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ORIGINAL PAPER

Degradation of ethylbenzene by free and immobilized *Pseudomonas fluorescens*-CS2

Suneetha Parameswarappa · Chandrakant Karigar · Manjunath Nagenahalli

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Abstract *Pseudomonas fluorescens*-CS2 metabolized ethylbenzene as the sole source of carbon and energy. The involvement of catechol as the hydroxylated intermediate during the biodegradation of ethylbenzene was established by TLC, HPLC and enzyme analysis. The specific activity of Catechol 2,3-dioxygenase in the cell free extracts of *P. fluorescens*-CS2 was determined to be 0.428 μmoles min^{-1} mg^{-1} protein. An aqueous-organic, Two-Phase Batch Culture System (TPBCS) was developed to overcome inhibition due to higher substrate concentrations. In TPBCS, *P. fluorescens*-CS2 demonstrated ethylbenzene utilization up to 50 mM without substrate inhibition on inclusion of *n*-decanol as the second phase. The rate of ethylbenzene metabolism in TPBCS was found enhance by fivefold in comparison with single phase system. Alternatively the alginate, agar and polyacrylamide matrix immobilized *P. fluorescens*-CS2 cells efficiently degraded ethylebenzene with enhanced efficiency compared to free cell cultures in single and two-phase systems. The cells entrapped in ployacrylamide and alginate were found to be stable and degradation efficient for a period of 42 days where as agar-entrapped *P. fluorescens* was stable and efficient a period of 36 days. This demonstrates that alginate and polyacrylamide matrices are more promising as compared to agar for cell immobilization.

Keywords Ethylbenzene · *Pseudomonas fluorescens* · Biodegradation · TPBCS · catechol · Immobilization

S. Parameswarappa · C. Karigar (✉) · M. Nagenahalli
Bioremediation Laboratory, Biochemistry Division, Department of Chemistry, Central College Campus, Bangalore University, Bangalore, Karnataka 560001, India
e-mail: cskarigar@yahoo.com

Introduction

Ethylbenzene is a toxic aromatic compounds found as a component of petroleum hydrocarbons. Often ethylbenzene enters the environment in the form of industrial discharges from petroleum refining, plastic, resins and pharmaceutical industrial effluents or oil spills. Ethylbenzene also finds wide application as starting material for the preparation of styrene that is used as a solvent for coatings, and in making rubber and plastic wrap. The US Environmental Protection Agency's (US EPA 1996) has found that short-term exposure of ethylbenzene at levels above 0.7 ppm causes drowsiness, fatigue, headache and mild eye and respiratory irritation. The long-term exposures to ethylbenzene can potentially damage the liver, kidneys, central nervous system and eyes. Hence, ethylbenzene is considered as one of the priority pollutant. Also, ethylbenzene binds moderately to aquatic sediment and soils thereby leading to ground water contamination if released to land. In the light of

aforesaid reasons, there is an urgency to eliminate such pollutants form the environment.

The conventional physical or chemical treatment methods that are used to decontaminate the ethylbenzene create secondary effluent problems and are not cost effective. Thus, biological treatment processes are probably the alternate effective and eco-friendly technologies for treating aqueous waste streams containing organic compounds (Thayer 1991). Although the biochemistry of the aerobic biodegradation of BTEX compounds is fairly well understood (Assinder and Williams 1990), some fungus like *Phanerochaete chrysosporium* and *Cladophialophora* spp. have been shown to carry out only biotransformation of BTEX (Prenafeta et al. 2002).

Various approaches have been developed to treat the ethylbenzene contaminated ground waters. Several studies have shown that an aqueous-organic system can be used for the bioconversion of substrates that have low solubility in water. Such a system reduces the toxic effects of the substrates and/or the products (Bar & Gainer 1987). Recently, Two-Phase Batch Culture System (TPBCS) has been employed for the degradation of organic compounds. It consists of mineral salt medium (MSM) as the aqueous phase and a biocompatible organic solvent as the organic phase. In two-phase system, the substrate is solubilized in the organic phase and diffuses into the aqueous phase. The micro-organisms transform or degrade the substrate at the interface and/or in the aqueous phase. This system can be successfully used for biodegradation processes.

