Optimization of \(o\)-Chlorophenol Biodegradation by Combined Mycelial Pellets Using Response Surface Methodology

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Abstract In the present study, the immobilizing fermentation characteristics and \(o\)-chlorophenol biodegradation of \textit{Rhodopseudomonas palustris} using mycelial pellets as a biomass carrier were investigated. To improve the \(o\)-chlorophenol degradation efficiency of the combined mycelial pellets, eight cultivation variables including glucose concentration, yeast extract concentration, spore inoculum size, pH, and agitation speed were optimized with an integrated strategy involving a combination of statistical designs. First, Plackett-Burman experiments identified glucose, yeast extract, and spore inoculum size as three statistically significant factors important for \(o\)-chlorophenol removal. Then, the steepest ascent method was used to access the optimal region of these significant factors. Finally, response surface methodology by Box-Behnken optimization was used to examine the mutual interactions among these three variables to determine their optimal levels. The ideal culture conditions for maximum \(o\)-chlorophenol removal according to a second-order polynomial model were as follows: 15.60 g/L glucose, 3.09 g/L yeast extract, and 9% (\(v/v\)) spore inoculum size, resulting in an expected \(o\)-chlorophenol removal rate of 92.60% with an \(o\)-chlorophenol initial concentration of 50 mg/L and 96-h culture time. The correlation coefficient \((R^2 = 0.9933)\) indicated excellent agreement between the experimental and predicted values, whereas a fair association was observed between the predicted model values and those obtained from subsequent experimentation at the optimized conditions.

Keywords \(O\)-chlorophenol · \textit{Rhodopseudomonas palustris} · Combined mycelial pellets · Biodegradation · Box-Behnken design · Optimization

1 Introduction

Chlorophenols (CPs) are a primary raw material for the production of insecticides, herbicides, and biocides by industrial and agricultural users worldwide (Pérez-Alfaro et al. 2013; Huang et al. 2014). These compounds also form as a by-product of chlorine formation during pulp bleaching and water disinfection (Moreno-Andrade and Buitrón 2012). Given their toxicity, carcinogenicity, and mutagenicity (Sharma et al. 2013), most CPs, including \(o\)-chlorophenol, 2,4-dichlorophenol, and pentachlorophenol, are considered priority pollutants by the United States Environmental Protection Agency (Forootanfar et al. 2013). Therefore, the efficient removal of these compounds is of great importance for environmental protection.

Physical adsorption, solvent extraction, chemical oxidation, electrochemical, and photocatalytic oxidation are traditional methods widely used to remove CPs from wastewater (Ghaffari et al. 2014; Kuśmiercz and Świątkowski 2015; Coteiro and De Andrade 2007; Shi...
et al. 2012; Arellano-González et al. 2016; Georgieva et al. 2011); however, the high-cost, low-efficiency, and resulting hazardous by-products limit the practical application of these remediation strategies (Wang et al. 2015b). As such, alternative biodegradation methods have received increased attention because of their many advantages, such as eco-friendliness, low expense, limited hazardous by-product production, and high efficiency (García-Peña et al. 2012; Durruty et al. 2011). The ability of bacteria (Arora and Bae 2014); fungi (Chen et al. 2013), particularly laccase-producing fungi (Menale et al. 2012; Pérez et al. 1997); and microalga (Petroutsos et al. 2007) to degrade CP pollutants has been extensively studied; however, only a limited number of reports have focused on the degradation potency of photosynthetic bacteria (Mutharasaia et al. 2012). 

*Rhodopseudomonas palustris*, a purple, non-sulfur photosynthetic bacteria, has been shown to degrade organic pollutants in flexible metabolic modes, including aerobically under chemoheterotrophic conditions or anaerobically under photoheterotrophic conditions (Zhao et al. 2011; Melton et al. 2014; Hu et al. 2010). However, the application of this species to organic wastewater treatment is limited because it is easily preyed upon by protozoa, difficult to sediment, and displays poor solid-liquid separation (Idi et al. 2015). One of the effective strategies to solve these problems is the improvement of cell density through bacterial cell immobilization. 

