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Metabolism of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818

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Abstract

The microbial degradative characteristics of butyl benzyl phthalate (BBP) were investigated by the *Gordonia* sp. strain MTCC 4818 isolated from creosote-contaminated soil. The test organism can utilize a number of phthalate esters as sole sources of carbon and energy, where BBP was totally degraded within 4 days under shake culture conditions. High performance liquid chromatography profile of the metabolites isolated from spent culture indicated the accumulation of two major products apart from phthalic acid (PA), which were characterized by gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy as mono-*n*-butyl phthalate (MBuP) and monobenzyl phthalate (MBzP). Neither of the metabolites, MBuP, MBzP or PA, supported growth of the test organism, while in resting cell transformation, the monoesters were hydrolyzed to PA to a very minor extent, which was found to be a dead-end product in the degradation process. On the other hand, the test organism grew well on benzyl alcohol and butanol, the hydrolyzed products of BBP. The esterase(s) was found to be inducible in nature and can hydrolyze in vitro the seven different phthalate diesters tested to their corresponding monoesters irrespective of their support to the growth of the test organism.
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Phthalic acid esters (PAE) used as plasticizers belong to the widespread, toxicological significant environmental pollutants [1–4]. Despite a half century of apparently safe use, during the last couple of years several environmental and health groups around the world expressed deep concern over the phthalates associated adverse effects on ecological problems including human health. They are able to induce hepatic peroxisome proliferation, hormonal disorders, reproductive toxicity, and hepatocellular tumours [5–8]. In recent years, phthalic acid diesters have appeared as important pollutants in environmental samples as endocrine disrupting chemicals which influence the genitals because they have binding activity for the estrogen receptor [9,10] and cause proliferation of MCF-7 cells [11]. Moreover, attention has also been turned to low-dose toxicity of phthalates during crucial windows of fetal development, and all these advances fundamentally changed the perception of potential health risks of phthalates.

Among others, butyl benzyl phthalate (BBP) was used primarily as plasticizers in flexible polyvinyl chloride (PVC) products and as cellulose resins in food packaging materials [12,13]. Humans are exposed to BBP mainly via food which is hydrolyzed in the gastrointestinal tract to mono-*n*-butyl and monobenzyl phthalates, which are absorbed, further metabolized, glucuronidated, and excreted in the urine [12]. BBP has been tested for developmental and testicular toxicity in mice and in rats apart from malformations and embryonic deaths [14–18]. In rats, mono-*n*-butyl phthalate (MBuP) and monobenzyl phthalate (MBzP) are formed as major metabolites from BBP [19,20] which participate in the induction of antiandrogenic and teratogenic effects and also in most frequent types of fetal malformations during major organogenesis, consistent with those induced by BBP [21, and references therein].

Metabolic breakdown of phthalate esters by microorganisms is considered to be one of the major routes of

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environmental degradation of these widespread pollutants. A number of studies have reported on the biodegradation of phthalate esters in natural water, wastewater, and in soils [22–32]. In the bacterial degradation of PAEs, some organisms can selectively hydrolyze only one ester bond to give monoalkyl phthalate and an alcohol where the latter compound is then used for growth. While other organisms are capable of complete mineralization of either the monoalkyl or dialkyl phthalates. MBuP was formed as the only metabolite from di-*n*-butyl phthalate (DBP) enriched pure isolates similar to the mammalian metabolism with the accumulation of monoesters of phthalates [33]. However, most of the studies on the biodegradation of PAEs by axenic microorganism are on dialkyl phthalates. Microbial degradation and fate of alkyl aryl phthalate namely, butyl benzyl phthalate, by pure culture remains largely unknown. Recently, Kim et al. [34] reported the enhanced degradation of BBP by fungal cutinase in comparison to yeast esterase and in the latter case an appreciable amount of MBuP was accumulated on the hydrolysis of BBP. In the present study, the degradation of BBP by a *Gordonia* sp. strain MTCC 4818 is reported. MBuP and MBzP have been characterized as the major accumulated metabolites in the catabolic pathway and the nature of the esterase(s) involved in the initial degradation of BBP has been demonstrated.

