Appl Biochem Biotechnol (2011) 163:497–518
DOI 10.1007/s12010-010-9057-2

Biodegradation of Chlorinated and Non-chlorinated VOCs from Pharmaceutical Industries

P. Balasubramanian · Ligy Philip · S. Murty Bhallamudi

Received: 24 May 2010 / Accepted: 9 August 2010 /
Published online: 27 August 2010

Abstract Biodegradation studies were conducted for major organic solvents such as methanol, ethanol, isopropanol, acetone, acetonitrile, toluene, chloroform, and carbon tetrachloride commonly used in pharmaceutical industries. Various microbial isolates were enriched and screened for their biodegradation potential. An aerobic mixed culture that had been previously enriched for biodegradation of mixed pesticides was found to be the most effective. All the organic solvents except chloroform and carbon tetrachloride were consumed as primary substrates by this mixed culture. Biodegradation rates of methanol, ethanol, isopropanol, acetone, acetonitrile, and toluene were measured individually in batch systems. Haldane model was found to best fit the kinetics of biodegradation. Biokinetic parameters estimated from single-substrate experiments were utilized to simulate the kinetics of biodegradation of mixture of substrates. Among the various models available for simulating the kinetics of biodegradation of multi-substrate systems, competitive inhibition model performed the best. Performance of the models was evaluated statistically using the dimensionless modified coefficient of efficiency (E). This model was used for simulating the kinetics of biodegradation in binary, ternary, and quaternary substrate systems. This study also reports batch experiments on co-metabolic biodegradation of chloroform, with acetone and toluene as primary substrates. The Haldane model, modified for inhibition due to chloroform, could satisfactorily predict the biodegradation of primary substrate, chloroform, and the microbial growth.

Keywords Biodegradation · Substrate inhibition kinetics · Organic solvents · Volatile organic compounds · Pharmaceutical industry

P. Balasubramanian · L. Philip (✉) · S. M. Bhallamudi
Environmental and Water Resources Engineering Division, Department of Civil Engineering,
IIT Madras, Chennai 600 036, India
e-mail: ligy@iitm.ac.in

Introduction

Pharmaceutical industries use a wide range of organic solvents as raw materials in their research, development, and manufacturing facilities [1]. All processes in a pharmaceutical industry, including chemical synthesis, formulation, filtering to concentrate or removal of impurities, extraction of valuable products, facilitation of waste-stream cleanup, and byproduct recovery, use organic solvents. The aqueous streams arising from aqueous/organic phase separations after organic syntheses have a cocktail of organic solvents. They contribute significantly to high organic load in the wastewater discharges from pharmaceutical industries. These wastewaters contain a wide variety of solvents at varying concentrations, generally too low for viable, cost-effective recovery [2]. Therefore, low-cost destructive technologies with maximum benefits and environmental acceptance are preferred in such instances. Biological treatment is still regarded as the most common and economical approach for the treatment of contaminants in wastewater. High-technology and low-cost bio-units have been successfully established for the treatment of variety of organic compounds found in domestic and industrial wastewaters [3].

Among more than 50 organic pollutants found in wastewater streams from pharmaceutical industry, more than 80% of the pollutant load is contributed by not more than ten key compounds. Therefore, studies concerning bulk carbonaceous pollutant elimination from such wastewater streams usually focus on key pollutants such as isopropanol, ethyl acetate, methanol, ethanol, n-heptane, tetrahydrofuran, toluene, dichloromethane, acetic acid, and acetonitrile [4]. Since most of these solvents, except toluene and n-heptane, are hydrophilic in nature, their discharge through wastewater to the environment needs to be controlled. Although a great deal of emphasis is placed on the efficient removal of bulk pollutant loads, degradation of chlorinated compounds and their influence on readily biodegradable pollutants needs to be explored.

The biotransformation of chlorinated compounds by bacteria has been shown to be either due to co-metabolism or primary metabolism. Several studies conducted during the last two decades demonstrated that aerobic mixed populations, grown on hydrocarbons such as methane, propane, butane, and phenol, can effectively degrade most chlorinated aliphatic hydrocarbons by means of co-metabolism [5]. Microorganisms have been shown to generate an extensive range of enzymes that enable them to degrade a wide variety of chlorinated compounds through different pathways [6, 7]. These chlorinated compounds may be co-metabolized in the presence of a more easily biodegradable non-chlorinated hydrocarbon serving as the primary carbon and energy source. Aerobic co-metabolism has been exploited extensively for bioremediation of chlorinated aliphatic compounds such as trichloroethylene in groundwater and in some cases for chlorinated benzenes and phenols [8].

It is important to understand the effects of co-metabolism and substrate inhibition among mixtures of volatile organic compounds (VOCs) in biodegradation processes because most industrial effluents contain a wide variety of compounds. It may be noted that co-metabolism and substrate inhibition associated with chlorinated VOCs during biodegradation have not been elucidated extensively. There is an urgent need to gain insight into the above mentioned aspects. This information will be helpful in the optimization of any biological process for treatment of industrial effluents and emissions containing mixtures of both easily biodegradable and recalcitrant compounds. In pharmaceutical effluents too, the presence of chlorinated compounds among other mixtures may increase the level of recalcitrance. Yet, relatively little is known about how the coexisting substrates affect the biodegradation kinetics of any individual organic constituent by a mixed microbial community.

Several mathematical models have been proposed for mixed homologous substrate consumptions and microbial growth in the past. For instance, Rogers and Reardon [9] investigated and modeled substrate interactions during the biodegradation of mixtures of toluene and phenol by *Burkholderia Sp*. JS150. Louarn et al. [10] modeled substrate interactions during aerobic biodegradation of a mixture of vinyl chloride and ethane using competitive inhibition model. Lendenmann et al. [11] have proposed and validated a model for the growth of *Escherichia coli* on six sugars. Bielefeldt and Stensel [12] modified a two-compound competitive substrate model to simulate the interactive effect of toluene, ethylbenzene, and xylene on benzene degradation. Guha et al. [13] studied substrate interactions in binary and ternary mixtures of naphthalene, phenanthrene, and pyrene and demonstrated the feasibility of using multi-substrate competitive model for predicting biodegradation kinetics of mixtures. Reardon et al. [14] developed a new Sum Kinetics with Interaction Parameters model for predicting the biodegradation kinetics of mixtures of substrates. This model is based on individual interaction parameters that have to be predicted using single-substrate biodegradation kinetics and with multiple substrate experimental observations. Knightes and Peters [15] studied the multi-substrate biodegradation kinetics for binary and complex mixtures of polycyclic aromatic hydrocarbons like naphthalene, methylnaphthalene, and phenanthrene and advocated the use of multi-substrate model with inhibition for predicting the biodegradation kinetics for mixture of substrates. It may be noted that although several models are available for simulating the biodegradation of binary and ternary mixtures of substrates, not many studies have been conducted to evaluate the interaction of various VOCs commonly present in pharmaceutical industry and validate the applicability of available multi-substrate models. Though studies on transformation of chloroform by pure or mixed cultures grown on methane, propane, butane, or toluene and by nitrifying bacteria have been well documented, not much work has been carried out on aerobic transformation of chloroform with other organic solvents commonly present in the liquid and gaseous effluents from pharmaceutical industry.

The objectives of the present study are to: (a) evaluate the co-metabolic biodegradability of chloroform in presence of pharmaceutical co-emissions of organic solvents like methanol, ethanol, isopropanol, acetone, acetonitrile, and toluene; (b) study the effect of substrate inhibition and substrate interactions; and (c) evaluate several multi-substrate mathematical models for the mixed biodegradation of homologous mixture of substrates commonly found in pharmaceutical industrial emissions.

Materials and Methods

Experimental Procedures

Nutrient Media for Bacterial Culture Enrichment

All bacterial strains were grown at 30 °C in minimal salt medium (MSM) prescribed for specific degradation of chlorinated solvents by Atlas [16]. All media were autoclaved at 121 °C and 15 psi for 15 min. The cultures were grown overnight and harvested when the medium optical density (OD_{600}) was between 0.1 and 0.2. The cells were collected by centrifugation, washed with MSM, and suspended in fresh MSM. The composition of MSM is as follows (quantity of chemicals are given in g/L in parentheses): $Na_2HPO_4{\cdot}2H_2O$ (3.5), KH_2PO_4 (1), $(NH_4)_2SO_4$ (0.5), $MgCl_2{\cdot}6H_2O$ (0.1), $Ca(NO_3)_2{\cdot}4H_2O$ (0.05), and trace

elements I (1 mL). Trace elements I contains: EDTA (0.5), $FeSO_4 \cdot 7H_2O$ (0.2), and trace elements II (100 mL). Trace elements II contains: H_3BO_3 (0.3), $MnCl_2 \cdot 4H_2O$ (0.03), $CoCl_2 \cdot 6H_2O$ (0.2), $ZnSO_4 \cdot 7H_2O$ (0.1), $Na_2MoO_4 \cdot 2H_2O$ (0.03), $NiCl_2 \cdot 6H_2O$ (0.02), and $CuCl_2 \cdot 2H_2O$ (0.01).