Bacterial cell immobilization by using natural or synthetic materials as carriers is a versatile and effective method for biological treatment of recalcitrant or toxic wastewater (Ma et al. 2016). In comparison with free-living cells, the immobilized microbial cells not only promote biodegradation but also provide many other advantages, such as high cell density, feasibility of continuous processing, cell stability, and lower costs of recovery (Chen et al. 2014; Park et al. 2013). The major concern is the selection of a compatible carrier for particular cells. Traditional immobilization carriers include natural substances such as wood chips and synthetic materials such as sodium alginite, polyvinyl alcohol (PVA), granulated activated carbon, and agar (Wani et al. 2016; Mahesh et al. 2015; Mita et al. 2015; Bilal et al. 2016). However, for practical industrial applications, some of these carriers may require a high initial expense and are difficult to recycle, contain toxic degraded monomers, or inhibit the biological activity of bacteria and thus affect the mass transfer rate of the system. The mycelial pellet, a type of biological matrix, is an alternative carrier for immobilizing functional bacteria. In particular, filamentous fungi can grow in submerged cultures and spontaneously form microbial particles known as mycelial pellets (Nair et al. 2016). These pellets have some exceptional features including better biocompatibility, shorter propagation time, faster sedimentation, and efficient solid-liquid separation, which is beneficial for reducing environmental contamination (Zhao et al. 2012; Villena et al. 2010). Therefore, the present study used mycelial pellets as a biomass carrier to immobilize *Rhodopseudomonas palustris* for CP removal in wastewater.

Given that both combined mycelial pellet formation and CP biodegradation are sensitive to variations in cultivation conditions, such as carbon and nitrogen sources, pH, temperature, and agitation speed, it is essential to optimize these conditions. Response surface methodology (RSM) is a group of statistical techniques that can be used to determine the optimal conditions for a given outcome and includes central composite design (CCD), Box-Behnken optimization, and Doehlert matrix (DM) (Suhaia et al. 2013; Abdelhay et al. 2008). Of these, Box-Behnken optimization is one of the most common RSM techniques, and consists of a three-level design based upon the combination of two-level factorial analyses (Box and Behnken 1960). This method has been successfully applied to bio-treatment processes, such as the optimization of culture conditions for methyl parathion biodegradation by *Fusarium* species (Usharani and Muthukumar 2013).

In the present study, a strain of o-chlorophenol degrading photosynthetic bacteria, *Rhodopseudomonas palustris* PSB-1D, was immobilized on the mycelial pellet to obtain combined mycelial pellet. To obtain the maximum o-chlorophenol removal rate by combined mycelial pellets, significant cultivation factors were identified using a Plackett-Burman design and further optimized by Box-Behnken optimization methodology. To the best of our knowledge, this is the first report using mycelial pellet as biomass carrier for *Rhodopseudomonas palustris* immobilization to degrade CP in wastewater.

### 2 Materials and Methods

#### 2.1 Chemicals and Reagents

Analytical grade o-chlorophenol (2-CP; Sinopharm Group Chemical Reagent Co., Ltd., Shanghai, China) was used as a biodegradation substrate. Chromatographic grade
methanol was purchased from Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade, commercially available, and used without further purification.

2.2 Microorganisms

*Phanerochaete chrysosporium* DH-1 was purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and formed mycelial pellets with submerged shaking cultivation. The *Rhodopseudomonas palustris* PSB-1D strain (GenBank Accession No. HM068966) used for o-chlorophenol biodegradation was isolated from shallow substrate sludge downstream of the sewage outfall of an insecticide factory in Shenyang, China (Hu et al. 2010).

2.3 Culture Medium

*Phanerochaete chrysosporium* DH-1 spores were maintained on potato dextrose agar (PDA) slants containing 4 g/L potato extract, 20 g/L glucose, and 15 g/L agar. PSB medium for *Rhodopseudomonas palustris* PSB-1D contained 2 g/L glucose, 0.6 g/L (NH$_4$)$_2$SO$_4$, 0.2 g/L yeast extract, 0.6 g/L KH$_2$PO$_4$, 0.4 g/L K$_2$HPO$_4$, 0.2 g/L CaSO$_4$, and 0.3 g/L MgSO$_4$·7H$_2$O. Combined mycelial pellet culture and biodegradation studies were conducted in basal medium containing 10 g/L glucose, 2 g/L yeast extract, 0.6 g/L KH$_2$PO$_4$, 0.4 g/L K$_2$HPO$_4$, 0.2 g/L CaSO$_4$, 0.3 g/L MgSO$_4$·7H$_2$O, and 0.1% (v/v) trace nutrient solution (Luo et al. 2014). All media were adjusted to an initial pH of 7.0–7.5 using 10% HCl and 10% NaOH, and then autoclaved at 121 °C for 20 min. 2-C-P was sterilized separately by filtration using a 0.2-μm regenerated cellulose membrane filter (Sartorius Stedim, Germany) and then added to the sterilized medium.