Materials and methods

Chemicals. All the seven phthalate diesters used in this study are of commercial grade where butyl benzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), dimethyl phthalate (DMP), diisobutyl phthalate (DIBP), and dicyclohexyl phthalate (DCHP) were obtained from Merck (Germany), di-(2-ethylhexyl) phthalate (DEHP) was from the Aldrich Chemical (Milwaukee, WI) while diphenyl phthalate (DPhP) was from Hi-Media (India). All other chemicals and reagent used in this study were of analytical grade and used without any purification.

Synthesis of phthalic acid monoesters. The method of Snell [35] for the synthesis of mono-(2-ethylhexyl)-phthalate that was subsequently modified by Suzuki et al. [36] was used for the synthesis of phthalic acid monoesters. For the synthesis of the monoesters, 10 mmol of phthalic anhydride and 15 mmol of *n*-butyl-, iso-butyl-, cyclohexyl-, 2-ethylhexyl-, or benzyl alcohol were heated at 100–120 °C for 3 h. However, for the synthesis of monomethyl phthalate, 10 mmol of phthalic anhydride and 10 ml of methanol were refluxed at 100 °C for 6 h and then the rest of the methanol was removed under vacuum. The residual liquid of each monoester was added to 200 ml of 1% sodium hydrogen carbonate solution and the phthalic acid diester and/or alcohol were removed by extracting twice with 50 ml of diethyl ether. Subsequently, phthalic acid monoesters were extracted by shaking twice with 50 ml of diethyl ether from the solution after acidifying to pH 4.0 with 5 N hydrochloric acid. The ether solution was dehydrated with anhydrous sodium sulfate and finally evaporated using rotary evaporator under reduced pressure at 40 °C. The purity of each monoester was ascertained from thin-layer chromatography (TLC) and by high performance liquid chromatography (HPLC).

Organism and culture conditions. The test organism used in the present study is a rod shaped Gram-positive bacterium isolated from a creosote-contaminated soil by enrichment on BBP as the sole carbon and energy sources. The biological systematic properties of this organism were determined by the Microbial Type Culture Collection and GenBank at the Institute of Microbial Technology, Chandigarh, India (MTCC Accession No. 4818) while fatty acid methyl ester (FAME) analysis and 16S rRNA compositional analysis were performed, respectively, by Microbial ID and MIDI Laboratories, Newark, USA.

Cells were grown in a liquid mineral salt medium (pH 7.0) containing (per liter) 3.34 g of K_2HPO_4, 0.87 g of NaH_2PO_4, 2.0 g of NH_4Cl, 123 mg of nitrilotriacetic acid, 200 mg of $MgSO_4{\cdot}7H_2O$, 12 mg of $FeSO_4{\cdot}7H_2O$, 3 mg of $MnSO_4{\cdot}H_2O$, 3 mg of $ZnSO_4{\cdot}7H_2O$, and 1 mg of $CoCl_2{\cdot}6H_2O$. Solid media contained 2% agar (HiMedia, India). Cells were grown by incubating them at 28 °C on a rotary shaker (180 rpm) in 100 ml Erlenmeyer flask containing 25 ml of mineral salt medium supplemented with different phthalate esters (1 g/L) individually as carbon source and incubated for different periods of time. Enzyme induction and protein synthesis on BBP hydrolysis were examined by resting cell transformation of BBP with induced and non-induced cells. BBP and glucose grown starter cultures were used for the growth of the experimental cultures. Cells were harvested in the late log phase by centrifugation (8000*g*, 10 min), washed twice by equal volume of potassium phosphate buffer (50 mM, pH 7.0), and finally resuspended in the same buffer to give an optical density (OD_{600}) of 1.0. BBP (1 g/L) was added with or without chloramphenicol (25 μg/ml) in BBP and glucose-grown cell suspensions, and incubated at 28 °C for different periods of time up to 96 h to monitor BBP hydrolysis. Unless stated otherwise, each experimental set was cultivated in triplicate.

Isolation of metabolites. After incubation, the cultures were extracted thrice with equal volume of chloroform. The aqueous parts were acidified to pH 1.5–2.0 by concentrated hydrochloric acid and extracted thrice with equal volume of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residues of combined chloroform and ethyl acetate extracts and/or standard acidic compounds were methylated with a boron-trifluoride–methanol solution (Merck, Germany) as needed prior to analysis.

