International Biodeterioration & Biodegradation 78 (2013) 17–23

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

# Enhanced biodegradation of 4-chlorophenol by *Candida tropicalis* PHB5 via optimization of physicochemical parameters using Taguchi orthogonal array approach

Bikram Basak [a], Biswanath Bhunia [b], Subhasish Dutta [a], Apurba Dey [a,*]

[a] Department of Biotechnology, National Institute of Technology Durgapur, Mahatma Gandhi Avenue, Durgapur 713209, India
[b] Department of Biotechnology, Bengal College of Engineering and Technology, Bidhannagar, Durgapur 713212, India

ARTICLE INFO

*Article history:*
Received 2 May 2012
Received in revised form
7 December 2012
Accepted 7 December 2012
Available online 4 January 2013

*Keywords:*
Optimization
Biodegradation
4-Chlorophenol
*Candida tropicalis*
Taguchi DOE methodology

ABSTRACT

*Candida tropicalis* PHB5 was able to grow on 4-chlorophenol and to metabolize this substrate. 4-Chlorophenol degradation proceeds through a pathway involving transient production of three key metabolites 4-chlorocatechol, 4-chloropyrogallol and 4-carboxymethelenebut-2-en-4-olide which were identified by HPLC analysis. The Taguchi orthogonal array design methodology was used to improve the 4-chlorophenol biodegradation ability of strain PHB5. At 3 levels, an orthogonal array was selected to analyze the effects of the different physicochemical process factors. Experiments were undertaken to study the effects of eleven factors affecting the growth of *C. tropicalis* on 4-chlorophenol and its degradation. Taguchi methodology predicted that biomass yield on 4-chlorophenol could be increased from 409.7 mg $l^{-1}$ to 1046.9 mg $l^{-1}$ and the amount of 4-chlorophenol degraded could be increased from 314.5 mg $l^{-1}$ to 949.7 mg $l^{-1}$. Based on Taguchi methodology, an overall enhancement of growth by 155.5% and of 4-chlorophenol degradation by 201.9% were predicted. The predicted values were validated by experimental data which showed than under optimal cultural conditions, the biomass yield was increased by 141.62% and 4-CP degradation by 183%.



## 1. Introduction

Environmental contamination by chlorophenolic compounds is a serious problem worldwide. Phenol and chlorophenols are ubiquitous pollutants that contaminate natural water resources from a variety of sources such as wood preservatives, incineration of waste, pesticides etc. Besides, they are generated as byproducts during bleaching of pulp with chlorine and during disinfection of water by chlorination (Saez and Rittmann, 1993; Kim et al., 2002; Song-hu and Xiao-hua, 2005; Tobajas et al., 2012). Owing to their persistence and toxicity they affect living organisms. Most of the chlorophenolics have been designated as priority environmental pollutants by the US Environmental Protection Agency (Wild et al., 1993; Caldeira et al., 1999). Removal of such pollutants is of practical significance as discharge of these compounds into the environment can pose severe risks to populations (Jiang et al., 2007).

Researchers have investigated the metabolism of 4-chlorophenol (4-CP) by several microbial strains, either co-metabolically (Jiang et al., 2007; Tobajas et al., 2012) or as sole source of carbon and energy (Brown et al., 1990; Sahoo et al., 2010; Li et al., 2011). Co-metabolic removal of toxicants is also a well-established method for their removal and phenol and glucose have been claimed as a good primary growth substrate in the biodegradation of 4-CP (Jiang et al., 2007; Tobajas et al., 2012).

Getting better insights about how the different influencing physicochemical parameters contribute to the effective removal of toxicants from wastewater is a prerequisite as each microorganism has its own individual physicochemical requirements for growth (Annadurai et al., 2008). Hence, it is considered worthwhile to study the effect of different media components and to consider various environmental factors that have influence on 4-CP biodegradation. The traditional "change-one-variable-at-a-time" (COVT) approach is time consuming and practically and/or economically not feasible if the number of factors is high (Prasad et al., 2005). Moreover, it ignores the interactions among various parameters involved in the optimization study (Abdel-Fattah et al., 2005). The application of statistical experimental design techniques in bioremediation studies can result in improved biodegradation of toxicants, thereby, reducing developmental time and process cost. Optimization of the biodegradation conditions is a prerequisite to large-scale applications of microbial biodegradation processes.