2.4 Combined Mycelial Pellet Culture

*Preparation of Spore Suspension* A spore suspension was used for inoculation as the spores could be easily quantified (Wang et al. 2013). Under sterile conditions, slant media from DH-1 cultures were washed three times with sterilized deionized water to obtain a spore suspension, and poured into a sterile flask after gentle shaking. A high-concentration suspension was obtained after several repetitions of this process and normalized to 5.0 × 10$^5$ spores/mL with sterilized deionized water.

*Preparation of PSB-1D Seed Culture* Preculturing was performed in a 100-mL Erlenmeyer flask containing 30 mL of PSB medium inoculated with approximately 1 × 10$^8$ CFU/mL PSB-1D cells from fresh slants, and incubated at 30 °C on a rotary shaker (MaxQ 5000, Thermo Fisher Scientific Corporation, America) at 140 rpm for 36 h. Then, 10 mL of culture was transferred to a 250-mL Erlenmeyer flask containing 100 mL of PSB medium with 50 mg/L 2-CP and incubated at 30 °C on a rotary shaker at 140 rpm for 72 h to obtain the PSB-1D seed culture.

*Combined Mycelial Pellet Culture and 2-CP Biodegradation Experiments* A 250-mL Erlenmeyer flask containing 100 mL of basal medium and 50 mg/L 2-CP was simultaneously inoculated with 10% (v/v) spore suspension and 10% (v/v) PSB-1D seed culture. The flask was incubated at 30 °C on a rotary shaker at 130 rpm for 96 h to form combined mycelial pellets. Sterile basal medium containing only 50 mg/L 2-CP was used as a control. At the end of the cultivation, 10-mL culture samples were withdrawn to analyze the 2-CP degradation rate.

2.5 Experimental Design

*Plackett-Burman Design* A Plackett–Burman experimental design was used to identify significant factors influencing 2-CP removal by combined mycelial pellets. Eight cultivation factors were examined: glucose concentration, yeast extract concentration, spore inoculum size, PSB-1D inoculum size, CaSO$_4$, initial pH, agitation speed, and culture temperature. Based on our previous results, in one-factor-at-a-time (OFAT) optimization experiments, the eight factors were chosen as independent variables at a high (+1) and low (−1) level as described in Table 1. Small (~3-mm diameter), spherical combined mycelial pellets formed after 96 h of incubation time.

The method of least squares was used to fit the experimental responses to the following first-order polynomial model, as Eq. (1) (Hii et al. 2015):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i$$

where $Y$ is the predicted response (2-CP removal rate in %), $\beta_0$ is the constant coefficient, $\beta_i$ is the regression coefficient, $X_i$ is the coded independent variable, and $k$ is the number of variables. Factors with regression analysis $p < 0.05$ were considered to have a significant effect on 2-CP removal and further optimized by Box–Behnken optimization.
A second-order polynomial model was then fitted to the experimental results by multiple regression, resulting in an empirical equation model for the response associated with the independent variables. The standard second-order polynomial equation used to predict the optimal condition is expressed as Eq. (3) (Kalali et al. 2011):

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \]  

where \( Y \) is the predicted response (removal rate of 50 mg/L 2-CP for 96 h of incubation time) and \( X_i \) is the coded value for the independent variable. The regression coefficients are as follows: \( \beta_0 \) is the intercept term, \( \beta_i \) is the linear coefficient, \( \beta_{ij} \) is the interaction coefficient, and \( \beta_{ii} \) is the quadratic coefficient. The quality of fit for the second-order model equation was expressed as the determination coefficient \( R^2 \), and its statistical significance was evaluated by \( F \) test. The optimal values were obtained from the regression equation optimization and 3D response surface plots.

**Statistical Analysis** Data represent the mean of three independent experiments unless otherwise indicated. Design Expert 8.0.6 software (Stat-Ease, Inc., Minneapolis, MN, USA) was used for the experimental design and regression analysis and to plot the response surface plots. Analysis of variance (ANOVA) was applied to evaluate the model’s statistical significance.

