Effect of Chloroform on Aerobic Biodegradation of Organic Solvents in Pharmaceutical Wastewater

Balasubramanian P*, Ligy Philip, S. Murty Bhallamudi

Abstract—In this study, cometabolic biodegradation of chloroform was experimented with mixed cultures in the presence of various organic solvents like methanol, ethanol, isopropanol, acetone, acetonitrile and toluene as these are predominant discharges in pharmaceutical industries. Toluene and acetone showed higher specific chloroform degradation rate when compared to other compounds. Cometabolic degradation of chloroform was further confirmed by observation of free chloride ions in the medium. An extended Haldane model, incorporating the inhibition due to chloroform and the competitive inhibition between primary substrates, was developed to predict the biodegradation of primary substrates, cometabolic degradation of chloroform and the biomass growth. The proposed model is based on the use of biokinetic parameters obtained from single substrate degradation studies. The model was able to satisfactorily predict the experimental results of ternary and quaternary mixtures. The proposed model can be used for predicting the performance of bioreactors treating discharges from pharmaceutical industries.

Keywords—Chloroform, Cometabolic biodegradation, Competitive inhibition, Extended Haldane model, Pharmaceutical industry.

I. INTRODUCTION

MANY drugs in the pharmaceutical industry are produced by chemical synthesis, involving a series of reactions and recovery processes [1]. Due to purity concerns, most of the solvents used in pharmaceutical manufacturing activities are not reused. As a result, pharmaceutical wastewater contains many recalcitrant chemicals; including a wide variety of organic solvents at varying concentrations, generally too low for viable, cost-effective recovery [2]. Most commonly found organic constituents include top ten priority pollutants such as isopropanol, ethyl acetate, methanol, ethanol, n-heptane, tetrahydrofuran, toluene, dichloromethane, acetic acid, and acetonitrile [3]. In particular, presence of recalcitrant chlorinated compounds, including common solvents such as perchloroethylene (PCE), trichloroethylene (TCE), chloroform (CF) and carbon tetrachloride (CT), makes the biological treatment of the pharmaceutical wastewater a challenging task.

Anaerobic cometabolic biodegradation of recalcitrant chlorinated compounds has been reported extensively [4-11].

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However, anaerobic treatment of chlorinated VOC emissions from pharmaceutical industry is not feasible because the flue gas contains large quantity of oxygen. Hence, there is an urgent need to explore the possibility of aerobic cometabolic degradation of chlorinated VOCs. Though much work has been carried out on aerobic biodegradation of TCE [12], literature on aerobic biodegradation of chlorinated aliphatic compounds other than TCE is scarce. Fully halogenated chloro-trifluoro-ethylene was shown to degrade in presence of oxygen by purified soluble methane monooxygenase of *Methylosinus trichosporium* [13]. Ryoo et al. [14] were the first to show that a fully chlorinated compound such as perchlorethylene (PCE) can be biodegraded aerobically by *Pseudomonas stutzeri OX1* through Toluene-o-xylene Monooxygenase (ToMo) enzyme.

Numerous studies have demonstrated aerobic cometabolism of chloroform with formate or methane grown *Methylosinus trichosporium*, [18, 21] with propane, [22] butane [20] or toluene grown cells of *Pseudomonas butanovora* and *Mycobacterium vaccae,* [17] and with ammonia by nitrifying bacteria *Nitrosomonas europaea* [15, 16, 19]. Methane monooxygenase [23-25], ammonia monooxygenase [16, 19] and butane monooxygenases were found to be responsible enzymes for CF co-oxidation [20]. In general, oxygenase enzymes responsible for ring oxidation and fission sustain the cometabolic activity. However, no studies have been reported on the aerobic biodegradation of fully chlorinated aliphatic compound such as carbon tetrachloride either in presence of a primary metabolic substrate or cometabolic substrate.

Multiple pollutants are encountered in most of the industrial emissions, including those from pharmaceutical industry. Rogers et al. [26] reported that utilization of a mixed culture consisting of two different aerobic pure cultures could enhance the biodegradation of mixed pollutants such as toluene, phenol, and benzene through interspecies interactions. Biodegradation of mixtures of TCE and CF has been studied with toluene oxidizing bacteria [17] as well as with a mixed methanotrophic culture [23]. The mixed methanotrophic culture could successfully degrade both CF and TCE. Chang and Cohen [18] evaluated the microbial degradation of chlorinated and non chlorinated methanes, ethanes and ethenes by a mixed methane-oxidizing culture, grown under chemostat and batch conditions. Further, they compared the efficiency of biodegradation by mixed methane oxidizing cultures with that by two pure methanotrophic strains: CAC1 (isolated from the mixed culture) and *Methylosinus trichosporium OB3b*. This study revealed that mixed culture had high transformation capacity than that of

pure cultures. It was also found that the toxicity of chlorinated aliphatic hydrocarbons in a mixture containing multiple compounds was cumulative. The degradation could be predicted using parameters measured from the degradation of individual compounds.