* Corresponding author. Tel.: +91 343 2755209; fax: +91 343 2547375.
*E-mail address:* apurbadey.bt@gmail.com (A. Dey).

0964-8305/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2012.12.005

In this study we used the Taguchi design of experiments (DOE) approach to optimize the 4-CP-degrading ability of the new yeast isolate *Candida tropicalis* PHB5. The parameters were screened for their significant effects using a 3-level factorial design Taguchi orthogonal array (OA) approach. We have also determined the kinetics of 4-CP degradation and we have identified some of the metabolites produced during the degradation process.

## 2. Materials and methods

### 2.1. Chemicals and analysis

Analytical and HPLC grade chemicals used were purchased from Sigma Aldrich (USA), Himedia (India) and Merck (India). Water used for the HPLC analysis was prepared by Ultrapure Water System (Arium$^R$, 611UF, Sartorius, Germany). The Qualitek-4 software package (Nutek Inc., MI, USA) was used to analyze the experimental design and the analysis of the experimental data.

### 2.2. Microorganism

*C. tropicalis* PHB5 was isolated from an effluent of steel plant wastewater in Durgapur, India. Identification of the organism was done by analysis of ITS1 (internal transcribed spacer), ITS2 and 18S rRNA gene (NCBI GenBank Accession number: JN542555). The microorganism was maintained by routine weekly transfer under aseptic conditions to a mineral salt medium providing 4-CP (at concentration 500 mg $l^{-1}$) as sole source of carbon and energy and stored at 4 °C after incubation at 30 °C for 48 h.

### 2.3. Preparation of the inoculums

The inocula were prepared for the biodegradation experiments using a mineral salt medium composed of (g $l^{-1}$) $K_2HPO_4$ 2.1, $KH_2PO_4$ 0.4, $NH_4NO_3$ 0.6, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.023 and $FeCl_3$ 0.002 (Alexander and Lustigman, 1966) with 500 mg $l^{-1}$ 4-CP. The medium was prepared and autoclaved at 121 °C for 15 min. After inoculation, the cultures were incubated at 30 °C in a New Brunswick Innova® 42 incubator shaker (New Jersey, USA) at 120 rpm for 48 h. The final cell suspension was standardized to an $OD_{600}$ of 0.2 to inoculate the experimental cultures.

### 2.4. HPLC analysis of metabolites of 4-CP degradation

In the cell free supernatant, 4-CP metabolites were identified and quantified by HPLC analysis. The HPLC system (Waters ™ 600, Milford USA) was equipped with UV/Visible detector at 280 nm and a $C_{18}$ hypersil column (4.6 mm × 250 mm; 5 μm particle size; Waters, USA). The mobile phase used was acetonitrile-water (70:30 v/v) at a flow rate of 1 ml $min^{-1}$ and 30 °C. The supernatant was filtered through a 0.22 μ filter and injected in the column at different time intervals during incubation. The metabolites concentrations throughout the course of the degradation process were monitored by comparing with standard curves prepared from chemicals obtained commercially.

### 2.5. Optimization methodology (Taguchi methodology)

Taguchi DOE involves the establishment of different experimental situations through OAs to reduce experimental errors and to enhance the efficiency and reproducibility of experiments. Robust design has been considered in this study because it helps to minimize the effect of noise factors in the process of optimization and leads to a dynamic or robust experimental design (Dehnad, 1989).

