Metabolic cooperation of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the utilization of butyl benzyl phthalate: effect of a novel co-culture in the degradation of a mixture of phthalates

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Degradation of butyl benzyl phthalate (BBP) by a co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY was investigated. In the degradation of BBP by the co-culture, the limitations of the individual species in metabolizing BBP were overcome, leading to the development of a consortium capable of complete utilization of this ester. In the degradation of BBP by the co-culture, the presence of multiple esterases was demonstrated in both species by activity staining of non-denaturing gels, indicating their roles in the degradation process. The esterases were found to be inducible, with unique or broad substrate specificities towards BBP and its monoesters. Moreover, a number of catabolic enzymes other than esterases identified in the metabolism of BBP-degraded intermediates facilitated the co-culture-mediated degradation process. The versatility of the co-culture was further established by the rapid and complete degradation of a mixture of phthalate esters of environmental concern.

INTRODUCTION

Phthalic acid esters (PAEs), commonly called phthalates, are a family of multifunctional chemicals that are widely used as plasticizers and in a variety of personal and consumer products (Nassberger *et al.*, 1987; Bradbury, 1996; Koo *et al.*, 2002). Structures of the most commonly used phthalate esters are depicted in Fig. 1; these include diethyl phthalate (DEP), di-n-butyl phthalate (DBP), diisobutyl phthalate (DIBP), butyl benzyl phthalate (BBP), diisononyl phthalate (DINP) and di-n-octyl phthalate (D-n-OP) (Koch *et al.*, 2003). These plasticizers have been shown to be eluted at a constant rate from plastic products to the environment (Latini, 2005). Consequently, they are widely distributed in the environment and have been described as being among the most abundant man-made environmental pollutants, accounting for a number of potential causes of human health problems including developmental and testicular toxicity as well as antiandrogenic and teratogenic effects (Bower *et al.*, 1970; Ema *et al.*, 1995; Soto *et al.*, 1995; Harris *et al.*, 1997; Nakai

et al., 1999; Aldert *et al.*, 2000; Uriu-Adams *et al.*, 2001; Liu & Chen, 2006).

In both aquatic and terrestrial systems, microbial action is thought to be the principal mechanism of PAE degradation (Staples *et al.*, 1997). Micro-organisms that degrade PAEs may be aerobic (Wang *et al.*, 1995), anaerobic (Wang *et al.*, 2000) or facultative (Zhang & Reardon, 1990). Previous studies have shown that the micro-organisms largely utilize an aerobic pathway for PAE degradation and the major enzymes that have been identified to catabolize phthalate esters include esterase(s), as well as enzymes to metabolize the side-chain alcohols and phthalic acid resulting from PAE-hydrolysis (Wang *et al.*, 2000; Kurane *et al.*, 1984; Ribbons *et al.*, 1984; Roslev *et al.*, 1998; Chatterjee *et al.*, 2005).

There are reports of isolation of several micro-organisms from the environment with the ability to degrade PAEs individually (Karegoudar & Pujar, 1984; Kurane, 1986; Nomura *et al.*, 1992). Degradation of BBP, an alkyl aryl phthalate, necessitates diverse metabolic machinery involving sets of distinct degradative genes. Thus, most of the literature on the degradation of BBP describes more than one bacterial species or undefined consortia, and very little is known about the role of individual organisms in BBP assimilation (Yuan *et al.*, 2002; Chang *et al.*, 2004; Li *et al.*, 2005; Xu *et al.*, 2006). On the other hand, several PAEs are

Abbreviations: BBP, butyl benzyl phthalate; DBP, di-n-butyl phthalate; DCHP, dicyclohexyl phthalate; DEHP, di-(2-ethylhexyl) phthalate; DEP, diethyl phthalate; DIBP, diisobutyl phthalate; DINP, diisononyl phthalate; DMP, dimethyl phthalate; D-n-OP, di-n-octyl phthalate; DPP, diphenyl phthalate; MBuP, monobutyl phthalate; MBzP, monobenzyl phthalate; PAE, phthalic acid ester; *p*-NPA, *p*-nitrophenyl acetate; TCA, tricarboxylic acid.

