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Jaykishan Songara
Division of Biological and Life Sciences, Ahmedabad University, Ahmedabad, Gujarat, India

Rishi Shanker
Division of Biological and Life Sciences, Ahmedabad University, Ahmedabad, Gujarat, India

Nirbhay Kumar Singh
Department of Microbiology, C.P. College of Agriculture, S.D. Agricultural University, Sardarkrushinagar, Gujarat, India

Correspondence
Jaykishan Songara
Division of Biological and Life Sciences, Ahmedabad University, Ahmedabad, Gujarat, India

Transformation of benzyl butyl phthalate by *Pseudomonas putida* and photocatalytic ZnO nanoparticles

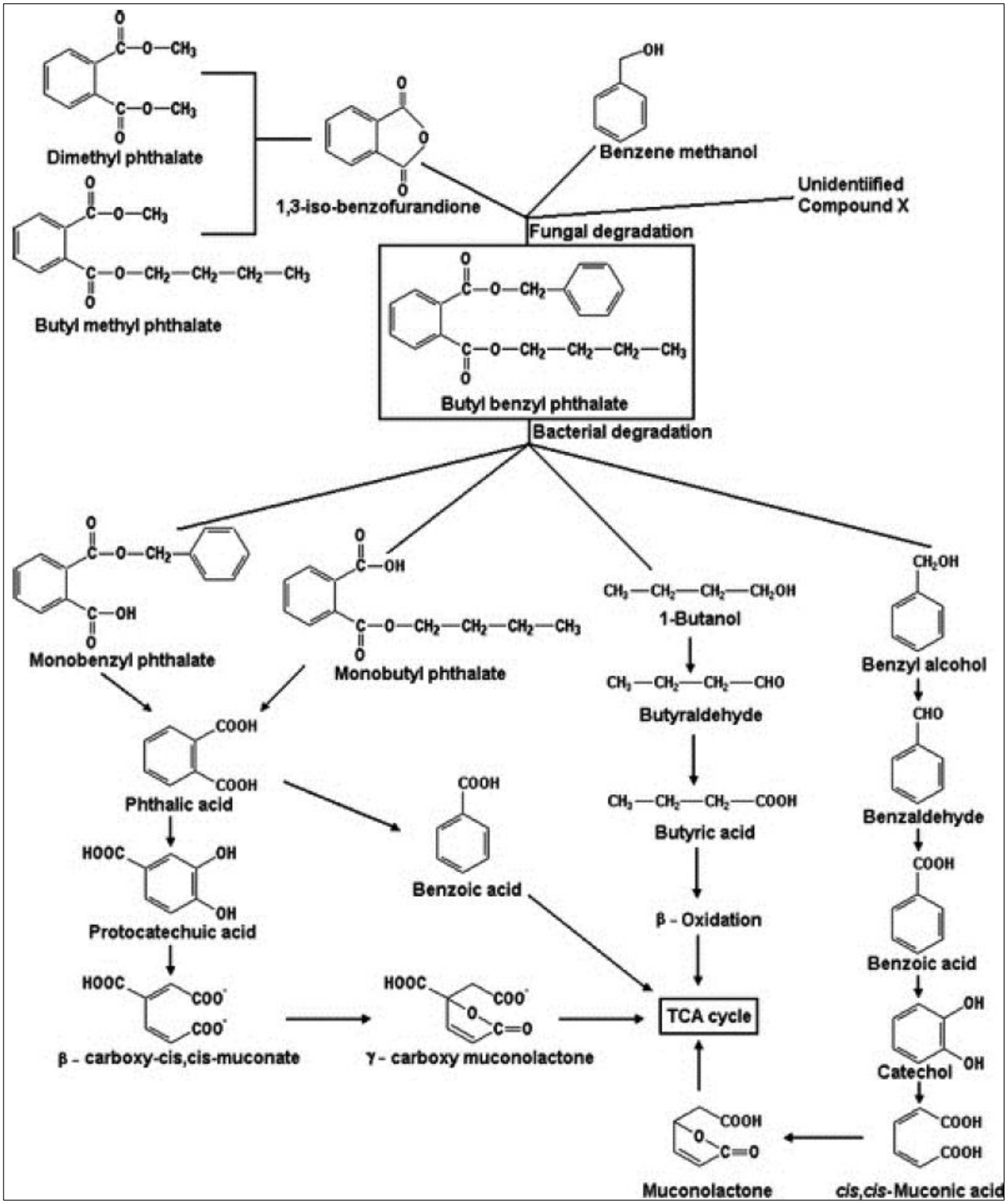
Jaykishan Songara, Rishi Shanker, Nirbhay Kumar Singh

