## ORIGINAL ARTICLE

# Optimization and kinetics studies on biodegradation of atrazine using mixed microorganisms

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**Abstract**   In this work, degradation of atrazine was carried out in batch reactors using mixed microorganisms obtained from pharmaceutical wastewater sludge. The effects of process parameters like pH, temperature, inoculum concentration, and agitation speed on atrazine degradation were studied and optimized using response surface methodology (RSM). The optimum condition for the maximum degradation of atrazine was pH – 6.7, temperature – 29.3 °C, inoculum concentration – 5%, and agitation speed – 137 rpm. At these conditions, the effect of atrazine concentration was studied. From the results, it was found that increase in atrazine concentration decreases the degradation efficiency. The maximum atrazine degradation was found to be 94.4%. Various cell growth models and substrate inhibition models were used to describe the atrazine degradation kinetics. From the results, it was found that Haldane model fits the data well with $R^2$ value of 0.9001.

## 1. Introduction

Atrazine is one of the most widely used herbicides in agricultural and forestry applications. The annual usage of atrazine ranges from 70,000 to 90,000 tonnes. It is used to control broadleaf and grassy weeds in crops such as maize, sorghum, and sugarcane [1]. Atrazine is a common pollutant of surface water, ground water, and soil. The biodegradability of atrazine was found to be low [2]. It is also proved that atrazine has toxic effects on algae, aquatic plants, insects, fishes, and mammals [3–5].

Atrazine is removed from soil and water by physical, chemical, and biological methods. In general, biological treatment processes have advantages over physical and chemical treatment methods. It is cost effective and environmental friendly. Few works are available on biodegradation of atrazine [6–8]. Most of these works are carried out using pure species and/or anaerobic conditions. Hence, this work is focused on the degradation of atrazine using mixed cultures in aerobic condition.

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models,

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evaluating the effects of factors, and searching for the optimum conditions. RSM is widely used in biotechnology, food technology, environmental engineering, etc. [9–13]. Till now, no work has been done, to study the interactive effects of process parameters on the degradation of atrazine using aerobic mixed microbial consortium. Hence, the objective of the present study is to degrade atrazine using mixed microbial consortium at aerobic conditions and to optimize the process parameters using response surface technology. Kinetic modeling was also carried out using various cell growth and substrate inhibition models.

## 2. Materials and methods

### 2.1. Chemicals

Atrazine was procured from the local market. The structure of atrazine is shown in Fig. 1. The mixed microbial consortium was obtained from the sludge taken from the wastewater treatment pond from a pharmaceutical industry, Shasun Chemicals and Drugs Ltd., Cuddalore, India. It was used as inoculum. Double distilled water was used throughout the experimental work. Experiments were carried out in 500 cc Erlenmeyer flasks.

### 2.2. Analysis

Atrazine concentration was measured in Bio-spectrophotometer (Model: BL-200, ELICO, India). Atrazine was extracted from sample by liquid extraction method. Dichloromethane was used as extractant.

Ten milliliter of sample was taken in a 50 ml conical separating funnel after centrifuging for 10 min. 5 ml of dichloromethane was added to the solution and shaken vigorously for 3 min. Excess pressure due to volatilization of dichloromethane was released by opening the bottom outlet keeping

upward. The solution was again shaken for 2 min and finally 1 min with releasing pressure after each shake. The whole content was allowed to stand quiet to separate the water dichloromethane layer. Dichloromethane being heavier remained in the bottom of the separating layer. It was allowed to pass through a filter paper topped with a bed of 2 gm of anhydrous sodium sulfate kept on glass wool. The filtrate was collected in a 25 ml volumetric flask. Above procedure was repeated twice, using 5 ml dichloromethane each time. Maximum absorbance for atrazine is observed at 228.8 nm [7]. Hence, the solution was then taken, and atrazine concentration was measured at 228.8 nm.

### 2.3. Experimental design by RSM

Response Surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. Box–Behnken design was used to study the effects of the variables toward their responses and subsequently in the optimization studies. This method was suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. The coded values of the process parameters are determined by the following equation

$$x_i = \frac{X_i - X_o}{\Delta x} \tag{1}$$

where $x_i$ – coded value of the $i$th variable, $X_i$ – uncoded value of the $i$th test variable, and $X_0$ – uncoded value of the $i$th test variable at center point.