The modifying groups, R_1 and R_2 are either methyl, ethyl, *n*-butyl, isobutyl, 2-ethyl hexyl, *n*-octyl, isooctyl, isononyl, isodecyl, cyclohexyl, phenyl or benzyl

Fig. 1. Chemical structure of phthalates commonly present in the environment.

often simultaneously present in the environment (Yuan *et al.*, 2002), and there is a surprising lack of information on the degradation of mixtures of PAEs (Yuan *et al.*, 2002; Chang *et al.*, 2004).

Our interest in the biodegradation of PAEs led us to isolate and characterize bacterial species that can degrade BBP. Among the species isolated, we have already reported that *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. WY could utilize BBP individually as sole source of carbon and energy (Chatterjee & Dutta, 2003, 2008). However, the growth of *Gordonia* sp. strain MTCC 4818 on BBP was supported solely by the utilization of BBP-hydrolysed alcohols, resulting in the accumulation of monobutyl phthalate (MBuP) and monobenzyl phthalate (MBzP) in the culture medium. Moreover, a small amount of phthalic acid generated due to inadequate hydrolysis of the phthalic acid monoesters was accumulated as a dead-end product in the degradation of BBP by the *Gordonia* strain (Chatterjee & Dutta, 2003). On the other hand, degradation of BBP by the *Arthrobacter* sp. strain WY was quite slow and the growth of the organism was observed to be based on the utilization of BBP-hydrolysed phthalic acid only, leaving the BBP-hydrolysed alcohols unutilized in the degradation process (Chatterjee & Dutta, 2008). Thus, both the *Gordonia* and the *Arthrobacter* strain were found to be metabolically restricted in their ability to assimilate the alkyl aryl phthalate completely (Chatterjee & Dutta, 2003, 2008). In the present study, a co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY was used for complete utilization of BBP, involving esterases and other enzymes in metabolic cooperation. Further, the co-culture was examined for its potential in the degradation of a mixture of phthalate esters widely present in the environment.

METHODS

Chemicals. All the phthalate diesters used in this study were of commercial grade and more than 98 % pure. Butyl benzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DIBP), dicyclohexyl

phthalate (DCHP), 1-butanol, catechol, *cis,cis*-muconic acid and protocatechuate were obtained from Merck. Di-(2-ethylhexyl) phthalate (DEHP), di-*n*-octyl phthalate (D-*n*-OP), benzyl alcohol, benzoic acid and phthalic acid were purchased from Sigma-Aldrich; diphenyl phthalate (DPP) was obtained from Hi-Media. The phthalate monoesters were prepared in the laboratory as described earlier (Chatterjee & Dutta, 2003). All other chemicals and reagents were of analytical grade and used without further purification.

Organism and culture conditions. The organisms, *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY, used in the present study were isolated from contaminated soil by enrichment culture technique with BBP as the sole source of carbon and energy (Chatterjee & Dutta, 2003, 2008). Cells of the individual organisms were grown in liquid minimal salt medium (pH 7.0) containing (l^{-1}) K_2HPO_4 , 3.34 g; NaH_2PO_4 , 0.87 g; NH_4Cl , 2.0 g; nitritotriacetic acid, 123 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $FeSO_4 \cdot 7H_2O$, 12 mg; $MnSO_4 \cdot H_2O$, 3 mg; $ZnSO_4 \cdot H_2O$, 3 mg and $CoCl_2 \cdot 6H_2O$, 1 mg, supplemented with BBP, 1 g l^{-1} (aqueous solubility, 2.7 mg l^{-1}). Solid media contained 2 % agar (HiMedia).

The inoculum for the co-culture was grown in a 250 ml Erlenmeyer flask containing 49 ml liquid mineral salt medium supplemented with 1 g BBP l^{-1} as sole carbon source and inoculated with 0.5 ml (OD_{660} 1.0) each of freshly grown culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY. Experimental co-cultures were grown in 250 ml Erlenmeyer flasks at 28 °C on a rotary shaker (180 r.p.m.) containing 49.5 ml liquid mineral salt medium supplemented with 1 g BBP l^{-1} as the sole carbon source and inoculated with 0.5 ml (OD_{660} 1.0) of a freshly grown inoculum co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY on BBP (1 g l^{-1}). At different time intervals, the contents of triplicate flasks were harvested to measure the growth of the co-culture (OD_{660}) and the cell density of the individual isolates in the co-culture by plate count, and also to analyse the detectable metabolites and the residual BBP present.

