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Optimization of the biodegradation of 17-α-ethynylestradiol (EE2) using response surface methodology

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Abstract: *17-α-ethynylestradiol (EE2) biodegradation was conducted using biofilm reactor in a medium containing both EE2 and glucose as organic carbon sources and in a medium without glucose and EE2 as the sole organic carbon source. The factors affecting the biodegradation are initial pH, initial EE2 concentration, initial nitrogen content, and biomass volume. Response surface methodology (RSM) and central composite design (CCD) were applied to determine the optimum operating conditions with the % degradation and the degradation efficiency as responses. The ANOVA revealed that EE2 concentration is the most significant factor that influences the % degradation and the biomass volume is the most significant factor that influences the degradation efficiency. Verification runs at optimum conditions showed that there were some peaks that appeared besides the peak of EE2 indicating that EE2 was biochemically transformed. The Monod kinetic and first order suit well for the EE2 biodegradation*.

Keywords: 17-α-ethynylestradiol (EE2), Response surface methodology (RSM), Verification runs

1. INTRODUCTION

Synthetic and natural estrogens are a group of environmental pollutants, which give their negative effects on aquatic organisms of receiving bodies of water as endocrine disrupting substances. Generally, the estrogen excretion by humans is estimated to be around 2.7 mg/L of urine daily (Zuo et al., 2006). The synthetic estrogen 17-α-ethynylestradiol (EE2) is the main component in the oral contraceptive pill and contains between 30 and 50μg per each pill (Guang Guo Ying et al., 2002). As well as, EE2 and 17-β-estradiol (E2) are detected in ecologically relevant amounts in, surface water, sewage effluents, bed sediments, river water, in digested and activated sludge (Baronti et al., 2000; Muller et al., 2008). In sewage effluents and surface waters, over 99% of the estrogenic activity is attributable to the presence of 17β-estradiol (E2) and EE2 due to insufficient removal during wastewater treatment (Clouzot et al., 2008). EE2 was shown to be capable of causing sex reversal (Lange et al., 2009) and reduction of fish populations at concentrations as low as ng/l (Kidd et al., 2007; Cloman Jamie et al., 2008).

Researches on the biodegradation of estrogen by heterotropic microorganisms showed that synthetic estrogens, such as EE2 in water can be reduced. In wastewater treatment, the microbial film process, which involves the use of biofilm reactor such as fluidized bed, was found to be highly efficient and useful for aerobic treatment (Nicollela et al., 2000). Thus, the study of the effectiveness of the fluidized bed reactor (FBR) and the optimization of degradation of EE2 using response surface methodology (RSM) is significant to determine the best parameters in reducing the synthetic estrogen concentration from the water. In addition, this research determined the presence of

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metabolites of EE2 biodegradation to validate the occurrence of the biodegradation process.

2. METHODOLOGY

2.1 Biofilm formation and acclimatization of the biofilm

Cement balls with diameter ranging from 212-300μm were used as the particles where biofilm formed in a continuously stirred tank reactor (CSTR). The effective volume is 3L. The feed compositions (g/L) are 0.28 glucose; 0.066 NaCl; 0.084 $MgSO_4.7H_2O$; 0.186 KH_2PO_4; 0.134 KCl; 0.817 NH_4Cl; and 0.914 $NaHCO_3$. The biofilm was fed every 24 hours for 5 months.

The biofilm obtained was acclimatized to EE2 by feeding into the reactor with 20 ppm EE2 and Tween 80 as surfactant to increase EE2 solubility in water. An EE2 stock solution of 1g EE2/L was prepared in methanol (Witters et al., 2003). EE2 from reaction broth was extracted using methanol and filtered using 0.45μm membrane filter. EE2 concentration was next determined using a high performance liquid chromatography (HPLC) LC-10AT equipped with UV spectrophotometric detector and LC 18 column. The process of acclimatization was conducted for 7 days. The biofilm was able to recover and resulted to an average of about 76.16% EE2 decrease for around 24 h.

2.2 Batch experimental runs

RSM and central composite design (CCD) were applied to optimize the initial pH, initial EE2 concentration, initial nitrogen content and biomass % volume that affect the EE2 biodegradation. A CCD was used in designing the experimental data. The design included 30 runs.