The use of immobilized micro-organism has many advantages over the conventional free cell system (Chien and Sofer 1985). Besides preventing washout of biomass in continuous flow reactors, immobilization facilitates easy separation and imparts greater operational flexibility. Immobilized cells can be much more tolerant to high concentrations of toxic chemicals (Westmeier and Rehm 1985; Dwyer et al. 1986). In addition, the cell density of immobilized cells is higher than that of the free cells, resulting in higher rates of biodegradation per unit volume of the reactor.

Numerous methods have been developed for immobilized biocatalyst preparation (Dwyer et al. 1986). Microbial cells may be flocculated or aggregated. They may be attached to a suitable carrier by adsorption or ionic bonding, or they may be entrapped in a polymer matrix. Among the most used adsorption carriers are activated charcoal, porous ceramics and porous silica supports, suitable polymers for entrapment of cells include alginate, polyacrylamide, agar and polyvinyl alcohol.

The constraint in the environmental management of the effluents remains the availability of the suitable micro-organisms that can overcome their culturing limitations from their natural habitats to the effluent conditions. In this context the use of efficient microbial systems offer many advantages.

This communication investigates the metabolic potential of *Pseudomonas fluorescens*-CS2 to degrade ethylbenzene. The metabolic efficiency of cells in single phase, two-phase and immobilized cultures has been assessed. The study provides valuable information for developing potential, efficient and eco-friendly biotechnology for the bioremediation of ethylbenzene from the contaminated or polluted environmental sites.

Materials and methods

Organism and growth

Pseudomonas fluorescens-CS2 degrading ethylbenzene was isolated from local automobile hydrocarbon contaminated site by enrichment culture technique (Suneetha et al. 2005). The MSM was amended with 0.2% (v/v) ethylbenzene as the sole source of carbon and energy (Ganji and Pujar 1992). The bacteria were grown in teflon capped 250 ml serum bottles containing 75 ml MSM as shake cultures at 25 ± 1°C on a rotary shaker (140 RPM). Yeast extract (0.05%) and peptone (0.05%) were additionally included into the MSM for the enzyme studies.

Degradation of ethylbenzene

The degradation rate was determined by culturing *P. fluorescens*-CS2 in serum bottles containing 50 ml MSM supplemented with 0.2% (v/v) ethylbenzene. The bacterial growth response was monitored photometrically at regular time intervals at 600 nm. The metabolic rate of the substrate was assessed by quantification of the key hydroxy metabolites catechol that accumulates in the aqueous spent medium by 4-amino antipyrine reagent (Collins and Daugulis 1997). The reddish brown to yellow color at pH 10 with absorbance maxima at 510 nm was directly proportional to the phenolic substance.

The rate of substrate degradation in aqueous phase of two phase culture system was monitored by HPLC analysis. Typically, 20 ml of aqueous spent medium was extracted with 5 ml of chloroform and analyzed by HPLC Shimadzu SCL-10A VP Model, C18 Column PHENOMENEX Make USA. A solvent system comprising of methanol and water [70:30 (v/v)], was used as mobile phase. The quantity of ethylbenzene that remained in organic phase during incubation was monitored by HPLC analysis of the organic phase.

Extraction and characterization of catechol

Accumulation of catechol in the spent medium during the growth of *P. fluorescens*-CS2 on ethylbenzene was isolated employing replacement culture technique. The acidified spent medium was extracted three times with diethyl ether (1:3 volumes). The extract was dried over anhydrous sodium sulfate and evaporated to dryness, the residue obtained was dissolved in methanol and further characterized by co-chromatography of authentic and isolated compounds on TLC (Alugram Sil G 0.20 mm thick layer, Macherey-Nagel GmbH & Co., Duren, Germany) and HPLC (Shimadzu SCL-10A VP Model C18 Column PHENOMENEX Make USA).

Catechol utilization assay by replacement culture technique

Pseudomonas fluorescens-CS2 cells degrading ethylbenzene as sole carbon substrate were harvested in their log phase. Cells were washed repeatedly with sodium phosphate buffer (50 mM; pH 7.0). An appropriate aliquot of the cell suspension (to obtain 0.02 OD at 600 nm) was inoculated into 50 ml of sterilized MSM supplemented with catechol (0.1% w/v). There after the bacterial growth was determined photometrically as already described. The catechol that remains in the spent medium was determined by 4-amino antipyrine reagent.

Biocompatibility experiments

The Biocompatibility of the bacterial strains for *n*-decanol was carried out according to Collins and Daugulis method (1996).