For resting cell transformations, cells grown in liquid mineral salt medium were harvested in the late-exponential phase by centrifugation at 8000 g for 10 min, washed twice, each time with an equal volume of potassium phosphate buffer (50 mM, pH 7.0), and finally resuspended in the same buffer to an OD_{660} of 1.0. BBP and possible pathway intermediates (1 g l^{-1}) were added individually to washed cell suspensions, and incubated at 28 °C for different time periods up to a maximum of 48 h. Unless stated otherwise, each experiment was performed in triplicate.

Degradation of mixture of phthalates by the co-culture. A stock of a PAE mixture (DMP, DEP, DBP, DIBP, DEHP, D-*n*-OP, DPP, DCHP and BBP) was prepared by the addition of 1 g equivalent of each PAE in an amber-coloured bottle, mixing thoroughly until the solid substrates dissolved completely. The co-culture was grown in 250 ml Erlenmeyer flasks containing 49.5 ml of liquid mineral salt medium supplemented with 1 g l^{-1} of PAE mixture and inoculated with 0.5 ml (OD_{660} 1.0) of a freshly grown co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY on the PAE mixture. The co-culture was incubated at 28 °C on a rotary shaker (180 r.p.m.) for 7 days. At different time intervals, the contents of triplicate flasks were harvested to measure the growth of the co-culture (OD_{660}) and the cell density of the individual isolates in the co-culture by plate count and also to analyse the residual PAE components present.

Preparation of cell-free extracts. Cells grown in mineral salt medium in presence of BBP or glucose (1 g l^{-1}) were harvested at the mid-exponential phase by centrifugation at 8000 g for 10 min at 4 °C. The pellet was washed twice with 10 vols 50 mM Tris/HCl buffer pH 8.0 and resuspended in an equal volume of either 50 mM

Tris/HCl buffer (pH 8.0) or 50 mM potassium phosphate buffer (pH 7.0) as desired. The cell suspensions were subjected to seven ultrasonic pulses generated with a sonicator (Labsonic-L, Braun Biotech International) for a total of 2 min at 4 °C. The resulting cell homogenates were centrifuged at 20 000 g for 20 min at 4 °C. The supernatants were used as sources of enzymes.

Non-denaturing gel and activity staining for esterase. BBP-grown cell-free extracts of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY were applied to non-denaturing 10% polyacrylamide gels prepared without SDS (Sambrook *et al.*, 1989). Esterase staining was carried out by incubating the gel for 20 min at 28 °C in substrate buffer mixture. The mixture contained 0.5% (w/v) Fast Blue BB salt (Hi-Media) and 5.38 mM α -naphthyl acetate (SRL, India) in 50 mM Tris/HCl buffer, pH 7.0 (Niazi *et al.*, 2001). Essentially, the esterase(s) present in the cell-free extract hydrolyse(s) α -naphthyl acetate to furnish α -naphthol, which further reacts with the Fast Blue BB salt to form a complex giving brownish colour bands to the sites of activity.

Isolation of esterases from native gel. After preparative gel electrophoresis, a strip of the gel was cut and stained for its esterase activity. The non-stained gel strips corresponding to esterase bands were cut, suspended in 3 vols 50 mM Tris/HCl buffer (pH 7.0), and homogenized in a glass tissue homogenizer. The supernatant was collected following centrifugation at 8000 g for 15 min at 4 °C, lyophilized and stored at -20 °C for further studies.