Abstract
Phthalic acid esters (PAEs) have been classified as endocrine disrupters and are considered one of the important and high priority pollutants. Microorganisms, especially bacteria are able to bio-degrade these compounds. However, the rate of degradation is very low due the poor bioavailability of these harmful chemicals to the microbes. Therefore, long term persistence of these compounds adversely affects human health and environment. In the present study, an attempt has been made to develop an eco-friendly rapid method for the degradation of benzyl butyl phthalate (BBP) using bacteria and photocatalytic nanoparticles. *Pseudomonas putida* was observed to degrade BBP. Preliminary observations indicated that photocatalytic activity of ZnO nanoparticles is dose and time dependent in transformation of BBP. However, the rate of transformation of photodegraded products of BBP by *P. putida* was similar to BBP transformation without photocatalytic activity of ZnO nanoparticles.

Keywords: Butyl benzyl phthalate, bioavailability, endocrine disruptors, photocatalytic, *Pseudomonas putida,* ZnO nanoparticles

Introduction
Phthalates or phthalate esters are the alkyl aryl or the dialkyl esters of 1, 2-benzenedicarboxylic acid. They are synthesized by the reaction of an appropriate alcohol (between 6-13 carbon atoms) with phthalic anhydride. They are odourless, colourless liquids which exhibit high solubility in oil and low solubility in water and possess low volatility and a high octanol-to-water partition coefficient [1].

Due to their low cost, sustainability, durability and performance, phthalates are the most widely used plasticizers. Phthalates allow the sliding of long polyvinyl molecules against one another upon addition to plastics; as a result of which the flexibility of plastics increases. Majorly, phthalates are used for converting polyvinyl chloride (PVC) from a hard plastic to a flexible plastic. They also act as a major component of flexible vinyl products like cables, wires, wall covering and flooring. Phthalates are also used in intravenous tubing and vinyl blood bags. Other uses of phthalates include their use as fixatives to retain the fragrances long time. However, Dimethyl phthalate (DMP) is mainly used in cellulose ester-based plastics like butyrate and cellulose acetate. It also acts as an ingredient of decorative clothes, lubricants, cosmetics, etc [1]. Di (2-ethylhexyl) phthalate (DEHP) is a predominantly manufactured phthalate which is largely used in toys, PVC medical devices and food packaging. Di-n-butyl phthalate (DBP) is used as a solvent for dyes, as a coalescing aid in latex adhesives and as a plasticizer in cellulose-based plastics [2]. The phthalates were manufactured for the first time in 1920s and in 1950s, the PVC was introduced which enhanced the manufacturing of phthalates in huge amounts. In the current scenarios, more than 55 different types of phthalates have been produced and incorporated in products [3]. The global annual production of phthalates has been crossed 5 million tons per annum [1]. More than 18 billion pounds of phthalates are being used as plasticizers in PVC products [4] and as inert components of pesticides, sprays and wood finishes [5]. The pathway for degradation of BBP by microbes is illustrated below [6].



Materials and Methods

Preparation of M9 medium

All the chemicals (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$, and Tween-80) used in the experiments were Analytical Reagent grade and procured from HiMedia (India). HPLC grade methanol was procured from HiMedia (India). Butyl benzyl phthalate was procured from Merck Millipore, India, and was ACS grade. All the stocks were prepared according to standard given in Maniatis *et al.* [7].

10X M9 Salts: Na_2HPO_4: 60 g, KH_2PO_4: 30 g, NaCl: 5 g and NH_4Cl: 10 g. 1 litter volume was made by adding dH_2O, pH was adjusted at 7.4, 1 M $MgSO_4$ was autoclaved separately and iron source ($FeSO_4$) – 5 g/l was filter sterilized. The final 1X M9-medium was prepared by adding the following chemicals from the prepared stocks: 100 ml M9 salts, 2 ml 1 M $MgSO_4$ and 10 ml of iron source and final volume of 1L was adjusted by adding dH_2O.

