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Construction of recombinant *Escherichia coli* for enhanced bioconversion of colchicine into 3-demethylated colchicine at 70 l bioreactor level

Kashyap Kumar Dubey[a], Shafiul Haque[b], Arshad Jawed[c], Bhanu P. Singh[d], B.K. Behera[e,*]

[a] University Institute of Engineering and Technology, M.D. University, Rohtak 124001, Haryana, India
[b] Gene Expression Laboratory, Department of Biotechnology, Jamia Millia Islamia (A Central University), New Delhi 110025, India
[c] Biochemical Engineering and Research Process Development Centre, Institute of Microbial Technology, Sec. 39-A, Chandigarh 160036, India
[d] Advanced Centre for Biotechnology, M.D. University, Rohtak 124001, Haryana, India
[e] Industrial Biotechnology Laboratory, Advanced Centre for Biotechnology, M.D. University, Rohtak 124001, Haryana, India

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ABSTRACT

A recombinant strain of *Escherichia coli* with CYP102A1 gene was developed for the demethylation of colchicine into their derivatives. The CYP102A1 gene responsible for demethylation was isolated from *Bacillus megaterium* ACBT03 and amplified using suitable primers. The amplified product was cloned into pET28a+ expression vector using host *E. coli* BL21(DE3) cells. The CYP3A4 (product of CYP102A1 gene) protein expression and other parameters like substrate toxicity, product toxicity and enzyme activity were optimized in shake flasks; and further scaled-up to 5 l bioreactor with 3 l working volume. In 5 l bioreactor, dissolved oxygen (DO) was optimized for maximum specific growth and enhanced 3-demethylated colchicine (3-DMC) production. The optimized conditions from shake flasks were scaled-up to 70 l bioreactor and resulted into ~80% conversion of 20 mM colchicine in 48 h with a volumetric productivity of 6.62 mg l^{-1} h^{-1}. Scale-up factors were measured as volumetric oxygen transfer coefficient (k_La) i.e., 56 h^{-1} and impeller tip velocity (V_{tip}) i.e., 7.065 m s^{-1}, respectively. The kinetic parameters K_m, k_{cat}, and k_{cat}/K_m of the CYP3A4 enzyme using colchicine as the substrate were determined to be 271 ± 30 μM, 8533 ± 25 min^{-1}, and 31.49 μM min^{-1}, respectively, when IPTG induced recombinant *E. coli* culture was used.

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1. Introduction

The alkaloid colchicine has an excellent anti-mitotic property and it is too toxic to be used as an anti-tumor drug in its native form. Various derivatives of colchicine, i.e., 3-demethylated colchicine (3-DMC), colchicoside, thiocolchicoside with improved therapeutic properties for anti-inflammatory and anti-tumor drugs have a good commercial demand as these compounds are used clinically for the treatment of certain forms of leukemia and solid tumors [1–3]. Due to limited availability of colchicine derivatives through colchicine producing plants, various efforts have been made to find the alternative routes for the production of 3-DMC and thiocolchicoside at industrial scale. The chemical conversion of colchicine into 2-, 3-demethyl colchicine is about 40–50% and not commercially viable. As an effective alternative to the chemical conversion process, biological methods involving CYP3A4 enzyme present in liver microsomes responsible for colchicine demethylation and belongs to cytochrome-450s family [4,5].

Still, very few reports are available on microbial demethylation of colchicine and its derivatives. Earlier reports have already proved that *Bacillus megaterium* is an effective microbe for regiospecific demethylation of colchicine at C-3 position in tropolone ring via CYP3A4 enzyme (product of CYP102A1 gene) [6,7]. This is the only bacterium having the CYP3A4 enzyme similar to the enzyme present in human liver and responsible for demethylation of colchicine [8,9]. The major disadvantage with this bacterium for the biotransformation of colchicine is its high PHB (poly-3-hydroxy butyrate) content [10–12], which carries serious problems during product extraction and purification. Poulev et al. [7] have had success with *Bacillus* IND-B375 strain in demethylation of colchicine, but due to poor yield the process was not encouraged for commercialization.

Cytochrome P450 monooxygenases (CYPs) are heme containing enzymes that catalyze various monooxygenation reactions in primary and secondary metabolism of microorganisms, animals and plants. CYPs are of great interest for the chemical and pharmaceutical industry, because of their high potential as catalysts for the selective introduction of molecular oxygen at even non-activated

* Corresponding author. Tel.: +91 1262 266665; fax: +91 1262 266665.
E-mail addresses: kashyapdubey@yahoo.com (K.K. Dubey), shafiul.haque@hotmail.com (S. Haque), arshadjawed29@gmail.com (A. Jawed), bhanuyadav7@rediff.com (B.P. Singh), bkbehera@ymail.com (B.K. Behera).

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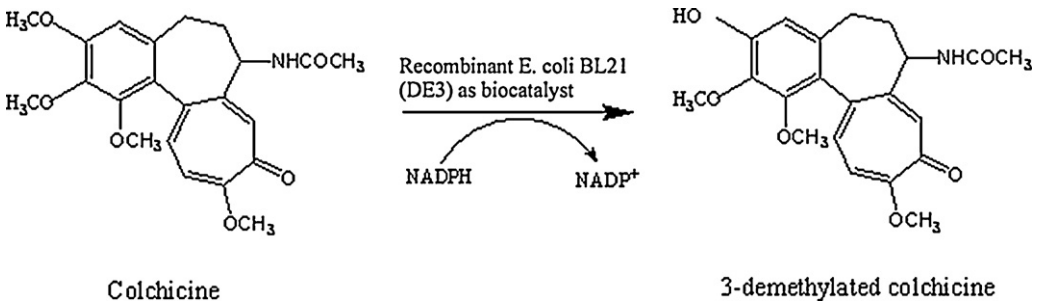

Fig. 1. Biotransformation of colchicine catalyzed by recombinant *E. coli* containing CYP102A1 gene.