Preparation of cell-free extracts and enzyme assay. Cells grown in mineral salt media in presence of BBP or glucose (1 g/L) were harvested at mid-exponential phase by centrifugation at 8000*g* for 10 min at 4 °C. The pellet was washed twice with 10 volumes of 50 mM potassium phosphate buffer, pH 7.0, and resuspended in 1–2 volumes of the same buffer. The cell suspensions were ultrasonicated for 2 min at 4 °C in seven pulses. The ultrasonicated cell suspensions were centrifuged at 20,000*g* for 20 min at 4 °C. The supernatants were used as cell-free enzymes for further studies.

Esterase activity towards *p*-nitrophenyl acetate was measured at 25 °C from the increase in A_{400} ($E = 13.6\,mM^{-1}\,cm^{-1}$) [37]. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, with an appropriate amount of enzyme and 100 μl of *p*-nitrophenyl acetate (25 mM in the same buffer) in a total volume of 1 ml. One unit of enzyme activity was defined as the amount which degraded 1 μmol of substrate per minute under the assay conditions, and the specific activity was defined as units per milligram of protein. Protein was measured by the method of Lowry with bovine serum albumin as the standard [38]. The hydrolytic activity towards phthalate diesters and monoesters was performed at 28 °C and the reaction mixture contained 60 μM of substrate in 50 mM potassium phosphate buffer, pH 7.0, in a final volume of 1 ml with an appropriate amount of enzyme for 30 min. The reaction was stopped by adding 50 μl of hydrochloric acid (2 N) and the solution was extracted as mentioned above. The extract was evaporated and the residue was dissolved in 40% methanol. A portion (25 μl) was put through HPLC to measure phthalate monoesters, phthalic acid, and residual phthalate diester. Quantitative estimation of individual components was made from the standard curve of the respective components created by HPLC under identical conditions.

Analyses. Metabolites obtained from spent culture and resting cell preparation were resolved by TLC on silica gel GF_{254} (Merck, India) plate using benzene:acetic acid (97:3) and detected by a UV lamp at

254 nm. However, the identity of resolved product(s) was determined occasionally by comparing with that of reference compounds developed identically. In addition, the metabolites were resolved by HPLC on an analytical Novapak C_{18} reverse-phase column attached to a Waters 515 solvent delivery system. The biodegraded products were eluted under isocratic mode with methanol–potassium phosphate buffer, 50 mM, pH 5.2 (40:60) as solvent system at a flow rate of 1 ml/min and detected by Waters 486 tunable absorbance detector at 254 nm. Yield of various metabolites so generated was calculated using Millennium Session Manager software package (version 2.15.01). For characterization of metabolites, preparative Novapak C_{18} reverse-phase column was used to separate and isolate the individual components. Collected fractions of individual product were combined, acidified to pH 1.5–2.0, and extracted with ethyl acetate or diethyl ether, and finally evaporated to dryness. The individual component so separated was tested for homogeneity by TLC and HPLC and subsequently used for NMR analysis.

An analysis of the biodegraded products of phthalate esters and/or its metabolites by gas chromatography–mass spectrometry (GC–MS) was performed by using a QP-5000 instrument (Shimadzu, Japan) fitted with a fused silica capillary column (DB-5, 30 m × 0.25 mm, J&W Scientific, Japan). The temperature program gave a 2-min hold at 50 °C, an increase to 300 °C at 6 °C/min, and a 16-min hold at 300 °C. The injection volume was 1 μl and the carrier gas was helium (1.5 ml/min). The mass spectrometer was operated at an electron ionization energy of 70 eV. Instrumental library searches, comparison with available authentic compounds, and mass fragmentation patterns were used to identify the metabolites.

For ^{1}H NMR analysis of the individual product, spectra were recorded by a DRX 500 NMR system (Brucker, Switzerland) operated at 500 MHz. The intensity of each chemical shift was determined relative to tetramethylsilane (TMS) as an internal standard, where deuterated dimethyl sulfoxide (d_6-DMSO) was used to dissolve each sample.