Very little information is available on cometabolic biodegradation of chlorinated compounds in presence of other classes of organic compounds, which co-exist in pharmaceutical wastewater, and can potentially serve as primary substrates. Compounds such as methanol, ethanol, isopropanol, acetone, acetonitrile and toluene predominantly occur as co-contaminants in pharmaceutical wastewater. Since oxygenases are involved in aromatic metabolism, it is expected that aerobic biodegradation of aromatics in general will induce oxygenase activity and thereby promote cometabolism of chloroform. However, relatively little is known about how the coexisting substrates affect the biodegradation kinetics of any individual organic constituent by a mixed microbial community. It is essential to gain insight into the above mentioned aspects so that biological process for treatment of industrial effluents and emissions containing mixtures of both easily biodegradable and recalcitrant compounds can be made effective.

Bielefeldt and Stensel [27] studied the degradation of a multisubstrate system consisting of benzene, toluene, ethylbenzene, o-xylene, and p-xylene, using mixed cultures from a manufactured gas site and modeled the system with competitive inhibition kinetics. Zhang and Bajpai [28] developed a comprehensive mathematical model to describe the cometabolic biotransformation of chlorinated solvent such as TCE by methanotrophs in a batch system at enzymatic level. Their simulation results indicated that the primary substrate dominated the key enzyme production, energy supply and microbial growth. Most of the models described in literature focused on the cometabolism of structurally similar compounds. Only a few researchers have used a general framework to model the cometabolism of structurally dissimilar substrates [29, 30]. Model predictions of cometabolism by Dikshit et al. [30] were based on parameters obtained from microbial growth on individual substrates. Kinetic models for aerobic cometabolism of chlorinated solvents with special emphasis on TCE were reviewed by Cohen and Speitel [31].

Overall biodegradability of chlorinated solvents and related chlorinated aliphatic hydrocarbons such as chlorinated methanes, chlorinated ethanes, chlorinated ethenes, chlorofluorocarbons (CFCs), chlorinated acetic acids, chlorinated propanoids and chlorinated butadienes was evaluated by Field and Alvarez [32]. Kim and Lee [33] developed a regression model using a quantitative structure–activity relationship technique for the prediction of the first-order rate constants (k), for the degradation of chlorinated aliphatic hydrocarbons. However, there have been very limited studies on aerobic cometabolic biodegradation of recalcitrant compounds in presence of multiple substrates. In specific, none of the studies dealt with aerobic cometabolic

biodegradation of higher order chlorinated methanes such as chloroform in presence of a mixture of easily biodegradable substrates.

Objectives of the present study are: (i) to assess the aerobic cometabolic biodegradation of chloroform in presence of pollutants such as methanol, ethanol, isopropanol, acetone, acetonitrile and toluene, which co-exist with chloroform in discharges from pharmaceutical industries; and (ii) to develop a mathematical model that describes the cometabolic conversion of chloroform along with other primary substrates, taking into account the effects of substrate inhibition, interactions among various substrates, and chloroform toxicity.

II. MATERIALS AND METHODS

A. Materials

Nutrient media for bacterial culture enrichment

All bacterial strains were grown at 30°C in minimal salt medium (MSM). The composition of MSM was same as that prescribed by Atlas [34] for the degradation of chlorinated compounds. All media were autoclaved for 15 min. at 121°C and 15 psi. Overnight grown cultures were harvested when the medium optical density (OD$_{600}$) was between 0.1 and 0.2. Cells were collected by centrifugation, washed with MSM, and re-suspended in fresh MSM.