### 2.6 Analytical Methods

**Determination of the 2-CP Removal Rate** The 2-CP removal rate was measured by removing cellular debris in the culture medium by centrifugation at 8000×g for 10 min and filtering the supernatant with a 0.22-μm membrane filter. The filtrate was then subjected to high-performance liquid chromatography (HPLC) to determine the 2-CP concentration in a Hitachi L-2000 apparatus (Tokyo, Japan) equipped with a Zorbax SB-18 ODS Spherex column (4.6 mm × 200 mm) and a UV detector. The operation conditions were as follows: mobile phase methanol and high-purity water (45:55, v/v); ultraviolet detection wavelength, 273 nm; flow rate, 1 mL/min; injection volume, 25 μL; and column temperature, 30 °C. The 2-CP removal rate was calculated as the difference between the initial and final concentrations.

**Scanning Electron Microscopy** Dried mycelial pellets were coated with gold under reduced pressure and

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**Table 1** Levels and actual values of cultivation factors for the Plackett-Burman design

| Code | Cultivation factors (variables) | Levels       |
|------|---------------------------------|--------------|
|      |                                 | -1 | 1  |
| \( X_1 \) | Glucose (g/L)                  | 5  | 15 |
| \( X_2 \) | Yeast extract (g/L)            | 1.0| 3.0|
| \( X_3 \) | Spore inoculum size (% v/v)    | 5% | 25%|
| \( X_4 \) | PSB-1D inoculum size (% v/v)   | 5% | 25%|
| \( X_5 \) | CaSO₄ (g/L)                    | 0.1| 0.5|
| \( X_6 \) | Initial pH                     | 6.0| 9.0|
| \( X_7 \) | Agitation speed (rpm)          | 100| 180|
| \( X_8 \) | Temperature (°C)               | 25 | 35 |
visualized with a scanning electron microscope (S4800, Hitachi High-Technologies Corporation, Japan) equipped with a gaseous secondary electron detector.

3 Results and Discussion

3.1 Internal Morphology of Combined Mycelial Pellets

Combined mycelial pellets formed during the first 3 days in basal medium containing 50 mg/L 2-CP and survived to maintain their own structure without further growth. The white pellets were hairy and fluffy with a regular and nearly spherical shape, and had an average diameter of approximately 3 mm.

The internal morphology of mycelia before and after immobilization was investigated by scanning electron microscopy as shown in Fig. 1. Notably, the mycelial pellets were composed of numerous smooth mycelia without any bacterial attachment (Fig. 1a). The pellets displayed a microstructure with abundant micropores and microchannels, indicating that the fungal pellets could establish the microecological environment necessary for functional bacteria by improving the culture rheology, resulting in improved nutrient and oxygen transfer into the biomass and reduced diffusional resistance (Bayramoglu et al. 2009). In contrast, a large quantity of rod-shaped PSB-1D bacteria was firmly aggregated on the mycelia after immobilization, and showed an uneven distribution (Fig. 1b). Stability analysis revealed that the combined mycelial pellets could survive and maintain their own structure in culture medium for over 20 days without disruption (Fig. 2). Moreover, the 2-CP removal rate by the combined mycelial pellets was 90.87% over an incubation time of 96 h, whereas those of free PSB-1D bacteria and Phanerochaete chrysosporium DH-1 were 78.14 and 55.86%, respectively. Therefore, these results demonstrate the superiority of mycelial pellets immobilized with Rhodopseudomonas palustris PSB-1D for 2-CP biodegradation.

To date, biological immobilization on mycelial pellet carriers has been reported for the production of hydrogen and bioflocculant fermentation (Nyman et al. 2013). For example, Aspergillus niger Y3 mycelial pellets were adopted as a biomass carrier to immobilize the hydrogen-producing bacterium Thermoanaerobacterium thermosaccharolyticum W16 to promote hydrogen yield (Zhao et al. 2012). Similarly, this carrier was also used to immobilize the bioflocculant-producing bacteria Agrobacterium tumefaciens F2 and Bacillus sphaericus F6 for bioflocculant fermentation (Wang et al. 2013). However, the present study represents the first attempt to use mycelial pellets as a biomass carrier for photosynthetic bacteria.