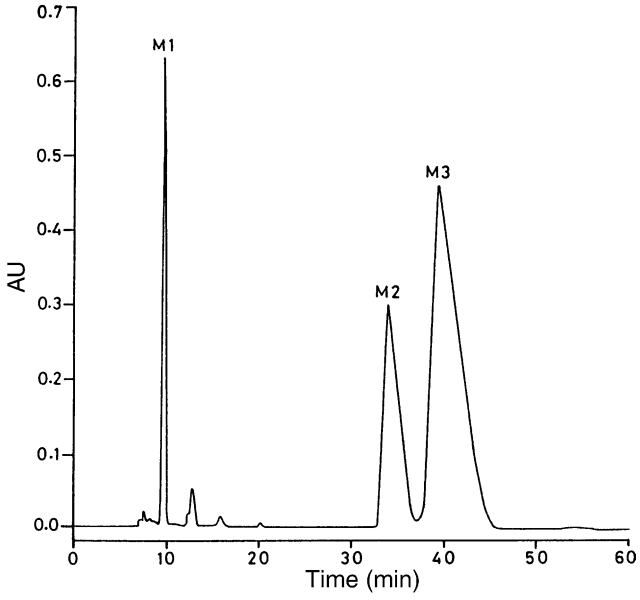

Fig. 1. Reverse-phase HPLC traces of butyl benzyl phthalate degraded products by *Gordonia* sp. strain MTCC 4818. M1, M2, and M3 are metabolites.

Results and discussion

Taxonomy

The test organism which grew on BBP as sole carbon source was identified as the genus *Gordonia* based on the morphological, nutritional, and biochemical characteristics of the organism [39]. Moreover, the major fatty acid methyl ester (FAME) analysis and a search with TSBA (version 4.0) database revealed strong correlation to *Gordonia* sp. with the best match to *Gordonia aichiensis* (similarity indices of 0.539). However, the partial 16S rRNA (500 bp) sequence analysis and comparison to GenBank data showed high degree of sequence homology to *Gordonia polyisoprenivorans* (98%) followed by *Gordonia bronchialis* (96.92%). Although, the combined analyses indicated a strong correlation in genus level but to have a confidence in species level or to give new species name full-length analysis of the 16S rRNA and further sequence comparison is necessary. Thus, at this point, the test organism has been identified as *Gordonia* sp.

Biodegradation of BBP and other phthalate esters

Gordonia sp. strain MTCC 4818 can grow individually on BBP, DBP, DIBP, and DPP as sole carbon sources among the phthalate esters tested. Moreover, the test organism can grow individually as carbon sources on benzyl alcohol, *n*-butanol or iso-butanol, the hydrolyzed products of the phthalate diesters. Analysis of metabolites obtained from spent culture and resting cell suspension on BBP by thin-layer chromatography (TLC) showed a major spot (R_f, 0.4) and a minor spot (R_f, 0.03) on silica gel G plate other than the starting material (R_f, 0.5), in case of incomplete degradation. However, HPLC analysis of the BBP degraded sample using reverse-phase analytical C_{18} column showed well-resolved peaks where the major spot (R_f, 0.4) in the silica gel G plate was resolved into two components. Fig. 1 shows HPLC profile of different metabolites obtained from spent medium when BBP was degraded completely. Two major metabolites of BBP assigned as M2 and M3 and a minor metabolite assigned as M1 showed retention times (RT) at 34.08, 39.51, and 9.8 min, respectively (Fig. 1). The analysis by HPLC revealed that few other metabolites in very minute amount were formed from BBP degraded sample, which could not be isolated for characterization. Products, M1, M2, and M3, were isolated by HPLC using reverse-phase preparative C_{18} column were found to be homogeneous.

Characterization of BBP-degraded metabolites

The ultra-violet spectrum of M2 and M3 showed absorption pattern similar to BBP and/or PA indicating the retention of the aromatic character of the metabolites. The peak M1 corresponds to authentic PA (RT, 9.8) by HPLC analysis. Moreover, this has been