Preparation of cell free extract and enzyme assay

The ethylbenzene grown *P. fluorescens*-CS2 cells in their log growth phase were harvested to obtain cell free extracts. After centrifugation of the medium to collect cells they were repeatedly washed with 50 mM sodium phosphate buffer, pH-7.0 and resuspended in 0.1 M KH_2PO_4 buffer (pH-7.8), containing 1mM ascorbic acid, acetone (10%), glycerol (10%), and 100 μM ferrous sulfate. This cell suspension was subjected to sonic disruption (Vibracell, Model VC 130, Sonics & Materials, CT, USA) for a total of 3 min. After sonicating the cell debris and unbroken cells were separated by centrifugation at 10,000 rpm for 20 min at 0–4°C. The resulting clear supernatant was used as the crude source of enzymes. The protein content in the crude enzyme was determined using Bovine Serum Albumin as the standard (Bradford 1976). Catechol 2,3-dioxygenase assay was carried out spectrophotometrically by monitoring the increase in absorbance at 375 nm (Nozaki et al. 1970).

Immobilization of micro-organism

The ethylbenzene degrading *P. fluorescens*-CS2 cells were harvested in their log phase of growth from 1 l of culture medium. The cell pellet obtained by centrifugation of the spent broth at 5,000 rpm for 10 min at 4°C, was used in immobilization experiments.

Immobilization of *P. fluorescens*-CS2 cells was carried out in alginate (Battmann and Rehm 1984), agar (Nilson et al. 1983), and polyacrylamide (Chibata et al. 1978) matrices. The bead stability was monitored by plating a known aliquot of spent media.

Scanning electron microscopic (SEM) examination of immobilized cells

The *P. fluorescens*-CS2 immobilized in alginate, agar and polyacrylamide matrices were processed for SEM observation. Briefly they were fixed in paraformaldehye (2%) and glutaraldehyde (2%) in 0.1 M phosphate buffer (pH 7.2–7.4) for 1–2 h. The fixed matrices were rinsed twice for 5–10 min each in sodium cacodylate buffer (pH 7.2–7.4) and post-fixed in 1–2% osmium tetraoxide buffer (pH 7.4) for 1–2 h at 4°C. The immobilized systems were then washed twice in sodium cacodylate buffer (pH 7.2–7.4) for 5–10 min each at room temperature and dehydrated

with a series of ethanol solutions (50, 75, 95, and 100%) at room temperature. Finally, the immobilized gel matrices were dried using the CO_2 by critical point technique (Balzers Critical Point Dryer-CPD 030) and sputter-coated with gold (Bal-Tec SCD005 Sputter Coater). Scanning electron micrographs were obtained using a JOEL JSM 5600LV Scanning Electron Microscope.

Batch culturing of immobilized cells

A 10 g each, of the alginate or agar immobilized beads containing 1.6×10^8 cfu g^{-1} of beads, were transferred to 500 ml capacity serum bottles containing 125 ml of MSM to which 0.6% (v/v) ethylbenzene was supplemented. The fermentation was carried out at 25 ± 1°C on a rotary shaker at 140 rpm for the desired incubation periods. Aliquots of spent culture broth were sampled each day. The degradation rate of ethylbenzene was monitored as described in Methods.

Repeat batch culture of immobilized cells

The long-term stability of the catabolic process was established by culturing the immobile cells as repeat batch cultures. The respective matrices with immobilized cells were incubated as above with the substrate for 2–3 days. The spent broth was decanted and residual beads were washed with sterile water and transferred into a fresh MSM with substrate. This process was repeated several times.

All the experimental data represents an averaged data obtained by conducting at least three independent experiments. Also appropriate control experiments were used in the studies.

Results

Comparison of growth and substrate metabolism between two phase and single phase systems

The *Pseudomonas fluorescens*-CS2 metabolized ethylbenzene from liquid cultures. The studies on the growth kinetics *P. fluorescens*-CS2 employing single and two-phase culture systems were indicative of an initial hydroxylation step in the degradation of ethylbenzene (Fig. 1). The ethylbenzene utilization