Microbial Consortia

The bacterial consortia used in this study were previously isolated and enriched by Ramakrishna and Philip [17] for the degradation of various groups of pesticides. Out of those bacterial isolates, lindane-degrading culture (LDC) and mixed pesticides (lindane, methyl parathion, and carbofuran) enriched culture (MEC) were utilized for this study. The major bacterial strains isolated from the mixed microbial consortium were identified as *Pseudomonas aeruginosa* (MTCC 9236), *Bacillus* sp. (MTCC 9235), and *Chryseobacterium joostei* (MTCC 9237). In addition, bacterial cultures from an activated sludge plant were also enriched with alcohols and chlorinated organic solvents by simultaneously decreasing the concentration of glucose from 1,000 mg/L and increasing the concentration of organic solvents from 0 to 100 mg/L. This freshly enriched bacterial culture was named as FEC. All the bacterial cultures were serially transferred once in a week (5 mL of bacterial culture) to fresh media (100 mL) containing the target pollutant along with other nutrients, and enriched further for 3 months.

Analytical Procedures

Measurement of Cell Density in Liquid Phase

The optical density (OD) method was used to measure biomass concentration under aerobic condition. OD was determined by turbid metric measurement in a spectrophotometer at 600 nm and correlated to dry cell weight. Cells were grown overnight, centrifuged, washed three times with physiological saline water, re-suspended in saline water, homogenized, and used as stock solution. Different dilutions were made from the above stock solution. A known volume of these solutions was filtered through 0.45-μm filter paper and weight of the dried cells was estimated. Corresponding absorbance was measured at 600 nm using a UV-Vis spectrophotometer (Techcomp, UK). This information was used for preparing a calibration curve between the dry cell weight and the absorbance. For unknown samples, the absorbance was measured at 600 nm and then it was converted to dry cell weight using absorbance versus dry cell weight calibration curve.

Gas Chromatographic Analysis

Liquid samples were analyzed by using PerkinElmer Clarus 500 gas chromatograph with flame ionization detector (GC-FID). GC was equipped with an auto sampler, an on-column, split/split less capillary injection system, and a capillary column (PerkinElmer Elite (PE)-624, 30 m×0.53 mm×0.5 mm film thickness). During the analysis, the column was held initially at a temperature of 50 °C for 20 min. Temperatures of injector and detector were maintained at 150 and 300 °C, respectively. Nitrogen was used as the make-up and carrier gas at a flow rate of 60 and 1.5 mL/min, respectively. Injections were made in the split mode with a split ratio of 1:20. Standard graphs for respective solvents were prepared individually by injecting known amounts of respective compound into a sealed bottle equipped with teflon septum as per the standard method [18]. The liquid samples were then

transferred to GC vials and analyzed by GC-FID. The intermediates were monitored using a GC-MS supplied by Agilent, USA, and the same temperature program was utilized.

Chemical Oxygen Demand

Chemical oxygen demand (COD) of liquid samples was estimated using closed reflex method as suggested in standard methods [18]. Closed reflux digestion was conducted in HACH COD digester (Model No 45600, USA).

Measurement of Free Chloride Ions

First, bacterial populations of the samples were estimated using the optical density method. Then the samples were centrifuged to remove the cells, and the supernatant fractions were analyzed for free chloride ions with a model DX-100 ion chromatograph (Dionex, Marlton, NJ, USA), equipped with a 4-mm (internal diameter) AS11 column and a conductivity detector, which was calibrated for chloride. Sodium hydroxide (0.5 mM) was used as the eluent and the flow rate was 1 mL/min. All samples were analyzed in duplicate.

Batch Biodegradation Studies

Batch studies were performed in 125-mL serum bottles. All the serum bottles were sealed with silicone/teflon septum; aluminum crimped and kept in orbital shaker at 120 rpm at 30 °C for the study. Sixty milliliters of MSM was taken in each serum bottle and autoclaved properly before the inoculation of concerned organic solvents, allowed to attain the equilibrium for 4 h, and the initial concentration in the liquid phase was noted. Then known amount of microbial inoculums were added so as to obtain an initial OD_{600} of approximately 0.1 to initiate the biodegradation. Gaseous oxygen was injected into the reaction bottle before taking liquid sample from the system in order to maintain aerobic conditions inside the reaction bottle. Liquid samples (4 mL each) were withdrawn at discrete time intervals and 1 mL from each sample was subjected to close centrifuging in order to remove microbes. Supernatant was then utilized for GC-FID analysis. Remaining 3 mL of each sample was used for measuring optical density, then centrifuged and further utilized for COD and chloride analyses. Both biotic and abiotic controls were employed. All the batch studies were conducted under aerobic conditions (dissolved oxygen levels remained above 3 mg/L) and without any pH control (pH remained in the range of 6.7–7.2). Purpose and description of various batch degradation studies are presented in Table 1.

Mathematical Models

Single-Substrate Biodegradation Kinetics

Several kinetic models available in literature (Edwards, Haldane (Andrews), Levenspiel, Monod and Webb) are employed to understand the relationship between specific growth rate and solvent concentration. Batch kinetic data were fitted to these models to determine the biokinetic parameters such as maximum specific growth rate (μ_{max}), yield coefficient (Y_T), half saturation concentration (K_s), and inhibition concentration (k_i) for growth of MEC in corresponding solvents. Governing equations of these models are presented in Table 2.

Table 1 Purpose and description of various batch degradation studies

No.	Purpose	Description
1	Screening of microbes	LDC, MEC, FEC
2	Screening of substrates	M, E, I, A, AN, T, CF, CT
Studies with single substrate		
3	Methanol biodegradation by MEC	M~0.5, 1, 2.5, 5, 7.5, and 10 g/L
4	Ethanol biodegradation by MEC	E~0.5, 1, 2.5, 5, 7.5, and 10 g/L
5	Isopropanol biodegradation by MEC	I~0.5, 1, 2.5, 5, 7.5, and 10 g/L
6	Acetone biodegradation by MEC	A~0.5, 1, 2.5, 5, 7.5, and 10 g/L
7	Acetonitrile biodegradation by MEC	AN~0.5, 1, 2.5, 5, 7.5, and 10 g/L
8	Toluene biodegradation by MEC	T~0.1, 0.2, 0.3, 0.4, and 0.5 g/L
Studies with multiple substrates		
9	Methanol–acetone mixture	M~2 g/L+A~2 g/L
10	Methanol–toluene mixture	M~2 g/L+T~0.3 g/L
11	Ethanol–acetone mixture	E~2 g/L+A~2 g/L
12	Ethanol–toluene mixture	E~2 g/L+T~0.3 g/L
13	Methanol–acetone–toluene mixture	M~2 g/L+A~2 g/L+T~0.3 g/L
14	Ethanol–acetone–toluene mixture	E~2 g/L+A~2 g/L+T~0.3 g/L
15	Methanol–ethanol–acetone–toluene mixture	M~2 g/L+E~2 g/L+A~2 g/L+T~0.3 g/L
Co-metabolic studies		
16	Screening of substrates favoring maximum dechlorination of chloroform by MEC	COD~2,000 mg/L with M, E, I, A, AN, T+CF~50 mg/L
17	Chloroform biodegradation by MEC	A~1,000 mg/L+CF~0, 5, 10, 25, 50, 100, 250, and 500 mg/L

Mixed Substrate Biodegradation Kinetics

The most common model used for describing microbial growth in substrate mixtures is one in which the specific growth rate is the sum of the specific growth rates on each substrate *i* (μ_i). In its simplest form, Monod expressions are used for each μ_i, yielding a model in which the presence of one substrate does not affect the biodegradation rate of the other. For

Table 2 Various substrate inhibition models and corresponding governing equations

Inhibition model	Governing equations	References
Edwards model	$\mu = \frac{\mu_{max} * S_e * e^{-(S_e/k_i)}}{K_s + S_e}$	[40]
Haldane model	$\mu = \frac{\mu_{max} * S_e}{K_s + S_e + \frac{S_e^2}{k_i}}$	[41]
Levenspiel model	$\mu = \mu_{max} * \left(1 - \frac{S_e}{k_i}\right) * \left(\frac{S_e}{K_s + S_e}\right)$	[42]
Monod model	$\mu = \frac{\mu_{max} * S_e}{(K_s + S_e)} * \left(\frac{K_i}{K_i + S_e}\right)$	[43]
Webb model	$\mu = \frac{\mu_{max} * S_e * \left(1 + \frac{S_e}{k_i}\right)}{K_s + S_e + \frac{S_e^2}{k_i}}$	[44]

a mixture with n substrates, (say i=1 to n), general governing equations for this no-interaction sum kinetics model are:

$$\frac{dM}{dt} = (M)^* \sum_{i=1}^{n} \left(\frac{\mu_{\max i}{}^* S_i}{Ks_i + S_i} \right) \tag{1}$$

$$\frac{dS_i}{dt} = -\left(\frac{M}{Y_{Ti}} \right)^* \sum_{i=1}^{n} \left(\frac{\mu_{\max i}{}^* S_i}{Ks_i + S_i} \right) \tag{2}$$

in which M is the biomass concentration in milligrams per liter, S_i is concentration of ith substrate in milligrams per liter, $\mu_{\max i}$ is the maximum specific growth rate of bacteria when only ith substrate is present in per hour, K_{Si} is the half saturation constant for the ith substrate in milligrams per liter when only that substrate is present, Y_{Ti} is the yield coefficient when only ith substrate is present, and t is the time from the start of the process.