C–H bonds in a regio-and/or stereospecific manner [13]. In order to increase the efficiency of *in vivo* system or to produce more P450 monooxygenase for *in vitro* catalysis, a more effective expression system has to be developed. The cytochrome P450 monooxygenase CYP102A1 from *B. megaterium*, also known also as P450 BM-3 or CYP3A4 is a 118 kD protein that catalyzes the subterminal hydroxylation (−1 to −3) of saturated and unsaturated fatty acids with a chain length of C12–C20 [14]. Many CYPs over-expression studies are still going on, but, the results achieved till now are having poor yield and might not be suitable for commercialization purpose. Keeping all the aforesaid facts in view, it can be assumed that the recombinant *Escherichia coli* system with CYP102A1 gene could be a better biocatalyst [15] for biotransformation of colchicine to obtain the respective regiospecific derivative (Fig. 1).

Previously we described the production of demethylated colchicine through microbial transformation and its scale-up process development with *B. megaterium* ACBT03 strain [6] and in the present paper, we reported the construction and application of a pET28a+ vector harbouring CYP102A1 gene with a strong T7 phage promoter in combination with *E. coli* BL21(DE3) for enhanced expression of CYP102A1 gene [16] and its large scale process development for the biotransformation of colchicine into their regiospecific derivatives. During the study, scale-up parameters like, volumetric oxygen transfer coefficient ($k_L a$) and impeller tip speed (V_{tip}) [6,17,18] have been estimated in a wide range of culture conditions, such as air flow rate, agitation and biomass concentration [19,20]. Our findings clearly showed that the production of colchicine derivatives via recombinant *E. coli* system can easily be scaled-up at commercial level.

2. Materials and methods

2.1. Bacterial strains and growth medium

Host *E. coli* DH5α cells (Invitrogen, USA) were used for plasmid multiplications. Expression vector pET28a+ (Novagen, USA) harbouring the CYP102A1 gene isolated from *B. megaterium* ACBT03 (available in author's lab) was transformed into host *E. coli* BL21(DE3) (Invitrogen, USA) for enhanced CYP3A4 enzyme production. Expression of CYP102A1 gene was under control of T7 strong promoter in the pET28a+ vector. Recombinant colonies were screened by adding kanamycin to the medium at a final concentration of 30 μg ml^{-1} (HiMedia, Mumbai, India). For DNA extraction, restriction digestion analysis and cloning experiments, standard methods were used as mentioned in Sambrook and Russell [21]. Different bacterial culture media and chemicals were purchased from HiMedia laboratories, Sigma and BDH chemicals. Restriction enzymes were obtained from New England BioLabs.

2.2. PCR amplification of CYP102A1 gene and its cloning into pET 28a+ expression vector

Oligonucleotide primers were designed by aligning the known reported sequences of CYP102A1 gene from NCBI GenBank database. The consensus sequences conserved in *B. megaterium* strain were taken for designing the gene specific primers for PCR amplification of the CYP102A1 gene from collected isolates. To facilitate the cloning of CYP102A1 gene into the bacterial expression vector pET28a+; desirable restriction sites i.e., *Bam*HI (5′ end) and *Eco*RI (3′ end) were introduced by the use of appropriately designed primers [forward primer 5″-CGGGATCC ATG ACA ATT AAA GAA ATG CCT C-3″; reverse primer 5″-GGAATTC TTA CCC AGC CCA CAC GTC-3″; underline bases shows the restriction sites for above mentioned restriction enzymes] from Microsynth, India. Plasmid mini preps were performed for the collected strains (QIAquick mini prep kit, Qiagen) and isolated plasmids were utilized as a template DNA for the amplification of CYP102A1 gene. Amplification conditions for CYP102A1 gene were, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min for 30 cycles. Initial denaturation and final extension conditions were 94 °C for 1 min and 72 °C for 10 min, respectively. Standard strain *B. megaterium* (MTCC*420) was used as a positive control for the CYP102A1 gene amplification reaction. PCR products of putative CYP102A1 (4957 bp) were gel purified (Gel extraction kit, Genei, Bangalore, India) and cloned into pET28a+ vector following the manufacturer's protocol. Recombinant vector pET28a+ harbouring CYP102A1 gene was further transformed into competent host *E. coli* BL21(DE3) cells. Transformants were selected primarily on antibiotic amended Luria broth (LB) agar plates and finally screened by CYP102A1 PCR amplification and restriction endonuclease digestion with *Bam*HI & *Eco*RI restriction enzymes.