The regression and graphical analysis with statistical significance were carried out using Design Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA). In order to visualize the relationship between the experimental variables and responses, 3D plots are generated from the models. The optimum values of the process variables are obtained from the response surface.

### 2.4. Experimental procedure

The range and level of the variables are given in Table 1. Experiments were carried out according to the Box–Behnken design shown in Table 2. The pH of the sample (150 ml) was adjusted to 5, 7, and 9 by adding acid or base as required. Sulfuric acid and sodium hydroxide were used as acid and base, respectively. The initial concentration of atrazine was varied to 5%, 10%, and 15%. The samples were kept in an incubated shaker (Lark, India), and the agitation speed and temperature were adjusted according to the BBD. After the degradation
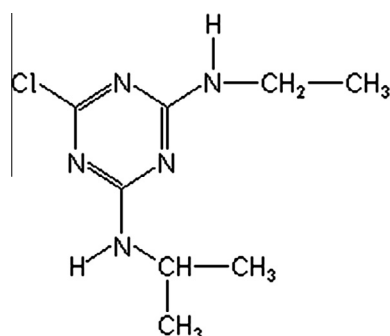


**Figure 1** Structure of atrazine.

**Table 1** Level of different process variables in coded and uncoded form for degradation of atrazine using mixed microbial consortium.

| Variables | Code | Levels | | |
|---|---|---|---|---|
| | | −1 | 0 | +1 |
| pH | A | 5 | 7 | 9 |
| Temperature, °C | B | 20 | 30 | 40 |
| Innoculum concentration, % | C | 5 | 10 | 15 |
| Agitation speed, rpm | D | 100 | 150 | 200 |

process, the samples were withdrawn and analyzed for atrazine.

The percentage degradation was calculated by

$$\%\text{degradation} = \frac{\text{initial atrazine concentration} - \text{final concentration}}{\text{Initial concentration}} \times 100$$

## 3. Results and discussion

Experiments were carried out to examine the combined effect of four different process parameters on the degradation of atrazine using mixed microbial consortium. The second order polynomial coefficients for each term of the equation were determined through multiple regression analysis using the Design Expert. The experimental and predicted values of percentage degradation of atrazine using mixed microbial consortium from pharmaceutical sludge are given in Table 2.

### 3.1. Experimental design and fitting of quadratic model

The second order polynomial Eq. (3) represents the mathematical model relating the percentage degradation using mixed microbial consortium from pharmaceutical sludge with the independent process variables.

$$
\begin{aligned}
\%\text{Degradation} = {} & 93.65 - 3.33A - 0.91B - 12.11C \\
& - 5.20D - 4.55AB + 0.25AC - 5.15AD \\
& - 1.40BC - 2.37BD - 0.92CD \\
& - 15.55A^2 - 11.46B^2 - 8.14C^2 \\
& - 9.45D^2
\end{aligned}
\tag{3}
$$

where $A$, $B$, $C$, and $D$ are the coded values of the test variables, pH, temperature (°C), inoculum concentration, and agitation speed (rpm), respectively.

The above model can be used to predict the percentage degradation of atrazine within the limits of the experimental factors. Fig. 2 shows that the actual response values agree well with the predicted response values.