Since the same metabolic pathway is used in the catabolism of both substrates, it may be possible that the two substrates compete for the active site on any of the enzyme in the pathway. A sum kinetics model incorporating purely competitive substrate kinetics was proposed by Yoon et al. [19]. For a mixture of substrates, general governing equation for microbial growth under competitive substrate biodegradation kinetics is:

$$\frac{dM}{dt} = (M)^* \sum_{i=1}^{n} \left(\frac{\mu_{\max i}{}^* S_i}{Ks_i + S_i + \sum_{j=1}^{n} \left(\frac{Ks_i}{Ks_j} {}^* S_j \right)} \right) \tag{3}$$

Another form of interaction between an enzyme and two substrates is noncompetitive inhibition, in which a non-reactive complex is formed when both substrates are simultaneously bound to the enzyme [20]. General governing equation for cell growth under noncompetitive interaction for a mixture of substrates is:

$$\frac{dM}{dt} = (M)^* \sum_{i=1}^{n} \left(\frac{\mu_{\max i}{}^* S_i}{(Ks_i + S_i)^* \left(1 + \sum_{j=1}^{n} \frac{S_j}{Ks_j} \right)} \right) \tag{4}$$

Uncompetitive enzyme inhibition is similar to noncompetitive inhibition except that one of the compounds (the inhibitor) can bind only to the enzyme–substrate complex and not to the free enzyme [20]. General governing equation for cell growth for a mixture of substrates under uncompetitive substrate interaction conditions is:

$$\frac{dM}{dt} = (M)^* \sum_{i=1}^{n} \left(\frac{\mu_{\max i}{}^* S_i}{Ks_i + S_i {}^* \left(1 + \sum_{j=1}^{n} \frac{S_j}{Ks_j} \right)} \right) \tag{5}$$

For all the above cases, the substrate depletion follows the same equation as that for biomass growth, modified with a factor $-\left(\frac{1}{Y_{Ti}}\right)$, similar to that in Eq. 2.

Co-metabolic Biodegradation Model

Haldane's model, modified for inhibition due to chloroform, was considered in the present study to describe the co-metabolic degradation of chloroform in presence of acetone. The mathematical model not only describes the chloroform biodegradation but also the temporal variations of primary substrate and biomass concentrations in the system. The model equations are:

$$\frac{dM}{dt} = M\left(\frac{\mu_{\max g} S_g}{Ks_g + S_g + \frac{S^2_g}{K_{ig}}}\right)\left(\frac{K_{ic}}{K_{ic} + C}\right) \tag{6}$$

$$\frac{dC}{dt} = -M(\eta)\left(\frac{1}{Y_T}\right)\left(\frac{\mu_{\max g} S_g}{Ks_g + S_g + \frac{S^2_g}{K_{ig}}}\right)\left(\frac{K_{ic}}{K_{ic} + C}\right) \tag{7}$$

where M is biomass concentration in milligrams per liter, S_g is concentration of primary substrate in milligrams per liter, C is concentration of chloroform in milligrams per liter, $\mu_{\max,\ g}$ is the maximum specific growth rate in the primary substrate, K_{ig} is the primary substrate inhibition constant for bacterial growth in milligrams per liter, K_{ic} is the chloroform inhibition constant for bacterial growth in milligrams per liter, $K_{s,g}$ is the half saturation constant for bacterial strain in milligrams per liter, η is milligrams of chloroform reduced per gram of substrate utilized by bacteria, and Y_T is the yield coefficient for bacterial strain.

Results and Discussion

Screening of Microbes

Major solvents used in pharmaceutical industrial operations comprise of alcohols, ketones, aromatic compounds, and chlorinated solvents like methanol, ethanol, isopropanol, acetone, acetonitrile, toluene, chloroform, and carbon tetrachloride. Preliminary studies were conducted to evaluate whether LDC, MEC, and FEC can grow on the above solvents as single substrates. Among all the enriched cultures studied, MEC exhibited the maximum degradation rate for all the organic solvents. Experimental studies showed that MEC could grow on all the above mentioned organic solvents, except on chloroform and carbon tetrachloride as single substrates.

Single-Substrate Biodegradation Studies

Batch experiments were conducted with MEC to determine kinetics of biodegradation of methanol, ethanol, isopropanol, acetone, acetonitrile, and toluene as sole carbon source. In these studies, concentrations of all the solvents except toluene were varied from 500 to 10,000 mg/L to achieve 1% *v*/*v*, while the concentration of toluene was varied from 100 to 500 mg/L based on solubility limit. It was observed in these studies that there was an increase in biomass concentration accompanied by a simultaneous decrease in residual substrate concentration. This indicates the biodegradation of substrate by microbes. The

biomass growth pattern and the methanol biodegradation patterns are shown in Fig. 1a and b, respectively. It can be clearly observed from Fig. 1b that the time taken for complete degradation of methanol depended on the initial concentration of methanol, and it varied from 3 to 12 days for a variation of initial methanol concentration from 500 to 10,000 mg/L. Similar trend was observed in case of biodegradation of other solvents by MEC. The time taken for complete degradation also depended on the solvent. Methanol, ethanol, isopropanol, and acetone took 12, 9, 13, and 11 days, respectively, for complete degradation, when the initial concentration was 10,000 mg/L. It is evident from the experimental results that acetonitrile could not be degraded completely when the initial concentration was more than 2,500 mg/L. Toluene was completely degraded in 4 days when the initial concentration

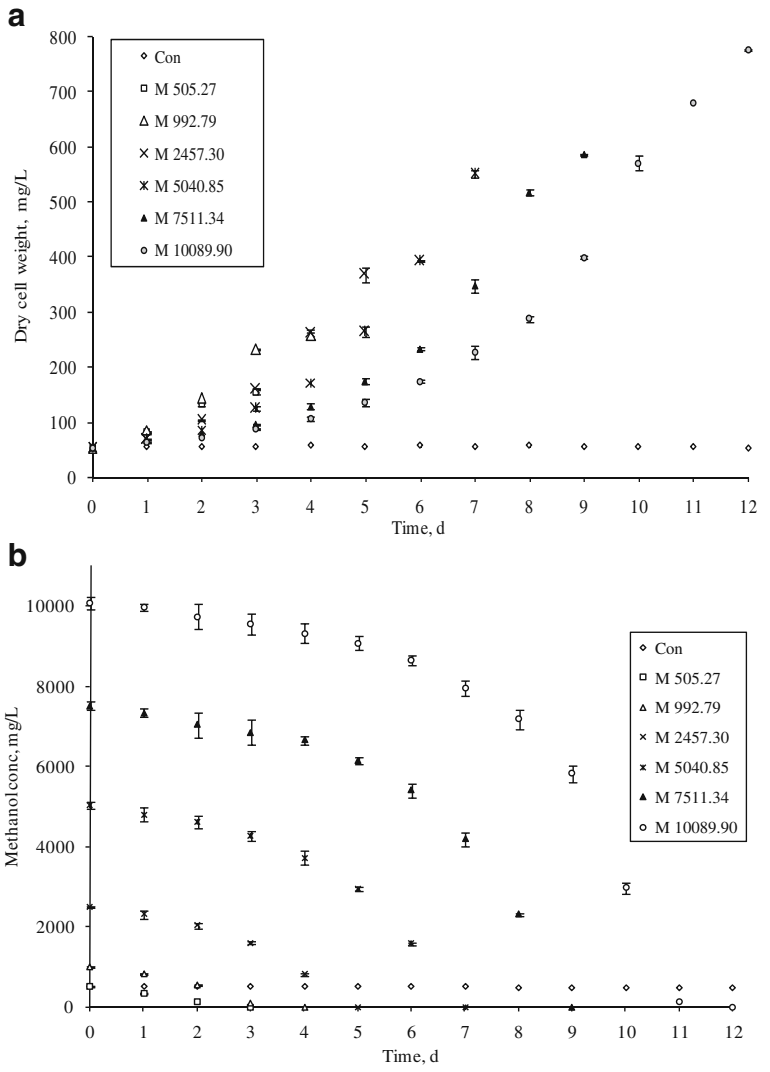


Fig. 1 **a** Growth of MEC for different initial concentrations of methanol. **b** Kinetics of biodegradation of methanol by MEC

was 500 mg/L. The biodegradation of various organic solvents was also verified by analyzing COD.