2.3. Over-expression of CYP3A4 enzyme

Bacterial cultures [*E. coli* BL21(DE3) cells with pET28a+ vector harbouring CYP102A1 gene] of LB medium containing 30 μg ml^{-1} kanamycin were incubated at 37 °C for overnight growth. Fresh medium (5 ml) was inoculated with 200 μl of overnight culture and grown for 1.5 h at 37 °C. The temperature was then reduced to 25 °C and 0.5 mM IPTG (isopropyl-β-D-thiogalactoside) was added to the grown culture. Shaking was continued for 4 h; afterwards, cells were pellet down by centrifugation and resuspended into 1 ml of 100 mM Tris–buffer (pH 8.0) containing 1 mg ml^{-1} lysozyme and 0.2% tween 80. The resuspended cells were frozen overnight at −20 °C and thawed at room temperature. Cell lysate was clarified by centrifugation at 11,000 × *g* for 10 min at 4 °C and protein concentration of the clarified cell lysate was determined by standard method [22], using BSA as a standard. Protein expression was analyzed by SDS-PAGE technique, using lysate samples normalized to 10 μg of protein.

2.4. Conversion of colchicine into 3-DMC

2.4.1. At shake flask level

The recombinant *E. coli* BL21(DE3) cells were subjected to submerged culture at shake flask level for biotransformation of colchicine into 3-DMC. The (Terrific broth) TB medium, containing 12 g l^{-1} tryptone, 24 g l^{-1} yeast extract, 4 ml l^{-1} glycerol, 2.3 g l^{-1} KH_2PO_4 and 12.2 g l^{-1} K_2HPO_4 was used for the growth of bacterium [23]; pH and temperature conditions were maintained to 7 and 37 °C, respectively. Filter sterilized kanamycin (30 mg l^{-1}) and colchicine (20 mM) were fed to the medium before inoculation. The flasks were inoculated with 10 ml of overnight culture and grown at 37 °C for 3-4 h; and 0.5 mM IPTG was added for induction purpose. The pH was maintained to 7.0 using 50% NH_4OH. Samples were collected at regular intervals from the inoculation time and analyzed by high-performance liquid chromatography technique (HPLC) [24].

2.4.2. At 5 l and 70 l bioreactor

Lab-scale level experiments were performed into 5 l bioreactor and pilot-scale microbial transformations were carried out in a 70 l (Applikon, Netherlands) bioreactor, using 3 l and 50 l working volume, respectively. Six-bladed turbine type impellers having 50 mm and 115 mm diameters were used in 5 l and 70 l bioreactor, respectively. The media components utilized were 10 g l^{-1} glucose, 2 ml l^{-1} glycerol, 15 g l^{-1} yeast extract, 5 g l^{-1} tryptone, 1.0 g l^{-1} KH_2PO_4, 5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} NH_4Cl, 2.5 g l^{-1} $Na_2HPO_4{\cdot}2H_2O$, 0.5 g l^{-1} $MgSO_4$, 30 mg l^{-1} kanamycin and 20 mM colchicine, for both, 5 l and 70 l bioreactors. Overnight grown recombinant *E. coli* culture was used as an inoculum to start the bioconversion. The bioreactor conditions were set to 400 rpm, 40% DO, 37 °C temperature and pH was maintained above 6.0. After 3 h of run, the temperature was reduced to 27.5 °C and 0.5 mM IPTG was added. Samples were collected at regular time intervals and analyzed by HPLC [24].

2.5. Fed-batch culture study

In a 5 l bioreactor, the working volume of fed-batch culture was increased from 3 to 3.5 l by feeding colchicine solution after 48 h. Colchicine was fed thrice at different time intervals, i.e., 10 h, 20 h and 36 h, respectively, using a peristaltic pump. The total colchicine added was 21 g in a fed-batch culture. The colchicine feed rate was chosen in such a way that it would not allowed to accumulate upto toxic levels which was based on a previously measured substrate and product toxicities (colchicine 7 g l^{-1} and 3-DMC ~5.5 g l^{-1}).

2.6. Determination of volumetric mass transfer coefficient (k_La) and impeller tip velocity (V_{tip})

The k_La value was determined according to the sulfite method as described by Maier and Buchs [25]. Impeller tip velocity was measured by formula of πND_i (where, N = no. of rpm; D_i = impeller diameter) as described in the earlier reports [17,26,27]. The DO concentration was kept constant (40–50% air saturation) by controlling the impeller speed and back pressure of the bioreactor. The DO concentration was measured by a sterilized galvanic electrode (Mettler-Toledo InPro 6000 series) and oxygen uptake rate (OUR) was determined via gas balance method.

2.7. CYP3A4 enzyme assay

Bacterial cells were harvested by centrifugation (10,000 × g at 4 °C for 10 min), followed by washing step with 100 mM potassium phosphate buffer (pH 6.5). Cells were disrupted by sonication and extracts were centrifuged at 20,000 × g for 1 h at 4 °C. The enzymatic activity of CYP3A4 was determined as described by Schwaneberg et al. [28].