**Table 2** Experimental conditions of Box Behnken design for atrazine degradation using pharmaceutical sludge.

| Run No. | pH | Temperature | Innoculum concentration | Agitation speed | % Degradation of atrazine | |
|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted |
| 1. | −1 | 0 | −1 | 0 | 88.5 | 89.9 |
| 2. | 0 | −1 | 1 | 0 | 63.0 | 65.1 |
| 3. | 0 | 0 | 0 | 0 | 93.7 | 91.6 |
| 4. | −1 | 0 | 0 | −1 | 75.3 | 76.8 |
| 5. | 1 | 0 | 1 | 0 | 52.3 | 57.0 |
| 6. | 0 | 1 | 0 | −1 | 78.3 | 82.5 |
| 7. | 0 | 0 | 0 | 0 | 93.8 | 91.6 |
| 8. | 0 | 1 | −1 | 0 | 86.4 | 89.3 |
| 9. | 0 | 0 | 0 | 0 | 93.6 | 91.6 |
| 10. | 0 | 0 | −1 | −1 | 90.1 | 99.1 |
| 11. | 0 | 1 | 1 | 0 | 62.2 | 65.1 |
| 12. | 0 | −1 | −1 | 0 | 81.6 | 93.9 |
| 13. | 0 | 0 | 0 | 0 | 93.7 | 91.6 |
| 14. | 0 | 0 | −1 | 1 | 87.6 | 90.4 |
| 15. | 0 | 0 | 1 | −1 | 67.5 | 78.6 |
| 16. | −1 | −1 | 0 | 0 | 68.5 | 66.4 |
| 17. | −1 | 0 | 1 | 0 | 61.2 | 60.9 |
| 18. | 0 | −1 | 0 | −1 | 74.6 | 80.1 |
| 19. | 1 | 0 | 0 | 1 | 50.2 | 55.6 |
| 20. | −1 | 0 | 0 | 1 | 65.8 | 65.3 |
| 21. | −1 | 1 | 0 | 0 | 71.2 | 69.5 |
| 22. | 0 | 0 | 1 | 1 | 61.3 | 57.8 |
| 23. | 1 | 1 | 0 | 0 | 56.8 | 57.6 |
| 24. | 0 | 0 | 0 | 0 | 93.5 | 91.6 |
| 25. | 0 | 1 | 0 | 1 | 66.5 | 63.1 |
| 26. | 0 | −1 | 0 | 1 | 72.3 | 70.2 |
| 27. | 1 | 0 | −1 | 0 | 78.6 | 81.0 |
| 28. | 0 | 0 | 0 | 0 | 93.6 | 91.6 |
| 29. | 1 | 0 | 0 | −1 | 80.3 | 73.6 |
| 30. | 1 | −1 | 0 | 0 | 72.3 | 65.4 |
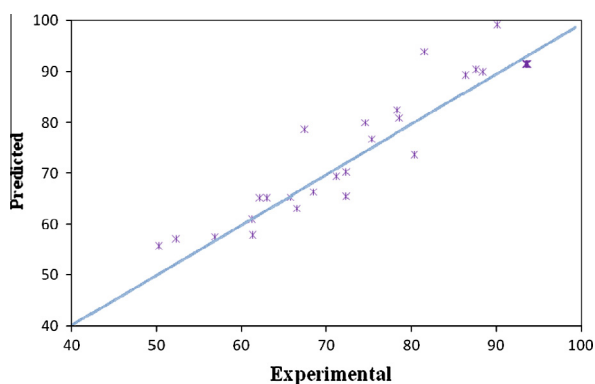
**Figure 2** Predicted value vs. experimental value of atrazine degradation.

### 3.2. Analysis of variance for response surface quadratic model

The adequacy of the models was further justified through analysis of variance (ANOVA). Lack-of-fit is a special diagnostic test for adequacy of a model that compares the pure error, based on the replicate measurements to the other lack-of-fit, based on the model performance. $F$-value, calculated as the ratio between the lack-of-fit mean square and the pure error mean square, is the statistic parameter used to determine whether the lack-of-fit is significant or not, at a significance level.

The results were analyzed by using ANOVA and are given in Table 3. The ANOVA of the quadratic regression model indicates that the model is significant. The Model $F$-value of 26.52 implies that the model is significant. Values of $P$ less than 0.05 indicate that the model term is significant. From the $P$ values, it was found that the variables, $A$, $C$, $D$, $AB$, $AD$, $A^2$, $B^2$, $C^2$, $D^2$, were significant model terms. The predicted $R^2$ of 0.7764 was in reasonable agreement with the adjusted $R^2$ of 0.9249. The fit of the model expressed by the coefficient of regression $R^2$ was found to be 0.9612, indicating that 96.12 percentage of the variability in the response could be explained by the model. This implies that the prediction of experimental data is quite satisfactory.

From the coefficient factors of Table 3, it was found that the interaction of pH and inoculum concentration has positive effect. The quadratic terms of pH, temperature, inoculum concentration, and agitation speed and interactions of pH – temperature, pH – agitation speed, temperature – agitation speed, inoculum concentration – agitation speed, and temperature – inoculum concentration have negative effect on atrazine degradation.