As shown in the SEM images of Fig. 1b, PSB-1D bacteria were successfully immobilized on the DH-1 mycelia, suggesting that the pellet’s polyporous structure provides sufficient surface area for the adhesion of PSB-1D bacteria and to establish a microecological environment suitable for bacterial growth (Zhang et al. 2011). Fungal mycelia are considered a superior biosorbent for heavy metals because of the abundance of different functional groups in the cell wall capable of metal adsorption (Wang et al. 2015a). For instance,
Phanerochaete chrysosporium mycelial pellets can serve as a biosorbent to remove Cr (VI), which enhanced the active sites for adsorption and provided advantageous conditions for further Cr (VI) adsorption (Chen et al. 2011). However, the mechanism underlying the joint effects of Rhodopseudomonas palustris in mycelial pellets deserves further study.

3.2 Screening of Significant Cultivation Factors Using a Plackett-Burman Design

Factors significantly influencing 2-CP degradation were identified by a Plackett-Burman experiment using eight independent process variables. The design matrix and resulting 2-CP removal rates are shown in Table 2, where \( X_1 \text{--} X_8 \) and \( D_1 \text{--} D_3 \) represent the coded independent variables and dummy variables, respectively. Three dummy variables were used to estimate the experimental error and measure the adequacy of the first-order polynomial model.

To determine the optimal response, a fitted first-order polynomial model equation for 2-CP removal \( (Y) \) was obtained from the Plackett-Burman experiment and defined as Eq. (4) as follows:

\[
Y_1 = 76.68 + 3.83X_1 + 9.76X_2 - 2.68X_3 - 2.43X_4 \\
+ 2.14X_5 + 1.63X_6 + 1.74X_7 - 1.37X_8
\]  

(4)

Statistical analysis of the selected factorial model indicated that the response equation provided a suitable model for the Plackett-Burman design \( (R^2 = 0.9851; \text{Adj } R^2 = 0.9455; P = 0.0116; \text{Table 3}) \). Factors with confidence levels > 95\% \( (P < 0.05) \) were considered to significantly affect the response and were selected for further optimization studies.

### Table 2 Plackett-Burman design matrix and corresponding 2-CP removal rate

| Run | Glucose | Yeast extract | Spore inoculum size | PSB-1D inoculum size | CaSO\(_4\) | pH | Agitation speed | Temperature | \( D_1 \) | \( D_2 \) | \( D_3 \) | 2-CP removal rate (%) |
|-----|---------|---------------|--------------------|---------------------|---------|----|----------------|-------------|--------|--------|--------|----------------------|
| 1   | −1      | −1            | −1                 | 1                   | 1       | 1  | 1              | −1          | 1      | 1      | 1      | 66.87                |
| 2   | −1      | −1            | 1                  | −1                  | 1       | 1  | 1              | −1          | 1      | 1      | −1     | 65.23                |
| 3   | 1       | −1            | 1                  | −1                  | 1       | 1  | 1              | −1          | −1     | −1     | 1      | 64.57                |
| 4   | −1      | 1             | 1                  | −1                  | −1      | 1  | 1              | −1          | −1     | −1     | 1      | 68.82                |
| 5   | −1      | 1             | 1                  | −1                  | 1       | 1  | 1              | −1          | −1     | −1     | −1     | 87.52                |
| 6   | 1       | 1             | −1                 | −1                  | −1      | 1  | 1              | 1           | −1     | −1     | −1     | 91.11                |
| 7   | 1       | −1            | −1                 | −1                  | 1       | 1  | 1              | 1           | −1     | −1     | −1     | 85.56                |
| 8   | 1       | −1            | 1                  | 1                   | 1       | 1  | 1              | −1          | −1     | −1     | −1     | 66.78                |
| 9   | −1      | −1            | −1                 | −1                  | −1      | 1  | 1              | 1           | −1     | −1     | −1     | 63.14                |
| 10  | 1       | −1            | −1                 | 1                   | −1      | 1  | 1              | 1           | −1     | −1     | −1     | 92.91                |
| 11  | 1       | 1             | −1                 | −1                  | 1       | 1  | 1              | 1           | 1      | −1     | −1     | 92.76                |
| 12  | 1       | −1            | −1                 | −1                  | 1       | 1  | 1              | 1           | −1     | 1      | 1      | 74.92                |