2.8. Standardization of optimal expression, affinity purification and SDS-PAGE analysis

Transformed *E. coli* BL21(DE3) cells with ligated vector were grown in Luria broth (LB) and induced with 0.5 mM IPTG. Pellet was collected at different time intervals and checked for protein expression on 12% SDS-PAGE and stained with Coomassie Brilliant Blue-R250. Different IPTG concentrations (0.5–1.5 mM) and different temperature combinations (28–37 °C) were used to standardize optimal expression. Fusion protein was purified under denaturating condition with N-terminally attached His_6-tag which allowed purification by immobilized metal chelate affinity chromatography (IMAC), utilizing a Ni-NTA-resin (Qiagen, Germany). Fractions of 0.5 mM IPTG induced culture was harvested at different time intervals and loaded on Ni-NTA column; and desired protein was eluted with buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing different concentrations of imidazole (60, 80, 100, 150, 200 and 250 mM). Desired elutants along with crude extract were checked on 12% SDS-PAGE as described by Laemmli [29] and fractions containing the desirable protein were subjected to dialysis. SDS-PAGE experiments were performed on Mini Protean3 assembly (Bio-Rad), and molecular weight markers were purchased from GmBH, Unigenetics Inc. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

3. Results

3.1. Effect of substrate and product toxicity on recombinant E. coli at shake flask level

The recombinant system was successfully constructed (as discussed in Section 2) and further exploited for biotransformation and scale-up studies. The effect of colchicine, thiocolchicine, 3-DMC, and colchicoside toxicities was evaluated by measuring the growth of recombinant *E. coli* in 250 ml shake flask. Fig. 2 clearly showed the effect of varying substrate (colchicine/thiocolchicine) concentrations ranging from 5 mM to 35 mM on the bacterial growth rate. It is evident from Fig. 2 that both the substrates were inhibiting the bacterial growth at 15 mM concentration, and significantly less growth was observed at 30 mM and 35 mM concentrations, respectively. Fig. 3 shows the product toxicity on recombinant *E. coli* cells, and during the experiment it was observed that at 10–15 mM concentrations 3-DMC and colchicoside inhibits

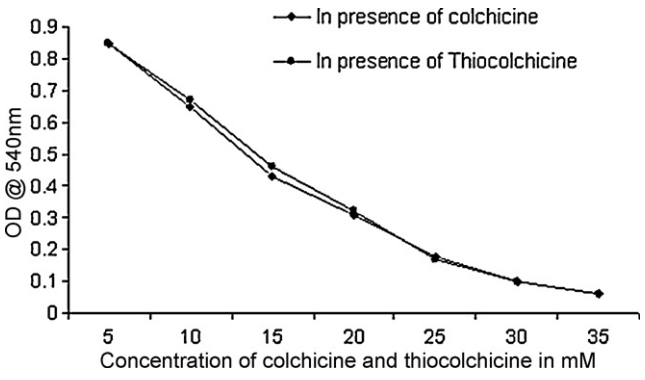

Fig. 2. Effect of different concentrations of substrate (colchicine and thiocolchicine) toxicity on growth rate of recombinant *E. coli*.

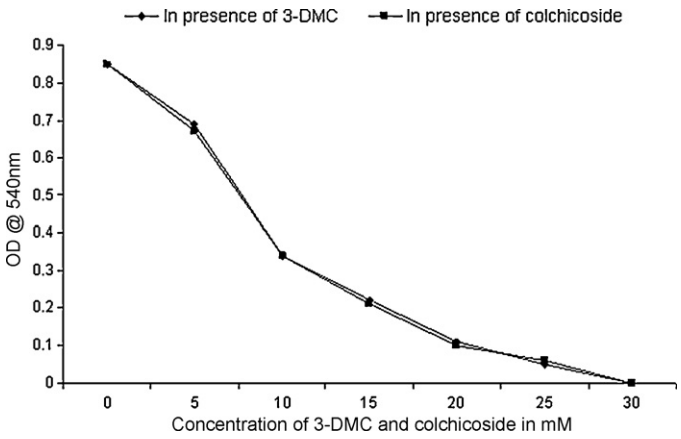

Fig. 3. Effect of different concentrations of product (3-DMC and colchicoside) toxicity on growth rate of recombinant *E. coli*.

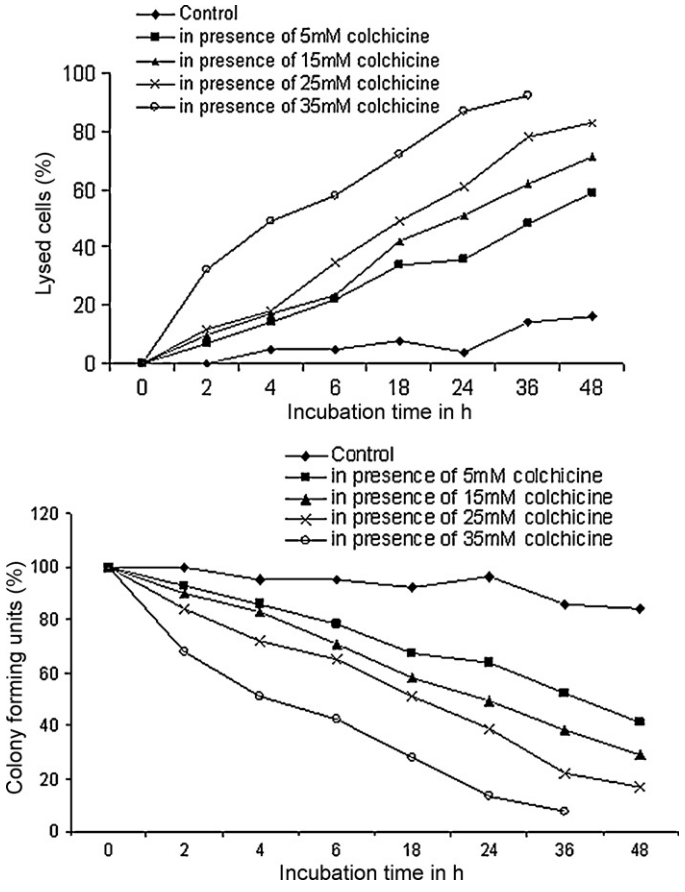

Fig. 4. Effect of different concentrations of colchicine on colony forming units (CFUs) and on recombinant *E. coli* cell lysis.