confirmed by mass spectrum of the methyl ester of peak M1. The m/z of major ion peaks (relative abundance) so obtained are: 194 (M^+, 5), 164 (7), 163 (100), 133 (5), 120 (2), 104 (10), 92 (14), 77 (34), 76 (22), and 50 (30) which correspond to dimethyl phthalate based on the match of the mass spectrum of the authentic sample. The GC analysis of the BBP degraded samples showed two detectable peaks with retention times at 11.05 and 15.88 min. Again, metabolites M2 and M3 isolated by preparative HPLC when injected individually in GC column showed identical retention times with M2 at 15.87 and M3 at 11.04 min. The mass fragmentation profile of the metabolite M2 (Fig. 2A) displayed molecular ion peak at m/z 222, i.e., at 90 mass units less than that of the parent compound BBP, indicating possible hydrolysis of the benzyl ester moiety of the molecule. Other discernible peaks in the mass spectrum of M2 are at m/z 149 (M^+–O–$(CH_2)_3$–CH_3), 148 (M^+–O–$(CH_2)_3$–CH_3–H), 104 (M^+–COO–$(CH_2)_3$–CH_3–OH, and/or M^+–O–$(CH_2)_3$–CH_3–COOH), 76 (M^+–COO–$(CH_2)_3$–CH_3–COOH). While the metabolite M3 displayed molecular ion peak at m/z 256, i.e., 56 mass units less than that of BBP, indicating possible hydrolysis of the butyl ester moiety of the parent compound (Fig. 2B). Other discernible peaks in the mass spectrum of M3 are at m/z 149 and 150 for (M^+–O–CH_2–C_6H_5) and its hydrogenated species, m/z 107 (O–CH_2–C_6H_5)$^+$, 108 (C_7H_7–OH)$^+$ as the base peak, m/z 91 (CH_2–C_6H_5)$^+$, m/z 79 (C_7H_7–OH–CO)$^+$, m/z 77 (C_6H_5)$^+$, and m/z 76 (C_6H_4)$^+$. Thus the m/z of major ion peaks (relative abundance) observed for M2 are 222 (M^+, 1.9), 163 (4.8), 149 (13.4), 148 (12.2), 133 (3.2), 105 (8), 104 (100), 77 (8.6), 76 (85), 75 (15.3), 74 (27.8), and 50 (71) and for M3 are 256 (M^+, 1.5), 150 (35), 149 (22), 148 (28), 108 (100), 107 (17.7), 105 (4.6), 91 (63), 90 (32.4), 79 (31), 77 (20.6), 76 (14), 65 (24.7), 50 (16), and 43 (84). The mass spectra (fragmentation and peak intensity) and GC retention times for M2 and M3 showed close match with data for authentic samples, mono-*n*-butyl- and monobenzyl phthalates, respectively. This was supported by 1D and 2D COSY ^{1}H NMR spectra of M2 and M3. In both cases of M2 and M3, the basic phthalate spectral features were retained as indicated by the aromatic proton signals (ca. δ 7.51–7.75) for phthalate ring. However, a broad signal was observed at δ 13.19 and 13.65 for M2 and M3, respectively, which indicated the possible presence of a carboxyl proton in either case. In deuterium oxide exchange for both samples, the observed peaks disappeared supporting the labile proton in M2 and M3. In case of M2, no signals were observed for the benzyl group but signals for the alkyl protons of BBP were retained while for M3, no signal was detected for the alkyl protons of BBP but the signals corresponding to the protons of the benzyl group are re-

