Microbial functional diversity plays an important role in the degradation of polyhydroxybutyrate (PHB) in soil

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Abstract

Towards bioremediation of recalcitrant materials like synthetic polymer, soil has been recognized as a traditional site for disposal and subsequent degradation as some microorganisms in soil can degrade the polymer in a non-toxic, cost-effective, and environment friendly way. Microbial functional diversity is a constituent of biodiversity that includes wide range of metabolic activities that can influence numerous aspects of ecosystem functioning like ecosystem stability, nutrient availability, ecosystem dynamics, etc. Thus, in the current study, we assumed that microbial functional diversity could play an important role in polymer degradation in soil. To verify this hypothesis, we isolated soil from five different sites of landfill and examined several microbiological parameters wherein we observed a significant variation in heterotrophic microbial count as well as microbial activities among the soil microcosms tested. Multivariate analysis (principle component analysis) based on the carbon sources utilization pattern revealed that soil microcosms showed different metabolic patterns suggesting the variable distribution of microorganisms among the soil microcosms tested. Since microbial functional diversity depends on both microbial richness and evenness, Shannon diversity index was determined to measure microbial richness and Gini coefficient was determined to measure microbial evenness. The tested soil microcosms exhibited variation in both microbial richness and evenness suggesting the considerable difference in microbial functional diversity among the tested microcosms. We then measured polyhydroxybutyrate (PHB) degradation in soil microcosms after desired period of incubation of PHB in soil wherein we found that soil microcosms having higher functional diversity showed enhanced PHB degradation and soil microcosms having lower functional diversity showed reduced PHB degradation. We also noticed that all the tested soil microcosms showed similar pattern in both microbial functional diversity and PHB degradation suggesting a strong positive correlation ($r=0.95$) between microbial functional diversity and PHB degradation. Thus, the results demonstrate that microbial functional diversity plays an important role in PHB degradation in soil by exhibiting versatile microbial metabolic potentials that lead to the enhanced degradation of PHB.

Keywords PHB · Bioremediation · Microbial functional diversity · Positive correlation

Introduction

Light weight, durability, and low making cost of plastic make them an integral part of our daily life. Plastic can be classified into two categories—biodegradable and non-biodegradable based on the presence of hydrolysable bonds in the polymer. Plastic materials derived from petrochemicals cause serious environmental problems due to their non-degradable nature. To this end, polyethylene, a widely used non-biodegradable polymer, includes nearly 64% of all synthetic plastics produced (Harshvardhan and Jha 2013). Since materials derived from polythene are non-biodegradable, they accumulate in the environment at an alarming rate of nearly 25 million tons per year (Balasubramanian

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et al. 2010) and thus pose a major threat to the environment (Zeng et al. 2013). The major threat associated with plastic waste arises from chemicals that leach from the plastic to the environment. There are different strategies such as land-fill disposal, burning, and recycling reported to reduce the load of these non-biodegradable plastics, but these processes are not environment friendly as the processes generate lots of greenhouse gases into the environment. Bioremediation happens to be the most efficient and environment friendly approach to protect the environment from the recalcitrant hazards (Shah et al. 2008; Tokiwa et al. 2009; Chen et al. 2017). Biodegradable plastics offer the best solution to protect the environment from hazards caused by the conventional non-biodegradable plastics (Tribedi et al. 2012). PHBs are macromolecules synthesized as reserve material by the bacteria when the bacteria grow under stress conditions (Galia 2010). Since PHB is having properties similar to various synthetic thermoplastic like polyethylene (PE) and polypropylene (PP), it could be used as a potential substitute of non-biodegradable polymers like PE and PP (Verlinden et al. 2007). PHB is a linear homopolymer having D-3 hydroxy butyric acid as its monomer. The advantage of PHB usage is that it has ester bonds and it could be targeted by microbial enzymes frequently. The presence of this ester bond in PHB makes the polymer biodegradable. Biodegradation is a biological process where living organisms degrade complex organic compounds to non-toxic products of lower molecular weights that are often utilized by microorganisms as their nutrient and energy source (Altaee et al. 2016). The biodegradation of PHB occurs either in aerobic or anaerobic conditions. In aerobic condition, PHB gets degraded to carbon dioxide (CO_2) and water (H_2O), while in anaerobic condition, PHB gets degraded to CO_2 , H_2O , and methane (CH_4) by microorganisms (Wang et al. 2014). Since enormous amounts of polymer wastes are dumped in landfills, the polymer wastes need to be removed from the landfill to regenerate the landfill. For the effective remediation of polymer from the target site, in situ bioremediation seems to be the best option. Composting has been used as a very promising in situ approach for effective bioremediation of plastic pollutants in soil (Chen et al. 2015). However, composting has also been used in stabilizing agriculture solid waste, as well (Huang et al. 2008; Yu et al. 2011). Microbial community plays an important role in composting as in one side, it degrades the solid-waste pollutant in soil; and on the other side, it increases the soil fertility and agricultural productivity. For soil bioremediation, diverse strategies of composting such as bioaugmentation, biostimulation, and natural attenuation have been widely used (Chen et al. 2015). Bioaugmentation represents that an isolated organism(s) having the capacity to degrade the polymer used to be added to the native soil from outside (Tribedi and Sil 2013). Biostimulation represents that the remediation of

