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EXPERIMENTAL ARTICLES

Structural and Metabolic Correlation for *Bacillus megaterium* ACBT03 in Response to Colchicine Biotransformation[1]

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Abstract—This study aims to evaluate the effects of colchicine on metabolic and structural changes in *Bacillus megaterium* ACBT03, enduring colchicine bioconversion. Electron microscopy examination of cells adapted to different concentrations of colchicine for its bioconversion to pharmacologically active 3-demethylated colchicine, endowed changes in cell shape, decreased cell wall and plasma membrane thickness. In line with microscopic studies, lipid and membrane protein contents were drastically reduced in bacterial cells adapted to higher concentrations of colchicine and resulting into decrease in cell membrane thickness. More numbers of polyhydroxybutyrate (PHB) rich inclusion bodies were found inside the colchicine adapted cells and presence of higher amount of PHB, a carbon source for generation of redox potential, indicates that it might be responsible for activation of P450 BM-3 enzyme and plays significant role in colchicine demethylation. The presence of dense ribosome like bodies in colchicine adapted cells showed higher biosynthesis of P450 BM-3. Reduction in cell wall and cell membrane thickness, presence of more inclusion bodies and ribosome like masses in colchicine adapted cells were some of the key interlinked phenomena responsible for colchicine bioconversion. This is the first study which reports that colchicine demethylation process severely affects the structural and metabolic functions of the bacteria.

Key words: *Bacillus megaterium,* colchicine, 3-demethylated colchicine, microbial transformation, P450-BM3.

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The role and importance of cytochrome P450 monooxygenases (CYPs or P450s) in drug development, biodegradation processes and biocatalysis have been already acknowledged because of their high potential as catalysts for the selective introduction of molecular oxygen at even non-activated C–H bonds in a regio- and/or stereo-specific manner [1]. P450 monooxygenases exhibits an extremely wide substrate spectrum which is the basis of their ability to activate or detoxify a large variety of target molecules. P450 monooxygenases have been isolated from bacteria, yeasts, insects as well as plants and mammalian tissues.

Various anti-cancer drugs like colchicine, taxol, vinblastin, vincristine and nocodazole are metabolized by CYPs present in human liver [2–4]. Colchicine is an alkaloid having an excellent anti-mitotic attribute and it is highly toxic to be used in pharmacological preparations in its native form. Derivatives of colchicine, i.e., 3-demethylated colchicine (3-DMC), colchicoside, thiocolchicocide with enhanced therapeutic properties for anti-inflammatory and anti-tumor drugs [5, 6] have a good commercial value. Demethylated colchicine showed about 35-fold less toxic as compared to parent molecule and equal anti-tumor activity to the thiocolchicine [7, 8]. Due to limited availability of colchicine derivatives through plants, diverse efforts have been made to find the alternatives for the production of 3-DMC and thiocolchicoside. Although, scanty reports are available on microbial demethylation of colchicine [9, 10], and they proved that *Bacillus megaterium* is an effective microbe for regio-specific demethylation of colchicine at C-3 position in tropolone ring via P450 BM-3 enzyme (product of cytochrome P450 monooxygenase CYP102A1 gene) [11, 12]. This is the only bacterium having the P450 BM-3 enzyme similar to the enzyme present in human liver (CYP3A4) and responsible for demethylation of colchicine [9, 13]. In *B. megaterium*, P450 BM-3 is a 119 kDa protein that catalyzes the sub-terminal hydroxylation of saturated and unsaturated fatty acids with a chain length of C_{12} to C_{20} [14]. From a technical point of view, microbial P450s are easier to handle than P450 enzymes from plants and animals, hence preferred for industrial applications.

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Bacillus spp. are industrially important due to their ability of microbial transformations of various natural [9, 15, 16] and synthetic compounds [17]. In such microbial transformation processes there may be possibilities of structural and physiological changes inside the bacterial cells. Studies on electronic microscopy of various Gram-positive bacteria have been well documented in connection with their structure and functions [18–20], and proved that a specific strain of *B. megaterium* ACBT03 is having potential for demethylation of colchicine and thiocolchicine [7, 9].

The current study is the extension of our previous reports, in which we described the production of demethylated colchicine through microbial transformation including its scale-up process development using *B. megaterium* ACBT03 strain [9] and construction of recombinant *E. coli* expressing CYP102A1 gene for enhanced biotransformation of colchicine into their regio-specific derivatives at 70 L fermenter level [7]. In the present study, we evaluated the membrane damage/metabolic profile by analyzing the lipid and membrane protein contents in conjunction with carbon, hydrogen and nitrogen (CHN) analysis by exposing the *B. megaterium* ACBT03 cells to increasing concentrations of colchicine for higher demethylation process. In addition, electron microscopic observations were performed to investigate the surface damage and structural changes of the cells adapted to different concentrations of colchicine. So, in the present study attempts have been made to evaluate the structural alterations of *B. megaterium* ACBT03 cells caused in response to the biotransformation process, and to elucidate and establish a significant correlation between the structural and metabolic profile of the bacterium undergoing the bioconversion process.