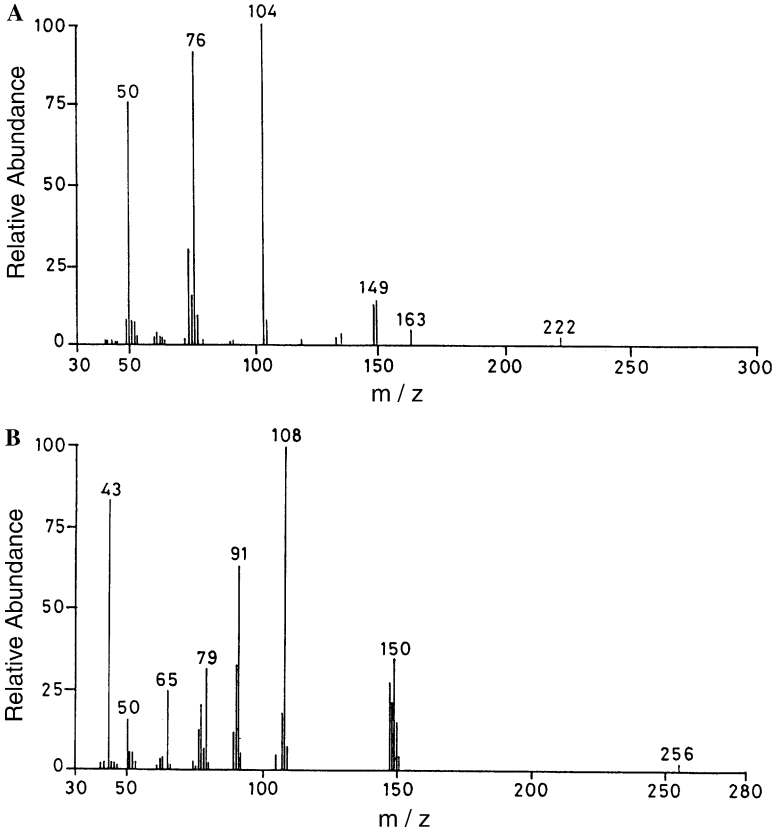

Fig. 2. Mass fragmentation patterns of the biodegraded products M2 (A) and M3 (B) of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818.

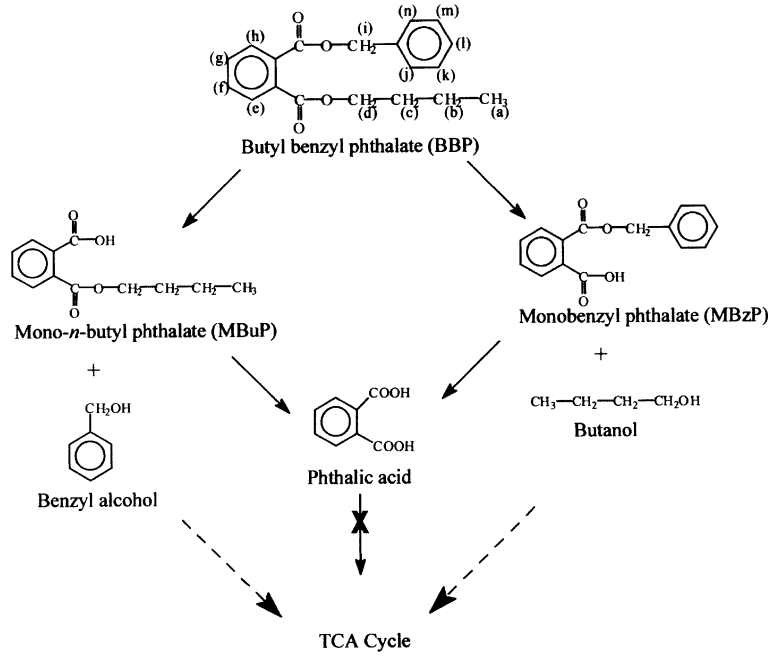

Fig. 3. Metabolic pattern of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818.

tained. The ^{1}H NMR data for M2 are: H_a (t, 3H, ca. δ 0.82–0.91), H_b (h, 2H, ca. δ 1.32–1.40), H_c (q, 2H, ca. δ 1.59–1.65), H_d (t, 2H, ca. δ 4.18–4.20), H_{e-g} (m, 3H, ca. δ 7.59– 7.60), H_h (m, 1H, ca. δ 7.71–7.73), and $H_{carboxyl}$ (br. s, 1H, δ 13.19). In case of M3, ^{1}H NMR data obtained are: H_i (s, 2H, δ 5.26), H_{j-n} (m, 5H, ca. δ 7.31–7.42), $H_{f,g}$ (m, 2H, ca. δ 7.60–7.65), H_e (m, 1H, ca. δ 7.69–7.70), H_h (m, 1H, ca. δ 7.74–7.76), and $H_{carboxyl}$ (br. s, 1H, δ 13.65). The protons assigned above were indicated in the structure of BBP (Fig. 3).