Preparation of 10X stock suspension of BBP

For proper solubility of compounds, 10X concentrated suspension of BBP system was prepared by mixing the ingredients in amount, 10X M9 salts – 100 ml, Tween-80 – 120 µl, BBP - 44.8 µl (for 50 ppm concentration of BBP). 1 M $MgSO_4$ and iron source ($FeSO_4$) were added while preparing (1X) working system. For MM + Tween-80 stock preparation BBP was not added.

Standardization of conditions for HPLC analysis of BBP

The linear dilutions of BBP were prepared in Acetonitrile and ammonium acetate (pH= 5.5, 10 mM) in the ratio of 60:40 (1, 5, 10, 25, 50, 100 and 200 ppm). Standards were characterized by HPLC (Shimadzu LC-2010HT) using Kromasil (100-5-SIL 4.6 × 250 mm, pore size: 100 Å, particle size: 5 µm) column. The column was procured from VDS optilab, Germany. Detection was done through PDA (254 nm)

detector. Acetonitrile and Ammonium Acetate (pH = 5.5, 10 mM) in the ratio of 60:40 were used as a mobile phase. Injection volume was set as 10 µl, run time set was 8 min. Correlation value was determined by plotting curve of peak area versus concentration of BBP (mg/L).

Characterization ZnO nanoparticles

ZnO nanoparticles (ZnO NPs) were kindly provided by CSIR-NPL, New Delhi (CSIR-National Physical Laboratory). The ZnO NPs were in the size range of 5-10 nm as observed in the TEM and the details were provided by the CSIR-NPL. Stock of 100 µg/ ml of ZnO NPs was prepared in the MQ and in the experimental medium (Minimal salt media). The stock suspension was sonicated using probe sonicator (Sonics Vibra cell, Sonics & Material Inc., New Town, CT, USA) for 10 min on ice (pulse, 15 sec on - 30 sec off, amplitude 30%). The sonicated suspensions of ZnO were kept at room temperature for cooling. Further, the stock was diluted to make 10 µg/ ml solution and the suspensions were finally characterized using Zetasizer (Model ZEN3600, Malvern instruments Ltd., Malvern, UK) facilitated with 4.0 mW 633 nm laser.

ZnO nanoparticles-mediated transformation of BBP

The suspension of BBP at a concentration of 50 ppm was prepared in minimal medium in two different tubes. One tube was kept as a control (ZnO nanoparticles were not added) and another was supplied with 10 ppm of ZnO nanoparticles. Suspensions were sonicated using probe sonicator for 10 min in ice (pulse, 15sec on - 30 sec off, amplitude 30%). 10 ml of suspensions from both the tubes were transferred into separate petridishes for UV-light exposure. Before exposing, sample from each suspension was taken for the reference of initial concentration then samples were exposed under UV for 10 min. After completion of UV-light exposure, the volume was adjusted to the equal of initial volume by adding the exposure medium. For HPLC analysis, 1 ml of sample was taken from each petridish and equal volume of Acetonitrile and ammonium acetate (60: 40) was added in samples. For filtration, tubes were centrifuged at 14,000×g for 5 minutes and transferred into the new tube.

Culture Conditions for P. putida

P. putida strain was procured from MTCC, Chandigarh (MTCC Number: 2445). All the system flasks were prepared in 100 ml volume in 250 ml Erlenmeyer flasks. All the flasks were kept overnight in 50% HNO_3 and autoclave before the use for experiments). BBP was provided as the sole carbon source. Microbial culture of *P. putida* was inoculated from glycerol stock in nutrient broth (NB) and the culture was kept overnight on incubator shaker at 30° C and 180 rpm. Next day, 1% culture was inoculated into fresh NB. When, the bacterial cells grown up to OD_{600} ~0.4, the 4 ml of culture was pelleted down by centrifuge at 4,000xg for 5 min and 4° C. Pellets of *P. putida* was resuspended in the BBP containing minimal medium. The flasks were kept on incubator shaker at 30° C and 180 rpm for 48 hr.

Transformation of BBP by P. putida

The adapted culture of *P. putida* cells were pelleted down by centrifuging at 12000xg for 5 min at 4°C. The pelleted cells were used as inoculum for microbial transformation of BBP. BBP transformation experiment was carried by preparation of control and treated flasks (inoculated with *P. putida*). All the flasks were kept on incubator shaker at 30°C and 180 rpm for 3 days.