Microbial consortia

The bacterial consortia enriched and isolated by Ramakrishna and Philip [35] for degradation of lindane (LDC) and mixed pesticides (MEC) were employed in this study. *Pseudomonas aeruginosa* (MTCC 9236), *Bacillus sp.* (MTCC 9235) and *Chryseobacterium joostei* (MTCC 9237) were the major bacterial strains isolated from the mixed microbial consortium. In addition, bacterial cultures from an activated sludge plant, referred to as Freshly Enriched Culture (FEC), were also enriched with alcohols and chlorinated organic solvents. This was achieved by simultaneously decreasing the concentration of glucose from 1000 mg/L and increasing the concentration of organic solvents from 0 to 100 mg/L. All the bacterial cultures were serially transferred once in a week (5 mL of bacterial culture) to fresh media (100mL) containing the target pollutant along with other nutrients, and enriched further for three months.

B. Analytical Procedures

Measurement of cell density in liquid phase

The optical density (OD) method was used to measure biomass concentration. 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-Visible spectrophotometer (Techcom,

UK). This information was used for preparing a calibration curve between the dry cell weight and the absorbance. This calibration curve was used for determining concentrations in unknown samples through measurement of absorbance at 600 nm.

Gas chromatographic analysis

Perkin Elmer Clarus 500 gas chromatograph with flame ionization detector (GC-FID) was used for analyzing residual VOC concentrations in liquid samples. GC was equipped with an auto sampler, an on-column, split/split less capillary injection system, and a capillary column (Perkin Elmer 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 twenty minutes. Temperatures of injector and detector were maintained at 150 and 300°C, respectively. Nitrogen was used as the make-up and carrier gas at flow rates 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. Liquid samples were then transferred to GC vials and analyzed by GC-FID.

Measurement of free chloride ions

Samples were centrifuged to remove cells, and supernatant fractions were analyzed for free chloride ions with a model DX-100 ion chromatograph (Dionex, Marlton, N.J.), 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, with a flow rate of 1 mL/min.

C. Experimental Procedures

Batch studies were performed in 125 mL serum bottles. All serum bottles were sealed with silicone/teflon septum; aluminium crimped and kept in orbital shaker at 120 rpm at 30^0C for the study. Sixty milliliters of MSM was taken in each serum bottle and autoclaved properly. Known volumes of concerned organic solvents were injected into the aqueous phase of the serum bottles with gas tight syringes (SGE, Australia). Serum bottles were allowed to attain equilibrium in four hours and the initial concentration in the liquid phase was noted down. Then a known amount of microbial inoculum was 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 samples from the system. Liquid samples (4 mL each) were withdrawn at discrete time intervals and one milliliter from each sample was subjected to close centrifuging in order to remove microbes. Supernatant was then utilized for GC-FID analysis. Remaining three milliliters 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 and all samples were analyzed

in duplicate. 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). All degradation/removal was assumed to be biological as abiotic systems yielded no significant removal. Purpose and description of various batch degradation studies are presented in Table I.

D. Mathematical Model

Single substrate biodegradation kinetics

Among several kinetic models available in literature (Edwards, Haldane (Andrews), Levenspiel, Monod and Webb), Haldane model [36] was found to be the most appropriate model for describing the degradation of single substrate [37]. Therefore, this model was employed to determine the biokinetic parameters such as maximum specific growth rate (μ_{max}), yield coefficient (Y_T), half saturation concentration (Ks), and inhibition concentration (K_i) for growth of MEC in corresponding organic solvents. The governing equation for this model is given in Eq. (1).

TABLE I
PURPOSE AND DESCRIPTION OF VARIOUS BATCH DEGRADATION STUDIES

No.	Purpose & Description	Initial concentrations
Cometabolic degradation of chloroform with single substrate		
1	Methanol + Chloroform	M ~ 1.35 g/L + CF ~ 0, 10, 25, 50, 75 and 100 mg/L
2	Ethanol + Chloroform	E ~ 1 g/L + CF ~ 0, 10, 25, 50, 75 and 100 mg/L
3	Isopropanol + Chloroform	I ~ 1 g/L + CF ~ 0, 10, 25, 50, 75 and 100 mg/L
4	Acetonitrile + Chloroform	AN ~ 1.6 g/L + CF ~ 0, 10, 25, 50, 75 and 100 mg/L
5	Toluene + Chloroform	T ~ 0.5 g/L + CF ~ 0, 10, 25, 50, 75 and 100 mg/L
Cometabolic degradation of chloroform with multiple substrates		
6	Methanol + Acetone + Chloroform	M ~ 2 g/L + A ~ 2 g/L + CF ~ 50 mg/L
7	Methanol + Toluene + Chloroform	M ~ 2 g/L + T ~ 0.3 g/L + CF ~ 50 mg/L
8	Acetone + Toluene + Chloroform	A ~ 2 g/L + T ~ 0.3 g/L + CF ~ 50 mg/L
9	Methanol + Acetone + Toluene + Chloroform	M ~ 2 g/L + A ~ 2 g/L + T ~ 0.3 g/L+ CF ~ 50 mg/L

M – Methanol, E – Ethanol, I – Isopropanol, AN – Acetonitrile, T – Toluene, CF - Chloroform

$$\mu = \frac{\mu_{max} * S}{K_s + S + \frac{S^2}{K_i}} \quad (1)$$

Where, μ is the specific growth rate and S is the substrate concentration.