Hydrolysis of butyl benzyl phthalate in vivo

The test organism neither utilizes PA as growth substrate nor can degrade or transform PA when incubated with resting cell suspension of BBP grown cells. Growth of the test organism on BBP is solely due to the utilization of side-chain alcohols of the BBP hydrolyzed products, i.e., benzyl alcohol and *n*-butanol (Fig. 3). While benzyl alcohol was degraded via benzaldehyde, benzoic acid, catechol, and *cis*,*cis*-muconic acid (data not shown). Quantitative analysis of the hydrolyzed products isolated from BBP-grown spent cultures at different times is presented in Table 1. It may be noted that the extraction efficiency of BBP was found to be $85 \pm 4\%$ while for MBuP, MBzP, and PA it was estimated as $98 \pm 1\%$ under the standard assay conditions. It has been observed that mono-*n*-butyl and monobenzyl phthalates are the major accumulated products. The molar concentration of the monoesters of BBP together with the trace amount of PA and non-degraded BBP, if any, corresponds to the total BBP added as substrate in the growing culture. The molar ratio of mono-*n*-butyl to monobenzyl phthalates was found to be close to 1:2 throughout the degradation process while BBP was completely degraded by 96 h of incubation. The rate of hydrolysis of the diester or the monoesters to PA is very low and only 3.8% (in μmol) of the latter was formed

Table 1
Biodegradation of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818

Substrate[a]/metabolite	Amount of metabolites (μmol)[b] accumulated from BBP grown culture with time (h)							
	24	36	48	72	84	96	120	168
Butyl benzyl phthalate	62.4	60.5	53.6	45.5	27.7	ND[c]	ND	ND
Mono-*n*-butyl phthalate	2.2	2.7	5.4	8.2	14.3	25.0	24.5	23.9
Monobenzyl phthalate	3.9	4.5	9.7	15.1	29.1	50.2	49.9	49.3
Phthalic acid	0.2	0.4	0.6	0.8	1.6	3.1	4.1	4.5

[a] Substrate concentration: 1 g/L in 25 ml of medium contains 80 μmol of butyl benzyl phthalate.
[b] Each value is the average of triplicate determinations.
[c] Not detected.

after 4 days of incubation when BBP was totally hydrolyzed, while 5.6% (in μmol) of PA was formed after 7 days of incubation. However, no significant change in the composition of the reaction products was observed on further incubation of the culture. Reports on the hydrolysis of phthalic acid diesters to phthalic acid with the intermediate accumulation of phthalic acid monoester in the culture medium are not many [32,33]. However, in those cases, phthalic acid was further metabolized via protocatechuic acid by a ring cleavage dioxygenase. Monoethyl phthalate and PA were also observed following biodegradation of diethyl phthalate by *Micrococcus* sp. and *Aureobacterium saperdae* NRRL B-14840 [40,41] while an alternative pathway was proposed for the degradation of diethyl phthalate in soil contaminated with methanol [25]. However, phthalic acid monoesters detected in river water were attributed to sewage discharge or microbial degradation of phthalic acid diesters [36]. But, in the microbial metabolism of DBP by a denitrifying bacterium, MBuP and PA were obtained as dead-end products [42].

Hydrolysis of butyl benzyl phthalate in vitro

Crude enzyme preparations from BBP grown cells of *Gordonia* sp. can hydrolyze BBP to MBuP, MBzP, and to a minor extent to PA. Hydrolysis of the monoesters of BBP to PA has also been detected when MBuP and MBzP were used individually as substrate with crude enzyme preparations of BBP grown cells. On the other hand, cell-free extract of glucose grown cells can transform BBP to a minor extent to its monoesters and PA while the latter has not been detected when MBuP and MBzP were used individually as substrate. Table 2 summarizes the esterase activity of *Gordonia* sp. towards BBP, MBuP, MBzP, and *p*-nitrophenyl acetate. Moreover, all the seven phthalate esters used in this study were transformed to their respective monoesters irrespective of their utilization as sole carbon source in mineral salt medium (Table 3) indicating broad substrate specificity of the esterase. Formation of phthalate monoesters was validated by the TLC analysis of the transformed products, which gave R_f values identical to those given by authentic phthalate monoesters.