Enzyme assays. Esterase activity towards *p*-nitrophenyl acetate (*p*-NPA) was measured at 25 °C from the increase in A_{400} ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$) (Pocker & Stone, 1967). The reaction mixture contained an appropriate amount of enzyme in 50 mM potassium phosphate buffer (pH 7.0) and 100 μl *p*-NPA (25 mM in the same buffer) in a total volume of 1 ml. The hydrolytic activity of the cell-free extracts and the eluted proteins from gels towards phthalate diesters and monoesters was assayed at 28 °C for 30 min. The reaction mixture contained 60 μM substrate in 50 mM potassium phosphate buffer (pH 7.0) with an appropriate amount of enzyme in a final volume of 1 ml. The reaction was stopped by adding 50 μl HCl (2 M) and the solution was extracted three times, each time with an equal volume of ethyl acetate. The extract was evaporated and the residue was dissolved in 40% methanol and an aliquot (25 μl) was subjected to HPLC to measure the amount of phthalate monoesters, phthalic acid and residual phthalate diester present. Quantitative estimation of the individual components was made from a standard curve of the respective component created by HPLC under identical conditions. The activity of catechol-1,2-dioxygenase was assayed spectrophotometrically by monitoring the increase in A_{260} due to the formation of *cis,cis*-muconic acid (Ngai *et al.*, 1990). Further metabolism of *cis,cis*-muconate by the crude extract was inhibited by the addition of EDTA disodium salt (1 mM final concentration) to the reaction mixture. Transformation of *cis,cis*-muconate by the cell-free extract was also measured spectrophotometrically by monitoring the decrease in A_{260} by *cis,cis*-muconate lactonizing enzyme. A molar absorption coefficient of $16\,800 \text{ M}^{-1} \text{ cm}^{-1}$ for *cis,cis*-muconate was used to calculate the enzyme activities (Dorn & Knackmuss, 1978). The activity of protocatechuate *ortho*-cleavage dioxygenase was assayed spectrophotometrically by monitoring the decrease in absorption maxima at 250 and 290 nm due to the formation of β -carboxy-*cis,cis*-muconate (Iwagami *et al.*, 2000; Paul *et al.*, 2004). One unit of enzyme activity was defined as the amount which degraded 1 μmol substrate min^{-1} under the assay conditions, and the specific activity was defined as units of enzyme activity (mg protein^{-1}). Protein was measured by the Lowry method with BSA as the standard.

Isolation of metabolites. The bacterial cultures or the incubation mixtures of resting cells were centrifuged at 8000 g for 10 min and the supernatants were acidified to pH 1.5–2.0 with 2 M HCl. These were extracted three times, each time with an equal volume of ethyl acetate. Following incubation and hydrolysis of BBP, MBuP and MBzP by the gel-eluted esterases, the reaction mixtures were also acidified and extracted similarly. The extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure in a rotary evaporator (Hahn Shin Science Co.). In the case of the biodegraded samples of the mixture of phthalates, the extracts were spiked individually with 0.2 mg equivalent of phenanthrene (as external standard) dissolved in ethyl acetate and evaporated under reduced pressure.

Analyses. Unconverted BBP and its metabolites obtained from spent culture and resting cell incubation mixture as well as the biotransformed products of various phthalate esters obtained from incubation with the cell-free extracts and the gel-eluted esterases of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY were resolved by TLC on silica gel GF₂₅₄ (Merck) plates using benzene/acetic acid (97:3, v/v) as the solvent system and detected under UV light at 254 nm. The identities of resolved products were determined occasionally by comparing with reference compounds developed identically.

Further, the unconverted BBP and its metabolites were resolved by HPLC using an analytical Nova-pak C₁₈ reverse-phase column (3.9 $\times$ 150 mm, particle size 4 μm , pore size 60 Å, end-capped) 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, v/v) as the solvent system at a flow rate of 1 ml min^{-1} and detected by a Waters 486 tunable absorbance detector at 254 nm. Metabolites were identified by comparison of their retention times with those of the authentic compounds or by co-elution under the same set of conditions. Yield of the metabolites was calculated using the Millennium Session Manager software package (version 2.15.01).

GC-MS analysis of the degradation products of BBP and phthalate mixtures was performed using a Varian 3800 GC apparatus equipped with a 30 $\text{m} \times 0.25 \text{ mm}$ (0.25 μm film thickness) HP-5MS capillary column and attached to a Saturn-2000 mass spectrometer (Varian). The temperature programme was held at 70 °C for 1.5 min, increased to 200 °C at an increment of 10 °C min^{-1} , further increased to 280 °C at an increment of 5 °C min^{-1} , and then held for 10 min at 280 °C. The injection volume was 1 μl , and the carrier gas was helium at a flow rate of 1.5 ml min^{-1} . The mass spectrometer was operated at an electron ionization energy of 70 eV. Instrumental library searches, comparison with authentic compounds and mass fragmentation patterns were used to identify the metabolites. Biodegradation of the mixture of phthalates was monitored by analysis of the individual residual phthalate esters using phenanthrene as an external standard, where all samples were dissolved in an equal volume of ethyl acetate prior to GC-MS analysis. A parallel non-biodegraded control of phthalate mixture was extracted and processed as above. Quantitative estimation by GC-MS analysis of the individual phthalates in the biodegraded samples of phthalate mixture was made relative to the peak area of the non-biodegraded control after normalization of the peak areas of each analysis with the external standard.