Fig. 2 Microscopic images of combined mycelial pellets at different incubation times: 2 days (a), 4 days (b), and 20 days (c). Scale bar = 2000 μm
Based on the results of our statistical analysis, glucose concentration \((P = 0.0189)\), yeast extract concentration \((P = 0.0013)\), and spore inoculum size \((P = 0.0476)\) were the factors that most significantly influenced 2-CP removal by combined mycelial pellets. Glucose is commonly utilized as a carbon source for providing energy for cell growth and as a co-metabolism substrate to enhance CPs removal (Lee and Lee 2007). Glucose has also been suggested to be an essential nutrient for providing the carbon backbone for the formation of mycelial pellets (Nair et al. 2016). Several studies have reported enhanced biodegradation of mono-chlorophenol by different microorganisms, such as *Pseudomonas putida* (Fakhruddin and Quilty 2005), *Comamonas testosteroni* (Tobajas et al. 2012), and *Rhodopseudomonas palustris* (Dong et al. 2011). Fakhruddin and Quilty (2005) reported that addition of more glucose could facilitate substrate removal by enhancing *Pseudomonas putida* CP1 growth and thereby attenuating the toxicity of 2-CP. Similar results were obtained in this study, where increasing the glucose concentration enhanced the removal of 2-CP accompanied by an increase in the amount of combined mycelial pellets. In addition to glucose, yeast extract may significantly influence 2-CP removal because it strongly affects cell division and the specific activity of the degrading microbe (Ahmed and Ahmed 2014). The size of the spore inoculum had significant negative effects on 2-CP removal, possibly because previous studies have concluded that using high concentrations of fungal spores might lead to the formation of dispersed mycelia, whereas low inoculum size is beneficial for pellet production for filamentous fungi (Espinosa-Ortiz et al. 2016).

Previous studies reported that calcium ions are a necessary medium component for mycelial pellet formation, as addition of calcium ions induces mycelial aggregation during fungal growth by changing mycelial surface charge properties and promoting bridge formation between cells (Nyman et al. 2013). Therefore, calcium sulfate was selected as an independent variable in the Plackett-Burman experiment. However, our study results indicated that the amount of combined mycelial pellets did not depend on the calcium sulfate concentration and that the calcium sulfate concentration did not significantly influence 2-CP removal in a concentration range of 0.1–0.5 g/L. Initial pH also did not significantly affect 2-CP removal in a range of pH 6–9. In addition to calcium sulfate and pH, other factors including PSB-1D inoculum size, agitation speed, and temperature did not significantly affect 2-CP removal and were thus all maintained at the middle level for further optimization procedures.

### 3.3 Path of Steepest Ascent Design

In the Plackett-Burman results, the coefficient estimates for glucose concentration and yeast extract concentration were positive (+3.83 and +9.76), whereas that for spore inoculum size was negative (−2.68), which meant that increasing the concentration of glucose and yeast extract and decreasing the inoculum size of the spores would result in a higher removal rate for 2-CP. The

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**Table 3 Statistical analysis of process parameters in relation to 2-CP removal rate**

| Variables                | Sum of squares | Degrees of freedom | Mean square | Coefficient | Standard error | F value | P value (prob > F) |
|--------------------------|----------------|--------------------|-------------|-------------|----------------|---------|-------------------|
| Model                    | 1622.30        | 8                  | 202.79      | 76.68       | 0.82           | 24.86   | 0.0116            |
| Glucose                  | 175.64         | 1                  | 175.64      | 3.83        | 0.82           | 21.53   | 0.0189*           |
| Yeast extract            | 1144.07        | 1                  | 1144.07     | 9.76        | 0.82           | 140.24  | 0.0013*           |
| Spore inoculum size      | 86.03          | 1                  | 86.03       | −2.68       | 0.82           | 10.55   | 0.0476*           |
| PSB-1D inoculum size     | 70.91          | 1                  | 70.91       | −2.43       | 0.82           | 8.69    | 0.0601            |
| CaSO4                    | 54.83          | 1                  | 54.83       | 2.14        | 0.82           | 6.72    | 0.0809            |
| pH                       | 31.79          | 1                  | 31.79       | 1.63        | 0.82           | 3.90    | 0.1429            |
| Agitation speed          | 36.44          | 1                  | 36.44       | 1.74        | 0.82           | 4.47    | 0.1250            |
| Temperature              | 22.61          | 1                  | 22.61       | −1.37       | 0.82           | 2.77    | 0.1946            |
| Residual                 | 24.47          | 3                  | 8.16        |             |                |         |                   |
| Total                    | 1646.77        | 11                 |             |             |                |         |                   |

\( R^2 = 0.9851, \text{ Adj } R^2 = 0.9455, \text{ Pred } R^2 = 0.7622 \)

*Statistically significant at 95% probability level \((P < 0.05)\)
experimental design and response of the steepest ascent experiment are shown in Table 4. The highest 2-CP removal rate was 92.36% when the glucose concentration was 15 g/L, the yeast extract concentration was 3.0 g/L, and the spore inoculum size was 10%, suggesting that this point was near the region of the maximal 2-CP removal response. This point was thus set as the center point in the Box-Behnken optimization.