Table 2
Specific activities of esterase(s) from cell-free extracts of *Gordonia* sp. strain MTCC 4818 grown on butyl benzyl phthalate vis-à-vis glucose

Substrate[a]	Esterase activity[b]	
	Butyl benzyl phthalate	Glucose
Butyl benzyl phthalate	30.3×10^{-3}	0.7×10^{-3}
Mono-*n*-butyl phthalate	1.3×10^{-3}	ND[c]
Monobenzyl phthalate	1.1×10^{-3}	ND
p-Nitrophenyl acetate	44.8×10^{-3}	13.5×10^{-3}

[a] Micromoles of substrate hydrolyzed per minute per milligram of protein under standard assay conditions.
[b] Each value is the average of triplicate determinations.
[c] Not detected.

Table 3
Phthalate esters used as growth substrate vis-à-vis esterase activity of the cell-free extracts of *Gordonia* sp. strain MTCC 4818

Phthalate esters	Growth	Esterase activity[a]
Dimethyl phthalate (DMP)	–	+
Di-*n*-butyl phthalate (DBP)	+	+
Di-iso-butyl phthalate (DIBP)	+	+
Di-(2-ethylhexyl) phthalate (DEHP)	–	+
Dicyclohexyl phthalate (DCHP)	–	+
Butyl benzyl phthalate (BBP)	+	+
Diphenyl phthalate (DPhP)	+	+

[a] Cell-free extracts of BBP grown cells were used for esterase activity.

Induction of esterase

The relationship between esterase induction and BBP degradation was examined with *Gordonia* sp. strain MTCC 4818 culture grown in the presence and absence of BBP and chloramphenicol. The culture grown with constant exposure to BBP was highly induced for BBP degradation. Under resting cell transformation, these cultures hydrolyzed 12% of BBP within 6 h, 34% within 24 h, and 56% within 96 h at 28 °C. Chloramphenicol, an inhibitor of bacterial protein synthesis, was added initially to cultures pre-grown with or without BBP to determine whether BBP-hydrolyzing enzyme(s) were constitutive or inducible. Cells grown for 2 days in the presence of glucose showed a longer lag phase in the hydrolysis when exposed to BBP and plateaued at 6% hydrolysis within 96 h. Induced cultures that were dosed with chloramphenicol at 0 h hydrolyzed 31% of the BBP during the first 24 h of exposure. However, further BBP hydrolysis by these cultures was not observed after 24 h. In contrast to induced cultures, the addition of chloramphenicol at 0 h to non-induced cultures inhibited BBP hydrolysis to less than 0.2% in 96 h. In both experiments, no abiotic hydrolysis of BBP was detected in sterile controls.

Moreover, in the cell-free enzyme preparations, 43- and 3.3-fold increase in esterase activity towards BBP and *p*-nitrophenyl acetate, respectively, has been observed (Table 2) when the cultures were grown in the presence or absence of BBP. On the other hand, the test organism grown on glucose in the presence of MBuP or MBzP when tested for BBP or its monoester hydrolysis showed that only the former culture enhanced BBP hydrolysis and also furnished trace amount of PA from both MBuP or MBzP. These observations indicate that BBP and/or MBuP may be the inducers of the esterase(s) responsible for the hydrolysis of BBP and its monoesters. Esterase purified from *Micrococcus* sp.

YGJ1 utilizing dialkyl phthalates as carbon source without any accumulation of monoalkyl phthalate in spent medium did not show hydrolytic activity on monoalkyl phthalate indicating the possibility of another esterase involved in the degradation of monoalkyl phthalates [43]. However, in this study, it is imperative that the esterase(s) involved in the hydrolysis of BBP to PA via MBuP and MBzP be inducible in nature. Esterases hydrolyzing di- and monoalkyl phthalates from *Nocardia* isolates were also shown to be induced by phthalate esters while the monoalkyl phthalate esterases of *Pseudomonas* and *Arthrobacter* isolates were reported to be induced by free phthalic acid [33]. In contrast, esterase involved in the phthalate ester catabolic pathway in *Arthrobacter keyseri* 12B was found to be constitutive in nature [24]. Thus, the metabolism of alkyl aryl phthalate by pure bacterial culture tested in this work is similar to the mammalian one with the accumulation of monoesters of phthalates [21,44]. Further studies on the genetic and biochemical characterization of esterase involved in the initial degradation of BBP from this bacterium may allow us to understand the structure–function relationship of this enzyme to its mammalian counterpart, which are in progress.

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