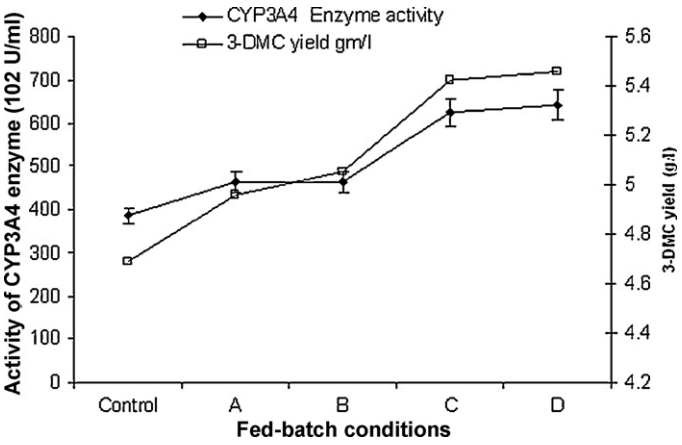

Fig. 5. Effect of fed-batch culture on CYP3A4 enzyme activity in shake flask (Control: *Bacillus megaterium* MTCC*420 grown in presence of 7 g l^{-1} colchicine; A: recombinant *E. coli* culture grown in presence of 7 g l^{-1} colchicine; B: recombinant *E. coli* culture grown in presence of 7 g l^{-1} thiocolchicine; C: recombinant *E. coli* culture grown in presence of 7 g l^{-1} colchicine +5 ml l^{-1} glycerol; D: recombinant *E. coli* culture grown in presence of 7 g l^{-1} thiocolchicine +5 ml l^{-1} glycerol).

the bacterial growth. It was also observed that during biocatalysis process the product transformed by recombinant *E. coli* was less toxic in comparison to the substrate added. Comparison of bacterial cell lysis and colony forming units made during incubation was also performed. The obtained results clearly indicate that more than 80% cells were lysed, and about 20% colonies were formed after the addition of 35 mM colchicine (Fig. 4). In addition to above, ~90% cells were lysed after 36 h incubation time at 35 mM colchicine. Similar results were obtained when thiocolchicine was used for cell wall permeability study (data not shown).

3.2. Effect of fed-batch culture on bioconversion efficiency

In order to check the activity of CYP3A4 enzyme in *B. megaterium* MTCC*420 strain and in recombinant *E. coli* system; 7 g l^{-1} colchicine and 7 g l^{-1} thiocolchicine were added in the culture (Fig. 5). Recombinant *E. coli* showed maximum CYP3A4 enzyme activity [650 (10^2 U/ml)] when glycerol was fed two times (after 24 h and 36 h) after incubation, at the rate of 1 ml l^{-1} and 0.5 ml l^{-1}, respectively. It was also observed that due to enhanced biotransformation activity of over-expressed CYP3A4 enzyme, the biocatalytic efficiency of the cells sampled from the bioreactor at different time intervals were increased 3–4-folds. High toxicity of colchicine resulted into recombinant *E. coli* cell lysis (inhibited CFU's); already shown in Section 3.2. Table 1 clearly shows that when 7 g l^{-1} colchicine was fed into batch mode then yield of 3-DMC was ~5.45 g l^{-1} and about 77% bioconversion was achieved. When colchicine was added in a repetitive fed-batch mode then ~5.96 g l^{-1} 3-DMC yield and about 85% bioconversion of colchicine was achieved. The 3-DMC yield was ~8% higher in fed-batch culture in comparison with batch culture mode.

Table 1
A comparative study of bioconversion efficiency of recombinant *E. coli* in batch and fed-batch culture (5 l bioreactor) mode for 3-DMC production.

Parameters	Mode of culture	
	Batch[a]	Fed-batch[b]
Dry cell weight (g l^{-1})	15.50 ± 1.24	17.25 ± 1.24
3-DMC yield (g l^{-1})	5.45 ± 0.15	5.96 ± 0.11
Bioconversion (%)	77	85

[a] Batch conditions: initial colchicine concentration, 7 g l^{-1}, dissolved oxygen 40%, agitation 450 rpm.
[b] Fed-batch conditions: feeding time of colchicine (10 h, 20 h and 36 h after inoculation), other conditions are similar as batch culture.

3.3. Kinetic parameters studied for CYP3A4 enzyme

Kinetic parameters (K_m and k_{cat}) of the purified CYP3A4 enzyme were studied for *B. megaterium* MTCC*420, *B. megaterium* ACBT03, IPTG induced recombinant *E. coli* and uninduced recombinant *E. coli* (Table 2) systems. IPTG induced recombinant *E. coli* showed the maximum turnover number (k_{cat}/K_m 31.49 μM min^{-1}), in comparison with *B. megaterium* MTCC*420, *B. megaterium* ACBT03 strain and uninduced recombinant *E. coli*. Maximum conversion of colchicine into 3-DMC was also achieved (~6.6 g l^{-1}) in induced culture of recombinant *E. coli*.