Spectral determinations. Enzyme-catalysed transformation of various substrates was monitored by recording changes in UV-visible spectra of the compounds using a Cary 100 Bio UV-visible spectrophotometer (Varian). Reactions were scanned in the range 220–400 nm for 0–30 min. Data were analysed by the Varian Cary Win UV Scan application software.

RESULTS AND DISCUSSION

Biodegradation of BBP by a mixed culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. WY

Gordonia sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY were previously reported to utilize BBP individually as sole source of carbon and energy, but both strains were found to be metabolically restricted in terms of their ability to degrade BBP completely (Chatterjee & Dutta, 2003, 2008). To compensate the individual metabolic deficiencies of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the degradation of BBP, co-culture of these two strains was used to develop a competent degradative system for BBP and other potential phthalate diesters of environmental concern.

The co-culture was found to utilize BBP completely as sole source of carbon and energy. Apart from BBP, the two strains could utilize a number of intermediate metabolites of BBP degradation (Table 1). The BBP metabolic profiles were evaluated by TLC, HPLC and GC-MS analyses of ethyl acetate extracts of resting cell incubations with BBP of BBP-grown co-cultures. TLC analysis revealed the presence of various polar metabolites in the reaction mixture, which were tentatively identified as MBuP and/or MBzP (R_F 0.4), benzoic acid (R_F 0.56), benzyl alcohol (R_F 0.46) and phthalic acid (R_F 0.03) based on comparison with the R_F values of the respective standard compounds. HPLC and GC-MS analyses of the organic extracts of the resting-cell-mediated BBP degradation products produced by the co-culture showed product profiles similar to the results of TLC analysis and their identities were confirmed by comparing the retention times, coelution profiles and mass fragmentation patterns of the metabolites with those of the authentic compounds analysed under identical conditions (data not shown). In addition, both HPLC and GC-MS analyses could resolve the spot (R_F 0.4) as observed in silica gel G plates into two components that were identified as

Table 1. Utilization of BBP-degraded intermediate metabolites by *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY individually as sole carbon source

+, Growth positive; –, growth negative.

Substrate	MTCC 4818	WY
Phthalic acid	–	+
Protocatechuic acid	+	+
Benzyl alcohol	+	–
Benzoic acid	+	–
Catechol	–	–
<i>cis,cis</i> -Muconic acid	–	–
<i>n</i> -Butanol	+	–
Monobutyl phthalate	–	+
Monobenzyl phthalate	–	+

MBuP and MBzP by both HPLC and GC-MS analyses. Thus the metabolites so characterized upon degradation of BBP by the co-culture support the BBP degradation profiles of the individual members (Chatterjee & Dutta, 2003, 2008) of the consortium.

BBP and metabolite concentrations during co-cultivation of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY and the growth of the co-culture are presented in Fig. 2(a). The co-culture was found to degrade BBP completely by 72 h while the growth of the bacteria reached stationary phase at 108 h. During growth, very low concentrations of BBP-hydrolysed products and other metabolic intermediates accumulated in the spent culture, indicating continuous utilization of intermediate metabolites by the co-culture during incubation. Although the monoesters MBuP and MBzP were found in relatively higher amounts during the first 36 h of incubation, with

Fig. 2. (a) Time-courses of BBP and metabolite concentrations during co-cultivation of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY and growth of the co-culture in mineral salt medium containing 1 g BBP l⁻¹. ▲, Growth of the co-culture (OD₆₆₀); ○, BBP concentration. Concentrations of metabolites: ●, MBuP; □, MBzP; △, phthalic acid; ■, benzoic acid. (b) Growth dynamics of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the co-culture utilizing BBP as sole carbon source. The initial amount of BBP in 50 ml medium was 140 μmol. Each value is the mean of triplicate determinations with the standard deviation in the range of ±5% of the mean.

time these monoesters were depleted and their degradation was complete by 108 h. The BBP-hydrolysed product benzyl alcohol was not detected in the reaction medium and was presumed to be immediately transformed *in vivo* by the action of alcohol and aldehyde dehydrogenases of the co-culture to benzoic acid, which could be detected early in the culture but was completely degraded by 108 h of incubation. The other BBP-hydrolysed product, 1-butanol, was not detected under the analytical conditions used but it was reported previously to be metabolized via butyraldehyde and butyric acid leading to the tricarboxylic acid (TCA) cycle using the β -oxidation pathway (Chatterjee *et al.*, 2005).