MATERIALS AND METHODS

Bacterial strain and chemicals. The microbe *B. megaterium* ACBT03 (already available in author's laboratory) [7, 9], a Gram's positive spore forming bacterium having properties like, no growth on mannitol salt, catalase positive, lecithinase negative and showing resistance towards penicillin [12]. The microbe was isolated from the industrial site of Alchem International Pvt. Ltd., New Delhi, India. Soil microbes were isolated, serially diluted and subcultured several times, in order to obtain pure colonies [21]. The Gram-positive (tested by Gram staining) bacteria having rod shape were tested for demethylation of colchicine as described by Poulev et al. (1995) [21] and stored as per the standard procedure. All the chemicals and media components were purchased from HiMedia laboratories, RFCL and Sigma.

Culture conditions and biotransformation of colchicines. The ability of bacterial culture to grow in contact with selective forces of various concentrations of colchicine (5, 7 and 10 g/l) was practiced on agar media comprising ammonium mono-phosphate (0.1%), yeast extract (1%), glucose (1%) and glycerol (0.5%). The culture medium was also containing potassium mono-hydrogen phosphate (0.8%) and potassium di-hydrogen phosphate (0.3%), and the incubation temperature was maintained at 28°C for bacterial growth. As we know that the colchicine is toxic to the microorganism, so, at the initial stages of adaptation 1.0 g/l colchicine was used as selective force for adaptation. Under such conditions the bacteria was grown for about 6 generations till the specific growth was obtained similar to the control unadapted bacterial cells. At the end of each generation the bacterial cultures were evaluated [21] for their biotransformation ability of colchicine into their respective 3-demethyl and glycosyl derivatives using thin layer chromatography (TLC) following the methodology given by Bodoki et al. (2005) [22], and through reverse phase high pressure liquid chromatography (RP-HPLC) following the modified methodology of Klein and Davis (1980) [23] using C18 column (245 nm wave length, 10 min retention time, 2 ml/min flow rate). Colchicine and demecolcine were used as a standard during HPLC experiment. The above practice was repeated for bacterial culture with gradual increase in selective forces of colchicine up to 10 g/l. As described above, the bacteria were grown in 250 ml shake flask, having 50 ml working volume, incubated on rotary shaker at 300 rpm at 28°C. At the end of each generation, the bacteria were tested for their potential of demethylation process.

Transmission Electron Microscopy (TEM). *B. megaterium* ACBT03 culture was harvested by centrifugation at 5000 rpm for 10 min. Bacterial samples were fixed in modified karnovsky's fluid [24] containing 0.1 M sodium phosphate buffer of pH 7.4. Fixation was done at 4°C for 10–18 h, after that the tissues were washed with fresh buffer, and post-fixed for 2 h in 1% osmium tetra oxide in the same buffer at 4°C. After several washes in 0.1 M sodium phosphate buffer, the specimens were dehydrated in graded acetone solution and embedded in CY 212 araldite. Ultra thin sections of 60–80 nm thickness were cut by using an ultra cut E (Reichert Jung) Ultra microtome, and the sections were stained in alcoholic uranyl acetate and lead citrate for 10 min each, before proceeding for examining the grids in a transmission electron microscope (Philips, CM-100) operated at 60–80 kV. The TEM studies were performed at Electron Microscopy facility of All India Institute of Medical Sciences (AIIMS), New Delhi, India.

Scanning Electron Microscopy (SEM). *B. megaterium* ACBT03 cultures adapted to various concentrations of colchicine were fixed in 2.5% glutaraldehyde for 2 h followed by three washes with 0.1 M phosphate buffer (pH 7.2). Afterwards bacterial samples were washed with double distilled water and air dried. Coating of cells were performed with Gold (thickness ~20 nm) and finally observed under Scanning Electron Microscope JEOL (JSM-6100). The samples

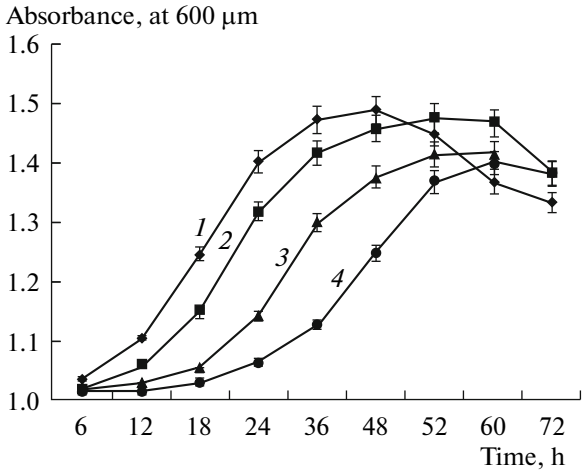

Fig. 1. Growth of control (*1*) and colchicine (*2*—5 g/l, *3*—7 g/l and *4* —10 g/l) adapted *B. megaterium* ACBT03 cells in shake flask at 28°C.

were processed for the SEM studies at Sophisticated Analytical Instruments Facility (SAIF) of Punjab University, Chandigarh, India.