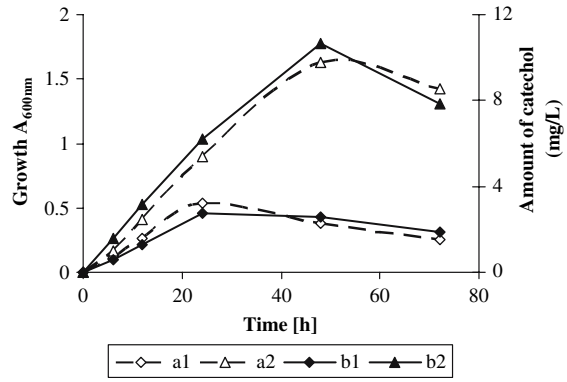

Fig. 1 Metabolism of ethylbenzene by *Pseudomonas fluorescens*-CS2; Growth response (a-1, a-2) and ethylbenzene degradation (b-1, b-2) in single and two phase culture systems, respectively

studies in TPBCS by *P. fluorescens*-CS2 by HPLC analysis demonstrates the unutilized substrate distributed in aqueous and organic phases at different point of times. Furthermore the complete disappearance of substrate was found to occur in 96 h of incubation period (Fig. 2). The *n*-decanol was selected as organic phase in TPBCS as it was found to be biocompatible to *P. fluorescens*-CS2 (Fig. 3) and it was not being degraded by the organism as no growth was found upon incubating with *n*-decanol as sole carbon and energy.

Ethylbenzene metabolic pathway of *P. fluorescens*-CS2

The insight into the metabolic pathway operating in *P. fluorescens*-CS2 was obtained by the isolation and characterization of metabolite produced during the

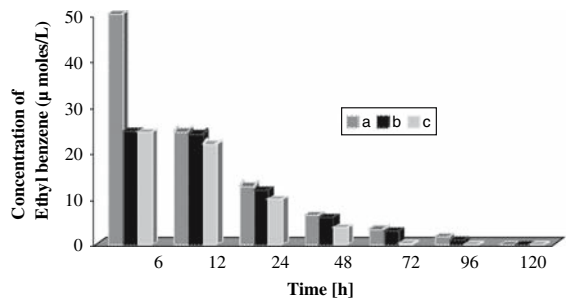

Fig. 2 The ethylbenzene utilization studies in TPBCS by *P. fluorescens*-CS2: (**a**) ethylbenzene in *n*-decanol, (**b**) ethylbenzene in aqueous medium (control). (**c**) Ethylbenzene in spent aqueous medium (test)

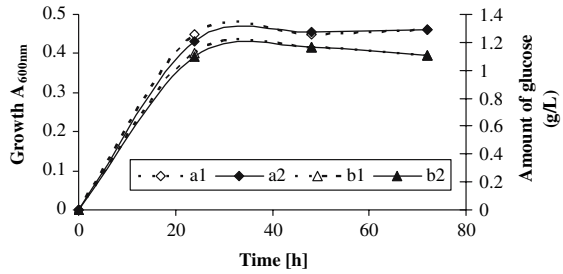

Fig. 3 *n*-Decanol biocompatibility analysis for *P. fluorescens*-CS2. Growth in the presence of *n*-decanol + glucose (a1-control, a2- test): Glucose utlization b1 (control), b2 (test)

growth of the organism on ethylbenzene as the substrate. Both the TLC and HPLC analysis of the spent medium established that catechol was a key metabolite. The metabolite showed identical R_f and R_t-values on co-chromatography with authentic catechol. The R_f and R_t-value of metabolite were 0.54 and 3.4 min, respectively. These observations were further supported by the experiments on the catechol utilization studies (Fig. 4) and the catechol 2,3-dioxygenase enzyme assay. The specific activity in the cell free extract was determined to be 0.428 μmoles min^{-1} mg^{-1} protein. Hence based on these studies catechol was established as the key intermediate in the degradation of ethylbenzene by *P. fluorescens*-CS2.

Ethylbenzene metabolism by immobilized bacterial cells

The immobilization of *P. fluorescens*-CS2 cells into alginate, agar and polyacrylamide matrices was evident in SEM micrographs of the beads (data not shown). The metabolism of ethylbenzene by immobilized cells was also established in terms of the accumulation of catechol in the spent medium (Fig. 5). Further the alginate and polyacrylamide gel beads were found to be stable up to 42 days after repeated use in batch cultures fermentation systems. The immobilized cells were able to sustain the substrate metabolic ability even in the order between 0.4 and 0.6% (v/v). The free cells however could metabolize ethylbenzene upto 0.2% only. The alginate and polyacrylamide immobilized *P. fluorescens*-CS2 produced catechol in repeated batch culture medium from ethylbenzene continuously for a 42-day period. On the other hand the agar immobilized cells were stable and retained the ethylbenzene metabolic activity only for 36 days. Thus, polyacrylamide and alginate are good matrices for immobilization of *P. fluorescens*-CS2 cells than agar as the matrix.