The response surfaces curves show the relative effects of two variables, by keeping the other variable at fixed level, on atrazine degradation. The 3D plots are shown in Figs. 3–8. pH is one of the important factors for the degradation process. From Fig. 3, it was found that increase in pH up to 6.7 increases atrazine degradation after that degradation decreases. pH tolerance of microorganism is quite important for degradation of atrazine. It was clear from Fig. 3 that atrazine degradation increases with an increase in temperature from 20 to 29.3 °C. The atrazine degradation decreases with further increase in temperature up to 40 °C. Atrazine degradation is significantly suppressed at higher temperatures. This may be due to the loss of cell viability of microorganism. This is clearly observed in Figs. 3, 5 and 7. Increase in inoculum concentration decreases atrazine degradation. At low inoculum concentration, the maximum degradation occurs (Figs. 4, 6 and 8). Increase in agitation speed up to 137 rpm increases the atrazine degradation. This may be due to intimate contact of microorganisms and the atrazine that enhances the degradation. Further increase in agitation leads to decrease in atrazine degradation (Figs. 5, 7 and 8). This is due to at high speeds, disruption of cells occurs which leads to poor degradation.

The results obtained showed that a pH of 6.7, temperature of 29.3 °C, inoculum concentration of 5%, and agitation speed of 137 rpm were the best conditions to obtain maximum atrazine degradation using mixed culture obtained from pharmaceutical wastewater sludge. The optimal values for the variables as predicted by MATLAB were found to be within the design region.

**Table 3** Analysis of variance for response surface quadratic model.

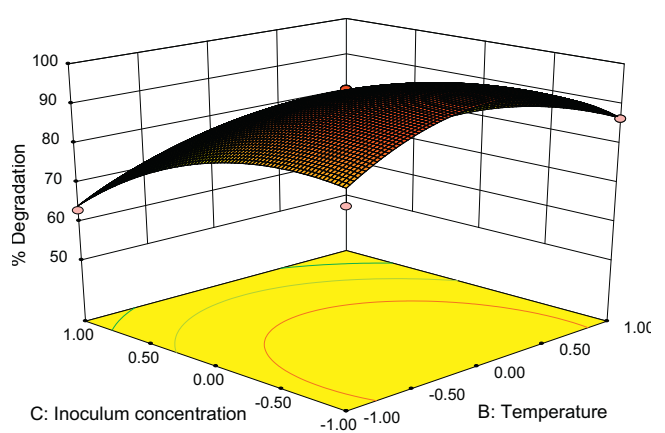| Source | Coefficient factor | Sum of square | Degree of freedom | Mean square | $F$-value | Prob $> F$ |
|---|---|---|---|---|---|---|
| Model | 93.65 | 5088.40 | 14 | 363.46 | 26.52 | <0.0001 |
| $A$ | −3.33 | 133.33 | 1 | 133.33 | 9.73 | 0.0070 |
| $B$ | −0.91 | 9.90 | 1 | 9.90 | 0.72 | 0.4087 |
| $C$ | −12.11 | 1759.34 | 1 | 1759.34 | 128.37 | <0.0001 |
| $D$ | −5.20 | 324.48 | 1 | 324.48 | 23.68 | 0.0002 |
| $AB$ | −4.55 | 82.81 | 1 | 82.81 | 6.04 | 0.0266 |
| $AC$ | 0.25 | 0.25 | 1 | 0.25 | 0.018 | 0.8944 |
| $AD$ | −5.15 | 106.09 | 1 | 106.09 | 7.74 | 0.0140 |
| $BC$ | −1.40 | 7.84 | 1 | 7.84 | 0.57 | 0.4612 |
| $BD$ | −2.37 | 22.56 | 1 | 22.56 | 1.65 | 0.2189 |
| $CD$ | −0.92 | 3.42 | 1 | 3.42 | 0.25 | 0.6245 |
| $A^2$ | −15.15 | 1658.07 | 1 | 1658.07 | 120.98 | <0.0001 |
| $B^2$ | −11.46 | 900.95 | 1 | 900.95 | 65.74 | <0.0001 |
| $C^2$ | −8.14 | 454.07 | 1 | 454.07 | 33.13 | <0.0001 |
| $D^2$ | −9.45 | 612.36 | 1 | 612.36 | 44.68 | <0.0001 |
| Residual | | 205.58 | 15 | 13.71 | | |
| Lack-of-fit | | 205.53 | 10 | 20.55 | 1868.43 | <0.0001 |
| Pure error | | 0.055 | 5 | 0.011 | | |
| Cor total | | 5293.99 | 29 | | | |
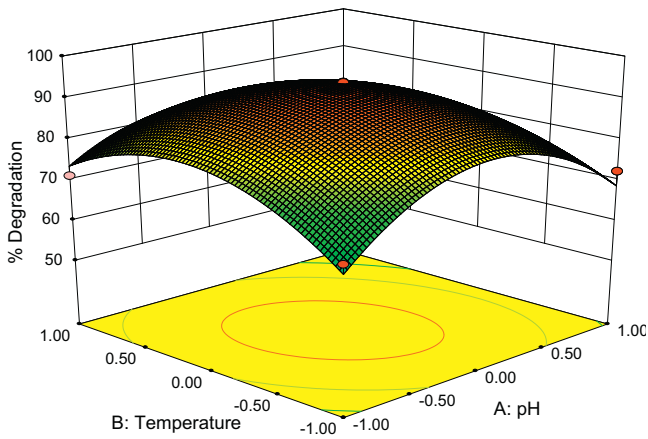
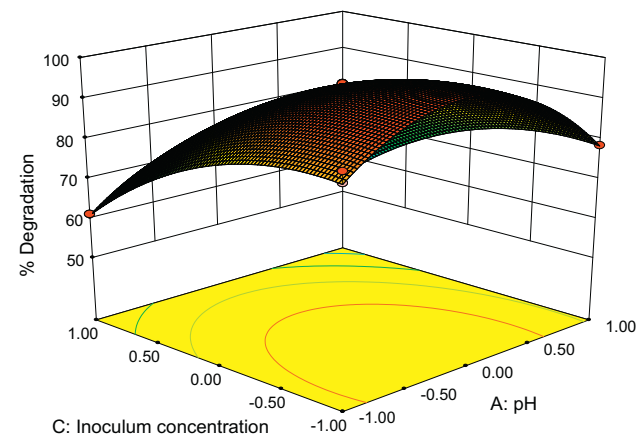**Figure 3** Effect of pH and temperature on atrazine degradation.