3.4 Box-Behnken Optimization

Based on the results from the Plackett-Burman experiment and steepest ascent analysis, glucose concentration (12.5–17.5 g/L), yeast extract concentration (2.5–3.5 g/L), and spore inoculum size (7.5–12.5%) were chosen as the critical independent variables and designated as \( x_1, x_2, \) and \( x_3 \), respectively. The variables \( x_i \) were coded as \( X_i \) according to Eq. (2), and each variable was assessed at three different levels, defined as low (−1), middle (0), and high (+1), as shown in Table 5.

A total of 17 experimental runs with a three-factor, three-level Box-Behnken design were performed. The design matrix for the independent variables and associated response for 2-CP removal are shown in Table 6.

The CP-2 degradation response data were used for the analysis of variance for each process factor as shown in Table 7.

A second-order polynomial regression model containing one block term, three linear terms, three quadratic terms, and three interaction terms was obtained to fit the experimental results by applying a multiple regression analysis to the experimental data. The quadratic model is given as Eq. (5):

\[
Y_2 = 92.20 + 1.57X_1 + 0.78X_2 - 1.08X_3 - 0.80X_1X_2 - 0.63X_1X_3 - 0.33X_2X_3 - 3.30X_1^2 - 1.92X_2^2 - 2.48X_3^2
\]

This analysis yielded a \( R^2 \) of 0.9933 with \( P < 0.0001 \). The “lack of fit F value” of 0.11 (> 0.05) implied that the lack of fit within the model could be attributed to pure error. Moreover, the predicted \( R^2 \) of 0.9819 was in reasonable agreement with the adjusted \( R^2 \) of 0.9846. Collectively, these findings indicate that the established quadratic polynomial model for 2-CP removal by combined mycelial pellets was adequate and reliable in representing the actual relationship between the independent variables and response.

Three-dimensional response surface plots obtained from the equation model \( (Y_2) \) were analyzed to determine the optimal value for each variable and assess intervariable interactions (Fig. 3a–c). Figure 2 shows the relative effect of the three variables (glucose, yeast extract, and spore inoculum size) on 2-CP removal rate. The center point coordinates indicate the optimal value for the respective variable. The optimal levels for the three variables obtained from the maximum point of the model were calculated in coded units to be at 0.24, 0.18, and −0.26 for \( X_1, X_2, \) and \( X_3 \), respectively. Thus, the optimal values for glucose, yeast extract, and spore inoculum size were 15.60 g/L, 3.09 g/L, and 9%, respectively, resulting in a maximum predicted 2-CP removal rate of 92.60%.

### Table 4

| Run | Glucose (g/L) | Yeast extract (g/L) | Spore inoculum size (%) | 2-CP removal rate (%) |
|-----|--------------|---------------------|-------------------------|----------------------|
| 1   | 10           | 2.0                 | 15                      | 75.77                |
| 2   | 12.5         | 2.5                 | 12.5                    | 83.49                |
| 3   | 15           | 3.0                 | 10                      | 92.36                |
| 4   | 17.5         | 3.5                 | 7.5                     | 85.21                |
| 5   | 20           | 4.0                 | 5                       | 83.51                |
3.5 Experimental Validation of the Optimized Conditions

Subsequent biodegradation experiments conducted with the predicted optimal conditions resulted in a 2-CP removal rate of 92.28%, which was nearly identical to the predicted value. This finding confirmed that Box-Behnken optimization is an accurate and reliable method to predict 2-CP removal rates by combined mycelial pellets.

