Hindawi Publishing Corporation
ISRN Chemical Engineering
Volume 2013, Article ID 865618, 6 pages
http://dx.doi.org/10.1155/2013/865618

Research Article

Evaluation of Packed-Bed Reactor and Continuous Stirred Tank Reactor for the Production of Colchicine Derivatives

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Received 6 June 2013; Accepted 1 August 2013

Academic Editors: G. Bayramoglu, A. Brucato, I. Poulios, and I. Suelves

Bioconversion of colchicine into its pharmacologically active derivative 3-demethylated colchicine (3-DMC) mediated by P450BM3 enzyme is an economic and promising strategy for the production of this inexpensive and potent anticancer drug. Continuous stirred tank reactor (CSTR) and packed-bed reactor (PBR) of 3 L and 2 L total volumes were compared for the production of 3-demethylated colchicine (3-DMC) a colchicine derivative using *Bacillus megaterium* MTCC*420 under aerobic conditions. Statistical optimization technique was utilized with the most significant variables, that is, dissolved oxygen (DO), colchicine concentration, and process time for optimization. The validation of the model was performed by experiments on the predicted values in an individual run, and the optimum parameters were DO (~50%), colchicine concentration (7.5 g/L), and process time (39 h) resulted in a maximum bioconversion of 3-DMC 3.36 g/L. The PBR reactor achieved much higher productivity (6.58 g/L/h) as reported by earlier researchers. This is the first report on the use of PBR for bioconversion of colchicine.

1. Introduction

Bioreactor design is of crucial importance in the development of bioprocesses. Once a microorganism is selected and the culture and/or production conditions are optimized at laboratory scale, the next issue is proceeding to larger scale so that bulk quantities of the desired product can be produced optimally in a cost-effective manner. The selection of a proper reactor type is critical to such processes and plays a major role in large-scale production. The bioreactors widely used in today's fermentation industry are mainly those developed in the past decades purely for chemical reactions [1, 2]. Among others, Stirred Tank Reactors (STRs) and Packed-Bed Reactors (PBRs) find the widest application. The application of these reactors for biological processes has been intensively studied with aerobic cultures [3–5].

Colchicine is a well-documented pseudo alkaloid obtained from *Colchicum autumnale* L. and *Gloriosa superba*, widely used in therapy for the treatment of gout pain persisting for a very long time [6, 7]. It is too toxic to be of value as an antitumor drug, in its native form. Derivatives of colchicine, that is, 3-demethylcolchicine, colchicoside, and thiocolchicoside with improved therapeutic properties for anti-inflammatory and anti-tumor drugs, have good commercial demand as these compounds are known to have clinical significance for the treatment of certain forms of leukemia and solid tumors [8, 9]. Colchicine and a number of its prepared derivatives cannot be used as such due to their high toxicity in terms of high-risk/benefit ratio [8]. As far as the use in the anti-inflammatory field is concerned, the only marketed colchicine derivative is thiocolchicoside, bearing a thiomethyl moiety at C-10 and a glucose molecule at the hydroxyl in C-10. The therapeutically uses of this derivative are related to the muscle relaxant and antiphlogistic effects [7, 10]. Demethylated colchicine at C-3 position of the ring-A shows about 35-fold less toxicity as compared to parent molecule, and it equally has good anti-tumor activity as compared to that of thiocolchicine. 3-demethylcolchicine

and its glycosides are present in small amount in the colchicine producing plants [8–10].

In a rule of thumb, the classical method for determining optimal production conditions in fermentation process is varying in one process parameter while keeping others at a constant level. This practice is time-consuming and generates a need for large number of experiments, and the results are not so accurate and reproducible. In such practice, interaction effect between the variables is not taken into consideration. In recent times, response surface methodology (RSM) a good statistical experimental design tool has proved its worth, and it is now commonly used in optimization experiments of fermentation processes using biological system [11, 12]. In the present work, we have employed RSM to provide a fast and efficient way to understand and identify the significant process variables, thereby saving time and maintaining key information on each component. RSM includes factorial design and regression analysis that helps in evaluating significant variables and plotting them simultaneously against each other to evaluate the interaction and effect on each other simultaneously.

In the present work, we investigated the demethylation of colchicine in a continuous-flow packed-bed reactor in an effort to better understand possible adverse effect of colchicine bioconversion associated with the use of stirred tank reactors. When this experiment was carried out in a packed-bed reactor, the dissolved oxygen was required to be 60% v/v prior to entering the reactor. Because of the necessity of controlling dissolved oxygen and pH and making the process smoother and homogeneous, another fermenter was used to control conditions for the dissolved oxygen and other parameters. It is highly desirable to employ reaction conditions that minimize undesired secondary reactions of the indicated types. Tubular packed-bed reactors offer a number of advantages with higher conversion per unit mass of catalyst, recyclability, continuous operation, and minimum product inhibition when compared to stirred tank reactors.