**Figure 4** Effect of pH and inoculum concentration on atrazine degradation.



**Figure 5** Effect of pH and agitation speed on atrazine degradation.



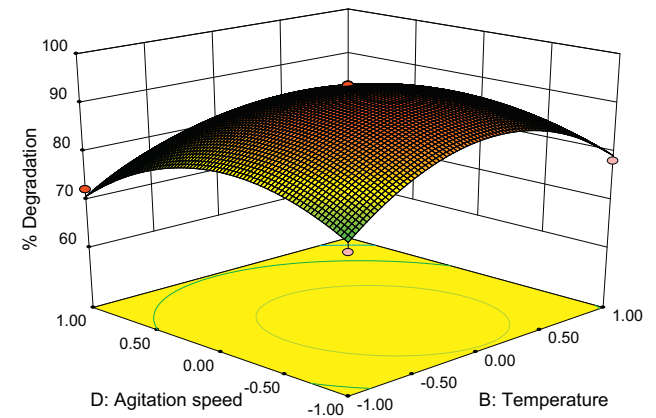**Figure 6** Effect of temperature and inoculum concentration on atrazine degradation.



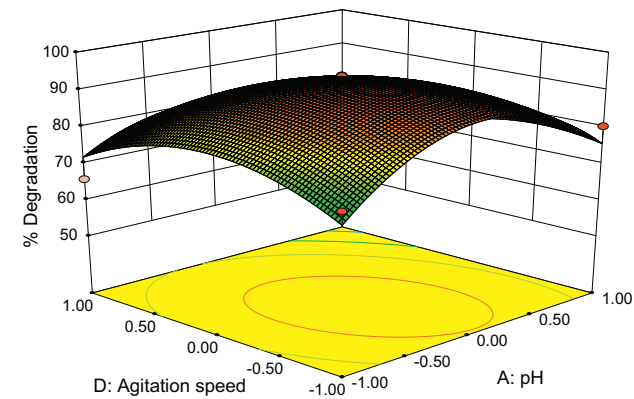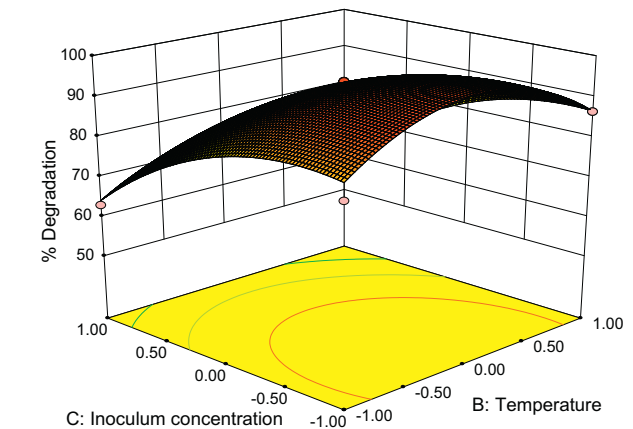**Figure 7** Effect of temperature and agitation speed on atrazine degradation.