polymer is accomplished by incorporating some important rate limiting nutrients to the soil (Tribedi and Sil 2013). Natural attenuation represents that the polymer would be remediated by the indigenous microbial community present in that given ecological niche of soil (Tribedi and Sil 2013). In case of bioaugmentation, the process suffers from some drawbacks as the introduced organism to the soil may either face competition or it may damage the pre-existing soil ecology as the microbial community in a given habitat is very sensitive to any perturbation of the ecosystem (Tyagi et al. 2011). In case of biostimulation, the addition of nutrient to the soil stimulates microbial growth including some dormant microbial population that might change the native microbial community ecological stability. Therefore, natural attenuation seems to be the most environments friendly, because the native microbial community is not challenged by anything. Thus, the polymer remediation at landfill site by the native soil microbes has been considered as a safer option (Silva et al. 2009). Soil is a heterogeneous and discontinuous natural environment where the microorganisms are distributed in discrete microhabitats (Stotzky 1997; Pietramellara et al. 2002). Environmental factors like carbon and other energy sources, ionic balance, temperature, pH, as well as intra and inter-species interactions among microorganisms extensively affect the soil microbial ecology (Nannipieri et al. 2003; Lauber et al. 2009). It was reported that each microhabitat has distinctive microbial community structure (Nannipieri et al. 2003), and thus, screening of proper microhabitat showing efficient degradation of a polymer is the most challenging issue (Venosa and Zhu 2003). Since bioremediation of polymer is dependent on the microbial metabolic diversity and metabolic potential of the microorganisms, microbial functional diversity could play an important role in landfill site selection for the effective remediation of the polymer. Therefore, in the current study, we have measured functional diversity of different landfill soils and tried to correlate this with the polymer remediation.

Materials and methods

Soil microcosm preparation

Soil samples (~10 cm depth) were separately collected from five different sites of Kolkata municipal solid-waste landfill ground (DHAPA). Thereafter, the isolated soil sample was homogenized and sieved through a 2-mm pore size sieve. After that, the soil samples were air-dried. Physicochemical properties of soil were determined and presented in Table 1. Five different microcosms: T1, T2, T3, T4, and T5 were prepared in this current study. For each soil microcosm preparation, 450 g of air-dried soil was separately taken in different sterile glass beaker. Thereafter, 100 mg of (2 piece of 50-mg

Table 1 Physicochemical properties of soil collected from landfill

Organic carbon (%)	Total nitrogen (%)	Total phosphorous (%)	pH
3.42	0.18	0.79	6.17

PHB film) the air-dried PHB films were added to each soil microcosm aseptically. Each microcosm was covered with aluminum foil and then incubated at 30 °C for 28 days. Before the addition of PHB films to the soil, all the films were made microorganism free by washing them three times with 70% ethanol. During the course of incubation, moisture content of each microcosm was maintained by adjustment with sterile Milli-Q water. After the incubation, soil samples and PHB films were separately isolated from each microcosm and analyzed for biotic activities and PHB degradation, respectively.