In case of isopropanol, a large proportion of it was initially oxidized to acetone at a very fast rate. Following this, the bioconversion of acetone to simpler innocuous compounds took time. These byproducts were subsequently utilized simultaneously during the exponential growth phase. No byproducts other than acetone (from isopropanol) were observed in the reaction mixture. This may be due to either the simultaneous utilization of the simpler compounds by microbes or their unstable nature.

Several mathematical models available in literature (Table 2) were employed to evaluate the biokinetic parameters. Batch kinetic data were fitted to these models to explain single-substrate inhibition on growth of the microbial culture. In these models, the yield can easily be estimated independently by linear regression of biomass and substrate measurements. The model performance was statistically evaluated using the dimensionless modified coefficient of efficiency,

$$E = 1 - \frac{\sum_{i=1}^{N} [|E(t_i) - O(t_i)|]}{\sum_{i=1}^{N} |O(t_i) - \overline{O}|} \tag{8}$$

where $E(t_i)$ is the numerically simulated value of a variable at time t_i, $O(t_i)$ is the experimentally observed value of the same variable at time t_i, and $\overline{O}$ is the mean value of the observed variable. E varies between -∞ and 1.0, higher values indicating better model prediction. Positive value of E represents an acceptable simulation, whereas $E>0.5$ represents a good simulation. E equal to one indicates a perfect simulation. Biokinetic parameters corresponding to each model were obtained using the observed data from batch experiments.

Variation of specific growth rate as a function of substrate concentration was predicted using the above derived biokinetic parameters (for all five models) and compared with the experimental data. It can be observed from Fig. 2 that Haldane model best describes the kinetics of methanol biodegradation. Among the single-substrate inhibition kinetic models evaluated in the study, the Haldane model showed the highest value of E (0.6832).

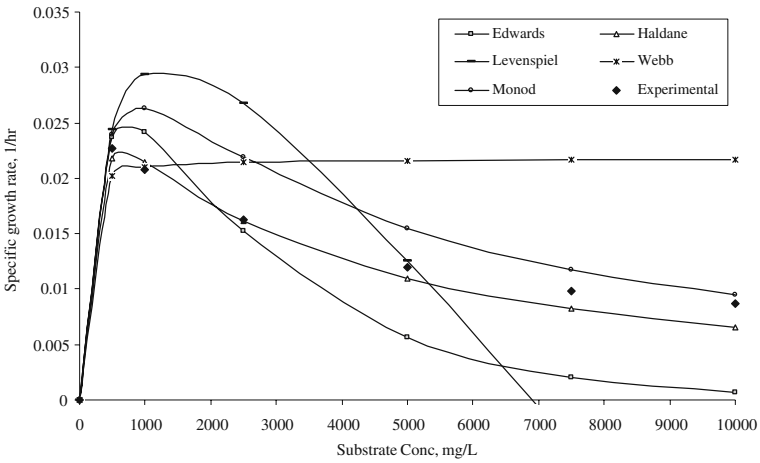


Fig. 2 Comparison of various model predicted specific growth rates for methanol biodegradation

Haldane model was used to estimate the biokinetic parameters for other solvents as it performed better than the other models for methanol biodegradation. Biokinetic parameters obtained by Haldane model for different solvents are presented in Table 3. Values of K_s indicate the ability of microbes to grow at low substrate levels and k_i values indicate the sensitivity of the culture to substrate inhibition. It can be observed from Table 3 that inhibition concentration and the biomass yield coefficient differ for various solvents. Microbes have grown at different rates in these organic solvents and the growth rate followed the order: ethanol > isopropanol > methanol > acetone > toluene > acetonitrile. Biokinetic parameters obtained in this study are compared with those reported earlier in literature [21–25]. The maximum specific growth rates of various microbial cultures for isopropanol biodegradation reported in literature varied from 0.0017 to 0.2/h. The maximum μ_{max} for the biodegradation of isopropanol was reported for *Bacillus pallidus* by Bustard et al. [25]. This pure bacterial culture is a solvent tolerant thermophile previously isolated from a phenol contaminated industrial effluent lake (Huntly, New Zealand). It may be noted that a mixed microbial consortium was used in the present study. The maximum specific growth rate obtained in the present study is comparable to the μ_{max} values for mixed cultures reported in literature [23].

Variation of specific growth rate with substrate concentration, as predicted by the Haldane model, for various substrates is shown in Fig. 3. These parameter values were used to validate the model with a set of independent experiments. Studies on single pollutant removal are insufficient for the establishment of generally applicable concepts concerning the simultaneous aerobic biodegradation of multiple organic solvents in a mixture, such as those occurring in waste discharges from pharmaceutical industries. Very few studies on utilization of mixed cultures, growing on multiple organic solvents, for the biodegradation of a mixture of organic solvents have been conducted in the past. In this work, studies on aerobic biodegradation of mixture of solvents, comprising methanol, ethanol, acetone, and toluene, were conducted with the initial concentrations less than their inhibition concentration. An attempt was made to model the biodegradation of these mixed substrate systems, using biokinetic parameters obtained from single-substrate biodegradation studies.

Mixed Substrate Biodegradation Studies

Mathematical models for biodegradation of multiple substrates can be basically classified into two categories namely, non-interactive models and interactive models. The assumption of the former model is that growth rate of bacteriological populations is controlled by only one limiting substrate at one time. While for the latter model, the growth rate of the bacteriological population depends on both the substrates at the same time. Furthermore, interactions among the mixed substrates were categorized into two distinct classes, either beneficial or detrimental [11–16]. The beneficial interactions may be due to the fortuitous

Table 3 Biokinetic parameters for various organic solvents (Haldane model)

Parameter	Symbol	Ethanol	Isopropanol	Acetone	Acetonitrile	Toluene
Maximum growth rate, 1/h	μ_{max}	0.0415	0.0393	0.0320	0.0243	0.0309
Half saturation constant, mg/L	K_s	179.86	378.01	398.09	144.75	44.69
Yield coefficient, mg/mg	Y	0.2561	0.3029	0.2543	0.2074	0.2959
Inhibition concentration, mg/L	k_i	3,347.6	2,747.5	4,242.5	847.4	147.4
Net coefficient of efficiency	E	0.6623	0.7493	0.6124	0.6613	0.7048

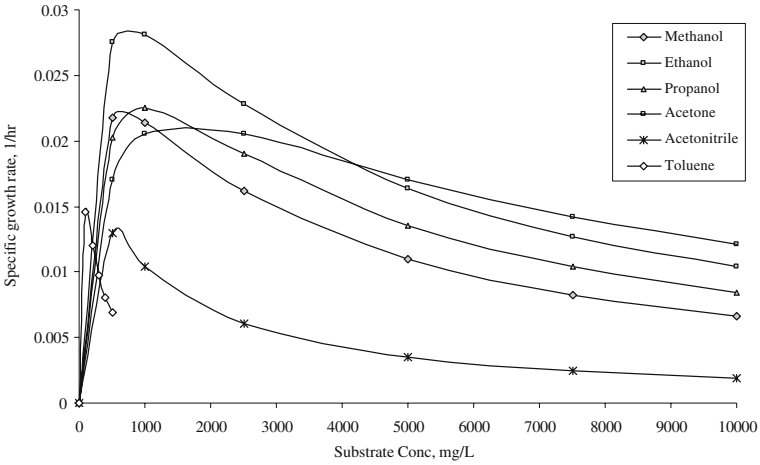


Fig. 3 Variation of specific growth rate of MEC for different substrates

growth of biomass in the presence of multiple substrates or may be due to the induction of essential enzymes required for degradation. On the contrary, interactions could be detrimental due to the competitive metabolism in which one substrate inhibits the utilization of another. Thus the biodegradation rate of an individual compound in a mixture may be enhanced or reduced relative to the comparable single-substrate biodegradation rate. These variations in biodegradation kinetics depend on type and nature of substrates present, their initial concentrations, the initial biomass concentration, respective biokinetic parameters, and their affinity constants. Significant substrate interactions were observed in all the experiments involving biodegradation of mixture of substrates. This was evident from the difference between the experimental substrate depletion rates and those predicted by the sole-substrate model.

The kinetic parameters determined in the single-substrate experiments were utilized in Eqs. 1 to 5 and kinetics of degradation of multiple substrates and growth of MEC were simulated. The model based on *no interaction* effect (results are not presented here) could not predict either the depletion of substrates or growth of biomass satisfactorily. No interaction model over predicted substrate depletion. This indicates that the presence of each substrate had an inhibitory effect on the degradation of the other.