**Figure 8** Effect of inoculum concentration and agitation speed on atrazine degradation.

To validate the prediction of the model, additional experiments in triplicate using shake flasks were performed at the optimized conditions. A maximum atrazine degradation of 94.4% was achieved. Good agreement between the predicted

and the experimental results verified the validity of the model and the existence of the optimal points. This showed that the model correctly explains the influence of the chosen variables on the degradation of atrazine by the mixed culture obtained

from pharmaceutical industry wastewater treatment plant sludge. The results obtained in this study were comparable with other species like *Rhizobium rhodococcus* (72%), *Klebsiella* sp., *Comamonas* sp. (83.3%), *Pseudomonas alcaligenes* (>80%), and *Agrobacterium radiobacter* (94%) [6,8,14,15]. Therefore, the mixed culture obtained from pharmaceutical sludge can prove useful in the development of improved process of atrazine degradation.

### 3.3. Effect of substrate concentration on atrazine degradation

At the optimized conditions, the atrazine concentrations were varied (2, 4, 6, 8, 10, 12, and 14 mg/L) to study the effect of atrazine concentration on degradation. The results obtained are shown in Fig. 9. It was clear from the figure that the time taken by the mixed culture to degrade atrazine depends on its initial concentration. For example, when the initial atrazine concentration was 2 mg/L, it took about 6 d to degrade completely, whereas it took about 11 d for degrading an initial atrazine concentration of 14 mg/L. The total degradation time taken at all initial atrazine concentration may be divided into two phases: initial lag phase and active degradation phase. The extents of two phases, in turn, depend on initial atrazine concentration.

### 3.4. Kinetic modeling of atrazine degradation using mixed culture

The utilization of environmental contaminants as substrates by the microbes has been studied by many researchers. These
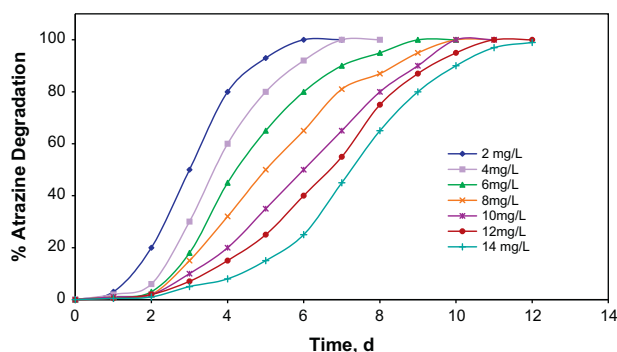
**Figure 9**  Effect of substrate concentration on atrazine degradation using mixed culture from pharmaceutical sludge.

**Table 4**  Kinetic models used for atrazine degradation.

| Model | Equation | Refs. |
|---|---|---|
| Monod | $\mu = \frac{\mu_{\max} S}{K_s + S}$ | [17] |
| Moser model | $\mu = \frac{\mu_{\max} S^2}{K_s + S^2}$ | [18] |
| Yano and Koga | $\mu = \mu_{\max} \frac{S}{K_s + S + \frac{S^2}{K_1} + \frac{S^3}{K_2}}$ | [19] |
| Teissier model | $\mu = \mu_{\max} \left[ \exp\left(\frac{-S}{K_i}\right) - \exp\left(\frac{-S}{K_s}\right) \right]$ | [20] |
| Webb model | $\mu = \mu_{\max} \frac{S\left(1 + \frac{S}{K_i}\right)}{K_s + S + \frac{S^2}{K_1}}$ | [20] |
| Haldane model | $\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_i}}$ | [21] |