Effect of metabolite compound on the rate of BBP transformation

As reported, benzoate is the metabolite of BBP [6]. Therefore, to assess its effect on the transformation rate of BBP the benzoate at a concentration of 25 mg/ L is provided with combination of BBP and the transformation of BBP were checked by HPLC. For HPLC, sample preparation was done by taking 1 ml of sample every day from each flask into 2 ml micro-centrifuge tubes and equal volume of Acetonitrile and ammonium acetate (60: 40) was added in each eppendorf tubes. For filtration, the tubes were centrifuged at 14,000×g for 5 minutes and the supernatant were transferred into the fresh tubes.

HPLC Analysis of samples

Analysis was done through HPLC (Shimadzu LC-2010HT) using Kromasil (100-5-SIL 4.6 × 250 mm, pore size: 100 Å, particle size: 5 µm) column (procured from VDS optilab, Germany). Detection was done through PDA (254 nm) detector. Acetonitrile and ammonium acetate (pH= 5.5, 10 mM) in the ratio of 60:40 were used as a mobile phase. Injection volume was set as 10 µl, run time set was 8 min and flow rate set was 1 ml/min.

Preparation of resting cells of P. putida

Adapted culture was used as inoculum for preparation of resting cells. The cells were grown in 1 liter flask which contained 400 ml of reaction medium and flask was kept on incubator shaker at 30°C and 180 rpm. After 48-72 hr, when the flask shown turbidity due to microbial growth, the whole culture medium were pelleted down at 12000xg for 5 min at 4°C, in pre-weighed 50 ml tubes. The supernatant was discarded from each tube and pellets of each tube were pooled into a single tube and repeated the centrifugation. The cells were stored at -80°C for the further use in the experiments.

Transformation of BBP by resting cells of P. putida

The experiment was performed in 10 ml volume in 50 ml erlenmeyer flasks. The flasks were kept overnight in 50% HNO_3 and autoclaved before the use in experiments. Frozen cells (-80° C) were thawed at (60-65° C) in water bath for 1-2 min followed by resuspending in BBP containing minimal medium (pre-chilled) by vortexing (kept on ice). Dead cells were removed by centrifugation at 1000×g for five minutes and the supernatant was collected into fresh tube. The initial OD_{600} of the medium after adding the cells was ~0.1. The photo-activated ZnO NPs were added into the respective flasks at a concentration of 10 mg/ L. The flasks were kept on water bath shaker at 30° C, and 60 rpm for 2 hr. 1 ml of samples were taken from each flask at 0 and 2 hr. Further, the samples were processed for HPLC by adding equal volume of Acetonitrile and ammonium acetate (60: 40).

Results and Discussion

Linearity curve of BBP standards

To assess the optical characteristics of BBP i.e. wavelength for maximum absorption, the standards of BBP were prepared in Acetonitrile and ammonium acetate (60: 40). Standards analysed by HPLC showed linear pattern. Maximum absorption wavelength observed was 230 nm which was supported by reported literature [8]. Retention time for BBP was 6.2 min. Correlation value (R^2) was found to be 0.99 as illustrated in figure 1, which indicated the suitability of instrument for the quantitative detection of BBP. The value of slope (m) = 10000 and intercept value (c) =6160.1.

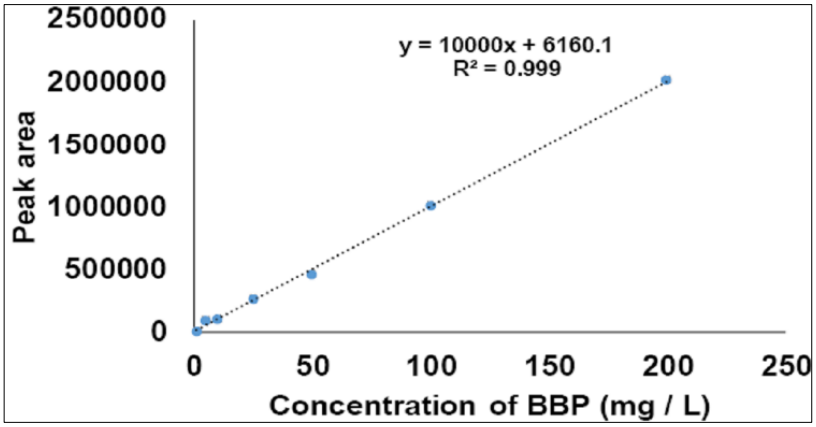


Fig 1: Linearity curve of BBP standards in HPLC.