The experimental design was based on the RSM involving a CCD in the form of 2^4 full factorial design. A 2^4 full factorial CCD for four factors was used giving a total number of 30 experimental runs. These experimental runs were done in 2 sets; one with the medium containing glucose and another without glucose. pH was varied at 7.5, 8, and 8.5; Initial EE2 concentration at 10ppm, 15ppm, 20ppm; initial nitrogen content at 0.817g NH_4Cl/L, 0.858g NH_4Cl/L, 0.899g NH_4Cl/L; and biomass values at 5%, 7.5%,10% in 1000mL solution, respectively. The % degradation and degradation efficiency are the responses. The % degradation is the percentage of EE2 removal and the degradation efficiency is the milligram of EE2 degradation per litter of biofilm per hour.

2.3 Verification runs

Verification run is the repeat run at optimum conditions. These experiments were performed to determine if the reduction in EE2 concentration was really due to biochemical transformation by detecting the intermediates formed during the biodegradation process. For detection of intermediates, samples with medium containing glucose and without glucose were prepared in 1L Imhoff cones and operated at the optimum conditions. Samples of both mediums were analyzed every 3 h for 2 days. Samples were extracted with methanol and analyzed using HPLC. The chromatogram obtained was used as bases for the determination of the presence of metabolic products as intermediates in the EE2 biodegradation.

2.4 Kinetic rate model

The actual data were used to determine the appropriate kinetic model (zero order, first order and monod kinetic) to describe the behaviour of the EE2 biodegradation. The values of the rate constants of zero order and first order kinetic were calculated from equations:

$$\text{zero-order}: -\frac{dS}{dt} = k \quad (1) \quad \text{and} \quad \text{first-order}: -\frac{dS}{dt} = kS \quad (2)$$

The intergral forms of these equations are shown below:

$$\text{zero-order}: S = S_0 - k_0 t \quad (3) \quad \text{and} \quad \text{first-order}: \ln\left(\frac{S_0}{S}\right) = k_1 t \quad (4)$$

The experimental data for EE2 concentrations are plotted in the form of (S_0-S) versus time for determining zero-order and ln(S_0/S) versus time for first-order kinetic. The slope of the straight line gives the rate constants for zero-order or first-order kinetic.

The monod kinetic was calculated from equation:

$$\frac{dS}{dt} = -\frac{1}{Y_{x/s}} \frac{\mu_{max} * S}{k_s + S} * X \qquad (5)$$

Where μ_{max} : maximum specific growth rate (time^{-1}), K_s : substrate saturation constant (i.e. substrate concentration at half μ_{max}) (mass/unit volume), S : substrate concentration (mass/unit volume), X: biomass concentration (mass/volume). The μ_{max} and K_s are constants for a given organism for a specified substrate. The μ_{max} is an indication of organism growth, how fast it can grow in conditions where all limiting substrates are in excess. The K_s is an indication of how quickly μ moves from μ_{max} to zero, which defines as the concentration of the limiting substrate emigrates towards 0.

3. RESULTS AND DISCUSSION

The data obtained from the experiments were put into the design expert software for further analysis. Without performing any transformation on the responses, examination of the fit summary output revealed that the two-factor interaction (2FI) model is statistically significant for % degradation (medium without glucose), the quadratic model is significant for % degradation (medium containing

glucose) and degradation efficiency (medium without glucose), and the linear model is significant for degradation efficiency (medium containing glucose).

Table 2 shows the ANOVA table for response surface 2FI model for % degradation. The value " Prob. > F " in Table 2 for the model is less than 0.05 which indicates that the model is significant. Moreover, it indicates that the term in the model have a significant effect on the response. The main effect of EE2 (B), the two-level interaction of pH and EE2 (AB), the two-level interaction of EE2 and biofilm (BD), and the two-level interaction of NH_4Cl and Biofilm (CD) are significant model terms. Other model terms are not significant. The lack-of-fit was found to be insignificant in this case. The R^2 value is high, close to 1, which is desirable. The predicted R^2 is in reasonable agreement with the adjusted R^2. Adequate precision is the comparison of the range of the predicted values at the design points to the average prediction error. A ratio greater than 4 is desirable. In this case, the value was well above 4. Meanwhile, the coefficient of variation (CV) as the ratio of the standard error of estimate to the mean value of the observed response, expressed as a percentage, is a measure of reproducibility and repeatabilty of the models (Chen et al., 2010). As a general rule, the coefficient of variation shoud not be greater than 10 %. A high coefficient of variation showed that variation in the mean value is high and does not satisfactorily develop an adequate response model (Daniel, 1991). From the results obtained, it showed that the coefficient of variation was less than 10 % for medium without glucose (3.33%), representing a better precision and reliability of the conducted experiments.