P450 BM-3 enzyme activity assay. The activity assay of P450 BM-3 enzyme was performed according to the method described by Schwaneberg et al. (1999) [25]. The formation of *p*-nitrophenolate was determined by measuring the absorbance at 410 nm [with extinction coefficient = 13.2 L / (mmol · cm)] wavelength. The enzyme activity was defined as the initial rate of *p*-nitrophenolate formation, with the unit activity of the enzyme that produced 1 μmol of *p*-nitrophenolate/min.

Estimation of membrane lipid and protein. The culture broths were centrifuged for 10 min at 8000 rpm and pellets were washed thrice with phosphate buffer and dried for 24 h at 75°C for obtaining constant weight. The dry cell biomass of each bacterial culture was incubated for 1 h at 60°C with sodium hypochlorite for cell wall lysis. Supernatants were obtained by centrifugation at 6000 rpm and then transferred to a soxlet system. Total cell lipid content was extracted by adding 5 ml of 96% ethanol and acetone (1 : 1 = v/v) [3, 37]. The total bacterial cell membrane proteins content was isolated by the method given by Scopes et al. (1994) [26].

Elemental CHN analysis. Carbon, hydrogen and nitrogen (CHN) analysis was performed on Elemental Analyser (Perkin Elmer CHN Analyzer, Model-2400) based on the principle of "Dumas method" which involves the complete and instantaneous oxidation of the sample by "flash combustion". The combustion products are separated by a chromatographic column and detected by the thermal conductivity detector (T.C.D.), which gives an output signal proportional to the concentration of the individual components of the mixture. The elemental CHN analysis was performed at Sophisticated Analytical Instruments Facility (SAIF) of Punjab University, Chandigarh, India.

RESULTS

Colchicine biotransformation and P450 BM-3 enzyme activity. In the growth system, after inoculation, the bacterial cells divided synchronously for 24 h till they came to steady state. The bacterial growth kinetics of *B. megaterium* ACBT03 was studied both in control and in presence of selective forces of colchicine (5, 7 hff and 10 g/l) (Fig. 1). As evidenced from Fig. 1, under optimal conditions the growth curve showed three distinct phases of bacterial growth, i.e., lag phase (6 ± 0.3 h), log phase (18 ± 0.28 h) and stationary phase (24 ± 0.13 h). The total time taken by the bacteria to achieve a stationary phase was ~24 h. At the stationary phase, the specific growth rate and generation time were 0.767 ± 0.185 and 0.433 ± 0.119 /h, respectively. The maximum concentration of colchicine acceptable for adaptation of *B. megaterium* ACBT03 cells was observed about 10 g/l. At steady state the packed cell volume (PCV) for control and colchicine adapted bacterial biomass was 12 ± 1.5%, and at that stage the maximum bioconversion was observed, and a gradual shift was noticed in the generation time for colchicine adapted cells. With reference to control unadapted cells, 10 g/l colchicine adapted bacterial cells had taken 48 h to attain the steady state. A significant difference was noticed between the specific growth rates of unadapted control and colchicine adapted bacteria. However, no significant change was noticed in PCV of control and colchicine adapted cells at steady state.

Table 1 shows the P450 BM-3 enzyme activity in control and colchicine adapted bacterial cells. The colchicine bioconversion and P450 BM-3 enzyme activity were found maximum in between 36–48 h of the bacterial growth, irrespective of control and colchicine adapted cells [9, 21]. Bacterial cell adapted to 7 g/l and 10 g/l colchicine showed about 3–4 fold increases in P450 BM-3 activity and proved that P450 BM-3 enzyme is responsible for demethylation process [9, 21, 27], and these results were in concordance with the data reported by the earlier workers [28]. TLC analysis was performed from the extract obtained from fermented broth and the R_f values of colchicine and 3-DMC were noticed as 0.42 and 0.77, respectively, and obtained data was comparable to the established results. HPLC analysis (Fig. 2) showed distinct peaks at retention time 3.5 min and 2.5 min and obtained results were comparable to the results of colchicine standard and 3-DMC (demecolcine), respectively.

Electron microscopic studies. Vegetative cells of the bacteria collected from the steady phase of the growth were examined and light microscopic studies showed that the bacterial cells were like typical bacilli and no differentiation was possible between vegetative cells of bacteria grown under control and colchicine rich

Table 1. Colchicine bioconversion with respect to P450 BM-3 enzyme activity at different time intervals (shake flask level at 28°C)