Characterization of ZnO NPs hydrodynamic size (D.NM)

The average hydrodynamic size of ZnO NPs was 329.7 nm in Mili Q water (MQ), while the suspension of NPs in minimal medium (MM) showed average hydrodynamic size of 1206.33 nm. Increase in size could be due to the high ionic strength of the dispersant medium. The higher ionic strength can induce aggregation of NPs [9]. When NPs were present in the medium and at same ionic strength but additionally contained Tween-80, a non-ionic surfactant, the average size observed was 940.33 nm. The size of the complex was more than in MQ but less than in MM alone. Thus, addition of surfactant can promote dispersion of NPs, so it can reduce interaction of salts with ZnO at some extent by creating stearic hindrance [10]. When BBP present in MM + Tween 80, the average size was 1006.46 nm which can be due to adsorption of BBP on NPs as illustrated in figure 2.

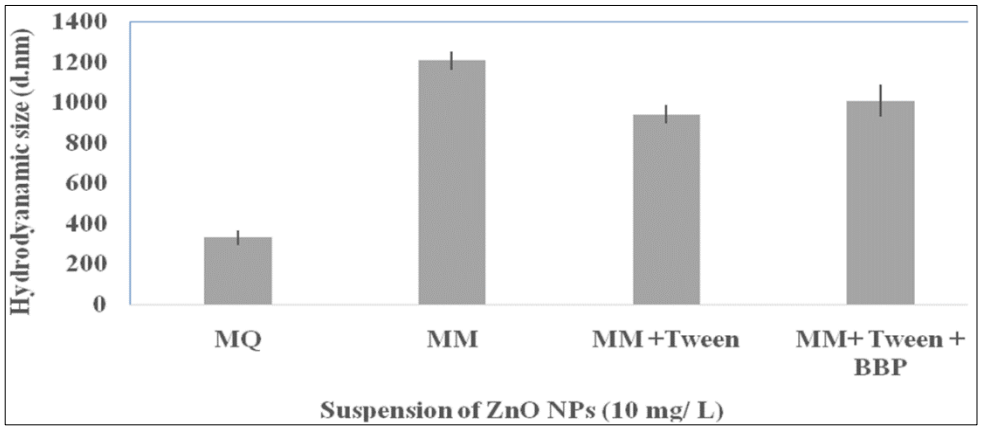


Fig 2: Hydrodynamic size of ZnO NPs (10 mg/ L) in different medium [MQ- Mili Q water, MM- Minimal medium; the bars show the average value of hydrodynamic size (d.nm) ± SEM]

Zeta Potential (mV)

The zeta potential of ZnO NPs was -19.7 ± 0.38 mV in MQ which decreased at -13.2 ± 1.59 mV in the presence of high ion concentration (in minimal salt medium). High salt concentration *i.e.* NaCl can reduce the zeta potential of the NPs [10]. The addition of Tween-80 also resulted in the decrease of zeta potential, which was found to be -7.35 ± 0.48 mV. When BBP was present in MM + Tween 80, the same trend was observed. The zeta potential was -6.44 ± 0.46 mV (Table 3). The obtained values of zeta potential suggest their incipient stability [11].

Table 1: Zeta potential of ZnO NPs in different media

ZnO NPs in different media	Zeta potential (mV)
Milli-Q water	-19.7 ± 0.38
Minimal medium	-13.2 ± 1.59
Minimal media + Tween 80	-7.35 ± 0.48
Minimal media + Tween 80 + BBP	-6.44 ± 0.46

Note: The value shown in Table 1 is the average value of zeta-potential (mV) ± SEM.