Table 1. ANOVA for response surface 2FI model for % degradation (medium without glucose).

Source	Sum of squares	DOF	Mean square	F-value	p-value Prob.>F
Model	512.14	10	51.21	9.17	< 0.0001 significant
A-pH	5.95	1	5.95	1.07	0.315
B-EE2	407.65	1	407.65	73.02	< 0.0001
C-NH_4Cl	1.69	1	1.69	0.30	0.589
D-Biofilm	7.68	1	7.68	1.38	0.255
AB	31.64	1	31.64	5.67	0.028
AC	0.10	1	0.10	0.018	0.894
AD	0.036	1	0.036	6.467E-003	0.937
BC	0.042	1	0.042	7.528E-003	0.932
BD	26.88	1	26.88	4.82	0.041
CD	30.47	1	30.47	5.46	0.031
Residual	106.07	19	5.58		
Lack of fit	91.75	14	6.55	2.29	< 0.185 not significant
Pure error	14.32	5	2.86		
Cor total	618.21	29			

Std. dev : 2.36 Mean : 70.90 C.V % : 3.33 R-squared : 0.828
Adj-squared : 0.738 Pred : 0.604 Adeq. Precision : 11.820

The same procedure is applied on response degradation efficiency for medium without glucose, on response % degradation and degradation efficiency for medium containing glucose. The resulting ANOVA tables for those responses are also calculated (not shown). For degradation efficiency (medium without glucose), the two main effects of EE2 (B) and biofilm (D), the two-level interaction of EE2 and biofilm (BD), the two-level interaction of NH_4Cl and biofilm (CD), and the second order effect of biofilm (D^2) are the significant model terms. The main effects of biofilm (D) is the most significant factors associated with biofilm degradation efficiency. This can be explained by the fact that the EE2 concentration increases with decreasing biofilm volume and giving high efficiency.

For % degrdation (medium containing glucose), the main effect of pH (A), EE2 (B), NH_4CL (C), biofilm (D), and the second order effect of EE2 (B^2) are the significant model term.

For degradation efficiency, the main effect of biofilm (D) is the significant model term.

The following equation is the final empirical model in terms of coded factors for:

- % degradation (medium without glucose):

$$\% \text{ degradation} = + 70.90 + 0.58*A - 4.67*B + 0.31*C - 0.65*D - 1.41*A*B + 0.080*A*C + 0.047*A*D - 0.051*B*C + 1.30*B*D + 1.38*C*D \quad (6)$$

The 3D surface graphs for % degradation (medium without glucose) are shown in Fig. 1-3. From the 3D surface plot (Fig. 1), it indicates that the EE2 concentration decreases

with decreasing biofilm volume and giving the high degradation. Moreover, the response curvature shows a maximum region towards minimum value of biofilm volume of 5% and minimum value of EE2 of 10 ppm. Also, the 3D surface graph in pH and EE2 (Fig. 2), and in NH_4Cl and EE2 (Fig. 3) were drawn. They show that highest degradation is at lowest value of NH_4Cl of 0.817 g/L and at the highest value of pH of 8.5.

Similar plots were drawn for degradation efficiency (medium without glucose), and % degradation and degradation efficiency for medium containing glucose (not shown). From 3D surface plots of % degradation (medium containing glucose), it is clear that highest degradation is at lowest value of EE2 concentration, nitrogen content as NH_4Cl, % biofilm volume and middle value of pH. The 3D surface plots for degradation efficiency (medium without glucose) showed that the response curvature proved a maximum region is at highest value of EE2 concentration, lowest value of % biofilm volume and pH, and middle value of nitrogen content. For degradation efficiency of medium containing glucose, the plots predicted results as same as those with medium without glucose, except for EE2 concentration at lowest value and nitrogen content at highest value.