2. Materials and Methods

2.1. Strain and Culture Medium. *Bacillus megaterium* MTCC*420 obtained from MTCC Chandigarh was used in this study. Culture methods and analytical methods were the same as previously reported by Dubey et al. [13, 14].

2.2. Fermentation Conditions

2.2.1. At Shake Flask Level. Shake flask experiments were carried out using 100 mL and 250 mL Erlenmeyer flasks containing 15 mL and 25 mL medium having 7 g/L colchicine respectively. After inoculation, flasks were incubated over night at 28°C, 200 rpm.

The culture was incubated for 72 h in the same conditions as described above, and every 12 h, samples were taken to evaluate the growth level and the 3-DMC production by HPLC [11, 15].

2.2.2. At Stirred Tank Reactor. All the fermenter operational conditions optimization trials were carried out in 5 L fermenter (Sartorious Inc., Germany) for bioconversion experiments [13]. The working volume of fermenters was 3 L. The impeller was 6-bladed Rushton turbine type having diameter 50 mm in 5 L fermenter. The pH was maintained in the fermenter by using peristaltic pump attached with 60% sugar solution. The cultivation time for growth was 72 h. Operational conditions were employed as reported by Dubey et al. [13].

2.2.3. Apparatus Used for Packed-Bed Reactor. The packed-bed reactor consisted of 20 cm of tubing (2.5 cm i.d.) containing 25 g of immobilized *B. megaterium* cells. The tubing was packed manually with the dry immobilized cells. The packing was then fixed in place using a mesh connected to stainless steel plugs. This tubing was submerged in a constant temperature water bath. A syringe pump was used to supply the colchicine.

Prior to initiating flow of colchicine to the reactor, nitrogen was passed through the packed bed for 5 min to remove air. Each experiment was initiated by quickly flushing the reactor with a total volume of the mixture of substrates equal to at least twice the void volume of the reactor. After quasi-steady-state operating conditions were achieved, several samples of the effluent stream were manually collected over a time frame corresponding to at least three reactor space times. (The reactor space time is the ratio of the void volume of the reactor to the total volumetric flow rate of the two feed stocks.) For a set of experiments corresponding to a specified operating temperature, the experiments at different space times were conducted in random order.

2.2.4. Determination of the Void Volume and the Space Time. The void volume (1.3 cm^3/g of catalyst) was calculated using the difference between the weights of the packed-bed reactor (tubing+catalyst) when filled with a colchicine of known density and the corresponding weight of the packed-bed reactor in the absence of this fluid. Corrections were made for the regions of the tubing outside the packed bed. Reactor space times were calculated as the ratio of the void volume of the reactor to the total volumetric flow rate of the two feedstocks.

2.3. Statistical Analysis. The optimization experiments were statistically designed and performed using response surface methodology (RSM) using Design-Expert from Stat-Ease, Inc. DO concentration, substrate concentration, and process time were considered as important variables in the experimental design and had been considered for optimization. The other process components were kept at constant levels throughout the experimental runs. The parameters, for example, DO, substrate conc., and process time, were simultaneously varied as depicted in Table 1. The results for substrate conversion were fed in the responses against each run (shown in Table 1). The experiments were designed according to the statistical design software Design-Expert 8.0 from Stat-Ease Inc. (Trial version).

The obtained data in real time was added in each set corresponding to the individual experiments. Design expert uses statistical designs and tools for predicting the significance and