Parameters	Culture conditions	Fermentation time (h)						
		12	24	36	48	56	68	72
Activity of P 450 BM-3 (10^{-3} U/ml)	Control (Un-adapted cells)	10.5 ± 0.32	14.3 ± 0.62	20.7 ± 1.18	17.5 ± 0.84	10.4 ± 1.13	7.1 ± 0.31	6.7 ± 0.26
	A	14.6 ± 0.16	22.5 ± 0.27	34.6 ± 1.52	32.3 ± 1.24	21.2 ± 1.72	14.6 ± 0.42	11.4 ± 0.84
	B	22.3 ± 0.23	33.5 ± 1.21	42.3 ± 2.31	49.2 ± 2.26	40.5 ± 1.68	36.7 ± 1.62	32.2 ± 1.42
	C	26.3 ± 0.39	40.7 ± 2.15	52.1 ± 1.85	67.3 ± 2.31	61.6 ± 2.13	58.7 ± 1.72	55.2 ± 1.72
Colchicine bioconver-sion (%)	Control (Un-adapted cells)	16.0 ± 0.18	21.0 ± 1.41	29.0 ± 1.14	24.0 ± 1.27	18.0 ± 0.32	NM	NM
	A	23.0 ± 1.21	35.0 ± 1.63	51.0 ± 1.52	48.0 ± 0.94	36.0 ± 1.31	29.0 ± 1.28	NM
	B	37.0 ± 1.05	54.0 ± 1.26	69.0 ± 1.84	73.0 ± 1.42	53.0 ± 2.11	45.0 ± 1.22	39.0 ± 0.84
	C	42.0 ± 1.14	65.0 ± 2.13	76.0 ± 1.74	82.0 ± 1.73	78.0 ± 1.78	65.0 ± 1.63	54.0 ± 1.32

* Values are the means ± standard deviations of three measurements.

Bacillus megaterium ACBT03 vegetative cells were collected at different time intervals during fermentation process (at shake flask level), and samples were analyzed by High Performance Liquid Chromatography.

NM = not measured; A = 5 g/l colchicine adapted cells; B = 7 g/l colchicine adapted cells; C = 10 g/l colchicine adapted cells.

Table 2. Image analysis of TEM of *Bacillus megaterium* ACBT03 adapted to various concentration of colchicine (at stationary phase)

Observation		Control	5 g/l colchicine adapted cells	7 g/l colchicine adapted cells	10 g/l colchicine adapted cells
Cell size (μm)	Length	1.247 ± 0.274	1.276 ± 0.208	1.278 ± 0.04	1.503 ± 0.384
Mean ± SD	Breadth	0.745 ± 0.135	0.752 ± 0.003	0.785 ± 0.122	0.984 ± 0.043
S-layer (μm) Mean ± SD		0.168 ± 0.053	0.112 ± 0.021	0.076 ± 0.024	0.084 ± 0.019 3
Cell wall (μm) Mean ± SD		0.046 ± 0.019	0.026 ± 0.0094	0.022 ± 0.006	0.014 ± 0.0037
Plasma membrane (μm) Mean ± SD		0.018 ± 0.014	0.015 ± 0.029	0.014 ± 0.018	0.013 ± 0.002
Number of Inclusion body		0–1	4	5	6–7

medium. However, TEM studies were carried out to analyze the fine structure of bacterial cells grown under controlled conditions and colchicine rich medium (Figs. 3a–3d). Whereas, SEM analysis clearly differentiates the shape of the control and colchicine adapted bacterial cells (Figs. 4a–4d).

Table 2 shows the changes occurred in the fine structure of *B. megaterium* ACBT03 adapted to different concentrations of colchicine in comparison to the control unadapted cells. Under controlled growth conditions the bacterial cell was appeared like cylindrical shape, having 1.247 ± 0.274 μm length and 0.745 ± 0.135 μm breadth (Fig. 3a) whereas bacterial cells adapted to 10 g/l colchicine concentration were slightly oval in shape with 1.503 ± 0.384 μm length and 0.984 ± 0.043 μm breadth (Fig. 3d). The shape of the bacterial cell was evidenced by the ratio of the length and breadth of the cell. However, the length and the breadth of the bacterial cell adapted to 7 g/l colchicine were comparable to unadapted control cells.

The thickness of S-layer (slim layer) of control unadapted cell was observed to be 0.168 ± 0.053 μm, whereas the thickness of S-layer was reduced ~2 fold in case of 10 g/l colchicine adapted bacterial cells. The cell wall thickness of control bacterial cell was about 0. 0.046 ± 0.019 μm, whereas the cell wall thickness of colchicine adapted cell was reduced to 0.014 ± 0.0037 μm (Figs. 3a–3d). A significant reduction in plasma membrane thickness was noticed in bacterial cells adapted to various concentrations of colchicine.