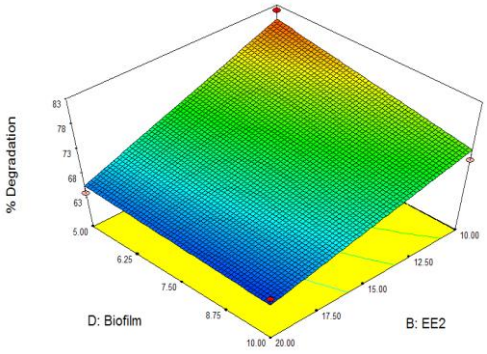

Figure 1. 3D surface graph in biofilm and EE2 for % degradation (without glucose).

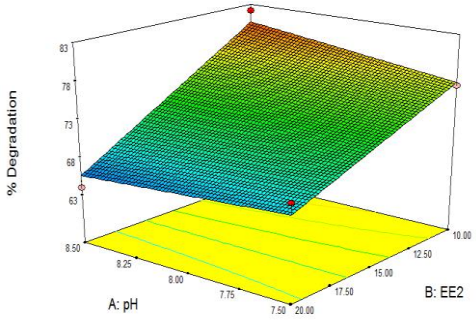

Figure 2. 3D surface graph in pH and EE2 for % degradation (without glucose).

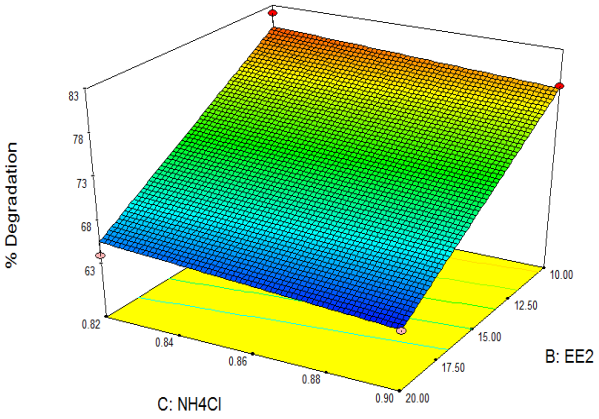

Figure 3. 3D surface graph in NH_4Cl and EE2 for % degradation (without glucose).

3.1 Verification runs

Verification run is the repeat run done at optimum conditions. The runs at the optimum conditions for the biodegradation of EE2 with glucose and without glucose were performed. Samples were taken every 3 h for 2 days and analyzed using HPLC.

HPLC chromatograph of EE2 samples with medium with glucose after 48 hours of biodegradation (not shown) did not show any peak besides the peak of EE2 even if the EE2 concentration decreased. This could be a proof that with the presence of glucose, the biofilm microorganisms used glucose as co-substrate and with its presence the biofilm organisms had enough energy and biological activity to fully degrade the EE2 and the resulting metabolites producing reaction product chromatograph with no detected intermediates. On the other hand, Fig. 4 showed that the chromatograph for the EE2 biodegradation using culture medium without glucose after 48 hours of biodegradation showed that besides the peak of EE2, there are other peaks that appeared which was not in the chromatogram of the original samples. These peaks could be the intermediates that were formed as biochemical reaction by-products during biodegradation using biofilms. Without the presence of glucose, the biofilm microorganism are forced to utilize the EE2 as the carbon source for its metabolism since there were no other organic carbon source present in the medium. Unlike in the previous case were there were glucose as co-substrate, the microorganisms were able to biochemically transform the mother molecule of EE2 but were not able to utilize the intermediates, thereby causing the intermediates to be present in the reaction broth even after 48 hours of biodegradation. The appearance of these peaks could be the proof that biochemical transformation of EE2 occurred during the biodegradation of EE2 by biofilm.

3.2 Kinetic rate model

The kinetic models namely, zero order, first order and monod model were considered to determine the best fit that will describe the EE2 concentration data obtained from the verification runs.

The appropriate zero order and first order model were determined through the use of the linear regression analysis in Microsoft excel.

