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ORIGINAL RESEARCH PAPER

INCREASE OF THE *TRAMETES VERSICOLOR* EFFICIENCY IN THE BIOREMEDIATION PROCESS FOR DICLOFENAC BIODEGRADATION IN AQUATIC ENVIRONMENTS

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Diclofenac (DCF) is a non-steroidal anti-inflammatory drug and, as pollutant, it represents a persistent residue hazard to health and to the environment. *Trametes versicolor* was previously selected for its ability in diclofenac biodegradation (up to 20%) during cultivation in submerged system under aerobic conditions at an initial DCF concentration of 10 mg L^{-1}. The influence of some factors such as nitrogen sources glucose, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, inoculum level, initial DCF concentration and incubation time, upon the biodegradation potential was examined by Plackett-Burman analysis. The parameters significantly influencing the DCF biotransformation were found to be yeast extract, glucose, $CuSO_4 \cdot 5H_2O$ and inoculum level. In these optimum conditions, the DCF biotransformation yield was 80%. This result was 60% superior in comparison with the medium without optimization. Analysis of variance exhibited a high coefficient of determination (R^2) value of 0.9987 and ensured that the polynomial model with the experimental data was a satisfactory one. Optimal conditions obtained in this work led to a solid foundation for further use of *Trametes versicolor* in biotreatment of high strength DCF pollutant effluents in water wastes.

Keywords: biodegradation, diclofenac, *Trametes versicolor*, Plackett-Burman design

Introduction

In the last years, the presence of emerging micropolluants such as pharmaceuticals, personal care products (PPCP) and endocrine disruptions chemicals in the environment has received much attention (Nakada *et al.*, 2006; Radjenovic *et al.*, 2007). The removal of many pharmaceuticals during municipal wastewater treatment has been found to be incomplete. As a result, residual amounts of these compounds have been detected ubiquitously in various environmental matrices in concentrations typically ranging from µg L^{-1} to ng L^{-1} (Rodriquez *et al.*, 2003;

Carballa *et al*., 2004; Weigel *et al*., 2004). It is important to highlight the potential impact of the release of pharmaceuticals into the environment because this type of compounds may cause increased aquatic toxicity and endocrine disruption (Ikehata *et al*., 2006; Jjemba *et al*., 2010). However, the real effect on the environment will depend on their concentration as well as on other physicochemical factors such as time of exposure, persistence, bioabsorption, bioaccumulation and biotransformation rate (Esplugas *et al.,* 2007).

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug used in human medical health as analgesic, antiarthritic and antirheumatic compound. This drug is a xenobiotic compound and presents relative persistence especially in the aquatic environments; its biodegradation depends on microbiota community, chemical composition and physical-chemical environment conditions (Bendz *et al.*, 2005).

Conventional treatment such as coagulation-flocculation and flotation, activated sludge and nitrification-denitrification reached variable degradation yields from 20 to 40% (Hofmann *et al*., 2007; Klavarioti *et al*., 2009, Kim *et al.*, 2009) but the main limitation is the formation of undesirable and sometimes toxic by-products (Negron-Encarcion and Arce, 2007). Nowadays there is still scarce information about the presence and fate of its metabolites in the environment. The biotransformation products can be generated by human metabolism or by microorganisms present in wastewater treatment plant and in natural waters, soils and sediments, which increases the probability to find them in the environment. Metabolites may have higher toxicity and, can be present in different aquatic bodies at higher concentration than parent compounds (Ferrando-Climent, *et al.*, 2012). The assessment of their fate is thus necessary in order to understand degradation mechanisms during wastewater treatment and to evaluate their potential environmental risk (Boxall *et al*., 2012).

Emerging technologies, such as those based on the use of white rot fungus or the ligninolytic enzymes have provided promising results, with degradation yields close to 100% (Marco-Urrea *et al*., 2010; Hata *et al.,* 2010).

Trametes versicolor belongs to the category of white-rot fungi (WRF), a cosmopolitan group of microorganisms with a high capability to degrade a wide range of xenobiotic and recalcitrant pollutants due to the complexity of extracellular (manganese and lignin peroxidase and laccase, versatile peroxidase) and intracellular (cytochrome P450 system) enzymes production (Gadd, 2001; Asgher *et al*., 2008).