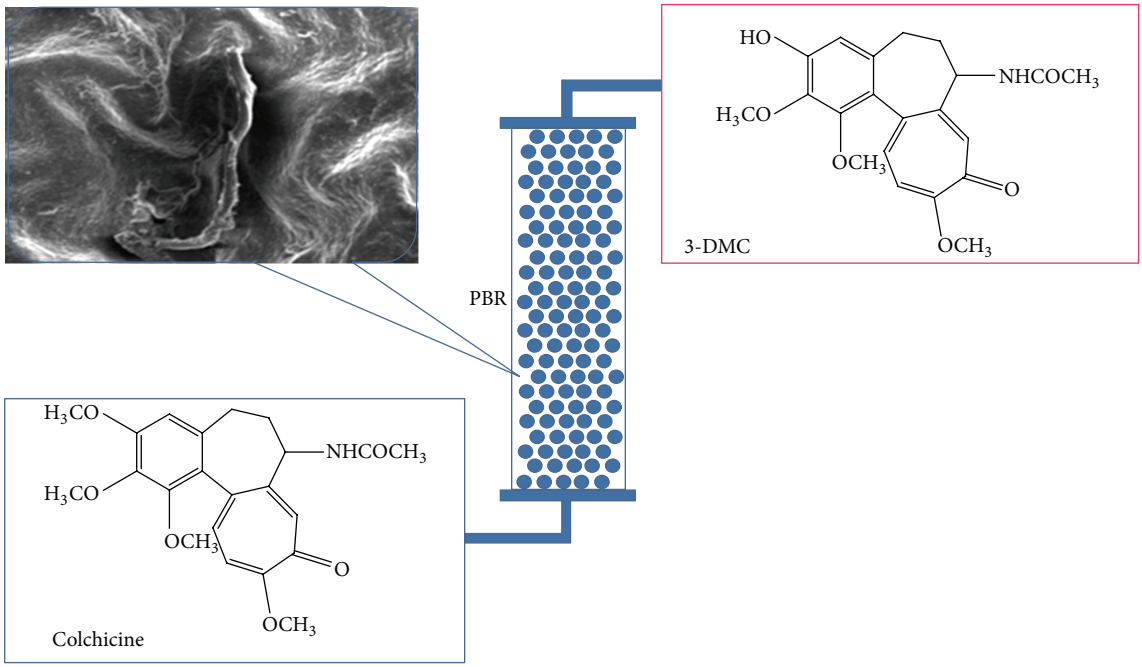

Figure 1: Schematic diagram showing biocatalysis process for conversion of colchicine to 3-demethylated colchicine through packed-bed reactor (PBR).

accuracy of the model as well as the predicted trends, in the attempted design space. The results were fitted into various RSM models out of which central composite design (CCD) predicted curves with better regression coefficients and non-significant lack of fit. Table 2 shows the maximum, minimum, central, and α value for dissolved oxygen, colchicine concentration, and process time, respectively. Data analysis was carried out using Design Expert software (free-trial version).

3. Results and Discussion

The present study, performed for the possible application of PBR system in biotransformed products. Previous researchers have published various research articles on the use of PBR system for biotransformation [16–18]. Author has also published report on submerged fermentation through *Bacillus megaterium* acting as biocatalyst for colchicine bioconversion [11–14]. In view of the facts and the demand of marketing, this study was performed to define a suitable reactor system which has less shear stress during biocatalysis mechanism. Earlier studies were carried out with CSTR with low productivity. In the current paper, we analyzed various important process parameters with special emphasis on dissolved oxygen, substrate concentration, and process time using statistical techniques for process optimization and control.

Our previous reports on bioconversion of colchicine, *B. megaterium* was successfully exploited for biotransformation, and effects of different concentrations (5 mM to 35 mM) of substrate/product (colchicine/3-DMC) toxicity on growth rate of *B. megaterium* have been studied [14] in CSTR. The similar results were achieved in PBR system with 2-3% variation (data not shown).

Table 1: Design matrix generated by DoE showing response of each run.

Run	A Dissolved oxygen (%)	B Colchicine (g/L)	C Process time (h)	Predicted 3-DMC (g/L)
1	50.00	5.00	48.00	4.32
2	32.50	7.50	39.00	4.60
3	32.50	7.50	39.00	4.60
4	15.00	5.00	48.00	1.45
5	32.50	7.50	39.00	4.60
6	32.50	7.50	39.00	4.60
7	32.50	11.70	39.00	3.18
8	32.50	7.50	39.00	4.60
9	32.50	7.50	39.00	4.60
10	50.00	10.00	30.00	4.60
11	32.50	7.50	54.14	5.10
12	15.00	10.00	48.00	2.74
13	50.00	10.00	48.00	7.90
14	32.50	3.30	39.00	2.43
15	61.93	7.50	39.00	5.95
16	15.00	10.00	30.00	0.91
17	15.00	5.00	30.00	1.12
18	3.07	7.50	39.00	0.67
19	32.50	7.50	23.86	2.04
20	50.00	5.00	30.00	3.10

Before starting the bioconversion experiments on PBR (Figure 1), the process was optimized at shake flask level

Table 2: Concentration ranges of independent process variables (dissolved oxygen, colchicine concentration, and process time) used in RSM.