For the zero order, the value of the rate constant and R^2 were found as given in Table 3. The value of the rate constant was calculated from equation (1) and (3). A plot of Time vs (S_0-S) data was shown in Figure 5. The low R^2 values show the inability of this model in describing the degradation of EE2 in batch reactor.

The first order model was applied to the experimental data to verify the performance. The values of the constants in the first order model for both mediums were calculated

Table 2. Summary data of the optimum operating conditions.

Factor	Medium containing glucose		Medium without glucose	
	% degradation	Efficiency	% degradation	Efficiency
pH	8	7.5	8.5	7.5
EE2 concentration (pm)	10	10	10	20
Nitrogen content, g NH_4Cl/L	0.817	0.899	0.817	0.84
% biomass volume	5	5	5	5

Table 3. Summary of R^2 and parameters for zero order, first order and monod model for both mediums.

Kinetic model	Medium with glucose	Medium without glucose
Monod model		
$Y_{x/s}$ (mg cell/ mg EE2)	0.75	0.5652
μ_{max}, (hr^{-1})	$0.148*10^{-2}$	$0.138*10^{-2}$
Ks, (mg EE2/L)	115.004	115
R^2	0.981	0.928
Zero order		
Ko, (mg EE2/L.hr^{-1})	0.178	0.185
R^2	0.893	0.659
1st order		
K_1, (hr^{-1})	0.041	0.048
R^2	0.975	0.959

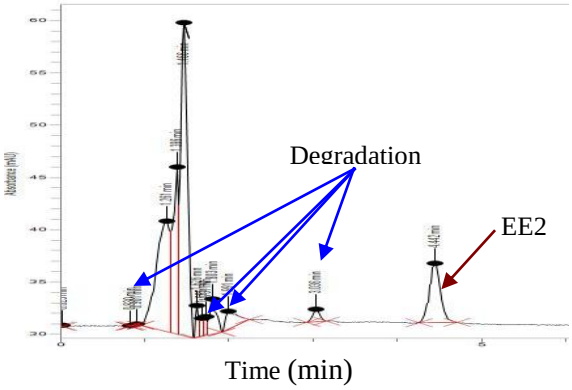

Figure 4. HPLC chromatograph of EE2 sample at time 48 (medium without glucose)

The constant values of first order model for both mediums are given in Table 3. Time vs ln(S_0/S) data were plotted for first order in Figure 6 .The R^2 values for the first order model were found to be good (≥ 0.9) for both mediums. Hence the biodegradation of EE2 using biofilm follows first order system.

The experimental values were also used to determine the parameters and to verify the performance of the monod model. The monod model also related the yield coefficient ($Y_{x/s}$) to the specific rate of biomass growth. So the substrate degradation rate is represented as:

$$\frac{dS}{dt} = -\frac{1}{Y_{x/s}} \frac{m_{max} * S}{k_s + S} * X$$

The substrate degradation rate equation was solved using ODE45, which is available in MATLAB. This equation was used to determine the parameters corresponding to the monod equation and the simulated response curve. The values of the kinetic parameters in the batch model for both mediums were estimated from the slope of the best fit lines and tabulated in Table 3. The EE2 reduction profile was well explained by the monod model

with high values of R^2 (0.9) for both mediums. The high R^2 values show the ability of this model in describing the batch kinetics of the biodegradation of EE2.

After determining the R^2 values, the EE2 biodegradation follows the first order and monod kinetic.

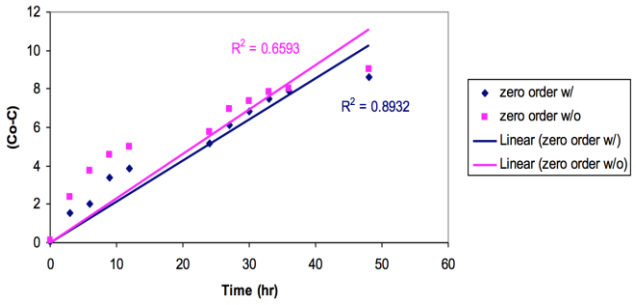

Figure 5. Zero order model in verification run kinetics of EE2 degradation.