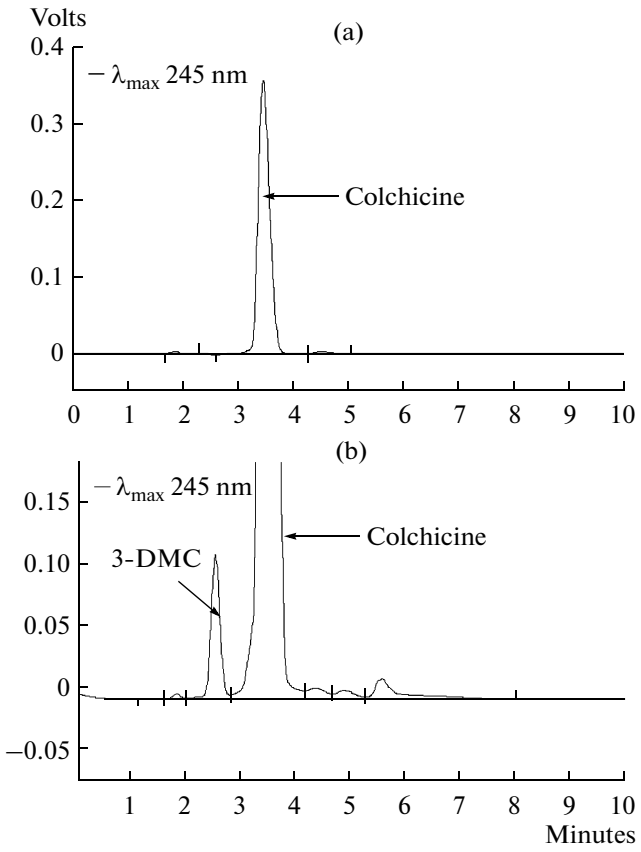

Fig. 2. HPLC chromatogram: (a) colchicine standard; (b) extracted sample.

The plasma membrane thickness of control bacterial cell was 0.018 μm whereas for 10 g/l colchicine adapted cell it was 0.013 μm. So, by considering the plasma membrane thickness of control cells as 100%, around 28% reduction in plasma membrane thickness was noticed for colchicine adapted cells (Figs. 3a–3d). A limited number of inclusion bodies were observed in vegetatively grown bacterial control cells. However, with increase in colchicine concentration in the culture medium, the number of inclusion bodies were increased inside the bacterial cells and reached about 6–7 (Figs. 3b–3d). The structure of the nucleus was distinguishable in vegetatively grown control bacterial cells whereas, nucleus was slightly indistinct in colchicine adapted cells. As reported earlier the location of mesosome was noticed to be mostly at peripheral region of the bacterial cells and bounded by membrane. No significant difference was noticed in the structure of the mesosome of control and colchicine adapted bacterial cells. However, the location of mesosome in colchicine adapted cells was more peripheral in comparison to control unadapted bacterial cells (Figs. 3a–3d).

Content of lipid and membrane protein. In line with microscopic studies the lipid and membrane proteins profile of *B. megaterium* ACBT03 cells adapted with different concentrations of colchicine along with control cells have been analyzed and it was found that the lipid and membrane protein contents were drastically reduced in bacterial cells adapted to higher concentrations of colchicine in 1 comparison to control cells (Table 3).

CHN elemental analysis of bacterial biomass. 'There were major changes noticed in the carbon and nitrogen content of the control and colchicine adapted bacterial cells during carbon, hydrogen and nitrogen (CHN) analysis. The results of CHN analysis showed an increase in carbon and nitrogen contents for the bacterial cells adapted to 5 g/l and 7 g/l colchicine in comparison to control unadapted cells, but the carbon and nitrogen content had been again decreased in 10 g/l colchicine adapted bacterial cells possibly due to reduced membrane thickness that occurs because of reduction in membrane protein content.

Table 3. Estimation of C, H, N, total membrane lipids and membrane proteins of cell wall of *B. megaterium* ACBT03 adapted to various concentration of colchicine at shake flask level

Type of culture	Membrane biomass (g/l)*	Percentage of carbon in membrane	Percentage of hydrogen in membrane	Percentage of nitrogen in membrane	Membrane lipids, mg/g DCW	Membrane proteins, mg/g DCW
Control	15.40 ± 0.04	31.44 ± 0.12	4.47 ± 0.08	4.87 ± 0.07	7.02 ± 0.06	6.5 ± 0.03
5 g/l colchicine adapted cells	17.20 ± 0.06	35.22 ± 0.09	5.32 ± 0.11	7.77 ± 0.10	12.28 ± 0.6	8.3 ± 0.12
7 g/l colchicine adapted cells	18.50 ± 0.03	36.00 ± 0.15	4.34 ± 0.05	7.08 ± 0.03	16.36 ± 0.11	10.24 ± 0.15
10 g/l colchicine adapted cells	17.50 ± 0.05	34.41 ± 0.17	5.74 ± 0.14	6.65 ± 0.05	20.72 ± 0.09	15.54 ± 0.19

* Values are the means ± standard deviations of five measurements.