3.4. Optimization of dissolved oxygen (DO) level

Batch cultivation was performed in 5 l bioreactor to optimize the DO for maximum conversion of colchicine into their respective derivatives. In addition to that, we have also evaluated the effect of DO on various parameters of recombinant *E. coli* culture, i.e., specific growth rate (μ_{max}), biomass substrate yield ($Y_{X/S}$) and product substrate yield ($Y_{P/S}$). Initially, the DO level was not controlled, but after 6 h incubation of the culture, the DO level was limited to 30%, 40%, 50%, 60% and 80%, by adjusting the agitation speed and back pressure (Table 3). The μ_{max}, $Y_{X/S}$, $Y_{P/S}$ and 3-DMC yield, all were recorded maximum at 40% DO level, i.e., 0.432 ± 0.07 h^{-1}, 2.57 ± 0.05 g g^{-1}, 0.77 ± 0.03 g g^{-1} and 5.5 g l^{-1}, respectively. So, for later experiments we had chosen 40% dissolved oxygen; and our experimental data showed that the DO plays a significant role in the bioconversion of colchicine into their respective derivatives.

3.5. Effect of colchicine addition on high density culture

In order to improve the volumetric productivity of the 3-DMC, the reaction was initiated at high cell density culture of 6 g l^{-1} (DCW) in a 5 l bioreactor. To meet the increased carbon and energy demand during fed-batch culture, the glucose feed rate was increased stepwise from 0.2–0.4 g l^{-1} h^{-1}. The cell density was increased linearly after initiation of biotransformation and finally reached at 16 g l^{-1} (DCW). The recombinant plasmid was present in ~95 ± 2% of the cells throughout the process as shown by the comparison of number of CFU's (colony forming unit) on LB agar media/kanamycin amended LB agar media; this clearly indicates that most of the high density cultures were showing higher transformation rate of colchicine in comparison to the low density culture.

3.6. SDS-PAGE analysis

Total cell protein from wild type *B. megaterium* ACBT03 along with His_6-tag purified uninduced recombinant *E. coli* BL21(DE3) and IPTG induced recombinant *E. coli* BL21(DE3) protein fractions were used for SDS-PAGE analysis in order to check the over-expression of the desirable protein. The molecular weight of CYP3A4 enzyme, i.e., 119 kD was well recorded in literature [23,30] and Fig. 6 shows the location of 119 kD band present in lanes 2, 3, 4 and 5, respectively. Protein expressed by induced and uninduced recombinant *E. coli* strains clearly indicates that the CYP3A4 enzyme expressed by IPTG induced *E. coli* was quite higher (lanes 4–5) in comparison to uninduced *E. coli* (lane 3).

Table 2
Kinetic parameters studied for demethylation of colchicine catalyzed by wild type *Bacillus megaterium* and recombinant *E. coli* (7 g l^{-1} colchicine used as substrate) at shake flask level.

Biocatalyst	K_m (μm)	k_{cat} (min^{-1})	k_{cat}/K_m (μm min^{-1})	Conversion (%)
B. megaterium MTCC*420	253 ± 20	6120 ± 65	24.18	43
B. megaterium ACBT03	234 ± 21	4956 ± 32	21.17	55
Recombinant *E. coli*	255 ± 19	5635 ± 18	22.1	74
Induced recombinant *E. coli*	271 ± 30	8533 ± 25	31.49	80

Table 3
Batch culture of recombinant *E. coli* performed at various aeration rates (DO) in 5 l bioreactor (five run sets at each aeration level); 7 g l^{-1} colchicine was added in the bioreactor (culture conditions have been already mentioned in Section 2).

Dissolved oxygen (% saturation)	30	40	50	60	80
μ_{max} (h^{-1})	0.342 ± 0.03	0.432 ± 0.07	0.412 ± 0.06	0.382 ± 0.04	0.321 ± 0.05
$Y_{X/S}$ (g_{DCW} $g_{colchicine}^{-1}$)	2.28 ± 0.07	2.57 ± 0.05	2.21 ± 0.11	1.85 ± 0.06	1.71 ± 0.05
$Y_{P/S}$ ($g_{3\text{-DMC}}$ $g_{colchicine}^{-1}$)	0.70 ± 0.06	0.77 ± 0.03	0.61 ± 0.04	0.50 ± 0.03	0.42 ± 0.06
3-DMC (g l^{-1})	4.90	5.5	4.3	3.5	3.0

Specific growth rate (μ_{max}) calculated from optical density (OD) data, after a calibration curve OD vs. dry cell weight; biomass substrate yield ($Y_{X/S}$); product substrate yield ($Y_{P/S}$); above data showed (average ± SD of five experiments); dissolved oxygen (DO) maintained constantly during process.

3.7. Optimization of process conditions for biotransformation of colchicine by recombinant E. coli BL21(DE3)

3.7.1. At shake flask (250 ml) level

Microbial biotransformation through recombinant *E. coli* BL21(DE3) was tested at shake flask level in batch culture mode and 20 mM colchicine was added after 6 h incubation; and 3-DMC production assay was carried out after 48 h of incubation with every 4 h interval time. Maximum 3-DMC production rate was observed at exponential phase (36 h) of bacterial growth (Fig. 7). The recombinant *E. coli* BL21(DE3) was capable for production of ~4235 mg l^{-1} of 3-DMC. At that stage, the specific 3-DMC content and 3-DMC productivity were 273.22 mg g^{-1} DCW and 5.67 mg l^{-1} h^{-1} measured, respectively (Table 4).

