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Production of demethylated colchicine through microbial transformation and scale-up process development

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Abstract

Mutant strain of *Bacillus megaterium* ACBT03 is capable of demethylation at third carbon position of colchicine and their derivatives. A wild strain of *B. megaterium* ACBT03 collected from field was grown at shake flask level and the most suitable sources of carbon and nitrogen were studied in order to increase the biotransformation process. Glucose in combination with glycerol having 2:1 ratio, and yeast extract plus peptone 1:3 ratio, at pH 7.0 and 28 °C incubation temperature were noticed to be the most suitable conditions for maximum biotransformation. The potential of demethylation of the wild strain was noticed to be very poor (20–25% of substrate supplied) when 0.1 g/l colchicine or thiocolchicine was used. In order to increase the potential for demethylation, *B. megaterium* ACBT03 was subjected to enrichment culture with higher concentration of colchicine. The bacterial cells were grown for 8–10 generations, in higher concentration of colchicine. The newly mutant developed through colchicine enrichment culture was named as *B. megaterium* ACBT03-M3. About 55% of colchicine and 60% of thiocolchicine were converted to their respective demethylation form, when this mutant was grown in 7 g/l colchicine and thiocolchicine, both at laboratory-scale (5-l-jar fermenter) and pilot-scale level (70-l fermenter). Under optimum culture conditions the key monitoring factors to scale-up the process of demethylation were dissolved oxygen (DO) level (2.5 vvm) of culture broth and impeller tip velocity (4710 cm/min).

Keywords: Microbial transformation; Scale-up; Colchicine; Thiocolchicine; Fermenter; 3-Demethylated colchicine; 3-Demethylated thiocolchicine; *Bacillus* spp

1. Introduction

Colchicine is a major alkaloid obtained from *Colchicum autumnale* and *Gloriosa superba* L., has an anti-mitotic property. It is too toxic to be of value as an anti-tumor drug, in its native form. Derivatives of colchicine, i.e., 3-demethyl colchicine, colchicoside, thiocolchicocide with improved therapeutic properties for anti-inflammatory and anti-tumor drugs [1–3] will have good commercial demand as these compounds were used clinically for the treatment of certain forms of leukemia and solid tumors [4]. Demethylated colchicine at C-3 position of the ring-A showed about 35-fold less toxicity as compared to parent molecule and equal to anti-tumor activity to that of thiocolchicine [5]. 3-Demethyl colchicine and its glucosides, however, are only minor constituents in the colchicine producing plants [4].

In order to commercialize these compounds, efforts have been made to find alternative routes for production of 3-demethylated colchicine and thiocolchicine. It has been noticed that chemical demethylation not only results for demethylation of the methoxy group at C-3 of the aromatic ring of colchicine but also leads to formation of a mixture of 1-, 2-, 3- and 10-mono-, di-, and tri-, demethyl derivatives. The chemical conversion of colchicine into 2-, 3-, demethyl colchicine is about 40–50% [6] and not viable for commercial production.

As an alternative to the chemical conversion process, biological methods involving CYP3A4 enzyme present in liver microsomes responsible for colchicine demethylation [7]. Besides this, it has also been noticed that few plant cell lines were able to produce demethylated colchicine, but in a very low quantity [8,4]. Plenty of reports were available on microbial demethylation process of both natural and synthetic [4,9,10] compounds.

However, scanty reports are available on microbial demethylation of colchicine and its derivatives. Poulev et al. [4] observed that a strain *Bacillus* IND-B375 demethylate colchicine (60%) and thiocolchicine (97%) when the substrate

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was added at a concentration of 0.1 mg/ml. Due to its poor yield, the process was not encouraged for commercialization. After a long gap, an US patent no. 6372458 (2000) claimed process for biotransformation of colchicone to 3-glycosyl derivatives at the rate of 70%, when 1 mg/ml thiocolchicone was added to broth containing *Bacillus megaterium*. Still, the above achieved results were having poor yield and might not be suitable for commercialization.

In this paper, we described a novel mutant of *B. megaterium* ACBT03, which was isolated in its wild-form from soil sample of an industry area, where *G. superba* L. is processed for colchicine production. This newly isolated mutant has potential for demethylation of colchicine and its derivatives. The *B. megaterium* is posses CYP3A4 enzyme responsible [11–13] for demethylation process at specific site [14–18]. This demethylation process was followed by glycosylation, due to the glycosyl transferase activity expressed by *B. megaterium* [19].

An attempt was made to produce 3-demethylated colchicine (3-DMC) and 3-demethylated thiocolchicine (3-DMTC) by microbial transformation process. Beside this, we have taken attempt to produce 3-DMC and 3-DMTC at scale-up level.

2. Materials and methods

2.1. Materials

The culture medium ingredients, i.e., carbon sources (glucose, glycerol), nitrogen sources (yeast extract, peptone) and salts (potassium mono-hydrogen phosphate, potassium di-hydrogen phosphate, ammonium chloride, magnesium sulphate, sodium chloride) used were obtained from Hi-Media (Mumbai, India). Solvents and reagents used for microbial culture, extraction, separation, product isolation, identification and purification were obtained from Qualigens (Mumbai, India). Colchicine and demethylated colchicine were purchased from Sigma and thiocolchicine was gifted by Sai Phytoceuticals, New Delhi, India.