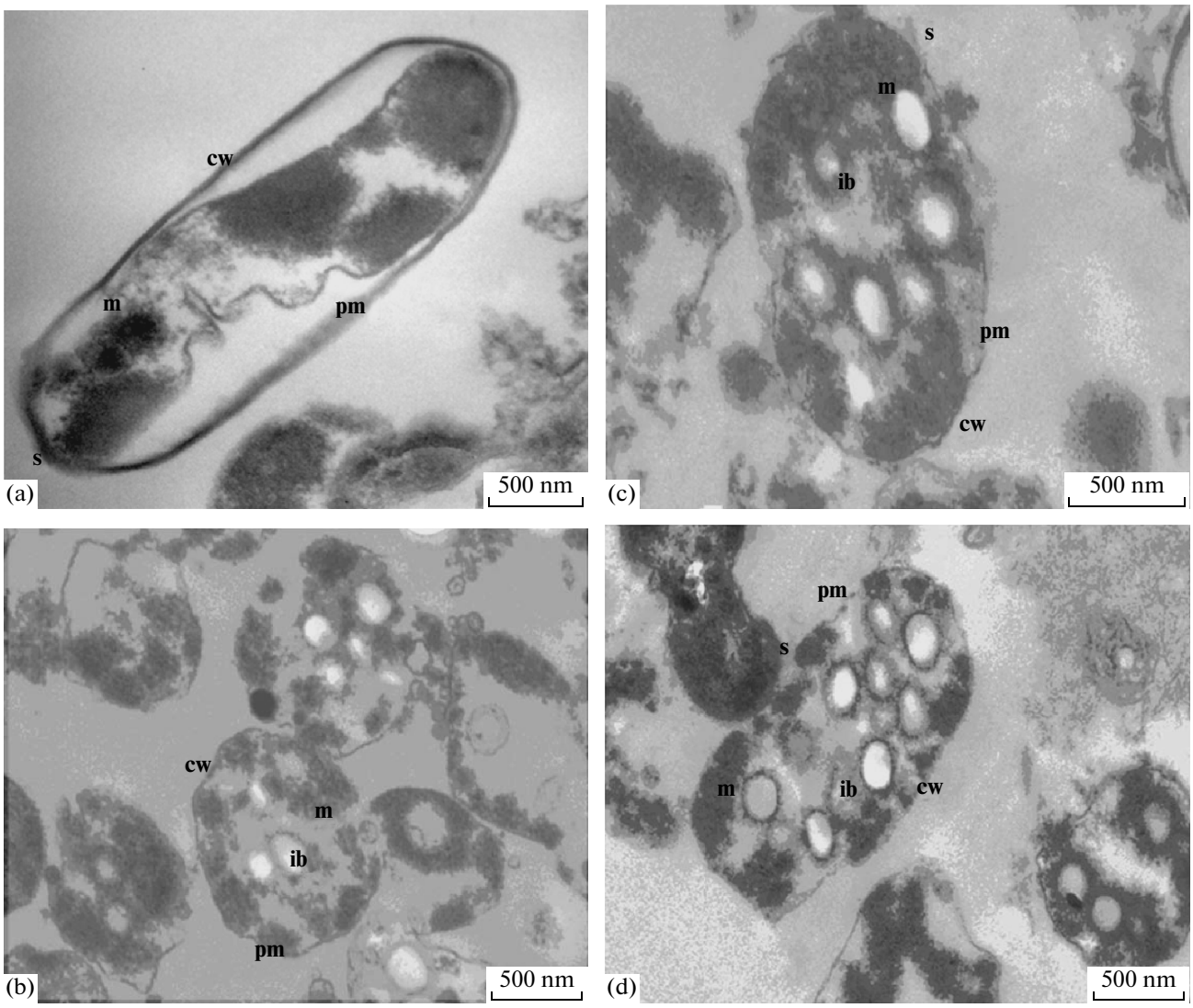

Fig. 3. Transmission electron micrograph of control (a; without colchicines treatment) and colchicine (b: 5 g/l, c: 7 g/l and d: 10 g/l) adapted *B. megaterium* ACBT03 (vegetative cells).
Slim layer (S-layer) = s, cell wall = cw, plasma membrane = pm, mesosome = m, inclusion body = ib.

The increase in carbon content in the bacterial biomass adapted to higher concentrations of colchicine might possible as a result of increase in number of inclusion bodies as evidenced from TEM studies (Figs. 3a–3d) which are mainly composed of carbon rich PHB (Polyhydroxybutyrate) granules. Moreover, the presence of residual colchicine/3-DMC (Table 3) might be another probable reason responsible for increase in carbon content in the microbial biomass adapted to 5–10 g/l colchicine. However, the increase in carbon content was observed to be in decreasing order of colchicine concentrations to which the bacterial cells were adapted. Since, nitrogen is an integral component of the bacterial plasma membrane building blocks, hence, similar to carbon content, major decrease in nitrogen content was also observed for *B. megaterium* ACBT03 cells adapted to higher concentrations of colchicine. It was also noticed that bacterial cells adapted to 10 g/l colchicine were having thin membrane accompanied by heavy loss in total membrane protein. The total hydrogen content of biomasses of control and colchicine adapted cells were remained unchanged and comparable.