**Table 5**  Value of kinetic parameters obtained from growth and inhibition models of atrazine biodegradation.

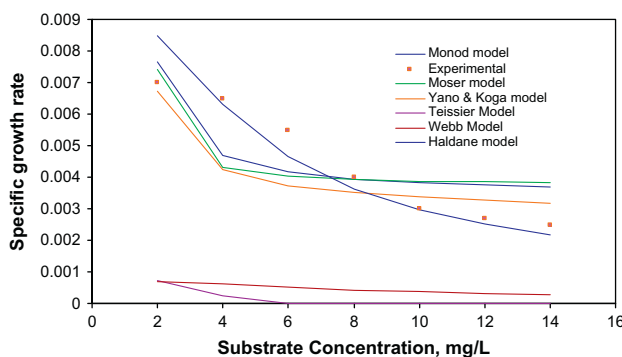| Model | $R^2$ | $\mu_{\mathrm{m}}$ (mg/mg/h) | $K_s$ (mg/L) | $K_i$ (mg/L) | $K_1$ (mg/L) | $K_2$ (mg/L) |
|---|---|---|---|---|---|---|
| Monod | 0.3044 | 0.0034 | −1.11 | | | |
| Moser | 0.0039 | 0.0038 | −1.949 | | | |
| Yano and Koga | 0.3804 | 0.0032 | −1.074 | | 171.2 | 679 |
| Teissier | 0.3146 | 0.4669 | 0.4599 | 0.5655 | | |
| Webb | 0.0911 | 0.0070 | 11.09 | 79.41 | 0.5109 | |
| Haldane | 0.9001 | 0.1918 | 18.58 | 0.1618 | | |

**Figure 10**  Comparison of various models with experimental values for atrazine degradation using mixed microorganisms.

are all aimed at detoxification of the environmental pollutants. Several microbial growth and biodegradation kinetic models have been developed, proposed, and used in biodegradation process [16]. In this work, the kinetics of atrazine degradation in the batch reactor was studied by various models like Monod model, Moser, Yano and Koga, Tessier, Webb and Haldane model and are given in Table 4. Biomass was measured as mixed liquid volatile suspended solids (MLVSS).

The experimental results of specific growth rate variation with initial atrazine concentration were fitted to various cell growth and substrate inhibition models like Monod model, Moser, Yano and Koga, Teissier, Webb and Haldane model. The model with the best fit was selected on the basis of highest correlation coefficient ($R^2$). The CF tool in MATLAB 7.0 was used to fit the models to different batch experimental data. The parameters of these models were found and given in Table 5. The specific growth rate was calculated and plotted against atrazine concentration as shown in Fig. 10. As seen from this figure, the specific growth rate ($\mu$) decreases as the atrazine concentration increases. Therefore, it seems that there is an influence of the initial atrazine concentration on the specific growth rate. The observation of substrate inhibition due to atrazine can be modeled using substrate inhibition models. The experimental results of specific growth rate variation with initial atrazine concentration were fitted to four inhibition models namely Yano and Koga, Teissier, Webb and Haldane model. The experimental values were compared with the predicted values and are shown in Fig. 10. The results from figures revealed that for the degradation of atrazine using pharma sludge, the Haldane ($R^2 = 0.9001$) model shows a better fit as compared to other models. Thus, Haldane model may be

proposed as the best model to describe the atrazine degradation using the pharma sludge.

## 4. Conclusions

Atrazine degradation studies were carried out using mixed microbial consortium obtained from pharmaceutical sludge. The process parameters namely pH, temperature, inoculums concentration, and agitation speed were varied and optimized using RSM. A maximum degradation of 94.4% was obtained at the optimum conditions. This is the first paper which describes the application of statistical design for the degradation of atrazine using mixed culture. From the results, it was observed that this optimization tool can be effectively used to maximize the degradation process. Effect of atrazine concentration on atrazine degradation was studied at the optimized conditions. It was found that at low atrazine concentrations, maximum degradation was found. Various growth and substrate inhibition models like Monod model, Moser model, Haldane model, Yano and Koga, Webb model, Teissier model were used to fit the experimental data. From the results, it was found that the Haldane model fits the data well for atrazine degradation using the sludge obtained from pharmaceutical industry wastewater treatment plant.

## Acknowledgements

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