2.2. Methods

2.2.1. Microbes isolation, culture and preservation

The microbes were isolated from the site located around Alchem International Pvt. Ltd., New Delhi who produces colchicine from *G. superba* L., at commercial level. Soil microbes were isolated and identified by serially diluted method as practiced in author's laboratory. The isolates were sub-cultured several times, in order to obtain pure colonies. The colonies were tested for Gram's test. The Gram-positive bacteria having rod-shape was tested for demethylation of colchicine by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) [20–23]. The isolated pure strain having property of demethylation was maintained in Petri dishes and repeatedly used for bioconversion experiments for further conformation. For long-term preservation, 30% glycerol was added to each microbial suspension prior to freeze sample in 2 ml cryo-vials at −196 °C.

2.2.2. Analysis of cell mass

The cell mass was determined by using a calibration curve relating optical density at 600 nm and dry cell weight (DCW). The optical density at 600 nm was measure with a spectrophotometer (Hitachi U-2000). The DCW was determined after the culture broth was filtrate through a 0.2 μm Millipore filter paper and dried at 121 °C overnight to a constant weight.

2.2.3. Culture conditions at flask level

The aliquot of culture of *B. megaterium* ACBT03 in cryo-tubes after thawing were used to inoculate 500 ml baffle type shake flask containing 150 ml of medium. After inoculation, flasks were incubated over night at 28 °C, 300 rpm. Five milliliters of pre-culture was transferred into 50 ml of the same medium to which colchicine and thiocolchicine were added to obtain 3, 5, 7 and 10 g/l final concentration. The culture were incubated for 3 days in the same conditions as described above, and every 6 h, samples were taken to evaluate the growth level (measured absorbance at 600 nm), the 3-DMC and 3-DMTC production by TLC and HPLC, the sterility on LB agar, and for the microscopic examination.

As colchicine is toxic to the microorganism, at the initial stage of adaptation, 0.1 g/l colchicine was used as selective force. Aqueous solution of colchicine was used to discharge the substrate in the culture broth, after 24 h of inoculation. As thiocolchicine is less soluble in aqueous medium, 70% ethyl alcohol was used to prepare thiocolchicine solution and mixed in culture broth on the basis of experiment designed and requirement. Under such conditions the microbes were grown for more than 10 generations till the specific growth rate was obtained same as in control. At the end of each generation the microbes were examined for its ability for biotransformation of colchicine and thiocolchicine to their respective 3-demethyl and glycosyl derivatives using thin-layer chromatography, and high pressure liquid chromatography [20–23]. The above practice was continued with gradually increasing in selective force of colchicine up to 10 g/l.

2.2.4. Fermentation conditions

Lab-scale experiments were carried out in 5-l-jar fermenter and pilot-scale microbial transformation experiments were carried out in 70-l (Applikon, The Netherlands) fermenter at 3-l and 50-l working volume, respectively. The impeller was 6-bladed turbine type having diameter 50 mm in 5-l and 115 mm in 70-l fermenter. The 5-l-jar fermenter was having two impellers and the 70-l fermenter was having three impellers. The sparger was ring type having 24 holes (size of hole 1.5 mm). The sparger ring was adjusted in such a way that the air is influx in the medium in the direction of the stirrer shaft. At the initial stage, the fermenter was having 45-l culture medium. The culture medium for fermentation was commercial type comprising glucose (10 g/l) + glycerol (5 ml/l), urea (2 g/l), di-ammonium phosphate (DAP) (2 g/l), starch (30 g/l); mureate of potash (MOP) (2 g/l), antifoaming agent (0.5 ml/l), and yeast extract (5 g/l) + peptone (15 g/l). The pH of broth was adjusted with sodium hydroxide and hydrochloric acid, and incubation temperature was maintained at 28 °C.

2.2.5. Fed-batch culture

2.2.5.1. At laboratory-scale. The working volume in a fed-batch culture at 5-l-jar fermenter increased from 2.5 to 3 l by feeding glucose and glycerol solution (where the percentage of glucose and glycerol 1.0% and 0.5%, respectively) after 72 h. When the carbon source was depleted, the feeding solution of 100 ml was added intermittently five times. The feeding solution was added by peristaltic pump coupled with pH probe. The total added carbon source was 45 g in fed-batch culture. The colchicine and thiocolchicine were fed after 36 h into the 5-l-jar fermenter containing *B. megaterium* ACBT03-M3. The total amount of the substrates (colchicine and thiocolchicine) added to was 21 g.

The *B. megaterium* ACBT03-M3 survived in 10 g/l colchicine was having poor biomass yield as compare to control. However, the high concentration colchicine adapted bacterial cells were grown relatively lower amount 7 g/l of colchicine containing medium showed luxury growth as noticed in control conditions. On the basis of above results *B. megaterium* ACBT03-M3 was used in subsequent experiments for production of 3-DMC and 3-DMTC.

2.2.5.2. At pilot-scale. For a 70-l fermenter, the first seed culture was prepared in 5-l-jar fermenter, as described previously and the same was used as inoculum for 70-l fermenter having working volume 50-l. The culture broth was cultivated for 24 h at 28 °C with 150 rpm and 1 vvm air before adding concerned substrates (colchicine or thiocolchicine). At this stage, the impeller tip velocity was 3140 cm/min. Subsequently, the tip velocity was gradually increased to 4710 cm/min after 36 h of fermentation and the same rate of impeller tip velocity was maintained till the 72 h when the fermentation process was terminated. The total amount of glucose + glycerol, and substrate (colchicine or thiocolchicine) feed to the 70-l fermenter in 72 h were 750, 350 g, respectively by the peristaltic pump coupled with pH probe automatically.

The culture samples were collected at various time intervals and subjected to analysis on requirement basis. The packed cell volume (PCV) was measured