DISCUSSION

The aim of this work was to evaluate the interaction, meant as an effect of colchicine with the microbial cell envelope for the structural alterations, and its correlation with the metabolic profile of the bacterial cell undergoing bioconversion process using P450 BM-3 enzyme. The current study demonstrates the

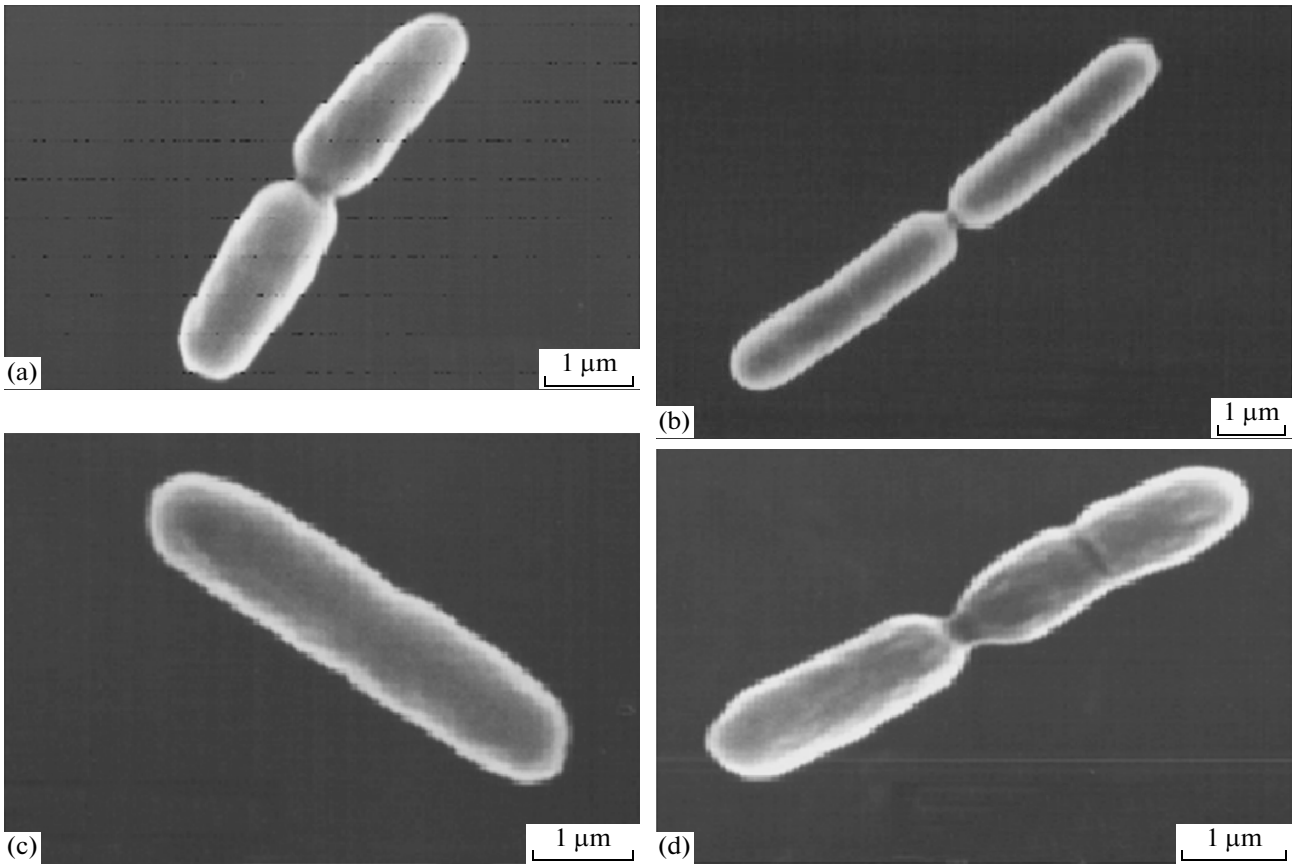

Fig. 4. Scanning electron micrograph of control (a: without colchicines treatment) and colchicine (b: 5 g/l, c: 7 g/l and d: 10 g/l) adapted *B. megaterium* ACBT03 (vegetative cells).

precise correlation existed between the structural and metabolic changes in the *B. megaterium* ACBT03 cells in response to high concentrations of colchicine during its demethylation process into pharmacologically active 3-DMC. However, no report is available till now, but there might be possibilities that the structural and metabolic changes occurs inside the bacterial cell due to colchicine toxicity. As stated earlier in the introduction part, the 3-DMC and its glucosides have shown to possess high quality pharmacological properties, accompanied with decreased toxicities, which makes these derivatives an important compound for anti-inflammatory and anti-cancer therapy [29, 30]. It has been already understood that P450 BM-3 is one of the most important enzyme responsible for demethylation of colchicine into 3-DMC in *B. megaterium* [27]. The P450 BM-3 enzyme is a membrane bound protein mostly attached to endoplasmic reticulum in eukaryotic system and having major role in demethylation of colchicine [28, 31], However, in prokaryotes the same enzyme is localized with the inclusion bodies and the presence of more densely distributed inclusion bodies like structures (Fig. 3) support as an evidence for synthesis of relatively more P540 BM-3 enzyme, which might be responsible for enhanced demethylation process in colchicine adapted bacterial cells.