Biodegradation model for binary mixtures with one primary substrate and chloroform

Haldane's model (Eq. 1) is modified for the biodegradation of a primary substrate in presence of chloroform to account for the inhibition due to chloroform. In this study, cometabolic biodegradation of chloroform was considered. The proposed mathematical model describes the biodegradation of primary substrate and chloroform, and growth of the biomass in the system. Biotransformation of chloroform depends on the biodegradation of primary substrates. Hence, biodegradation of chlorinated solvents (chloroform) is represented by an efficiency factor, η. The modified Haldane's model is given in Eqs. (2) – (4).

$$\frac{dS}{dt} = \left(\frac{X}{Y_T}\right) * \left(\frac{\mu_{max}S}{K_s + S + \frac{S^2}{K_i}}\right) * \left(\frac{K_{ci}}{K_{ci} + C}\right) \quad (2)$$

$$\frac{dX}{dt} = (X) * \left(\frac{\mu_{max}S}{K_s + S + \frac{S^2}{K_i}}\right) * \left(\frac{K_{ci}}{K_{ci} + C}\right) \quad (3)$$

$$\frac{dC}{dt} = \left(\frac{X * \eta}{Y_T}\right) * \left(\frac{\mu_{max}S}{K_s + S + \frac{S^2}{K_i}}\right) * \left(\frac{K_{ci}}{K_{ci} + C}\right) \quad (4)$$

Where, X is biomass concentration in mg/L, C is concentration of chloroform in mg/L, K_{ci} is the chloroform inhibition constant for bacterial growth in mg/L and η is mg of chloroform degraded / g of substrate utilized by bacteria.

Biodegradation model for mixtures with multiple primary substrates and chloroform

The model presented in Eqs. (2) – (4) are further modified when multiple primary substrates are present along with chloroform. The proposed model accounts for competitive biodegradation of primary substrates along with inhibition due to chloroform. Governing equations of the proposed model are given in Eqs. (5) – (7).

$$\frac{dS_i}{dt} = \left(\frac{X}{Y_{Ti}}\right) * \left(\frac{\mu_{max}S_i}{K_{si} + S_i + \frac{S_i^2}{K_{ii}} + \sum_{j=1}^{n-1}\left(\frac{K_{si}}{K_{sj}} * S_j\right)} * \left(\frac{K_{cii}}{K_{cii} + C}\right)\right) \quad (5)$$

$$\frac{dC}{dt} = \sum_{i=1}^{n} \frac{dS_i}{dt} \eta_i \quad (6)$$

$$\frac{dX}{dt} = \sum_{i=1}^{n} \frac{dS_i}{dt} Y_{Ti} \quad (7)$$

Where, subscript i refer to the ith primary substrate.

III. RESULTS AND DISCUSSION

A. Preliminary Studies

Batch experiments were conducted to assess whether LDC, MEC and FEC can grow on organic solvents such as methanol (M), ethanol (E), isopropanol (I), acetone (A), acetonitrile (AN), toluene (T), chloroform (CF) and carbon tetrachloride (CT), as single substrates for their carbon and energy source through heterotrophic metabolism. Among all the cultures screened, MEC exhibited maximum degradation rate for all the organic solvents. MEC could grow on all solvents, except on CF and CT, as single substrates for their carbon and energy source. Haldane model was found to satisfactorily simulate the biodegradation process in the above preliminary studies. Biokinetic parameters, estimated from single-substrate experiments, as determined earlier by Balasubramanian et al. [37] are presented in Table II.

Earlier studies by authors [37] on binary mixtures focused mainly on chloroform and acetone. Experimental data were used to determine biokinetic parameters for cometabolic degradation of chloroform using the modified Haldane's model (Eqs. 2 to 4). Herein, experiments were conducted to determine biokinetic parameters for cometabolic degradation of chloroform in the presence of other non-chlorinated solvents, commonly found in the emissions from pharmaceutical industries.