Fig. 2(b) depicts the individual growth of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the co-culture utilizing BBP as sole carbon source. It may be mentioned here that the pigmentation patterns of strains MTCC 4818 and WY in MSM utilizing phthalate diester(s) as sole carbon source or on Luria agar plates are peach and creamy white, respectively, which allows the growth of the individual species from the co-culture to be distinguished. The growth profile (Fig. 2b) of the individual species indicated an exponential phase of growth of both strains in the co-culture. The density of the *Gordonia* strain was greater than that of *Arthrobacter* strain throughout the incubation. However, the ratio of the *Gordonia* strain was higher during the initial hours of incubation in comparison to the later stages of the degradation process, indicating its ability to metabolize BBP more rapidly than the other member of the co-culture and subsequent utilization of the hydrolysed alcohols for its growth.

Spectrophotometric determination of enzyme activity in the metabolism of protocatechuate and catechol

Utilization of phthalic acid by *Arthrobacter* sp. strain WY and its metabolism via protocatechuic acid followed by the *ortho*-cleavage pathway, ultimately leading to the TCA cycle, was reported earlier (Chatterjee & Dutta, 2008). *Gordonia* sp. strain MTCC 4818 was reported to be unable to metabolize phthalic acid or utilize it as a growth substrate (Chatterjee & Dutta, 2003), but could utilize protocatechuic acid for growth (Table 1). The strain could metabolize protocatechuic acid *in vitro* by protocatechuate 3,4-dioxygenase as determined by spectral changes [specific activity $104 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], similar to *Arthrobacter* sp. strain WY (Chatterjee & Dutta, 2008). On the other hand, strain WY could not utilize catechol (Table 1) as sole carbon source but could transform catechol *in vitro* by catechol 1,2-dioxygenase [specific activity $142 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] followed by *cis,cis*-muconate lactonizing enzyme [specific activity $3.4 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], similar to *Gordonia* sp. strain MTCC 4818 (Chatterjee & Dutta, 2003). Metabolism of catechol and *cis,cis*-muconic acid by strain WY may be attributed to the broad substrate specificities of the

enzymes involved in protocatechuate metabolism. Very similar specific activity profiles of protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase and *cis,cis*-muconate lactonizing enzyme activities were observed by the cell-free extract of BBP-grown co-culture. Thus, combined effects appear to be involved in the metabolism of the intermediates protocatechuic acid and catechol in the degradation of BBP by the co-culture.

Analysis of esterases

In the bacterial degradation of phthalate diesters, esterases are the most important class of enzymes, accounting for the hydrolysis of the ester bond(s) to furnish monoester(s), phthalic acid and the hydrolysed alcohols for further utilization. To understand the involvement of esterase(s) in the degradation of BBP by the co-culture, presence of hydrolytic enzymes in *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY was analysed individually by activity staining (Fig. 3). Two distinct activity bands (E1 and E2) were observed in the cell-free extract of BBP-grown cells of the *Gordonia* strain analysed on non-denaturing gel stained for esterase (Fig. 3, lane 2). A single band of similar mobility to that of E2 with much lower intensity was observed in the extract of glucose-grown cells of the *Gordonia* strain, indicating the inducible nature of both the esterases. On the other hand, three distinct activity bands (E3, E4 and E5) were observed in the extract of BBP-grown cells of *Arthrobacter* sp. strain WY (Fig. 3, lane 3), which were absent in the extract of glucose-grown cells of the strain. However, a distinct activity band (E6) of faster mobility was observed in the extract of glucose-grown cells of strain WY, which was absent in the extract of BBP-grown cells of this strain, indicating substrate-specific

Fig. 3. Esterase profile in native PAGE from cell-free extracts of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY. Lanes 1 and 2, cell-free extract of *Gordonia* sp. strain MTCC 4818 grown in the presence of glucose (1) and BBP (2); lanes 3 and 4, cell-free extract of *Arthrobacter* sp. strain WY grown in the presence of BBP (3) and glucose (4). Each lane contained $15 \mu\text{g}$ protein.

inducible esterases. It may be mentioned here that the activity bands (E1, E2, E3, E4 and E5) were observed in the cell-free extract of BBP-grown co-culture, indicating the expression of all five essential esterases under co-culture conditions.