The electron microscopic analysis of steady state bacterial cells showed that, high colchicine concentration adapted cells were slightly oval in shape accompanied with decreased cell wall and plasma membrane thickness and it can be predicted that the higher concentrations of colchicine might be responsible for reduction in the thickness of bacterial cell wall and plasma membrane. It has been already proved that plasma membrane is composed of phospholipid bilayer and different types of proteins, and according to the Figs. 3a–3d, the length of phospholipid is almost constant, so the thickness of plasma membrane might change by the amount of proteins. The reduction in thickness of bacterial cell membrane is possible due to loosening of weakly immobilized components of membrane protein or inhibition of membrane protein synthesis [32] in bacterial cells adapted to high concentrations of colchicine [33].

The passage of colchicine through bacterial cell mainly depends on the structural configuration of bacterial plasma membrane. The reduction in total carbon and membrane lipid (Table 3) might have resulted

in decrease in the cell wall thickness as evident from electron microscopy study (Figs. 3a–3d). Generally, the extrinsic and intrinsic membrane proteins act as cementing material to keep the bacterial cell membrane intact. Reduction in total protein content of cell membrane (Table 3), as observed in higher colchicine adapted cells might have brought loosening in structure of bacterial membrane. The decrease in cell membrane thickness accompanied with loosening of membrane structure might be responsible for influx and efflux of colchicine and its derivatives more frequently in comparison to control unadapted cells. The numbers of inclusion bodies in colchicine adapted bacteria were remarkably more in comparison to control unadapted cells. Earlier reports have already proved that the inclusion bodies of *Bacillus* spp. are rich in PHB [32, 33] and PHB granule formation is a stress induced phenomena in *B. megaterium* [33]. During the PHB biosynthesis process one molecule of NADP is generated and finally converted to NADPH by NADP-Cytochrome P450 reductase [34]. As we know that P450 enzyme activity requires NADPH as a cofactor and NADP-Cytochrome P450 reductase as a coenzyme [34, 35]. Hence, the cytochrome P450 reaction involves transfer of electrons from NADPH to NADPH-cytochrome P450 reductase and then to cytochrome P450 [34]. This leads to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate. Virtually all subsequent chemical changes (e.g., demethylation) for this initial step. The basic reaction can be follows as $RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$ (where RH is the drug). The ability to catalyze such reactions that are difficult to achieve chemically with high selectivity, especially in water, at room temperature and under atmospheric pressure, makes P450 enzymes attractive for biotechnological applications [36]. So, it can be hypothesized that the PHB granules not only act as a reservoir of carbon and energy [32] but also work as a sink of reducing power, and probably considered as a redox regulator within the bacterial cell and help in P450 based demethylation process.

Although, no report is available till now which shows the direct role of PHB granules for enhanced activity of P450s enzymes, but as per the mechanism mentioned by Cribb (2003) and Dragon et al. [35, 34], which shows that when PHB degrades/utilized by bacteria, then two NADH molecules released and it can be speculated that the released NADH molecules might be utilized by P450 enzymes for its activation and enhance enzymatic activity. The formation of more number of inclusion bodies in colchicine adapted cells, as evidenced from electron microscopic studies, might have responsible for enhanced activity of P450 enzyme which ultimately resulted in increase in the rate of colchicine demethylation process.

Our study clearly demonstrated that regio-specific demethylation of colchicine caused a partial collapse/loosening of the integrity of the cytoplasmic membrane, leading to excessive leakage of metabolites and enzymes from the cell and finally alteration of the cell structure. The major alterations of the bacterial cells tested in the presence of colchicine suggests that this compound acts on the membrane, altering its lipid and protein profile, loosening the membrane components, and altering its structure [37]. However, it is also able to penetrate into the deeper parts of the cell, leading them major alteration of bacterial cell structure. Hence, it might be predicted that biotransformation process severely affects the metabolic profile and morphology of the bacteria, and these changes inside the bacterial cell caused mainly due to enhanced activity of P450 enzymes and there is a strong correlation exists between the structural and metabolic profile of the bacterial cell undergoing demethylation process. The novelty of the study is that this is very first time we have reported the effect of biocatalysis process on the bacterial cell undergoing the colchicine bioconversion process, and elucidated a correlation between the structural alterations with the metabolic profile of the bacterium.

The mechanism of microbial demethylation of colchicine is still unclear. Based on past literatures as stated earlier and present findings it has been concluded that reduction in bacterial cell wall thickness, appearance of more inclusion bodies (PHB granules), presence of denser peripheral mesosomes and ribosome like mass, and increase in P450 enzyme activity at steady state in colchicine adapted bacterial cells, are some of the key interlinked phenomena responsible for demethylation of colchicine into pharmacologically important 3-DMC. Hence, our electron microscopic and metabolomic studies fully endorsed that the colchicine bioconversion process adversely affects the metabolome of the bacterial cell which ultimately reflects into its structural modifications. The mechanism of interaction of colchicine with bacterial cell implicates the alteration of membrane lipid content along with alteration of the cell envelope structure. A better understanding of the interaction between colchicine and cell target molecules is going to be fundamental to work out the best environmental conditions to be used to ensure an effective demethylation process.

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