In case of binary substrate systems studied, for instance, the ethanol—acetone mixture, both the no-interaction sum kinetic model and the competitive interaction model showed positive coefficients of efficiency for both substrate utilization and microbial growth. However, the competitive model simulated the experimental results better than the no-interaction sum kinetic model. The overall E value obtained for competitive model for ethanol–acetone binary systems was as high as 0.89, compared to no interaction model where E value was only 0.61. Experimental and model (multi-substrate competitive inhibition) predicted biodegradation kinetics for binary mixture of ethanol and acetone is shown in Fig. 4. Similar results were obtained for other binary systems also (results are not shown). In most cases, coefficients of efficiency of simulations using uncompetitive and noncompetitive models were negative, indicating that the multiple substrate utilization in the present systems does not follow these models. E values for the simulations using various multiple substrate biodegradation kinetic models are presented in Table 4.

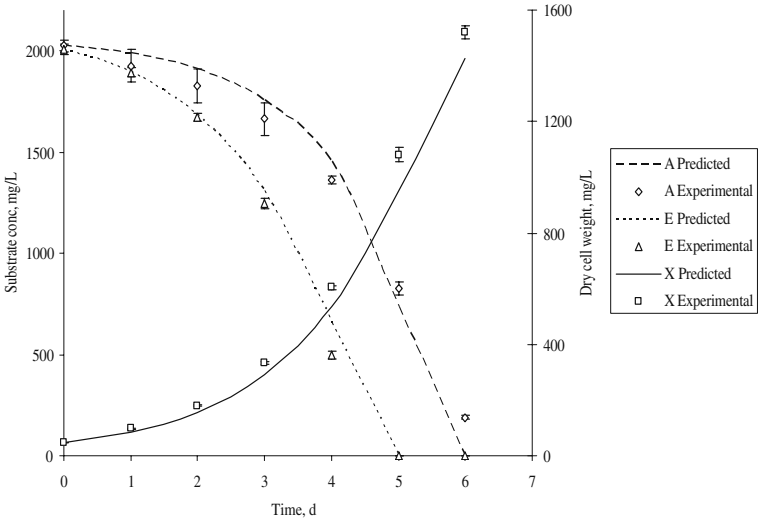


Fig. 4 Experimental and model (competitive inhibition) predicted biodegradation kinetics in a binary mixture of ethanol and acetone (*E* ethanol, *A* acetone, *X* biomass)

Though the no-interaction sum kinetics model showed positive coefficient of efficiency for binary substrate systems, it failed to simulate the microbial growth in ternary and quaternary systems (*E* values are negative: Table 4). The growth of biomass and the substrate biodegradation in ternary mixtures of methanol–acetone–toluene and the competitive model predicted values for this mixture are shown in Fig. 5. Although the toluene biodegradation and biomass growth were well predicted by the model, there were marginal deviations in the predicted methanol and acetone biodegradations from the corresponding measured values. Significant acetone consumption did not begin until the toluene concentration was nearly zero and the methanol concentration was low. Similar trend was observed in the ternary system of ethanol–acetone–toluene mixture (results not shown). This further confirms that acetone is the least preferred substrate for bacterial growth among the VOCs present in the mixture.

Biodegradation of the quaternary system consisting of methanol–ethanol–acetone–toluene was also studied. The competitive inhibition model was able to satisfactorily predict the biomass growth, and the consumptions of methanol and ethanol as evident from Fig. 6 and *E* values presented in Table 4. Although the trend of acetone consumption was simulated by the model satisfactorily, the model predicted values deviated from the observed values (E=0.75).

To summarize, microbial population grew more in a mixture of substrates as compared to when only one substrate was present. This ruled out the possibility of non-interactive model as an appropriate model to describe the biodegradation kinetics of mixture of substrates. Uncompetitive and noncompetitive models also failed to simulate the performance of ternary and quaternary systems satisfactorily. Only the competitive model performed satisfactorily for the simulation of ternary and quaternary systems, with value of coefficient of efficiency greater than 0.5 in all the cases. Though the compounds were simultaneously degraded, their degradation rates varied with respect to substrates.

Table 4 Modified coefficients of efficiency (*E*) obtained while evaluating performance for various multiple substrate biodegradation models

Model	Mixture	Modified coefficients of efficiency (*E*)				
		Degradation of substrate				Biomass growth
		I	II	III	IV	
No-interaction sum kinetics	MA	0.41	0.78			0.41
	MT	0.65	0.81			0.57
	EA	0.78	0.50			0.57
	ET	0.66	0.86			0.51
	MAT	0.46	0.54	0.59		−0.77
	EAT	0.62	0.48	0.74		−1.32
	MEAT	0.19	0.33	0.22	0.28	−0.63
Competitive	MA	0.42	0.57			0.79
	MT	0.80	0.73			0.85
	EA	0.96	0.83			0.88
	ET	0.81	0.67			0.88
	MAT	0.60	0.70	0.95		0.77
	EAT	0.84	0.68	0.77		0.76
	MEAT	0.80	0.94	0.75	0.84	0.61
Non competitive	MA	0.12	0.44			−0.26
	MT	0.64	0.92			0.53
	EA	−0.19	0.70			−0.11
	ET	0.67	0.97			0.23
	MAT	−0.10	−0.21	−0.42		−0.33
	EAT	−0.21	−0.16	−0.69		−0.32
	MEAT	−0.17	−0.16	−0.13	−0.48	−0.20
Un competitive	MA	0.07	−0.37			−0.26
	MT	−0.20	−0.68			0.27
	EA	−0.25	−0.14			−0.13
	ET	−0.20	−0.83			−0.11
	MAT	−0.09	−0.20	−0.36		−0.32
	EAT	−0.19	−0.14	−0.62		−0.30
	MEAT	−0.17	−0.15	−0.19	−0.44	−0.12

Initial conditions were mentioned earlier as in Table 1

M methanol, *E* ethanol, *A* acetone, *T* toluene

Biodegradation of Chloroform

Screening of primary substrates favoring aerobic co-metabolic biodegradation of chloroform in pharmaceutical emissions was carried out. Primary substrates employed in the study were methanol, ethanol, isopropanol, acetone, acetonitrile, and toluene as these are the major non-chlorinated organic solvents used in pharmaceutical operations. Laboratory batch kinetic studies were conducted to determine maximum degradation rates of these primary substrates in the presence of chloroform. Initial concentration of

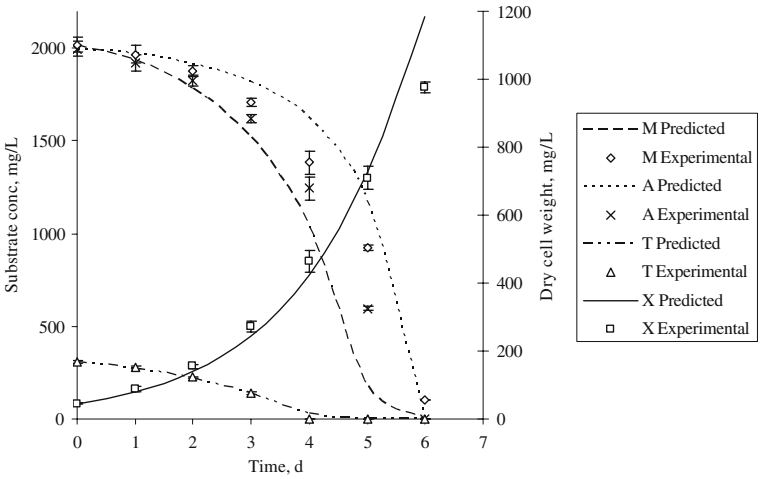


Fig. 5 Experimental and model (competitive inhibition) predicted biodegradation kinetics in ternary mixtures of methanol, acetone, and toluene (*M* methanol, *A* acetone, *T* toluene, *X* biomass)

chloroform in liquid phase was maintained around 50 mg/L. Bacterial (MEC) growth and COD degradation patterns were almost similar to those in studies without chloroform (results not shown). However, there was a slight decrease in the maximum biomass concentration as compared to the system without chloroform. This may be due to the toxicity of chloroform to microbes. Also, the time taken for attaining stationary phase got extended in almost all the reactors when chloroform was present.