Preparation of PHB film

For the preparation of PHB film, the conventional solvent-cast technique was used as described by Rawia et al. (2013). The PHB films were prepared by dissolving the polymer (0.5 g) in chloroform (10 ml) in a glass beaker with magnetic stirring for 30 min. The mixture was poured into glass petridishes (9 cm in diameter) as the casting surface. The petridishes were covered with puncture aluminum sheets and left in the dark at 30 °C for 24 h to allow complete evaporation of chloroform. Complete evaporation resulted in the formation of PHB films. The films were aged for 1 week prior to analysis to reach equilibrium crystallinity. The solution cast PHB films were then cut into smaller pieces as per the requirement of the experiment. The film was then sterilized by washing three times with 70% ethanol.

Preparation of PHB plate

The selection and culture of the isolated organism were carried out in medium containing PHB as sole carbon source. For the preparation of the medium, 1 g of PHB powder was dissolved in 1 l of basal medium containing 100-mg yeast extract, 1-g (NH₄)₂SO₄, 200-mg MgSO₄·7H₂O, 100-mg NaCl, 20-mg CaCl₂·2H₂O, 10-mg FeSO₄·7H₂O, 0.5-mg Na₂MoO₄·2H₂O, 0.5-mg Na₂WO₄·2H₂O, 0.5-mg MnSO₄, 1.6 g K₂HPO₄, and 200-mg KH₂PO₄. Thereafter, it was next sterilized by autoclaving. For solid medium, agar (20 g l⁻¹) was added prior to autoclaving.

Screening of PHB degrading organism

One gram of soil sample from different microcosms was separately added to 9 ml of 0.85% NaCl in sterile test tubes. After that, a series of dilution from 10⁻² to 10⁻⁵ were

prepared in 0.85% NaCl. An aliquot (0.1 ml) of the appropriate dilution from each microcosm was spread aseptically onto agar plates containing PHB as sole carbon source. The PHB plates were incubated at 30 °C for 7 days. Microorganisms developing clear zone around the colony on PHB plate after the desired period of incubation are being considered as PHB degrading microorganisms.

Determination of heterotrophic microorganisms in soil

One gram of soil from each soil microcosm was kept in sterile tube, diluted with 9 ml of 0.85% NaCl, and spread onto sterile Luria agar (LA) plates for the record of heterotrophic organisms. LA plates were incubated at 32 °C for 2 days.

Fluorescein diacetate (FDA) hydrolysis assay

FDA hydrolysis assay was performed by following the protocol of Adam and Duncan, (2001). In brief, 5-g soil sample collected from each soil microcosm was mixed with sterile 15-ml phosphate buffer (pH 7.6). Thereafter, 0.2 ml of FDA (1 mg ml⁻¹) in acetone was added to each tube separately. After the incubation of 20 min at 32 °C, fluorescein was extracted with an organic solvent mixture containing chloroform–methanol (2:1). Fluorescein concentration was then measured spectrophotometrically at 490 nm.

Physiological profiles of soil microbial community

Physiological profile of soil microbial community was assessed by BiOLOG ECO plates (Choi and Dobbs 1999; Tribedi and Sil 2013). To test the physiological profiles of soil microbial community, 1-g soil from each soil microcosm was kept in 9 ml of sterile Milli-Q water and the abundance of heterotrophic microorganisms was determined. 150 µl of soil suspension containing nearly 1000 colony forming unit (CFU) from each soil microcosm was separately added to BIOLOG ECO plates. Thereafter, BiOLOG ECO plates were incubated at 32 °C for 60 h and absorbance of each well of the ECO plate was measured at 590 nm. The Shannon diversity index (*H*), a popular index of functional diversity, was calculated using the following equation: $H = - \sum p_i \ln p_i$, where p_i is the ratio of the catabolism on each substrate (Absorbance_{*i*}) to the sum of catabolism of all substrates ($\sum \text{Absorbance}_i$) (Tribedi et al. 2015). Subsequently, the Lorenz curve was plotted using the data of metabolic fingerprinting from BiOLOG ECO plate. This curve was used to derive the Gini coefficient (*G*) using the formula:

$$G = 1 - 2 \int_0^1 L \, dF,$$

where L is the Lorenz curve and F is the standardized cumulative distribution of the standardized population.

PHB degradation analysis

PHB degradation was analyzed by measuring the weight loss of PHB films by following the protocol described by Tribedi and Sil (2013). The weight loss of PHB represents the extent of reduction in weight of PHB films from the initial weight to final weight of PHB films.