B. Biodegradation of binary mixtures with one primary substrate and chloroform

Experiments were conducted to study cometabolic biodegradation of chloroform in presence of various primary substrates such as methanol, ethanol, isopropanol, acetonitrile

TABLE II
BIOKINETIC PARAMETERS FOR VARIOUS ORGANIC SOLVENTS (BALASUBRAMANIAN ET AL., 2010)

Parameter	Symbol	Methanol	Ethanol	Isopropanol	Acetone	Acetonitrile	Toluene
Maximum growth rate, *1/hr*	μ_{max}	0.0343	0.0415	0.0393	0.0320	0.0243	0.0309
Half saturation constant, *mg/L*	K_s	183.30	179.86	378.01	398.09	144.75	44.69
Yield coefficient, *mg/mg*	Y_T	0.2026	0.2561	0.3029	0.2543	0.2074	0.2959
Inhibition conc, *mg/L*	k_i	2385.6	3347.6	2747.5	4242.5	847.4	147.4

and toluene. In these experiments, chloroform concentrations were varied from 10 mg/L to 100 mg/L. Maximum biodegradation of chloroform was observed in toluene fed batch reactors, followed by acetone fed batch reactors. Chloroform biodegradation up to 25 mg/L was observed in toluene, acetone and isopropanol fed batch reactors, while chloroform up to a concentration of only 10 mg/L could be degraded in methanol, ethanol and acetonitrile fed batch reactors. No batch system, other than the toluene fed system, showed any significant biodegradation when the concentration of chloroform was more than 50 mg/L. It was also noticed that chloroform concentrations above 50 mg/L significantly inhibited the consumption of toluene also. An extended lag phase was observed before the onset of chloroform degradation. As expected, degradation rates for primary substrates are significantly higher than those for chlorinated solvents. Formation of intermediates was not observed except in isopropanol fed reactors, where acetone accumulation was noticed initially. However, generated acetone disappeared subsequently. As discussed earlier [37], biodegradation of chloroform was verified with the release of free chloride ions into the medium. Variation of concentration of free chloride ions as a function of time in each of the reactors is presented in Fig 1.
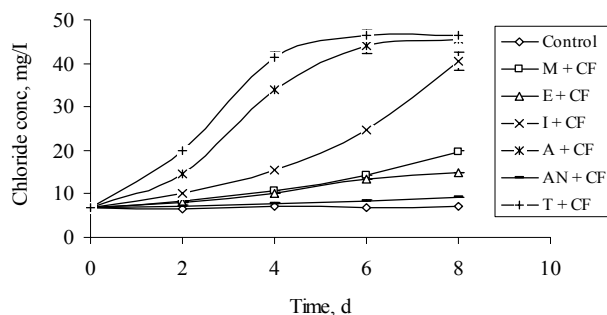

Fig. 1 Concentration of free chloride ions released during the cometabolic biodegradation of 50 mg/L of chloroform in presence of various primary substrates (M: methanol; E: ethanol; I: isopropanol; A: acetone; AN: acetonitrile; T: toluene; CF: chloroform)

Initial concentration of the primary substrate in these experiments was maintained around 2000 mg/L, expressed as COD. Among the primary growth substrates evaluated for the cometabolic biodegradation of chloroform, around 85% release of free chloride ions to the medium was observed in toluene, acetone, and isopropanol fed reactors, when chloroform concentration was not more than 50 mg/L. It was observed that chloroform was not degraded (nor was chloride detected) in the abiotic controls. Similarly, free chloride ion was not detected in any of the carbon tetrachloride fed batch reactors, indicating that there was no significant biodegradation of carbon tetrachloride.

Equations (2) to (4) were used along with the experimental data for cometabolic biodegradation of chloroform to estimate the biokinetic parameters such as chloroform inhibition

constant, K_{ci} and the efficiency factor, η. Other biokinetic parameters such as μ_{max}, K_s, Y_T and primary substrate inhibition constant K_i, were determined from the single substrates biodegradation experiments [37]. Experimental data obtained during the biodegradation of binary mixtures of primary substrates along with 50 mg/L of chloroform were utilized for calibration and determination of unknown parameters K_{ci} and η. These calibrated parameter values were used to validate the model with a set of independent experiments with various concentrations of chloroform ranging from 10 mg/L to 100 mg/L. Calibration and validation of the proposed model for cometabolic biodegradation of chloroform in presence of methanol are presented in Fig. 2.