Chloroform biodegradation during the log growth phase, in presence of a primary substrate, indicated that chloroform was biodegraded during the degradation of primary substrate. Maximum biodegradation of chloroform was observed in toluene fed batch reactors, followed by acetone fed batch reactors as shown in Fig. 7a. No other system

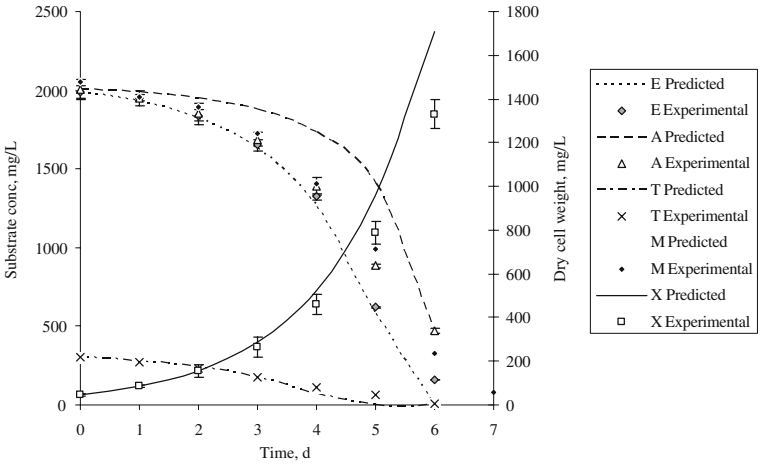


Fig. 6 Experimental and model (competitive inhibition) predicted biodegradation kinetics in quaternary mixtures of methanol, ethanol, acetone, and toluene (*M* methanol, *E* ethanol, *A* acetone, *T* toluene, *X* biomass)

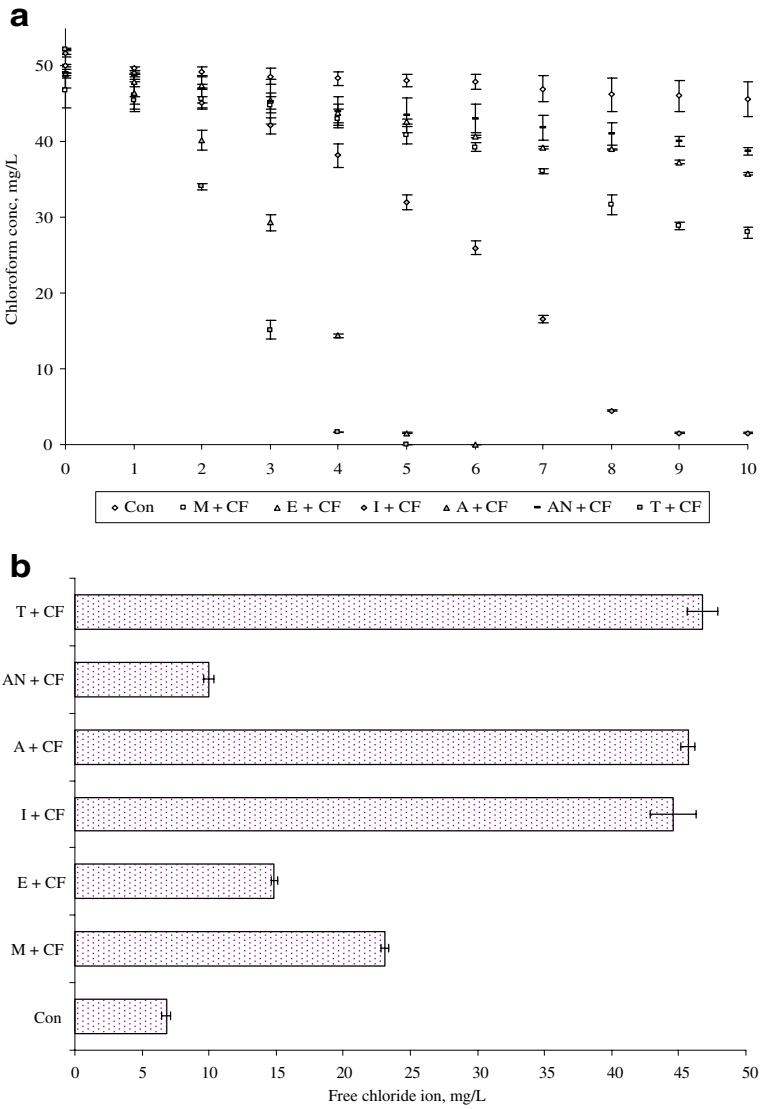


Fig. 7 **a** Biodegradation of chloroform by MEC in presence of various primary substrates (*M* methanol, *E* ethanol, *I* isopropanol, *A* acetone, *T* toluene, *CF* chloroform). **b** Release of free chloride ions during the co-metabolic biodegradation of chloroform in presence of various primary substrates (*M* methanol, *E* ethanol, *I* isopropanol, *A* acetone, *T* toluene, *CF* chloroform)

showed complete degradation of chloroform during this study. The co-metabolic biodegradation depends on the enzymes involved in the degradation of primary substrate. Among the primary substrates studied, biodegradation of toluene and acetone might have triggered the secretion of monoxygenases enzyme which simultaneously biodegraded chloroform. Similar observations were reported by other researchers for biodegradation of chloroform with toluene as a growth substrate [26, 27]. GC-MS analysis revealed the presence of acetol as a metabolite during the biodegradation of acetone, further confirming

the secretion of monoxygenases enzyme. Similar observations were made by Kotani et al. [28]. Kirchner et al. [29] observed the biodegradation of acetone occurs via acetol pathway and further concluded that this reaction requires molecular oxygen. Thus the oxygen-dependent acetone monooxygenase was known to biodegrade acetone via acetol pathway

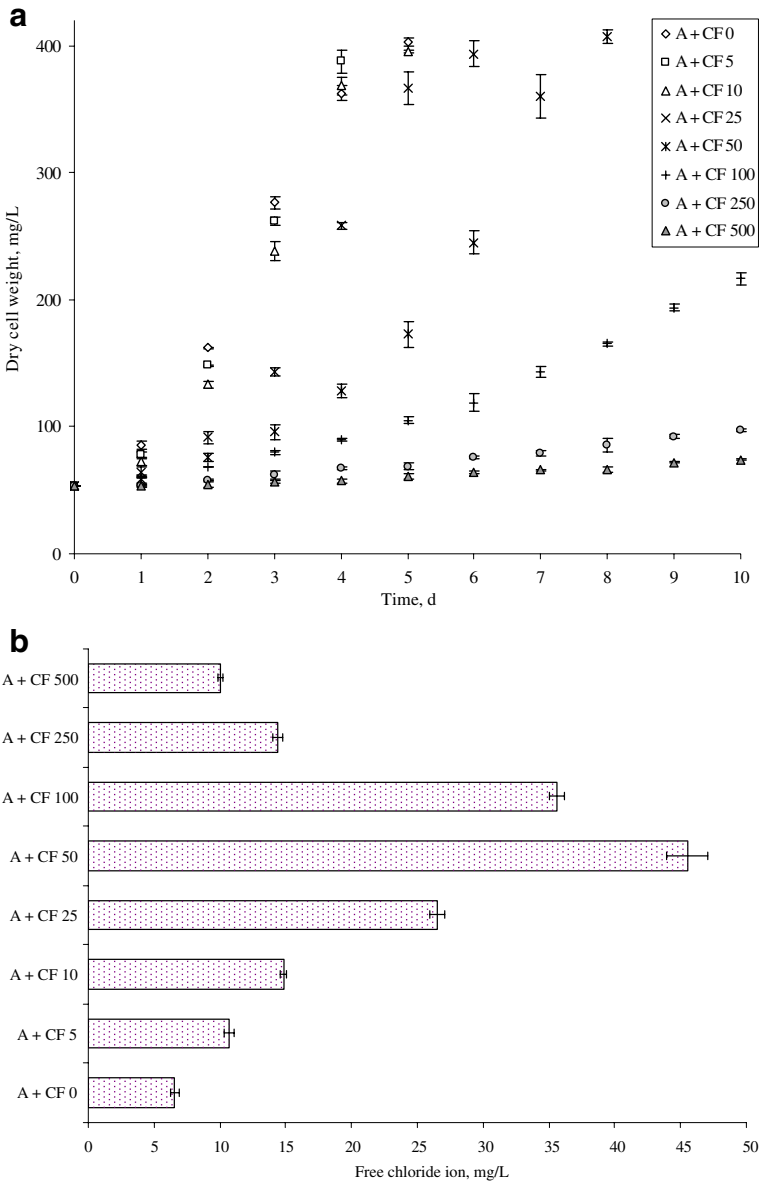
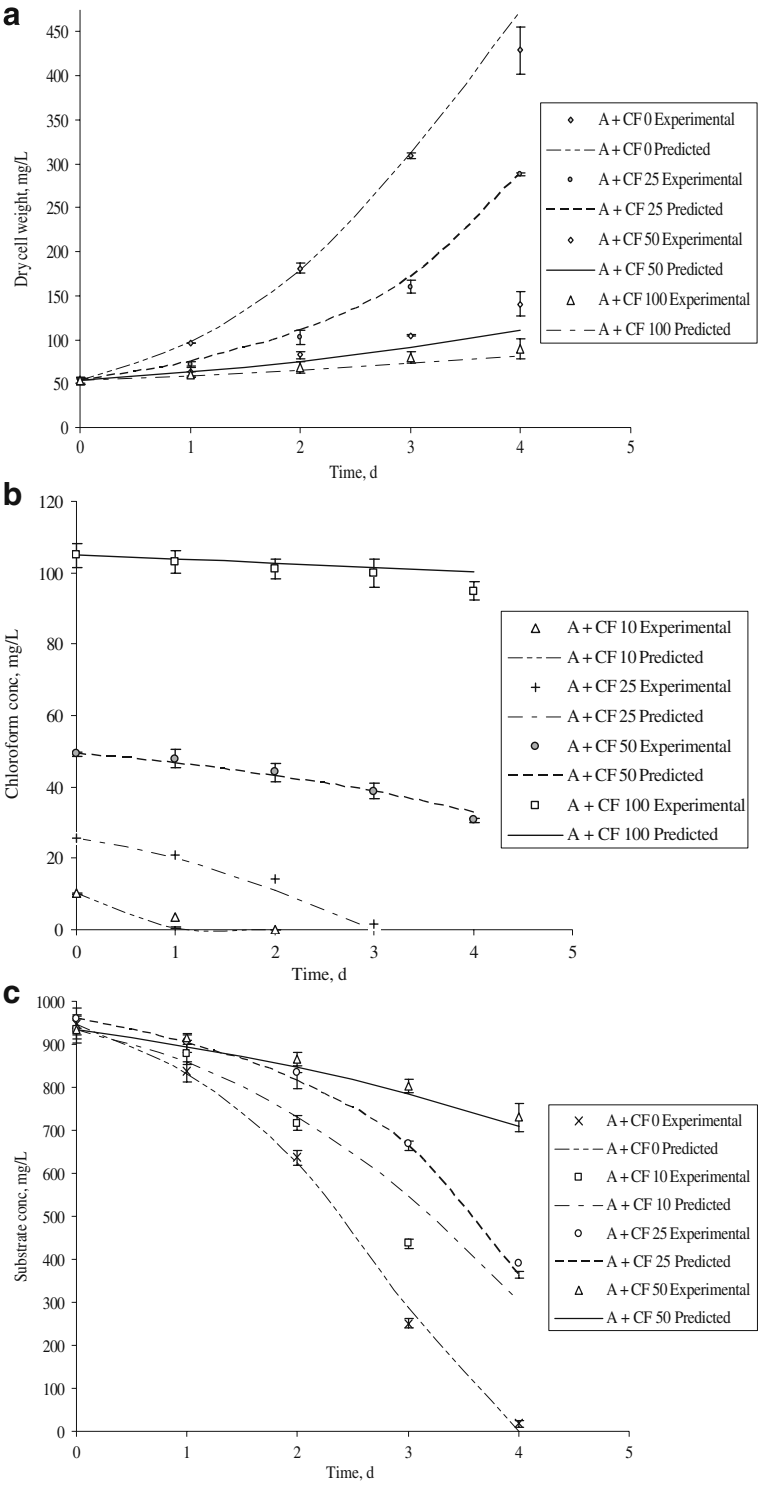