Discussion

Release of petroleum hydrocarbons in the environment is a widespread occurrence. One particular concern of this release is the contamination of drinking water resources. The microbial degradation of these compounds in aquatic environments can therefore serve as a significant attenuation mechanism (Prenafeta et al. 2001; Hutchins et al. 1990). However, in situ biodegradation of BTEX compounds of which ethylbenzene is the member is not ubiquitous. The bacteria metabolizing BTEX components have been characterized from an automobile contamination site by us (Suneetha et al. 2005). Instances of this nature once again demonstrate the development of

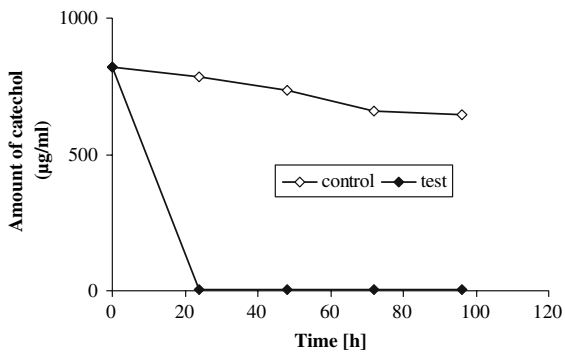

Fig. 4 Catechol utilization by *Pseudomonas fluorescens*-CS2. Catechol metabolism (test), abiotic oxidation (control)

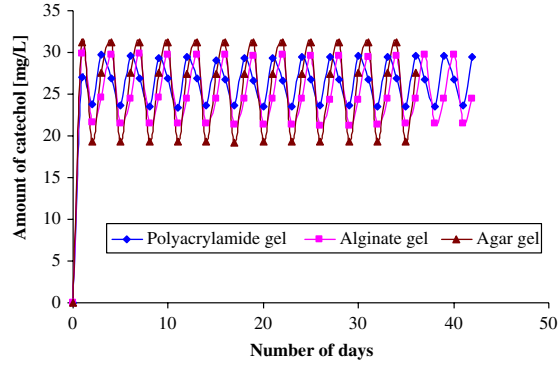

Fig. 5 Ethylbenzene metabolism by immobilized *Pseudomonas fluorescens*-CS2 in repeated batch cultures

biodegradation abilities by the indigenous microflora in order to carry on the usual elemental cycles that are operating in the environment. Among the various isolates *Pseudomonas fluorescens*-CS2 possessed exceptional ability to metabolize ethylbenzene. However higher concentrations of ethylbenzene provided in the medium behaved as inhibitory substrates.

There are several instances that describe the strain specific relationships between the substrate specificity and the extent of degradation. The common types of substrate interactions exhibited by microorganisms in presence of BTEX mixtures involve cometabolism and competitive inhibition. Most of the reported works have focused on biodegradation of BTEX mixtures (Burback and Perry 1993; Oh et al. 1994). The studies on the biodegradation of the individual components; like benzene, xylene, and toluene have been conducted but the reports on the efforts to understand biodegradation of ethylbenzene are few. So far only one such study was undertaken (Bestetti and Galli 1984). In the current work we have concentrated on understanding the metabolic pathway of ethylbenzene in *P. fluorescens*-CS2. Also an attempt is made to analyze the metabolic rates of ethylbenzene in single, two phase and immobilized cell culture systems.

The metabolic pathway of ethylbenzene in *P. fluorescens*-CS2 proceeds through an initial hydroxylation reaction. The two principle aerobic pathways that lead to the mineralization of aromatic hydrocarbons are; a dioxygenase attack on the aromatic ring which is referred to as the *tod* pathway, and a monooxygenase attack on the methyl substituents that is referred to as the *tol* pathway (Marcelo et al. 2005; Yoshinori et al. 2001). Since *P. fluorescens*-CS2 in culture medium generates catechol as one of the metabolic intermediate, as well as it elaborates a catechol 2,3, dioxygenase enzyme the bacterium metabolizing ethylbenzene follows the *tol* path way. The facts such as catechol ring cleavage that is evidenced by its complete utilization experiments and the presence cleaving enzyme in cell-free extracts help us to conclude that ethylbenzene is completely metabolized as sole carbon and energy source.