Similarly, calibration and validation of the proposed model were carried out also for the cometabolic biodegradation of chloroform in presence of ethanol, isopropanol, acetonitrile and toluene. (Results are discussed in Table III). It can be observed from Fig. 2 that the biodegradation of methanol, cometabolic degradation of chloroform, and the biomass growth are well predicted by the proposed mathematical model. Biokinetic constants in the proposed mathematical model for cometabolic biodegradation in binary mixture of acetone and chloroform were presented in our earlier study and the model performance was evaluated statistically using the dimensionless modified coefficient of efficiency (E) [37]. Biokinetic constants for other binary mixtures, obtained in the present study, are presented in Table III.

$$ E = 1 - \frac{\sum_{i=1}^{N} \left[\left| E(t_i) - O(t_i) \right| \right]}{\sum_{i=1}^{N} \left| O(t_i) - \bar{O} \right|} \tag{5} $$

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 $\bar{O}$ is the mean value of the observed variable. Positive value of E represents an acceptable simulation, whereas E > 0.5 represents a good simulation and E equal to one indicates a perfect simulation. E values for the calibration and validation runs for binary mixtures of a primary substrate and chloroform are given in Table III. It can be observed from Fig. 2 as well as from Table III that the proposed mathematical model for cometabolic biodegradation of chloroform, in presence of a primary substrate, performed well (E > 0.5).
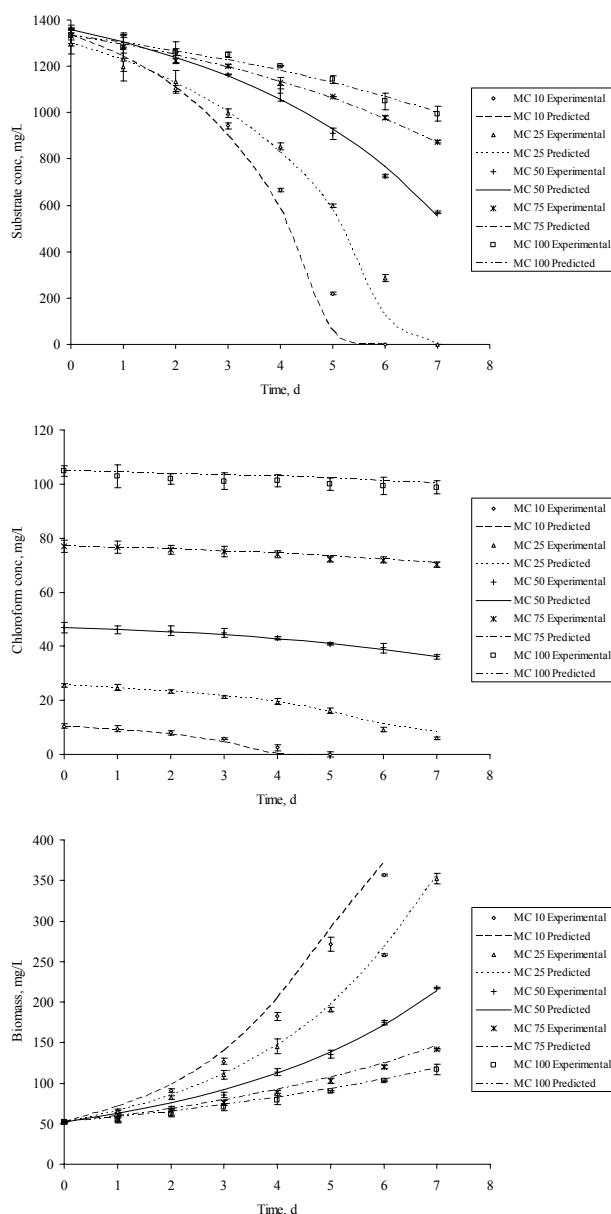
Fig. 2 (a) Experimental and model predicted methanol concentrations for cometabolic biodegradation of chloroform with methanol; (b) Experimental and model predicted chloroform concentrations for cometabolic biodegradation of chloroform with methanol; (c) Experimental and model predicted biomass concentrations for cometabolic biodegradation of chloroform with methanol. (initial methanol concentration = 2000 mg/L as COD; initial chloroform concentrations: 10, 25, 50, 75, and 100 mg/L; x in MCx represents chloroform concentration in mg/L)

C. Biodegradation of multiple primary substrates along with chloroform

Emissions from pharmaceutical industries normally contain several volatile organic compounds along with chlorinated compounds such as chloroform. Therefore, experiments were conducted to understand biodegradation of multiple primary