Fig. 8 **a** Growth pattern of MEC in presence of acetone for different initial concentrations of chloroform (*A* acetone, *CF* chloroform). **b** Release of free chloride ions at the end of study using acetone as primary substrate during the co-metabolic biodegradation for different initial concentrations of chloroform (*A* acetone, *CF* chloroform)


a
Dry cell weight, mg/L
Time, d
A + CF 0 Experimental
A + CF 0 Predicted
A + CF 25 Experimental
A + CF 25 Predicted
A + CF 50 Experimental
A + CF 50 Predicted
A + CF 100 Experimental
A + CF 100 Predicted
b
Chloroform conc, mg/L
Time, d
A + CF 10 Experimental
A + CF 10 Predicted
A + CF 25 Experimental
A + CF 25 Predicted
A + CF 50 Experimental
A + CF 50 Predicted
A + CF 100 Experimental
A + CF 100 Predicted
c
Substrate conc, mg/L
Time, d
A + CF 0 Experimental
A + CF 0 Predicted
A + CF 10 Experimental
A + CF 10 Predicted
A + CF 25 Experimental
A + CF 25 Predicted
A + CF 50 Experimental
A + CF 50 Predicted

◀ **Fig. 9** **a** Experimental and model predicted biomass concentrations for co-metabolic biodegradation of chloroform with acetone. **b** Experimental and model predicted chloroform concentrations for co-metabolic biodegradation of chloroform with acetone. **c** Experimental and model predicted acetone concentrations for co-metabolic biodegradation of chloroform with acetone

with the bacterial strains of *Rhodococcus* sp. and *Pseudomonas* sp. The aerobic pathway of chloroform biodegradation by methane oxidisers was reported by Bartnicki and Castro [30]. They observed that the biodegradation processes starts by insertion of oxygen into the molecule resulting in the formation of phosgene (dichloroformaldehyde) that subsequently decomposes abiotically to CO_2. The known metabolites of chloroform degradation such as trichloromethanol and phosgene were not observed in the present study. This may be due to the highly unstable nature of these compounds. However, the release of free chloride ions (Fig. 7b) to the medium confirmed the biodegradation of chloroform. Among the primary growth substrates evaluated for the co-metabolic biodegradation of chloroform, around 85% release of free chloride ions in to the medium was observed in toluene, acetone, and isopropanol fed reactors at the end of the study. It was observed that chloroform was not degraded (nor was chloride detected) in the abiotic controls.

During aerobic degradation of chlorinated compounds, it is common that toxic intermediates are formed which may cause damage to the bacterial cells involved. This has been a general problem in aerobic biodegradation of chlorinated compounds. In the presence of oxygen (oxidative pathway), chloroform undergoes oxidative dechlorination to form trichloromethanol, which spontaneously dehydrochlorinates to form phosgene. Subsequent hydrolysis of phosgene forms hydrochloric acid and the main metabolite is carbon dioxide [31]. Phosgene may also react with cellular macromolecules (such as enzymes, proteins, or the polar heads of phospholipids) which may result in loss of cellular function and cell death [32].

Effect of Initial Concentration of Chloroform on Co-metabolic Biodegradation

The effect of initial concentration of chloroform on biodegradation of 1,000 mg/L of acetone was studied for a better understanding of inhibition effect of chloroform on microbial growth. Chloroform concentrations in this study were varied from 0 to 500 mg/L (5, 10, 25, 50, 100, 250, and 500 mg/L). Microbial growth profiles for various concentrations of chloroform and the corresponding release of free chloride ions are shown in Fig. 8a and b, respectively.

Chloroform, up to a concentration of 25 mg/L, did not affect the microbial growth considerably. However, beyond this concentration, there was a remarkable decrease in microbial growth. Although, all the reactors had the same amount of primary substrate (acetone), differences in biomass growth pattern resulted due to different chloroform concentrations. An increase in chloroform concentration might have caused toxicity to microbes and inhibited the microbial growth. Biodegradation of chloroform in the concentration range of 5 to 50 mg/L occurred within 3 to 8 days. Analyses of concentration of free chloride ions (Fig. 8b) revealed that the biodegradation of chloroform occurred up to a concentration of 50 mg/L.

Biokinetic parameters such as maximum specific growth rate ($\mu_{max,g}$), yield coefficient (Y_T), half saturation concentration ($K_{s,g}$), and inhibition concentration (k_{ig}) for growth of MEC in acetone were utilized to find out the inhibition constant for chloroform (k_{ic}) using Eqs. 6 and 7. Model-simulated results matched well with the experimental results as shown in Fig. 9a–c. The model-predicted inhibition concentration for chloroform was 33.87 mg/L and the specific chloroform degradation rate was 0.16 L/mg day. The specific chloroform

degradation rate is compared with the values reported in the literature [33–39]. The specific chloroform degradation rate constant ranged from 0.0005 to 32. It may be noted that the rate constant differs for various microbial populations, depending on additional substrates and their concentrations, apart from other environmental and experimental conditions.

Conclusions

Studies were conducted to understand the biodegradation of major organic solvents such as methanol, ethanol, isopropanol, acetone, acetonitrile, toluene, chloroform, and carbon tetrachloride used in pharmaceutical industries. Studies were conducted for biodegradation of these solvents when they occur individually as well as in mixtures. Among the various microbial isolates enriched and screened, an aerobic mixed culture that had been previously enriched for biodegradation of mixed pesticides was found to be most effective. All the organic solvents except chloroform and carbon tetrachloride could act as primary substrates for this mixed culture. Chloroform was co-metabolically biodegraded with acetone and toluene as primary substrates. Haldane's inhibition model was found to best fit the kinetics of biodegradation of individual substrates. Biokinetic parameters obtained from the experiments for single substrates could be successfully used in a competitive inhibition model for predicting the kinetics of biodegradation of multi-substrate systems. The Haldane model, modified for inhibition due to chloroform, could satisfactorily predict the biodegradation of primary substrate, chloroform, and the microbial growth. Performance of the mathematical model was evaluated in terms of the dimensionless modified coefficient of efficiency (E). The single substrate biodegradation kinetics failed to predict the biodegradation of same substrate in a mixture of substrates, indicating that substrate interactions occurring in the system cannot be ruled out.

