examples which indicate that *E.coli* is able to complement missing ferredoxin reductase subunits leading to active initial dioxygenases (Simon *et al* 1993, Eaton *et al*. 1998, Moser and Stahl 2001). However the above two hypothesis needs to be verified with respect to the recombinant *E.coli* generated in this study.

CONCLUSIONS

There are number of biotic and abiotic factors which affect the microbial degradation of hydrocarbons in the environment (Doyle *et al*., 2008). One of the critical abiotic factors is the poor aqueous solubility of hydrocarbons which greatly influences its bioavailability and thus impacts the effectiveness of bioremediation (Wang *et al*., 2005). Cyclodextrins formed during the action of CGTase on starch enhances the bioavailability of hydrocarbons. The recombinant *E.coli* containing catabolic gene and CGTase gene was tested for its enhanced degradation in the presence of naphthalene. In the presence of starch, recombinant *E.coli* showed significant degradation of naphthalene when compared to the biotic control. Therefore we conclude that the expression of CGTase gene together with a catabolic gene in *E.coli* will enhance bioavailability and hence increases biodegradation which could be an economically viable option for bioremediation.

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