Factor	Name	Units	Type	Min.	Max.	−1 actual	+1 actual	Mean	Std. Dev.
A	DO	(%)	Numeric	3.07	61.93	15	50	32.5	14.46
B	Colchicine	(g/L)	Numeric	3.3	11.7	5	10	7.5	2.07
C	Process Time	(s)	Numeric	31.91	52.09	36	48	42	4.96

Table 3: ANOVA for response surface quadratic model to verify whether developed model is significant or nonsignificant.

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value (prob > F)
Model	66.63	6	11.1	176.23	<0.0001*
A-DO	45.96	1	45.96	729.41	<0.0001
B-Colchicine	13.71	1	13.71	217.54	<0.0001
C-Process time	3.37	1	3.37	53.51	<0.0001
AB	2.82	1	2.82	44.76	<0.0001
AC	0.56	1	0.56	8.83	0.0108
BC	0.21	1	0.21	3.3	0.0923
Residual	0.82	13	0.063		
Lack of fit**	0.82	8	0.1		

*significant, **nonsignificant.

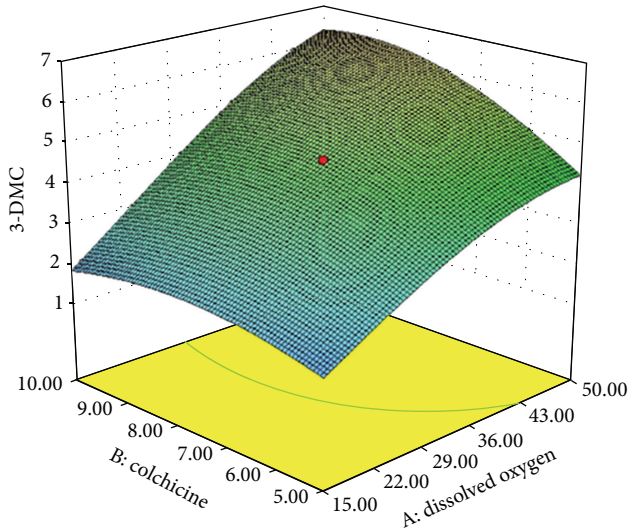

Figure 2: Response surface methodology showing bioconversion of colchicine to 3DMC with response to dissolved oxygen (DO).

which was carried forward to PBR system. In this study, various parameters were considered and were attempted for optimization by classical approach. The first step was to optimize the initial substrate concentration, within the selected range of 3 g/L to 10 g/L colchicine. The shake flasks were added with colchicine in an increment of 1 g/L colchicine at 0 h of the process. The incubation was done at 28°C for 72 h.

3.1. CSTR versus PBR

3.1.1. Response Surface Analysis for Optimization of Bioconversion Parameter. The optimization of bioconversion parameter was analyzed by response surface methodology. Table 1 represents the design matrix of the variables (DO, substrate conc., process time) in coded units along with the 3-DMC yield in mg/L. Table 3 shows the least square fit and significance of regression coefficient of variable parameter individually and in combination. The level of significance of each of the coefficient was checked by its respective P-value to confirm the interaction between the tested variables. It was noticed that with the decrease of the magnitude of the P-value, the corresponding coefficient was in increasing tendency. This result was in confirmation with the earlier findings by Dubey et al. [10, 14]. From the response surface data analysis, that the empirical relationship between 3-DMC yield (mg/L) and the tested variables (X_i) in the coded units through the following regression equation was confirmed:

$$\begin{aligned} \text{3-DMC}\,(\text{g/L}) = {} & 3.36 + 1.83 +^{*} \text{A} + \text{B} \\ & + 0.5^{*}\text{C} + 0.59^{*}\text{A}^{*}\text{B} \\ & + 0.26^{*}\text{A}^{*}\text{C} + 0.16^{*}\text{B}^{*}\text{C}. \end{aligned} \quad (1)$$

The contour graphs were plotted to determine the interaction of the variables and to determine the optimum level of each variable for maximum 3-DMC recovery (Figures 2 and 3). The model predicted maximum 3-DMC yield at dissolved oxygen 40%, substrate concentration (colchicine) 7.5 g/L, and process time 39 min.

Comparison of CSTR as well as PBR was performed by taking significant parameters for the study, namely, intial cell conc. (X_0 g/L), maximum cell conc. (X_m g/L), and Initial colchicine conc. (S_0 mM), productivity (g/L/h) and bioconversion time (h) (Table 4). Table 4 shows that maximum productivity was obtained in PBR system, that is, 6.58 g/L/h, in respect to earlier published reports on CSTR, that is, 4.7 g/L/h [13].