Substrate specificity and activity profiles of all the BBP-inducible esterases of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY, isolated from the native polyacrylamide gel, were determined individually towards *p*-NPA, BBP, MBuP and MBzP as substrate (Table 2). Band E1 of strain MTCC 4818 showed 5-fold greater BBP-hydrolytic activity than band E2; the latter, on the other hand, showed almost 2-fold greater activity with MBuP as substrate as compared to band E1. Interestingly, band E2 showed no activity with MBzP as substrate, which was only very weakly hydrolysed by band E1. On the other hand, the activity band of slowest mobility (E3) of strain WY showed nearly 2- and 10-fold greater hydrolytic activity with BBP as the substrate than bands E5 and E4, respectively. When MBuP was used as the substrate, bands E3, E4 and E5 showed higher hydrolytic activity as compared to E1 and E2. For hydrolysis of MBzP, both bands E4 and E5 showed reasonably good activity while band E3 did not show any activity with this substrate. The activity with *p*-NPA, a universal substrate for esterases, was very similar for all the gel-eluted esterases.

The *in vitro* activity profiles of the esterases indicated that BBP-hydrolysing activity of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY was similar, but the slow *in vivo* degradation of BBP by strain WY might be due to the poor transport behaviour of the hydrophobic substrate (Chatterjee & Dutta, 2008). The poor hydrolysing activity of the esterases present in the extract of BBP-grown cells of *Gordonia* sp. with the substrates MBuP and MBzP correlated with the accumulation of these monoesters in the spent culture (Chatterjee & Dutta, 2003). On the other

hand, much higher monoester-hydrolysing activity in the extract of BBP-grown cells of *Arthrobacter* sp. strain WY than that of *Gordonia* sp. strain MTCC 4818 provides the co-culture with a pool of hydrolytic enzymes that can hydrolyse BBP and its monoesters completely. Thus, it is apparent from the results of the *in vivo* degradation of BBP by the co-culture that the strains complemented each other enzymically in the degradation of BBP in the co-culture conditions, with cumulative effects of certain enzyme activities present in both the strains. As a consequence, an increase in growth rate as well as the extent of growth of the co-culture and absence of any detectable metabolite(s) in the spent medium after 108 h of incubation was observed as a result of rapid utilization and complete degradation of BBP by the co-culture. Based on the biochemical analyses, metabolic pathways employed in the degradation of BBP by either of the strains in the co-culture are summarized in Fig. 4.

Degradation of mixture of phthalates by the co-culture

Apart from BBP, *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY could utilize a number of phthalate esters individually as sole source of carbon and energy. Among the phthalate esters tested, DEP, DBP, DIBP, DPP and DCHP (in the pH range 8.2–8.8) were found to be utilized by the *Gordonia* sp., while DMP, DEP, DBP, DIBP, and DCHP were utilized individually by the *Arthrobacter* sp. as sole carbon source. However, the esterases present in the BBP-grown cell-free extract of the *Gordonia* sp. could transform all the phthalate diesters, although the BBP-grown cell-free extract of the *Arthrobacter* sp. was unable to transform DEHP, DPP and D-n-OP. Thus, neither strain can utilize DEHP and D-n-OP as sole carbon source, but the cell-free extract of BBP-grown culture of *Gordonia* sp. can transform both

Table 2. Specific activities of the crude enzymes and gel-eluted enzymes from cell-free extracts of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY grown on BBP

Cells were grown in mineral salt medium in the presence of BBP (1 g l^{-1}). Each value is the mean of triplicate determinations [μmol substrate hydrolysed $\text{min}^{-1} (\text{mg protein})^{-1}$ under standard assay conditions]. ND, Not detectable.