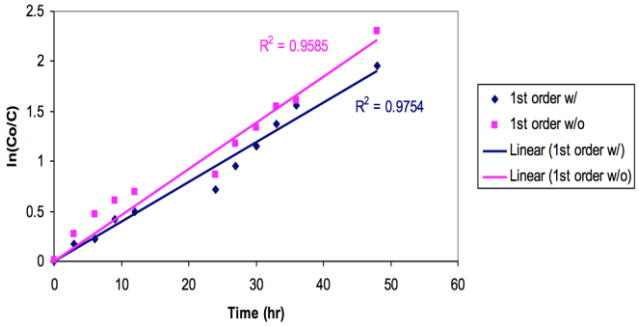

Figure 6. First order model in verification run kinetics of EE2 degradation.

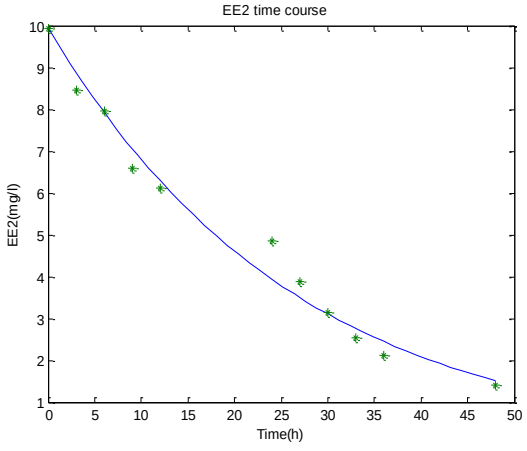

Figure 7. Simulation using monod kinetics for verification run with medium containing glucose (initial pH:8, initial EE2 concentration: 10ppm, initial nitrogen content: 0.817g/l, % Biomass: 5%).

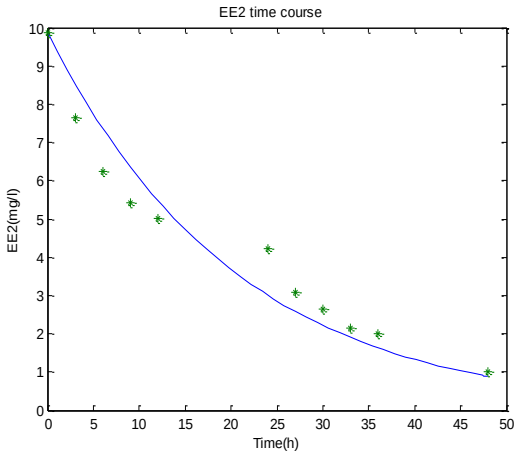

Figure 8. Simulation using monod kinetics for verification run with medium without glucose (initial pH:8.5, initial EE2 concentration: 10ppm, initial nitrogen content: 0.817g/l, % Biomass: 5%).

4. CONCLUSIONS

This paper presents the findings of an experimental investigation into the effect of initial pH, initial EE2 concentration, initial nitrogen content and biomass volume on the % degradation and degradation efficiency of EE2 biodegradation. The optimum conditions for the runs on the % degradation of medium containing glucose were initial pH of 8, initial EE2 concentration of 10ppm, initial nitrogen content of 0.817g NH_4Cl/L, and 5% biomass volume. The optimum conditions for the runs using medium without glucose were the same as those with glucose, except for initial pH of 8.5. Moreover, the optimum conditions of EE2 biodegradation for degradation efficiency were found to be 7.5 of initial pH, 10ppm of initial EE2 concentration, 0.899g NH_4Cl/L, and 5% of biomass volume for medium containing glucose. For medium without glucose, the optimum conditions were the same as those with glucose, except for EE2 concentration of 20ppm and nitrogen content of 0.84g NH_4Cl/L. **T**he ANOVA revealed that EE2 concentration is the most significant factor influencing the % degradation and the biomass volume is the most significant factor influencing the degradation efficiency. At the optimum conditions, the results showed that there were some peaks that appeared besides the peak of EE2 indicating that EE2 was biochemically transformed. Both the monod model and first order rate of reaction suit well for EE2 biodegradation. Future study maybe done in which the intermediates at the optimum conditions will be identified using GC-MS.

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