Transformation of BBP by photocatalytic ZnO NPs

To analyse the transformation of BBP by photocatalytic ZnO NPs, the suspension of MM + Tween 80 + BBP was treated with ZnO NPs at the concentration of 10 mg/ L [12]. The initial concentration (0 hr) of BBP in both the suspensions, control (without ZnO) and treated (with ZnO) was found to be ~ 50

mg/ L. After 10 min UV exposure (as suggested by the provider CSIR-NPL) and 2 hr shaking on incubator shaker at 60 rpm and 30^{O}C, the concentration of BBP remained same in the control flask as shown in figure 3. This occurred because BBP is an extremely inert material to degrade by only UV radiation [13]. On the other hand, the concentration of BBP reduced to 39.5 mg/ L in the treated flask which indicates the transformation of BBP by ZnO (~21%), as shown in figure 3. The less transformation of BBP could be due to the incipient stability of ZnO NPs, higher concentration of substrate, presence of ions or insufficient concentration of ZnO for the substrate (BBP) [11, 13].

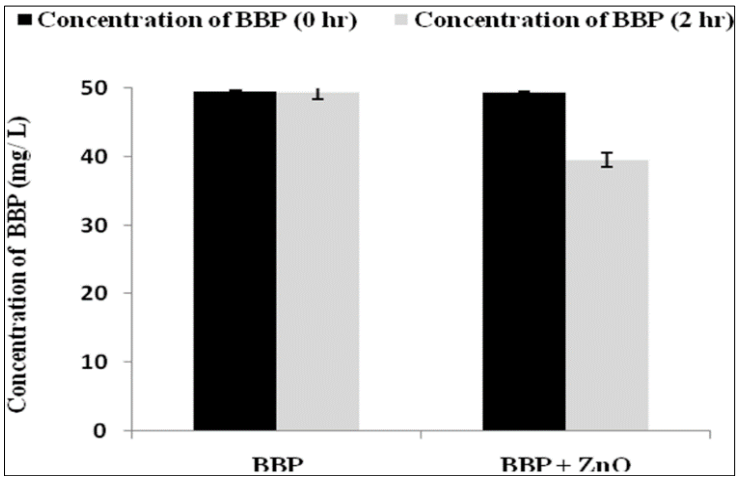


Fig 3: Transformation of BBP by ZnO NPs (Bars showing average concentration of BBP ± SEM)

Transformation of BBP by P. putida

The biotransformation of BBP was assessed by a three day experiment. The suspension of MM + Tween 80 + BBP was used as the culture medium for *P. putida*. The concentration of BBP was ~50mg/ L in both the flasks, control (without *P. putida*) and treated (with *P. putida*) on day 0. On day 1, none of the two samples showed any significant change in the concentration of BBP because there was no significant transformation of BBP. Microbes first synthesize enzymes for some time which will transform BBP (the available carbon source). On day 2, the concentration of BBP was found to be ~27 mg/ L in the treated flask (46% decrease) which is an indicator for the synthesis of phthalate-transforming enzymes (phthalate esterase) [14]. On day three of the experiment, the concentration of BBP in the treated flask was 0.6 mg/ L. For the control flask, the concentration of BBP on day 3 was almost equal to that on day 0 as shown in figure 4. This indicates that ~98 % of BBP was transformed by *P. putida* due to a high level of enzyme synthesis and that BBP is an inert compound without any treatment.

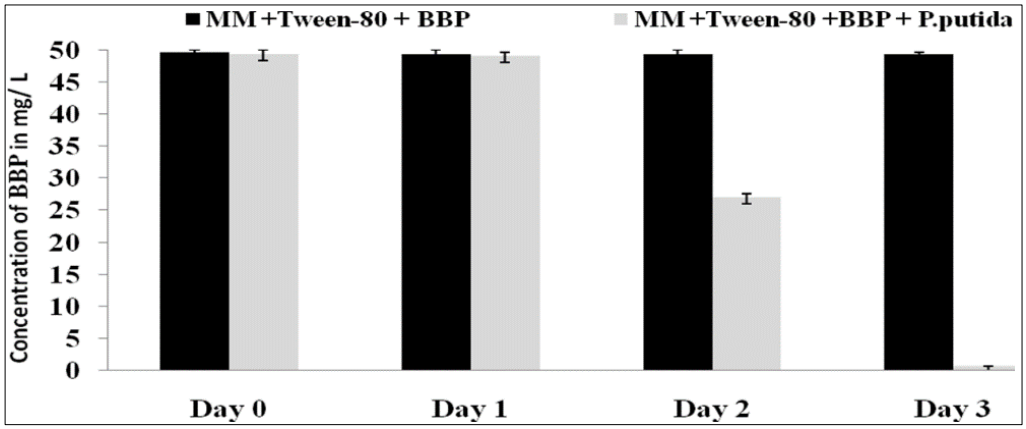


Fig 4: Transformation of BBP by *P. putida* by 3 day experiment [suspension of MM + Tween 80 + BBP treated with *P. putida* (attuned culture). Bars showing average concentration of BBP ± SEM].