References

1. Santos, F. M., & Lo Biundo, G. (1999). Treatment of pharmaceutical industry process wastewater using the extractive membrane bioreactor. *Environmental Progress, 18*(1), 34–39.
2. Bitzi, U., Egli, T., & Hamer, G. (1991). The biodegradation of mixtures of organic solvents by mixed and monocultures of bacteria. *Biotechnology and Bioengineering, 32*, 1037–1042.
3. Alaton, A., & Caglayan, A. E. (2006). Toxicity and biodegradability assessment of raw and ozonated procaine penicillin G formulation effluent. *Ecotoxicology and Environmental Safety, 63*, 131–140.
4. Constable, D. J. C., Gonzalez, C. J., & Henderson, R. K. (2007). Perspective on solvent use in the pharmaceutical industry. *Organic Process Research and Development, 11*, 133–137.
5. Arp, D. J., Yeager, C. M., & Hyman, M. R. (2001). Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene. *Biodegradation, 12*, 81–103.
6. Krooneman, J., Sliekers, A. O., Gomes, T. M. P., Forney, L. J., & Gottschal, J. C. (2000). Characterization of 3-chlorobenzoate degrading aerobic bacteria isolated under various environmental conditions. *FEMS Microbial Ecology, 32*, 53–59.
7. Olaniran, O., Bhola, V., & Pillay, B. (2008). Aerobic biodegradation of a mixture of chlorinated organics in contaminated water. *African Journal of Biotechnology, 7*(13), 2217–2220.
8. Cecen, F., Kocamemi, B. A., & Aktas, O. (2010). Metabolic and co-metabolic degradation of industrially important chlorinated organics under aerobic conditions, in Xenobiotics in the urban water cycle, mass flows, environmental processes, mitigation and treatment strategies in environmental pollution, edited by Kassinos, D.F., Bester, K. & Kummerer, K. Springer Netherlands.
9. Rogers, J., & Reardon, K. F. (2000). Modeling substrate interactions during the biodegradation of mixtures of toluene and phenol by Burkholderia species JS150. *Biotechnology and Bioengineering, 70* (4), 428–435.

10. Louarn, E., Aulenta, F., Levantesi, C., Majone, M., & Tandoi, V. (2006). Modeling substrate interactions during aerobic biodegradation of mixtures of vinyl chloride and ethane. *Journal of Environmental Engineering, 132*(8), 940–948.
11. Lendenmann, U., Snozzi, M., & Egli, T. (1996). Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. *Applied and Environmental Microbiology, 62*, 1493–1499.
12. Bielefeldt, A., & Stensel, H. D. (1999). Modeling competitive inhibition effects during biodegradation of BTEX mixtures. *Water Research, 33*, 707–714.
13. Guha, S., Peters, C., & Jaffe, P. (1999). Multisubstrate biodegradation kinetics of naphthalene, phenanthrene, and pyrene mixtures. *Biotechnology and Bioengineering, 65*, 491–499.
14. Reardon, K. F., Mosteller, D. C., Rogers, J. B., DuTeau, N. M., & Kim, K. H. (2002). Biodegradation kinetics of aromatic hydrocarbon mixtures by pure and mixed bacterial cultures. *Environmental Health Perspectives, 110*(6), 1005–1011.
15. Knightes, C. D., & Peters, C. A. (2006). Multisubstrate biodegradation kinetics for binary and complex mixtures of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry, 25*(7), 1746–1756.
16. Atlas, M. R. (1995). Handbook of Media for environmental microbiology, Second edition.
17. Ramakrishna, K., & Philip, L. (2009). Biodegradation of mixed pesticides by mixed pesticide enriched cultures. *Journal of Environmental Science and Health, 44*(1), 18–30.
18. APHA. (1998). *Standard methods for examination of water and wastewater* (20th ed.). New York: American Public Health Association.
19. Yoon, H., Klinzing, G., & Blanch, H. W. (1977). Competition for mixed substrates by microbial populations. *Biotechnology and Bioengineering, 19*, 1193–1210.
20. Segel, I. H. (1975). *Enzyme kinetics*. New York: Wiley.
21. Mohammad, B. T., Wright, P. C., & Bustard, M. T. (2006). Bioconversion of isopropanol by a solvent tolerant *Sphingobacterium mizutae* strain. *Journal of Industrial Microbiology & Biotechnology, 33*, 975–983.
22. McEvoy, E., Wright, P. C., & Bustard, M. T. (2004). The effect of high concentration isopropanol on the growth of a solvent-tolerant strain of *Chlorella vulgaris*. *Enzyme and Microbial Technology, 35*, 140–146.
23. Bustard, M. T., McEnvoy, E. M., Goodein, J. A. S., Burgess, J. G., & Wright, P. C. (2000). Biodegradation of propanol and isopropanol by a mixed microbial consortium. *Applied Microbiology and Biotechnology, 54*, 424–431.
24. Bustard, M. T., Meeyoo, V., & Wright, P. C. (2002a). Kinetic analysis of high concentration of isopropanol biodegradation by a solvent tolerant mixed microbial culture. *Biotechnology and Bioengineering, 78*(6), 708–713.
25. Bustard, M. T., Whiting, S., Cowan, D. A., & Wright, P. C. (2002b). Biodegradation of high concentration isopropanol by a solvent tolerant thermophile, *Bacillus pallidus*. *Extremophiles, 6*, 319–323.
26. Chauhan, S., Barbieri, P., & Wood, T. K. (1998). Oxidation of trichloroethylene, 1, 1-dichloroethylene, and chloroform by toluene/o-xylene monooxygenase from pseudomonas stutzeri OX1. *Applied and Environmental Microbiology, 64*(8), 3023–3024.
27. Pareles, R. E., Parales, J. V. Pelletier, D. A., & Ditty, J. L. (2008). Diversity of microbial toluene degradation pathways. In *Advances in Applied Microbiology, 64* edited by Laskin, A. I. Academic.
28. Kotani, T., Yurimoto, H., Kato, N., & Sakai, Y. (2007). Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. Strain TY-5. *Journal of Bacteriology, 189*(3), 886–893.
29. Kirchner, K., Wagner, S., & Rehm, H. J. (1996). Removal of organic air pollutants from exhaust in the trickle-bed bioreactor. Effect of oxygen. *Applied Microbiology and Biotechnology, 45*, 415–419.
30. Bartnicki, E. W., & Castro, C. E. (1994). Biodehalogenation—rapid oxidative-metabolism of monohalomethanes and polyhalomethanes by methylosinus trichosporium OB3b. *Environmental Toxicology and Chemistry, 13*, 241–245.
31. USEPA, (U.S. Environmental Protection Agency), (2001). Toxicological Review of Chloroform.
32. IPCS, (International Programme on Chemical Safety). (2004). *Chloroform. Concise international chemical assessment document 58*. Geneva: WHO.
33. Aziz, C. E., Georgiu, G., & Speitel, G. E., Jr. (1999). Cometabolism of chlorinated solvents and binary chlorinated solvent mixtures using *M. trichosporium* OB3b PP358. *Biotechnology and Bioengineering, 65*, 100–107.
34. Ely, R. L., Williamson, K. J., Hyman, M. R., & Arp, D. J. (1997). Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects and bacterial response. *Biotechnology and Bioengineering, 54*, 520–534.
35. Vlieg, J. E., Koning, W., & Janssen, D. B. (1997). Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. *Applied and Environmental Microbiology, 63*, 4961–4964.
36. Oldenhuis, R., van der Oedzes, J. Y., Waarde, J. J., & Janssen, D. B. (1991). Kinetics of chlorinated hydrocarbon degardaiton by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Applied and Environmental Microbiology, 57*, 7–14.

37. Alvarez-Cohen, L., & McCarty, P. L. (1991). A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environmental Science & Technology, 25*, 1381–1386.
38. Thompson, R. C. (1990). Aerobic biodegradation of chloroform by mixed and pure cultures of methanotropia in batch and continuous flow experiments. Master's thesis, University of Texas at Austin.
39. Leeson, A., & Bouwer, E. J. (1989). Aerobic biotransformation of halogenated aliphatic compounds. In Proceedings of Annual Conference of American Water Works Association, Los Angeles, pp. 805–813.
40. Haldane (Andrews), J. F. (1968). A mathematical model for the continuous culture of microorganisms utilizing inhibitory substance. *Biotechnology and Bioengineering, 10*(6), 707–723.
41. Edwards, V. H. (1970). The influence of high substrate concentration on microbial kinetics. *Biotechnology and Bioengineering, 12*, 679.
42. Levenspiel, O. (1980). The Monod equation: a revisit and a generalization to product inhibition situations. *Biotechnology and Bioengineering, 22*, 1671.
43. Monod, J. (1949). The growth of bacterial cultures. *Annual Revision of Microbiology, 3*, 371.
44. Webb, J. L. (1963). *Enzyme and metabolic inhibitors*. Boston: Academic.