3.7.2. At 5 l bioreactor level

Table 5 shows the operation conditions for the production of 3-DMC in a 5 l bioreactor using recombinant *E. coli* as a biocatalyst. About 77% biotransformation of colchicine was achieved when 20 mM colchicine (6 h incubation) was added to the reaction broth. In order to enhance the bioconversion process, rotation speed (rpm), impeller tip velocity, and volumetric oxygen transfer coefficient (k_La) were optimized at 450 rpm, 7.065 m s^{-1} and 56 h^{-1}, respectively. The recombinant *E. coli* had been capable of producing about 5450 ± 7.53 mg l^{-1} of 3-DMC. At that stage the specific 3-DMC content and 3-DMC productivity were measured 294.5 mg g^{-1} DCW and 6.13 mg l^{-1} h^{-1}, respectively (Table 4). The carbon dioxide evolution rate (CER) was also noticed to be maximum (Fig. 7) at 36 h of incubation.

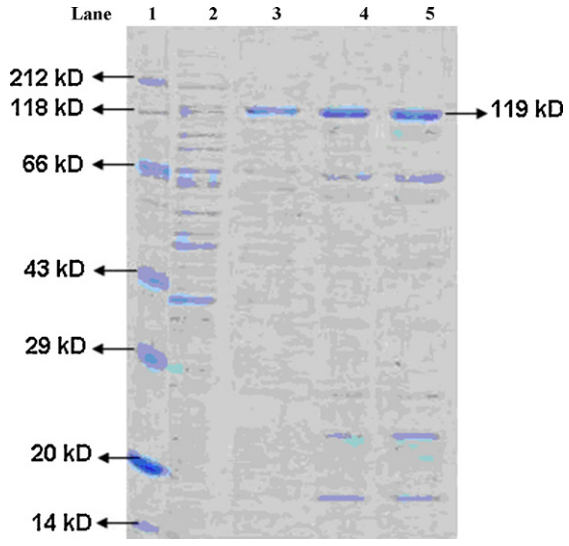

Fig. 6. SDS-PAGE analysis showing over-expression of CYP3A4 protein (119 kD) from recombinant *E. coli* system over wild type *B. megaterium*. Lane: 1 (protein molecular weight marker); lane 2 (protein extracted from wild type culture of *B. megaterium*ACBT03); lane 3 (IMAC purified protein extracted from recombinant *E. coli* BL21(DE3) cells); lanes: 4 and 5 (IMAC purified protein extracted from recombinant *E. coli* BL21(DE3) cells after induction with 0.5 mM IPTG).

3.7.3. At 70 l bioreactor level

The scale-up parameters for 3-DMC production, i.e., impeller tip velocity (V_{tip}) and volumetric oxygen transfer coefficient (k_La) [31,32] were studied. It was found that at about agitation of 450 rpm (equivalent to 7.065 m s^{-1} impeller tip velocity) and k_La value of 56 h^{-1} at 40% DO level (Table 4), the 3-DMC yield was maximum (~6300 mg l^{-1}) after 48 h of incubation. These results contradicted with the earlier reports, where, vigorous agitation about 900–1000 rpm and low rate of aeration resulted into relatively high rate of demethylation [33]. The obtained results in a 5 l bioreactor were utilized to optimize the operation parameters for the bacterial growth and colchicine bioconversion in a 70 l bioreactor. Twenty test trials were taken for the stability of recombinant system in 5 l and five trials were taken at 70 l bioreactor for scale-up process. The obtained results for 3-DMC bioconver-

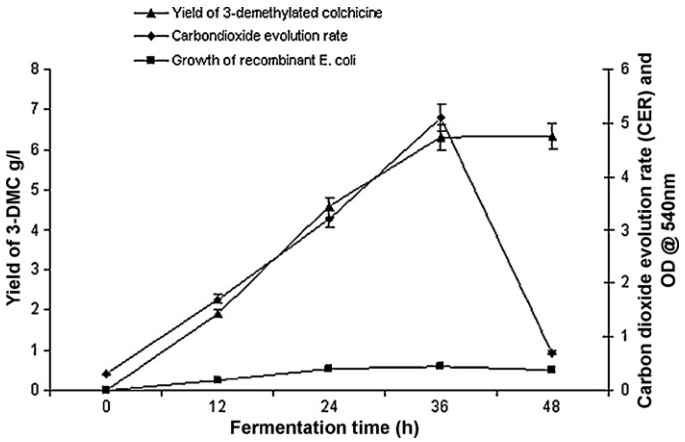

Fig. 7. Relationship between carbondioxide evolution rate (CER), *E. coli* growth rate and bioconversion yield of colchicine into their derivatives at 70 l bioreactor.

Table 4
Production of 3-demethylated colchicine (3-DMC) by recombinant *E. coli* under optimum conditions.

Nature of culture conditions	Total 3-demethylated colchicine (mg l^{-1})	Specific 3-demethylated colchicine yield (mg g^{-1} DCW)	3-Demethylated colchicine productivity (mg l^{-1} h^{-1})
Shake flask level	4235 ± 5.21	273.22	5.67
5 l bioreactor level	5450 ± 7.53	294.5	6.13
70 l bioreactor level	6300 ± 16.39	318.2	6.62

The pH (7.0) and temperature (37 °C) were maintained for all the cultures. The impeller tip velocity (7.065 m s^{-1}) and DO (40%) were maintained constant for 5 l and 70 l bioreactor.