Before fungi can be used for the bioremediation of contaminated sites and also for bioaugmentation, their concentration and metabolic activity in naturally contaminated sites, and the fundamental factors (environmental and nutritional requirements) that affect biodegradation must be first studied in laboratory. Mathematical modelling and statistical analysis methods are versatile techniques for the investigation of multiple process variables because it makes the process easily optimized with fewer experimental trials (Bajaj *et al.*, 2009). The Plackett Burman design method (PBD) is an effective screening design which considerably diminishes the number of experiments and gives as much information as possible

for the evaluation of the target factors. Only the most significant factors with positive influence are selected for further optimization. The less significance or high negative effect on response value would be omitted for further experiments (Liu and Wu, 2007).

The objective of this study was to identify significant variables influencing the diclofenac biodegradation by a selected strain of *Trametes versicolor* by optimizing the biotechnological parameters in order to improve the rate of biotransformation.

Materials and methods

Chemicals and fungal strain

Analytical grade chemical reagents, diclofenac sodium salt, acetonitrile (HPLC grade) and ingredients for culture media formulation were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The white-rot fungal strain *Trametes versicolor* was provided from the Cultures Collection of the Faculty of Biology, Alexandru Ioan Cuza University of Iași. The strain was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at 4°C. Subcultures were routinely made every 30 days.

Fungal inoculum preparation

A mycelial suspension of white-rot fungal was obtained by inoculation of three plugs (6 mm in diameter) of agar plugs, from the growing zone of fungal on plates, in 150 mL of malt extract medium which was shaken (135 rpm) at 25^0C for 4-5 days. Then the dense mycelial mass was aseptically blended with a homogenizer to obtain homogenous mycelial inoculum. This mycelium was then aseptically inoculated in basal liquid medium.

Diclofenac biodegradation

The biodegradation experiments were performed by submerged cultivation in basal liquid medium (MM) containing (g L^{-1}): glucose 5, yeast extract 5, peptone 20, $MnSO_4 \cdot H_2O$, 0.50; $CuSO_4 \cdot 5H_2O$, 0.50, pH 5.5. The cultivation took places on orbital shaker SI-300R Incubator Shaker (Jeio Tech, Korea) at 135 rpm and temperature of 25ºC, for many days.

For optimization studies, the following independent variables were chosen to be studied: nitrogen sources (yeast extract and peptone), glucose, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, the inoculum concentration, initial DCF concentration and incubation time. These varied according to the design of experiments (as presented in Table 1), and their effect on the DCF biodegradation yield (considered as response) was analyzed. In all the biodegradation experiments, the samples were taken at different time intervals and evaluated for biomass yield (expressed as dry weight) and residual DCF concentration.

Mycelium dry weight

Mycelium dry weight was determined by vacuum filtering the cultures through preweighed glass filters (Whatman GF/C, Maidstone, England). The filters containing the mycelial mass were placed in glass dishes and dried at 100 ^{0}C (Drying Oven Sanyo, Japan) at constant weight.

Diclofenac quantification

The crude culture supernatants after biomass separation by centrifugation at 10000 rpm for 10 minutes were analyzed for quantification of residual DCF content. Analysis was performed using an HPLC Agilent 1200 Series (Santa Clara, Ca, SUA) equipped with a photodiode array (PDA) detector at wavelength of 278 nm. The separation took place by a reverse phase BDS Hypersil C18 column (150 mm x 4.6 mm, particle 5 µm).

The DFC separation were performed under isocratic conditions with 70/30 acetonitrile/ultrapure water acidified with 0.1% (v/v) acetic acid. The retention time was 2.6 minutes and the instrumental quantification limit (LOQ) for DCF was < 0.2 mg L^{-1}.

DFC concentration (expressed as mg L^{-1}) was calculated using the calibration curve, which was carried out by using standard solution with known DCF concentration (in the range of 10-30 mg L^{-1}).

Biotransformation yield of DFC was calculated as percentage of the target compound concentration in crude supernatants at the tested cultivation time in ratio with the initial concentration.