3.1.2. Reusability of Biocatalyst. On the other hand, a set of triplicate experiments were performed in which a proposed

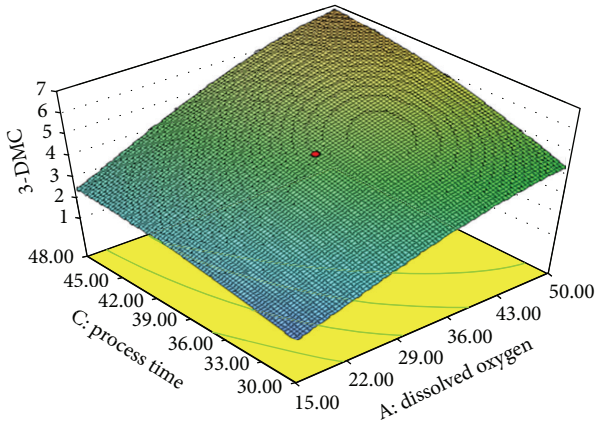

Figure 3: Response surface methodology showing bioconversion of 3DMC with response to dissolved oxygen (DO) and process time.

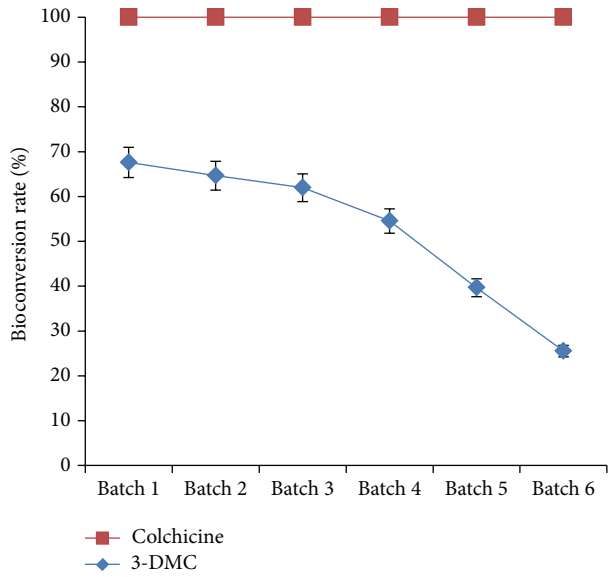

Figure 4: Showing batch-wise reuse of PBR system having constant flow of colchicine, that is, 35 mM converted into 3-DMC.

PBR system worked out for bioconversion efficiency for continuous six batch experiments in which 35 mM colchicine concentration used initially. Batch-wise experiments were performed (Figure 4) in which batch 1 shows ~67% colchicine converted to 3-DMC followed by ~60% as batch 3, and batch 4, the ~40% conversion rate was obtained, and very poor conversion was found at batch 6. Results showed loosing of biocatalytic/enzymatic activity after completion of five batch experiments in PBR.

4. Conclusion

The results here presented comprise a new approach to the bioconversion of colchicine into their respective derivative, that is, 3-demethylated colchicine (3-DMC). Furthermore, experimental design has provided an influential tool not only to study but also in optimization of bioconversion conditions

Table 4: Performance comparison of STR and PBR of lab scale for the production of 3-DMC at an equal DO level.

Parameters	CSTR	PBR
X_0 (g/L)	1.2	25
X_m (g/L)	180	25
S_0 (mM)	35	35
Productivity (g/L/h)	4.78 [13]	6.58
Bioconversion time (h)	72	32

(CSTR) continuous stirred tank reactor; (PBR) packed-bed reactor; X_0: initial cell concentration; X_m: maximum cell concentration; S_0: initial substrate (colchicine) concentration g/L.

that allows a significant enhancement of decisive feature of this process. Scanty reports are available on the PBR as an alternative form for CSTR mainly for the cultivation of the microorganisms which are sensitive to shear stress. In the present study, experimental design has been employed to evaluate the performance of continuous PBR system designed for the attainment of high bioconversion rate. Hence proposed continuous PBR reactor illustrates highly selective 3-DMC production achieved, which is considerably higher (Table 4) than the previous reports. The stability of operational and biocatalyst established the suitability and applicability of the proposed PBR. It was understood that the activity of enzyme was not altered significantly by operations as demonstrated by reuse of the same bed for five batches. Proposed system is presently being tested in the other stereospecific compounds with pharmacological interest.

Acknowledgment

Kashyap Kumar Dubey sincerely acknowledges UIET, M.D. University, for providing the facilities for research work.

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