Effect of benzoate on the transformation of BBP by P. putida

To assess the effect of benzoate, one of the metabolites of BBP [6], on the biotransformation of BBP, the suspension of MM + Tween 80 + BBP + Benzoate was used as the culture medium for *P. putida*. The concentration of BBP in both the flasks, control and treated, on day 0 was ~50 mg/ L. On day 1, none of the samples showed any significant change in the concentration of BBP. On day 2, the concentration of BBP was found to be ~27 mg/ L (30% decrease). On day 3, BBP was not found which indicates ~100 % transformation of BBP by *P. putida*. From this experiment, it was concluded that there was no significant effect of benzoate on the transformation of BBP by *P. putida* as illustrated in figure 5. The reason behind this could be that *P. putida* may be following the pathway where benzoate is not an intermediate and that it may be transforming phthalic acid into protocatechuate as shown in degradation pathway.

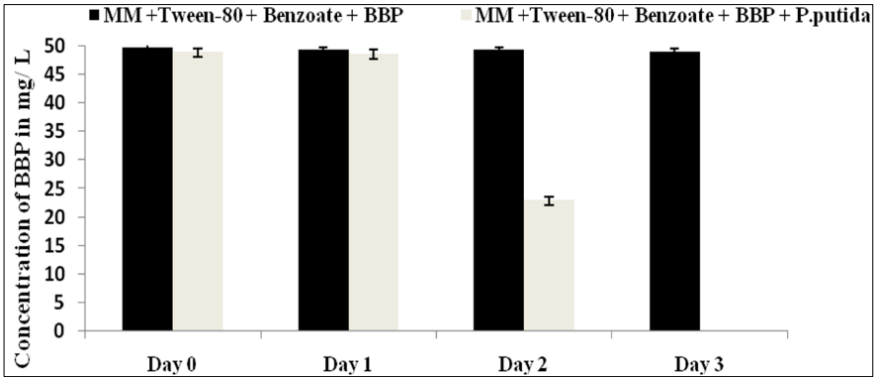


Fig 5: Effect of benzoate on transformation of BBP by *P. putida* (Bars shows average concentration of BBP ± SEM)

Resting Cells Experiment

The transformation of BBP by different routes: bio-route (BBP + *P. putida*), nano-route (BBP + ZnO NPs) and bio-Nano route (BBP + *P. putida* + ZnO NPs) was assessed by treating the MM + Tween 80 + BBP suspension accordingly.

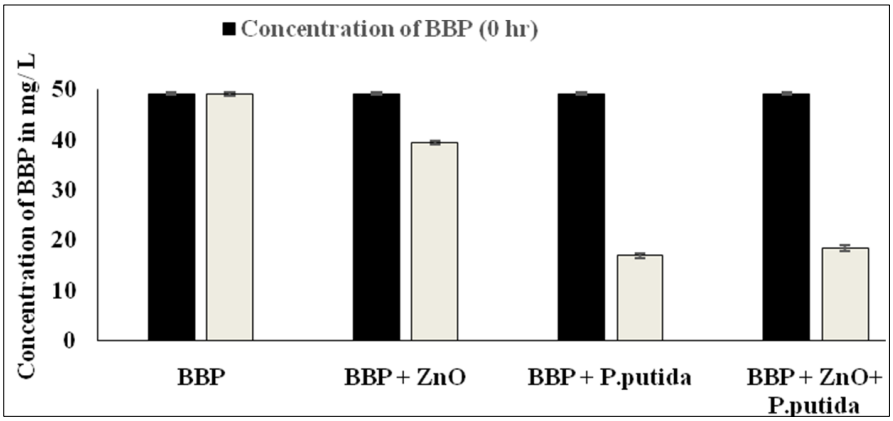


Fig 6: Resting cell experiment, performed by treating suspension of MM + Tween 80 + BBP with harvested resting cells of *P. putida* and for Photo transformation of BBP suspension of MM + Tween 80 + BBP was treated with ZnO NPs. Suspension of MM + Tween 80 + BBP treated with *P. putida* and ZnO NPs together for assessment of their combined effect. Bars showing average concentration of BBP ± SEM