Mathematical modelling methodology

The Plackett Burman experimental design (PBD), an effective technique for medium-component optimization (Li *et al*., 2008; Pan *et al*., 2008), was used to study the influence of some biotechnological parameters (independent variables) upon DFC biotransformation yield (response).

For mathematical modelling, a first-order polynomial model was used as follows:

$$Y = \beta_0 + \Sigma \beta_i \chi_i \quad (1)$$

where Y is the predicted response (DFC biotransformation yields, %), β_0 is the model intercept, β_i is the linear coefficient and χ_i is the level of the independent variable.

The experimental design (independent variables and tested range) used in this work is shown in Table 1. All the variables were evaluated at two levels, considered as low and high, which are denoted by (-1) and (+1), respectively.

All tests were performed in duplicates. Determination of DFC concentration was carried out in triplicate and the average value was taken as the response.

The variables with confidence levels above 95% ($P < 0.05$) were considered to have significant effect on DFC biotransformation and the variables with positive influence were used for further optimization of biotechnological conditions.

Statistical analysis

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fischer's F-test, its associated probability p (F), the correlation coefficient (R), and the determination coefficient (R^2) which measures the goodness of fit of regression model.

Results and discussion

The influence of the analyzed independent variables upon the DCF biotransformation in submerged cultivation system of a selected strain of *Trametes versicolor* was studied by using Plackett-Burman (PBD) experimental design. The

PBD design for 12 trials with two levels of concentrations for eight different variables was carried out according to the experimental matrix as shown in Table 2. The response variables (Table 2) indicated a wide variation in DCF biodegradation yields by selected fungus, in the range of 20-80%. The variation suggested that the analysis was important for improving the efficiency of the DCF biodegradation.

Table 1. The Plackett-Burman design matrix for screening the important variables for DFC biodegradation by *Trametes versicolor*

Independent variables	Units	Symbol	Levels of variation of the independent variable (χ_0)	
			-1	+1
Concentration of glucose	g L^{-1}	A	5.0	10.0
Concentration of yeast extract	g L^{-1}	B	1.0	5.0
Concentration of peptone	g L^{-1}	C	10.0	20.0
$CuSO_4 \cdot 5H_2O$	g L^{-1}	D	0.1	0.5
$MnSO_4 \cdot H_2O$	g L^{-1}	E	0.1	0.5
Inoculum concentration	% v/v	F	0.5	2.0
Concentration of diclofenac	mg L^{-1}	G	10.0	15.0
Incubation time	days	H	7.0	14.0

Table 2. The Plackett-Burman design matrix of independent variables for evaluating the biotechnological factors with influence on DCF biodegradation by *Trametes versicolor*

Run	Coded levels of variables								Biodegradation yield of DCF, (%)
	A	B	C	D	E	F	G	H	
1	-1	1	1	1	-1	1	1	-1	40.00
2	-1	-1	-1	-1	-1	-1	-1	-1	50.00
3	-1	-1	-1	1	1	1	-1	1	70.00
4	1	-1	-1	-1	1	1	1	-1	30.00
5	1	1	1	-1	1	1	-1	1	80.00
6	1	1	-1	1	1	-1	1	-1	30.00
7	-1	1	-1	-1	-1	1	1	1	30.00
8	1	-1	1	1	-1	1	-1	-1	55.00
9	1	-1	1	- 1	-1	-1	1	1	70.00
10	-1	-1	1	1	1	-1	1	1	35.00
11	-1	1	1	-1	1	-1	-1	-1	20.00
12	1	1	-1	1	-1	-1	-1	-1	30.00

The main effects consist in the possibility to determine the effect of each constituent. A large contrast means, either positive or negative, indicates that a factor has a large impact on DCF biotransformation; while a mean close to zero means that the factor has little or no effect. As shown in Figure 1, the quantitative

effect of peptone (C), $MnSO_4 \cdot H_2O$ (E), the initial DCF concentration (G) and the incubation time (H) have a negative influence on biodegradation process. Also, the concentration of glucose (A), the yeast extract (B), $CuSO_4 \cdot 5H_2O$ (D) and inoculum (F) have a strong positive effect for pollutant biodegradation and will be included in the next optimization experiments.