Table 5
Yield of 3-demethylated colchicine (3-DMC) in fed-batch culture of recombinant *E. coli*.

Rotational speed (rpm) at 5-l-jar fermenter	Impeller tip velocity m s^{-1} for 5 l and 70 l fermenter	Rotational speed (rpm) at 70 l fermenter		k_La (h^{-1}) for 5 l and 70 l fermenter	Yield of 3-demethylated colchicine mg l^{-1}
		Calculated	Observed		
150	2.355	65	70 ± 2	25	3347 ± 18.27
300	4.710	130	130 ± 5	45	5731 ± 12.65
450	7.065	195	200 ± 2	56	6300 ± 13.52
600	9.420	260	250 ± 2	78	3532 ± 17.29
750	11.775	326	320 ± 2	97	1732 ± 8.85

Scale-up parameters, i.e., impeller tip velocity and volumetric oxygen transfer coefficient (k_La) both were kept constant for 5 l and 70 l bioreactor.

sion at lab scale as well as on pilot scale were having variation of 8–10%.

4. Discussion

Previous researchers have already proved the potential of recombinant *E. coli* BL21(DE3) cells, when it was used as biocatalyst [20,34–36]. High cell density, good expression and easy purification methods are the main reasons for the utilization of *E. coli* cells as an expression host organism [19,23,37]. In the present work we have developed a recombinant *E. coli* system for colchicine derivatives (3-DMC and colchicoside) production, which have a great economic value and high market demand. Other wild/mutant type systems also involved in demethylation mechanism, like, *B. megaterium* can also be exploited by making this strain more promising for the biocatalysis of a variety of pharmaceutically important agents [7,23,38], but still no significant progress has been achieved to commercialize the process.

We investigated the effect of substrate and product toxicity on the developed recombinant *E. coli* system and found that both were highly toxic for bacteria at the concentrations of 30 mM and 15 mM, respectively (Figs. 2 and 3). Above results also show the effect of high concentration of colchicine on cell viability; and it was found that, on increasing the colchicine concentration from 15 to 20 mM >80% cells were lysed (Fig. 4). The possible reasons for decreasing bacterial growth rate include cell wall degeneration; an electron microscopic study also showed that cell wall loss occurred due to increased concentration of colchicine and thiocolchicine [39]. The enzyme deactivation, cofactor loss over permeable membrane and regulatory phenomenon might have been responsible for decrease in conversion rate. In other words, the demethylation of colchicine during this study may be described cofactor regeneration rates caused by increasing concentration of toxic substrates (colchicine and thiocolchicine) with its adverse effects on cell membrane, metabolism and viability. Earlier findings proved that, for the activation of CYP3A4 system, NADPH is an essential component, whenever NADPH is depleted, it might limit the biocatalysis process [16,40]. It is also crucial to ensure an adequate oxygen supply to the media. For *E. coli*, as a most popular expression host, it is well known that oxygen availability badly affects cellular yield [41]. DO is one of the key factors which dramatically affects the cell growth and recombinant protein production through recombinant *E. coli* [42,43] system.

At 70 l bioreactor level, bacterial cultivation showed significant impact of aeration, k_La and impeller tip velocity, which endorsed the study performed by earlier workers [44]. An increased oxygen intake could be responsible for better expression of the CYP3A4 gene [19,23,45], and as a result the production of 3-DMC was significantly high. At 5 l and 70 l bioreactor level, an agitation of 450 rpm has been proved to be critical for optimum results. Besides that, it was also observed that the angle of aeration plays an important factor for sufficient availability of oxygen to bacterial cells [45]. It was observed that the recombinant protein production in a bioreactor was significantly higher as compared to the shake flask level; that can be possible only because of the linear motion of the culture broth resulting into homogenous distribution of air, nutrient condition and pH maintenance. During scale-up process, it is very critical for careful selection of an array of appropriate biochemical characterization methods to assess product comparability at each stage of process scale-up. As shown in the results section, the quantitative comparison of the bioreactor performance supports the compatibility of the process maintained during scale-up [46]. The conversion efficiency of colchicine through *B. megaterium* ACBT03 based on whole cell biocatalysis was significantly increased in case of recombinant *E. coli* system. The outcomes of our kinetic parameter study for CYP3A4 enzyme activity, clearly showed that the conversion of colchicine into their derivatives (3-DMC), had been noticed maximum in case of recombinant *E. coli*, and k_{cat}/K_m value is quite high in this case as compared to other cultures of *B. megaterium* ACBT03 and *B. megaterium* MTCC*420 (Table 2). In addition to that, it was also noticed that until the recombinant *E. coli* was induced with IPTG, the biotransformation efficiency of the recombinant *E. coli* was quite similar to the *B. megaterium* ACBT03 soil isolate. In addition to that the productivity was limited by substrate and product toxicity, and catabolite repression. This is the first report demonstrating the significant improvement (~2 times in comparison to earlier reports) [6,7] of the biotransformation of colchicine through heterologous protein expression by recombinant *E. coli* system at 70 l bioreactor level which has a potential for the commercialization of the process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2010.03.014.

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