Initial concentration (0 hr) of BBP was ~50 mg/ L. Final concentration (2 hr) of BBP in the BBP + ZnO flask was found to be 39.5 mg/ L (~21% transformation). In the flask treated only with *P. putida*, the concentration of BBP was found to be 17.02 mg/ L. The resting cells of *P. putida* were able to transform ~66% BBP within 2 hr. The usage of ZnO NPs along with *P. putida* did not show any significant difference compared to using *P. putida* alone as shown in figure 6. This could be due to the adsorption of ZnO NPs on the cells of *P. putida*.

Conclusions

Phthalic acid esters (PAEs) can be degraded by many microbes, but the main difficulty is their lower bio-availability and the rate of degradation. There are few other reported nanoparticles-based methods but have nearly all have limitations of high dosage of NPs and lower substrate concentration. The present study was designed to overcome this problem by application of nanobiotechnology-based approach. It was observed that BBP was transformed efficiently by *P. putida*. The photocatalytic ZnO NPs were also able to degrade ~20% BBP but the combination of *P. putida* and ZnO NPs did not show any significant enhancement under the experimental conditions tested. The present observations indicated that different concentrations of NPs, light sources and time exposure be tested to derive a procedure for NP mediated rapid bacterial degradation. This can lead to the development of a nanobiotechnology-based environment friendly approach for the degradation of environmental micro-pollutants like phthalate esters.

References

1. Baikova SV, Samsonova AS, Aleshchenkova ZM, Shcherbina AN. The intensification of dimethyl phthalate destruction in soil. Eurasian Soil Science. 1999; 32(6):701-704.
2. Li J, Chen J, Zhao Q, Li X, Shu W. Bioremediation of endocrine disruptor di-n-butyl phthalate ester by Deinococcus radiodurans and Pseudomonas stutzeri. Chemosphere 2006; 78:342-348.
3. Lu Y, Tang F, Wang Y, Zhao J, Zeng X, Luo Q. Biodegradation of dimethyl phthalate, diethyl phthalate and di-n-butyl phthalate by Rhodococcus sp. L4 isolated from activated sludge. Journal of Hazardous Material. 2009; 168:938-943.
4. Staples CA, Peterson DR, Parkerton TF, Adams WJ. The environmental fate of phthalate esters: a literature review. Chemosphere. 1997; 35:667-749.
5. Mackintosh C, Maidonado J, Ikonomou MG, Gobas FPC Sorption of phthalate esters and PCBs in a marine ecosystem. Environmental Science and Technology. 2006; 40:3481-3488.
6. Chatterjee S, Karlovsky P. Removal of the endocrine disrupter butyl benzyl phthalate from the environment Applied Microbiology and Biotechnology. 2010; 87:61-73.

7. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning: A Laboratory Manual, 1st ed., Cold Springs Harbor Laboratory, Cold Springs Harbor, NY, 1982, 68.
8. Optimizing Analysis of Phthalates in Plastic http://www.chem.agilent.com/Library/applications/5991-2784EN.pdf
9. Kim KM, Kim TH, Kim HM, Kim HJ, Gwak GH, Peak SM *et al*. Colloidal Behaviors of ZnO Nanoparticles in Various Aqueous Media. Toxicology and Environmental Sciences. 2012; 4(2):121-131.
10. Khan Y, Durrani SK, Mehmood M, Ahmad J, Khan MP, Firdous S. Low temperature synthesis of fluorescent ZnO nanoparticles. Applied Surface Science. 2010; 257:1756-1761.
11. Greenwood R, Kendall K. Electroacoustic studies of moderately concentrated colloidal suspensions. Journal of the European Ceramic Society. 1999; 19(4):479-488
12. Kumar A, Pandey A, Shingh S, Shanker R, Dhawan A. Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells, Chemosphere. 2011; 83:1124-1132.
13. Xu X, Li S, Li X, Gu J, Cheu F, Li X *et al*. Degradation of n-butyl benzyl phthalate using TiO2/UV. Journal of Hazardous material. 2008; 164:527-532
14. Nomura Y, Harashimaya S, Takada N, Oshima Y. Phthalate Degradation in Pseudomonas putida. Enzyme engineering. 1990; 613:776-780.