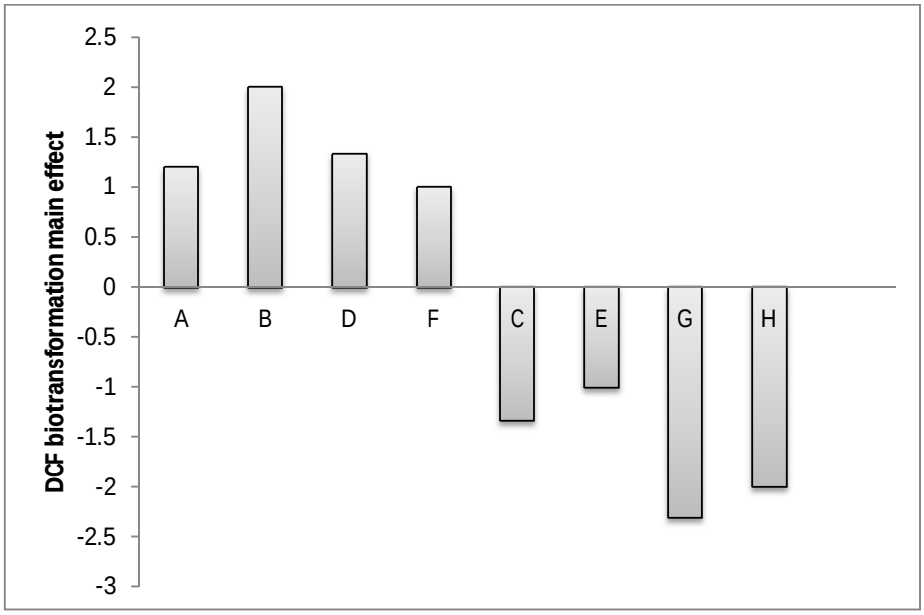

Figure 1. Effect of independent variables on DCf biotransformation by *Trametes versicolor* based on Plackett-Burman design results

Moreover, it can be noticed that the yeast extract concentration and the initial DCF concentration are the parameters which shows the most significant effects (positive and negative) on DCF biodegradation. A recent study by Popa *et al*. (2014) showed the considerable effect of the initial pollutant concentration on the carbamazepine removal; the carbamazepine removal decreased concomitantly with the increase of the initial concentration of the pollutant.

Also, Suflita (1989) reported that increasing the concentration of a pharmaceutical compound as residue pollutant may lead to a toxic effect, to the decrease of the available oxygen, and water potential of the medium upon the physiologic activity of the microorganisms, or to lowering the contact between the active biomass and nutrients.

The results obtained were in agreement with previous results which showed that glucose, yeast extract, inoculum size and volume of the medium have a significant effect on carbamazepine biodegradation by selected strain *Streptomyces* MIUG 4.89 (Popa *et al*,. 2014).

Zhou *et al*. (2011) reported that inoculum concentration was an important factor for the xenobiotic compounds degradation. A similar result was observed by Ghanem *et al*. (2012) for the chloroxylenol degradation by *Aspergillus niger*.
Nitrogen sources like yeast extract has been proved to support a rapid growth of cells and metabolites biosynthesis, as well as extracellular enzymes production (Suutari *et al*., 2002).

Based on the statistical analysis of confidence level of 8 variables (Table 3), yeast extract and the initial DCF concentration had confidence levels above 95% ($p<0.05$) and hence they were considered the significant parameters which influence DCF biotransformation. The yeast extract concentration is the positive significant variable affecting DCF elimination from liquid medium, while the initial DCF concentration was the negative significant parameter in DCF elimination.