#### 2.5.1. Design of experiments (phase 1)

The various factors to be optimized, that have significant effect on the biodegradation of 4-CP were identified in a first series of preliminary experiments that are not reported here. Each variable was investigated within the feasible range so that the variation inherent to the process did not mask the factor effect (Prasad et al., 2005). In this study, eleven physicochemical parameters (Table 1) were considered as important factors for growth and degradation of 4-CP by *C. tropicalis* PHB5. All the variables were investigated at three widely spaced levels shown in Table 1 chosen on the basis of preliminary experiments. Selection of the most suitable sources of nitrogen, phosphate and metal ions was done by the COVT method (data not shown). In the next step, a matrix was designed with the appropriate OAs for the selected parameters and their levels. Taguchi provides many standard OAs and corresponding linear graphs for this purpose (Taguchi, 1986). In the present study, three levels of eleven factors (Table 1) were considered and the size of experimentation was represented by symbolic array of L-27 (which indicated 27 experimental trials) (Table 2).

#### 2.5.2. Growth and phenol biodegradation by C. tropicalis with selected factors and levels (phase 2)

Freshly prepared cells suspensions ($OD_{600} \approx 0.2$) were used to inoculate 50 ml medium (composition shown in Table 1) in 250 ml Erlenmeyer flasks, containing 950 mg $l^{-1}$ of 4-CP. Biodegradation studies were carried out at different concentrations of each ingredient and for different physical parameters (Table 1). After incubation, 4 ml of each culture were collected and centrifuged at 15,000 × *g* for 10 min at 4 °C. The cell pellets were harvested and dried at 105 °C to a constant weight for 48 h in a hot air oven. The biomass was expressed as dry weight (mg $l^{-1}$) of culture. The supernatant was used for determination of residual 4-CP concentration. Residual 4-CP concentration was determined using the same HPLC system and conditions described above.

#### 2.5.3. Data analysis and prediction of performance (phase 3)

The data obtained from the experiments were processed using Qualitek-4 software (Nutek Inc., MI, USA) to evaluate the influence of individual factors, the multiple interactions of the selected factors, the determination of the optimal conditions and the process performance on growth of the organism and 4-CP degradation. In the present study *S/N* analysis was employed with bigger-is-better performance characteristics for all the experimental cases to estimate the performance (biomass increase and 4-CP removal) under the optimal cultural conditions. In the Taguchi method, the term 'signal' represents the desirable values (mean) and the term 'noise'

**Table 1**
Selected factors and their assigned levels taken under investigation.

| Serial no | Factor codes | Factor | Level-1 | Level-2 | Level-3 |
|---|---|---|---|---|---|
| 1 | A | $MgSO_4 \cdot 7H_2O$ (g $l^{-1}$) | 0.1 | 0.2 | 0.3 |
| 2 | B | Phosphate ion[a] (g $l^{-1}$) | 0.4 | 0.5 | 0.6 |
| 3 | C | NaCl (g $l^{-1}$) | 0.04 | 0.05 | 0.06 |
| 4 | D | Yeast extract (g $l^{-1}$) | 0.5 | 1 | 1.5 |
| 5 | E | $CaCl_2 \cdot 2H_2O$ (g $l^{-1}$) | 0.01 | 0.02 | 0.03 |
| 6 | F | Temperature (°C) | 28 | 30 | 32 |
| 7 | G | pH | 5 | 6 | 7 |
| 8 | H | Inoculums size (% v/v) | 1 | 2 | 3 |
| 9 | I | Incubation time (h) | 50 | 55 | 60 |
| 10 | J | Agitation (RPM) | 120 | 150 | 180 |
| 11 | K | Trace element solution[b] (% v/v) | 1 | 2 | 3 |

[a] Phosphate ion contains 1:1 proportion of $K_2HPO_4$ and $KH_2PO_4$.
[b] Trace element solution contains 0.3 g $l^{-1}$ $FeSO_4.7H_2O$; 0.05 g $l^{-1}$ $MnSO_4.H_2O$; 0.1g $l^{-1}$ $CoCl_2.6H_2O$; 0.034 g $l^{-1}$ $Na_2MoO_4.2H_2O$; 0.04 g $l^{-1}$ $ZnSO_4$ and 0.05 g $l^{-1}$ $CuSO_4.5H_2O$.