TABLE III
BIOKINETIC PARAMETERS FOR COMETABOLIC BIODEGRADATION OF CHLOROFORM ALONG WITH VARIOUS PRIMARY SUBSTRATES AND CORRESPONDING MODIFIED COEFFICIENTS OF EFFICIENCY (E) VALUES
(S: SUBSTRATES; CF: CHLOROFORM; X: BIOMASS)

	Methanol	Ethanol	Isopropanol	Acetonitrile	Toluene
K_{ic}	35.3393	46.2871	36.9848	60.6479	41.2950
η	0.0133	0.0101	0.1574	0.0091	0.2109
E values for 50 mg/L chloroform mixture					
S	0.93	0.96	0.70	0.77	0.76
CF	0.94	0.79	0.97	0.70	0.83
X	0.67	0.95	0.74	0.77	0.70
E values for 10 mg/L chloroform mixture					
S	0.57	0.81	0.79	0.87	0.64
CF	0.87	0.83	0.88	0.82	0.95
X	0.87	0.88	0.55	0.51	0.75
E values for 25 mg/L chloroform mixture					
S	0.83	0.79	0.85	0.86	0.54
CF	0.75	0.63	0.75	0.70	0.96
X	0.87	0.74	0.61	0.69	0.83
E values for 75 mg/L chloroform mixture					
S	0.74	0.74	0.77	0.67	0.84
CF	0.82	0.54	0.89	0.53	0.84
X	0.83	0.90	0.86	0.64	0.68
E values for 100 mg/L chloroform mixture					
S	0.85	0.61	0.87	0.77	0.61
CF	0.86	0.60	0.84	0.42	0.61
X	0.85	0.90	0.67	0.46	0.65

substrates (easy to degrade VOCs) in presence of chloroform. It is expected that both the interactions among the primary substrates and the inhibition effect due to high concentrations of chloroform play a significant role in the biodegradation of all the compounds. Interactions among the primary substrates in the absence of chloroform have been studied earlier [27, 37-39] and the interactions could be modeled satisfactorily using the competitive interaction model.

Biodegradation kinetics of ternary mixtures of (i) methanol-toluene-chloroform (MTC), (ii) methanol-acetone-chloroform (MAC) and (iii) acetone-toluene-chloroform (ATC), along with the biomass growth, are presented in Figs. 3, 4 and 5 respectively. These figures present the comparison of simulated results, obtained using Eqs. (5) to (7), with the experimental data. It can be observed from Fig. 3 for the case of MTC mixture that the proposed model predicted the biodegradation of the primary substrates, chloroform as well as the growth of biomass satisfactorily as indicated by E values presented in Table 4 (E > 0.5). Similar results were obtained with other ternary mixtures (Figs. 4 and 5). These results indicate that the proposed model satisfactorily simulates the interaction between primary substrates and chloroform.
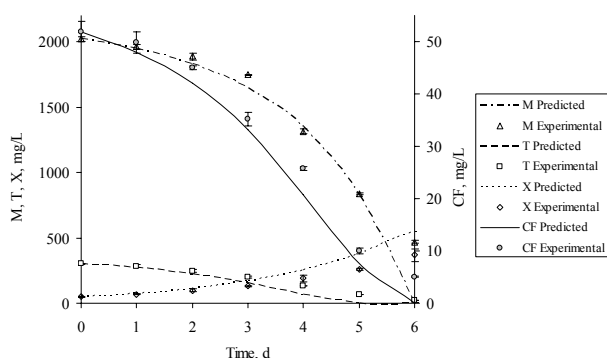
Fig. 3 Experimental observations and model predictions for the cometabolic biodegradation of ternary mixture of methanol, toluene and chloroform (M: methanol concentration; T: toluene concentration; X: biomass concentration; CF: chloroform concentration)
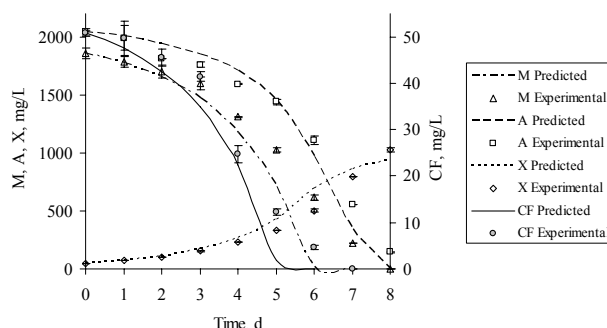