Table 3. Statistical analysis of the independent variables for DCF biodegradation

Variables	Effect	Coefficient	Standard error	p-value	Confidence level (%)
Intercept	-	45.00	-	-	-
Concentration of glucose	1.20	4.17	0.48	0.013	95
Concentration of yeast extract	2.00	5.00	0.68	0.018	97.5
Concentration of peptone	-1.33	-0.28	0.56	0.667	68
$CuSO_4 \cdot 5H_2O$	1.33	4.72	0.56	0.013	95
$MnSO_4 \cdot H_2O$	-1.00	-0.28	0.56	0.421	76.6
Inoculum concentration	1.00	3.83	0.68	0.014	95
Concentration of diclofenac	-2.30	-4.72	0.56	0.656	53.3
Incubation time	-2.00	-4.17	0.68	0.393	60.7

C.V. % -3.70; R^2-0.9987; adjusted R^2- 0.9928

The R^2 values (multiple correlation coefficients) closer to 1 denoted high agreement between the experimental and predicted responses and indicate that the mathematical model is very reliable in the present study. The coefficient of variation (CV) indicated the degree of precision in the comparison of experiments. A lower reliability of the experiment is usually indicated by high value of CV; in the present case the low value of CV (3.70) showed that experiments conducted were precise and reliable.

After applying the ANOVA statistical test, the polynomial model equation was established to describe the DCF biodegradation (Eq.2):

$$Y= 45.00+41.7\ A+5.00B+4.72D+3.83F-4.72G \quad (2)$$

where *Y* was the predicted diclofenac biotransformation yield (%), *A* the concentration of glucose (g L^{-1}), *B* the concentration of yeast extract (g L^{-1}), *D* the

quantity of $CuSO_4 \cdot 5H_2O$ (g L^{-1}), *F* the inoculum concentration (%, v/v), *G* the initial DCF concentration (mg L^{-1}).

The parity plot (Figure 2) showed a satisfactory correlation between the experimental and predicted values (obtained from Eq.2) of percentage of DCF degradation, whereas the points cluster around the diagonal line indicated an optimal fit of the model, since the deviation between the experimental and predicted values was minimal.

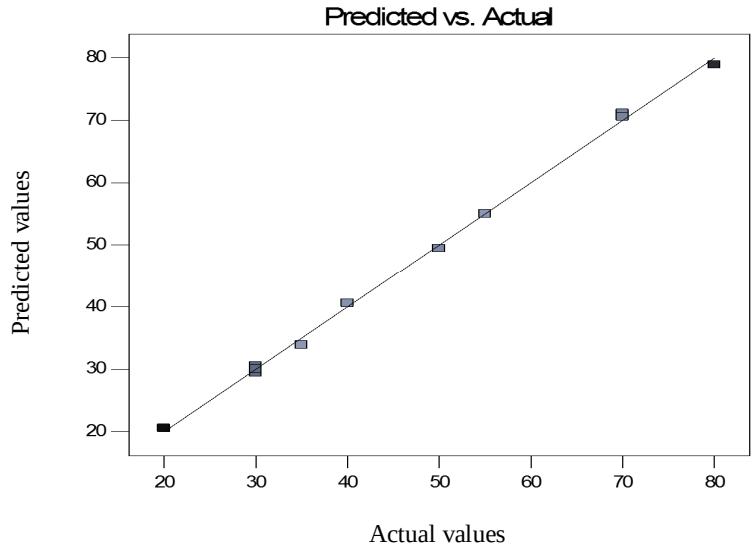

Figure 2. Parity plot showing the distribution of experimental vs. predicted values of DCF biotransformation

Conclusions

This paper investigated the effect of some biotechnological parameters on the biodegradation efficiency of DCF by a selected strain of *Trametes versicolor* by using a statistical analysis strategy of design experiments methodology to get the maximum results with a minimum of experiments. The results show clearly that the experimental conception is an appropriate method for screening the biotechnological factors with significant influence on DCF biotransformation.

The applied design led to a first-order mathematical model whose statistically significant coefficients are related to the most influential factors on the response. The most influent factors with impact in bioremediation of aquatic media contaminated with DCF, when *Trametes versicolor* is implied in biodegradation, are the yeast extract, glucose, $CuSO_4 \cdot 5H_2O$ and inoculum concentration.

Further, an experiments optimization strategy will be developed in order to increase the bioremediation efficiency. Future experiments will target real systems in multiple cultures, activated sludge and selected fungal cultures, under similar conditions to those in wastewater treatment system.

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