Protein	Esterase activity towards substrate			
	<i>p</i> -NPA	BBP	MBuP	MBzP
MTCC 4818				
Crude	44.2×10^{-3}	29.36×10^{-3}	1.35×10^{-3}	1.12×10^{-3}
E1	4.48	1.88	10.64×10^{-3}	8.2×10^{-3}
E2	3.44	0.366	22.37×10^{-3}	ND
WY				
Crude	33.48×10^{-3}	13.75×10^{-3}	12.27×10^{-3}	11.0×10^{-3}
E3	3.74	1.06	0.385	ND
E4	2.87	0.103	0.134	0.251
E5	3.66	0.674	0.213	0.311

Fig. 4. Suggested pathway for the complete degradation of BBP depicting the role of individual isolates of the co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY. BBP, butyl benzyl phthalate; MBuP, monobutyl phthalate; MBzP, monobenzyl phthalate; *, relatively weak *in vivo* activity.

DEHP and D-*n*-OP *in vitro*. The esterases responsible for the degradation of DEHP or D-*n*-OP are not expressed in the presence of either of the phthalate esters but the BBP-induced esterases possibly have broad substrate specificities to transform DEHP and D-*n*-OP. Formation of phthalate monoesters was validated by TLC analysis of the organic extract of the reaction mixture of the transformation of various individual phthalate diesters by cell-free extracts of BBP-grown cells of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY, which gave R_F values identical to the corresponding authentic phthalate monoesters (data not shown). Based on the ability to utilize and transform various phthalate diesters, it is obvious that the co-culture would be a potential consortium for the degradation of mixed phthalate diesters present in the environment.

The biodegradative potential of the co-culture was validated by using a mixture of nine phthalate esters as carbon source in mineral salt medium incubated at 28 °C. The growth of the consortium and the fate of the individual phthalate diesters with time is shown in Fig. 5(a). Fig. 5(b) shows the growth of individual species in the co-culture during the degradation of the phthalate mixture; the growth pattern is similar to that seen when BBP alone was used as sole carbon source (Fig. 2b). Utilization of the mixture of phthalate esters (total initial concentration 1 g l⁻¹) as carbon source was observed from the growth of the co-culture, where the stationary phase was reached within 5 days of incubation in mineral salt medium. In the degradation of the mixture of phthalates, BBP was rapidly degraded, followed by the low-molecular-mass phthalates, DMP, DEP and DBP, which were totally degraded within 120 h. It took 144 h for

complete degradation of DPP and DIBP. It has been observed that the co-culture-mediated degradation of the high-molecular-mass phthalates, DEHP, DCHP, D-*n*-OP, and the branched-chain phthalate DIBP took relatively longer (Hu & Wan, 2006). Thus, the temporal separation of consumption of different phthalate esters is possibly the consequence of substrate specificity and specific activity of expressed esterase(s) in the co-culture towards various phthalate esters grown on the mixture of phthalates. Apart from that, the temporal separation of consumption of various phthalate esters may be related to their degree of hydrophobicity and permeability factors. The above results support an apparent correlation between increasing length of the side-chain and decreasing biodegradability of phthalates (O'Grady *et al.*, 1985). Recently, degradation of phthalates was monitored individually for *Corynebacterium* sp. and *Sphingomonas* sp. and also as a co-culture of the two and it was observed that the degradation rates were enhanced when the phthalates were present as a mixture as well as under co-culture conditions (Chang *et al.*, 2004). In the present study, a mixture of nine phthalate esters of environmental concern was found to be completely degraded within 7 days by the co-culture. However, not only were the individual strains unable to utilize all the nine phthalate esters individually, but it also took much longer for the co-culture to completely degrade most of the individual phthalate esters (data not shown). Thus, for the enhanced degradation of a phthalate mixture under co-culture condition, it seems that there are cooperative effects allowing the micro-organisms to produce essential enzymes that are not produced by either of the strains alone or in the presence of a single substrate.

Fig. 5. (a) Time-courses of individual phthalate ester concentration during co-cultivation of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY and growth of the co-culture in mineral salt medium containing 1 g l⁻¹ of phthalate mixture. ◆, Growth of the co-culture (OD₆₆₀). Degradation of individual phthalate esters with time (per cent remaining): ○, DMP; ●, DEP; □, DBP; ■, DEHP; △, DIBP; ▲, BBP; ▽, DPP; ▼, DCHP; ◇, DOP. (b) Growth dynamics of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the co-culture utilizing a mixture of phthalates as sole carbon source. The initial culture (50 ml) contained equivalent amounts of the nine different phthalate esters with a total final concentration of 1 g l⁻¹. Each value is the mean of triplicate determinations with the standard deviation in the range of ±5% of the mean.

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