The problem of substrate inhibition was overcome by inclusion of second phase like *n*-decanol in TPBCS for ethylbenzene degradation. *n*-Decanol phase was thought to eliminate the substrate toxicity by way of the controlled delivery of substrate to aqueous phase in high substrate concentration medium. *n*-Decanol did not influence the physiology of the bacterium as it was chosen as the organic phase based on criteria such as biocompatibility, non-degradability and least solubility in aqueous medium (Collins and Daugulis 1997). The organic phase of TPBCS also avoids or minimizes the substrate evaporation, thereby making it more available to biodegradation. There are several reports on the application of TPBCS to degrade various toxic or inhibitory high-substrate concentrations. The selection of mixed microbial populations degrading some chlorinated and non-chlorinated xenobiotic compounds (Ascon-Cabrera and Lebeault 1993) and the biodegradation of polynuclear aromatic hydrocarbons (Vanneck et al. 1994).

More recently, 2-undecanone has been used as the organic phase in a two-phase system for the degradation of phenol in batch and fed-batch bioreactors (Collins and Daugulis 1997). The TPBCS could be successfully applied to clean up benzene, toluene, and p-xylene (BTX) from contaminated sand (Collins and Daugulis 1999). In our investigations the selection of *n*-decanol as the second phase for culturing *P. fluorescens*-CS2 in TPBCS are based on the results of biocompatibility and non-degradability experiments, non-degradability experiment revealed no growth on *n*-decanol. The ethylbenzene metabolism was enhanced by five times in TPBCS when compared to single phase system. However, the principle metabolic pathway of ethylbenzene degradation in TPBCS did not vary from the single phase system. Thus, TPBCS promoted the enhancement of ethylbenzene metabolic rates. The accumulation of catechol in the spent medium in all these batch culture experiments could be due to oxygen limiting conditions during the incubation.

The method of cell immobilization seems to be promising in the development of the biotechnology for the removal of various xenobiotic bearing effluents (Murugesan 2003). It was not only possible for us to immobilize *P. fluorescens*-CS2 successfully into three different matrices; the alginate, agar and polyacrylamide but also to retain the biodegradation ability in the immobilized beads. The immobilized cell systems reveal a drastic increase in the rates for ethylbenzene metabolism. Although, the rate of increase in the ethylbenzene metabolism in an immobiled cell systems with that of others could not

be comparable as the initial cell density various with other systems but which could be better compared with single and two phase systems. Moreover, these immobilized systems could be continuously used for several days without any loss in the biodegradation property. Since entrapped cells remain viable for a considerable duration they would be a better alternative against free cells for the bioremediation applications of variety of toxic organics from effluents (Kochar and Kahlon 1995; Hojae and Shang-Tian 1999).

Polyacrylamide, collagen–polyacrylamide, elastin–polyacrylamide, and gelatin–polyacrylamide have been used to immobilize organisms to treat cellulose waste (Petre et al. 2000). Sodium alginate coated with 1.0% chitosan has been used to treat petroleum derived hydrocarbons (Elzbieta et al. 2005), ploypropylene fibers immobilized systems were used to treat crude oil effluents (Diaz et al. 2002) and agar has been used to immobilize bacteria to treat phenol (Karigar et al. 2006). In our experiments alginate and polyacrylamide matrices were found to be more promising compared to agar with respect to their gel bead stability and ethylbenzene metabolism. As the cells entrapped in ployacrylamide and alginate were found to be stable and degradation efficient for a period of 42 days where as agar-entrapped *P. fluorescens* was stable and efficient a period of 36 days. This demonstrates that alginate and polyacrylamide matrices are more promising as compared to agar for cell immobilization.

The indigenous microflora member biodegradation potentials of this nature can be harnessed for developing biotechnologies for decontamination of polluted sites as well as water bodies. As *P. fluorescens*-CS2 capable of metabolizing ethylbenzene under hypoxic conditions and also with stands toxic substrate concentration. Thus, such immobilized bacterial cells adapted to higher ethylbenzene concentrations can play an important role in the development bioreactors to treat industrial waste streams containing high concentrations of ethylbenzene components with out the lose of metabolic activity of the cells.

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