Thus, the optimal medium compositions and culture conditions for maximum 2-CP removal (50 mg/L initial concentration) were as follows: 100 mL of liquid media (pH 7.5) consisting of 15.60 g/L glucose, 3.09 g/L yeast extract, 9% (v/v) spore inoculum content, 15% (v/v) PSB-1D inoculum content, 0.6 g/L KH2PO4, 0.4 g/L K2HPO4, 0.3 g/L CaSO4, 0.3 g/L MgSO4*7H2O, and 0.1% (v/v) trace element content in a 250-mL flask with a 30 °C culture temperature, 140 rpm agitation speed, and 96 h culture time. After optimization, the 2-CP removal rate increased to 92.28%, and was approximately 12% higher than that obtained with the original culture conditions (80.87%). The statistical experimental design provided an effective and feasible approach for 2-CP biodegradation. Additionally, the rate of removal of 2-CP by combined mycelial pellets was higher than that of Pseudomonas sp. CF600 (Nowak and Mrozik 2016), Rhodococcus rhodochrous DSM6263 (Hou et al. 2016), and Alcaligenes sp. A7–2 (Menke and Rehm 1992), but slightly lower than that of Pseudomonas putida CP1 (Farrell and Quilty 2002).

Thus, the present results demonstrate that using Phanerochaete chrysosporium mycelial pellets as a

Table 6 Box-Behnken optimization for independent variables and 2-CP removal response

| Run | Glucose | Yeast extract | Spore inoculum size | 2-CP removal rate (%) |
|-----|---------|---------------|---------------------|----------------------|
| 1   | 0       | 0             | 0                   | 92.52                |
| 2   | 0       | 1             | 1                   | 87.29                |
| 3   | 0       | 0             | 0                   | 92.05                |
| 4   | −1      | 1             | 0                   | 86.95                |
| 5   | −1      | 0             | −1                  | 85.39                |
| 6   | 1       | −1            | 0                   | 88.61                |
| 7   | 0       | −1            | 1                   | 86.31                |
| 8   | 0       | 1             | −1                  | 89.96                |
| 9   | 1       | 0             | −1                  | 89.78                |
| 10  | 0       | 0             | 0                   | 92.66                |
| 11  | 0       | 0             | 0                   | 92.27                |
| 12  | 1       | 1             | 0                   | 88.49                |
| 13  | 0       | −1            | −1                  | 87.64                |
| 14  | −1      | −1            | 0                   | 83.86                |
| 15  | −1      | 0             | 1                   | 84.32                |
| 16  | 0       | 0             | 0                   | 91.49                |
| 17  | 1       | 0             | 1                   | 86.17                |

Table 7 ANOVA for the fitted quadratic polynomial model of 2-CP removal by combined mycelial pellets

| Source | Sum of squares | Mean squares | F values | P value (prob > F) | Significant |
|--------|----------------|--------------|----------|--------------------|-------------|
| Model  | 135.50         | 15.06        | 114.90   | < 0.0001          | Significant |
| X1     | 19.63          | 19.63        | 149.78   | < 0.0001          |
| X2     | 4.91           | 4.91         | 37.50    | 0.0005            |
| X3     | 9.42           | 9.42         | 71.88    | < 0.0001          |
| X1X2   | 2.58           | 2.58         | 19.66    | 0.0030            |
| X1X3   | 1.61           | 1.61         | 12.31    | 0.0099            |
| X2X3   | 0.45           | 0.45         | 3.43     | 0.1066            |
| X12    | 45.93          | 45.93        | 350.53   | < 0.0001          |
| X22    | 15.49          | 15.49        | 118.18   | < 0.0001          |
| X32    | 25.90          | 25.90        | 197.68   | < 0.0001          |
| Residual | 0.92          | 0.13         |          |                    |
| Lack of fit | 0.072 | 0.024 | 0.11 | 0.9479 | Not significant |
| Pure error | 0.85 | 0.21 | | | |
| Cor total | 136.42 | | | | |

$R^2 = 0.9933$, Adjusted $R^2 = 0.9846$, Pred $R^2 = 0.9819$
biomass carrier to immobilize *Rhodopseudomonas palustris* should be an excellent candidate for the treatment of CP wastewater on an industrial scale.

### 4 Conclusion

The statistical experimental design enabled quick identification and optimization of key cultivation factors and their respective interactions. The present study examined eight variables using a Plackett-Burman design, which led to identification of three significant factors (glucose concentration, yeast extract concentration, and spore inoculum size) for 2-CP removal by combined mycelial pellets. The path of steepest ascent analysis was used to determine the optimal range for each factor, whereas Box-Behnken optimization was employed to identify the optimal level of these factors. A quadratic model was generated to accurately predict the value of each variable necessary for maximum 2-CP removal, which was then validated by further experimentation. Collectively, these findings demonstrate that the Plackett-Burman design and Box-Behnken optimization are accurate and reliable methods to design bioreactors for continuous 2-CP degradation.

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