Fig. 4 Experimental observations and model predictions for the cometabolic biodegradation of ternary mixture of methanol, acetone and chloroform (M: methanol concentration; A: acetone concentration; X: biomass concentration; CF: chloroform concentration)
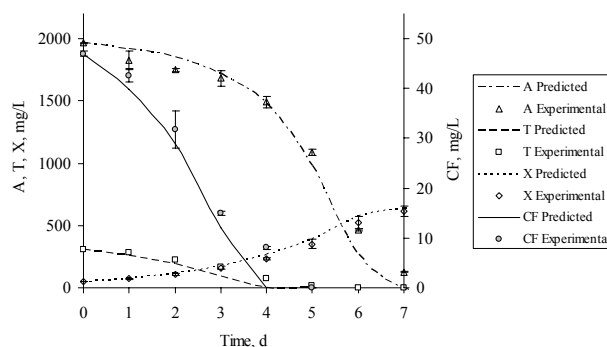

Fig. 5 Experimental observations and model predictions for the cometabolic biodegradation of ternary mixture of acetone, toluene and chloroform (A: acetone concentration; T: toluene concentration; X: biomass concentration; CF: chloroform concentration)

The performance of the proposed mathematical model was also tested with the kinetic data for biodegradation and biomass growth in a quaternary mixture of Methanol –

Acetone – Toluene – Chloroform (MATCF) (Fig. 6). As evident from Figs. 3, 4, 5 and Table 4 for E values, it can be concluded that the proposed model satisfactorily simulates the biodegradation process of mixture of primary substrates in presence of chloroform.
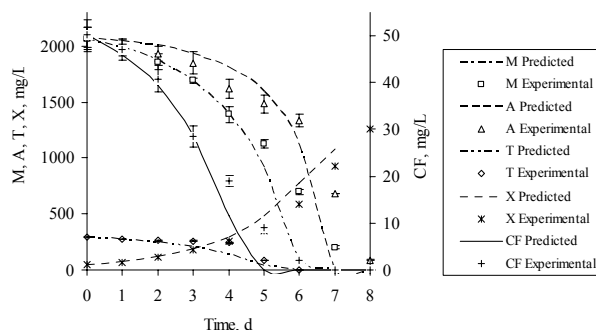

Fig. 6 Experimental observations and model predictions for the cometabolic biodegradation of quaternary mixture of methanol, acetone, toluene and chloroform (M: methanol concentration; A: acetone concentration; T: toluene concentration; X: biomass concentration; CF: chloroform concentration)

TABLE IV
MODIFIED COEFFICIENTS OF EFFICIENCY (E) OBTAINED WHILE EVALUATING PERFORMANCE FOR VARIOUS MULTIPLE SUBSTRATE MIXTURES (M: METHANOL; A: ACETONE; T: TOLUENE; S: SUBSTRATES; CF: CHLOROFORM; X: BIOMASS, INITIAL CONDITIONS WERE MENTIONED EARLIER AS IN TABLE I)

| Mixture | Modified coefficients of efficiency (E) | | | | |
	S1	S2	S3	CF	X
M-A-CF	0.60	0.55		0.89	0.60
M-T-CF	0.67	0.68		0.98	0.54
A-T-CF	0.73	0.88		0.85	0.71
M-A-T-CF	0.62	0.67	0.80	0.91	0.63

IV. CONCLUSIONS

Experimental studies were conducted with mixed pesticide enriched cultures (MEC) constituting mainly of *Pseudomonas aeruginosa, Bacillus sp. and Chryseobacterium joostei* to understand the cometabolic biodegradation of chloroform in presence of major organic solvents such as methanol, ethanol, isopropanol, acetone, acetonitrile and toluene, commonly used in pharmaceutical industries as binary, ternary and quaternary mixtures. Chloroform was cometabolically biodegraded with toluene and acetone as primary substrates very effectively. Haldane's inhibition model was used to evaluate the biokinetic parameters for the biodegradation of individual substrates. Biokinetic parameters obtained from the experiments for single substrates could be successfully used in the modified Haldane model for cometabolic biodegradation of chloroform in presence of a primary substrate to 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 model for binary mixtures was extended to ternary and quaternary mixtures by incorporating the competitive inhibition between various primary substrates and chloroform. This general model was validated using the experimental results of ternary and quaternary mixtures and the model performance was satisfactory. The proposed model will be useful for predicting the performance of bioreactors treating the discharges from pharmaceutical industries.

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