Statistical analysis

For multivariate analysis, loading plot of principal component analysis (PCA) was constructed. Correlation value was calculated between functional diversity and weight loss of PHB films. PCA and correlation coefficient were measured using the software Minitab 16. All results were subjected to statistical analysis of one-way analysis of variance (ANOVA) at 5% level. Each experiment was repeated three times to gain statistical confidence.

Results and discussion

Variation in the distribution of microbial population and microbial activity among different soil microcosms collected from landfill

Since distributions of soil microorganisms vary in different microhabitats, we examined the abundance of heterotrophic microbial population among different microcosms collected from different sites of landfill. We observed that there is significant difference in the abundance of heterotrophic microbial population among different microcosms, of which the lowest microbial count was observed in microcosm T3 and T5 (Fig. 1a). However, the highest microbial count was observed in microcosm T2 (Fig. 1a). Efforts were also given to measure soil microbial activity as it is directly related to microbial abundance. The existing literature showed that fluorescein diacetate (FDA) hydrolysis assay can be used to measure microbial activity in soils (Adam and Duncan 2001). Fluorescein diacetate (3',6'-diacetyl-fluorescein) (FDA) is a colorless compound where fluorescein is conjugated to two acetate radicals. The enzymes accountable for FDA hydrolysis are both free (exo-enzymes) and membrane bound enzymes such as proteases, lipases, and esterase that are abundant in the soil environment (Green et al. 2006). Thus, in the current study, FDA hydrolysis assay has been adopted to measure the soil microbial activity among different soil microcosms. The result showed that there is a significant variation in the microbial activity among different

Fig. 1 Variation in heterotrophic organisms and microbial activity among different microcosms. **a** Heterotrophic microorganisms were collected from soil and counted on Luria agar plate. Error bars indicated standard deviation ($\pm$ SD). Mean values with different letters are significantly different among the experimentally used samples. **b** Microbial activity of the soil microcosm was determined by fluorescein diacetate hydrolysis assay as described in the “Materials and methods”. Error bars indicated standard deviation ($\pm$ SD). Mean values with different letters are significantly different among the experimentally used samples

microcosms of which the lowest level of microbial activity was observed in T3 and T5 (Fig. 1b). On contrary, the highest level of microbial activity was observed in microcosm T2 (Fig. 1b). The pattern of microbial abundance showed similarity with the pattern in microbial activity (compare Fig. 1a, b). The difference in microbial activity profile of the microcosms was also observed with respect to carbon source utilization pattern of BiOLOG ECO plate. In this assay, microcosm T2 and T4 showed maximum and minimum levels of average well-color development (AWCD) (Fig. 2). AWCD is directly related to the total biological metabolic activities of a microcosm (Tribedi and Sil 2013). Higher AWCD of a microcosm reveals more biological metabolic activity associated with soil microcosm and vice versa (Tribedi and Sil 2013). To better understand these variations in C-source utilization

Fig. 2 Carbon source utilization pattern by the microbial communities present in different microcosms. The average well-color development (AWCD) assay was performed to examine the metabolic potentiality of different microcosms as metabolic potentiality is directly related to AWCD. Error bars in the diagram indicated the standard deviation ($\pm$ SD). Mean values with different letters are significantly different among the experimentally used samples

Fig. 3 Principal component analysis (PCA) of soil microbial metabolic profile. Loading plot was constructed to measure the similarity and differences among microbial communities in different soil microcosms using carbon source utilization spectrum of BiOLOG ECO plates

by different microcosms, we performed multivariate analysis using results obtained from BiOLOG ECO plates. In multivariate analysis, loading plot of principal component analysis (PCA) was performed. PCA is a multivariate technique that analyzes a data table in which observations are described by several inter-correlated quantitative dependent variables. The result of PCA showed that microbial communities of microcosms T1 and T3 are nearly similar (Fig. 3); and the other microcosms T5, T4, and T2 are dissimilar and different from the microbial communities of other microcosms (Fig. 3) based on the carbon source utilization pattern of BiOLOG ECO plates. Taken together, it can be stated that with respect to C-source utilization pattern, there exists similarities and differences in the microbial communities among different soil microcosms.

Thus, the result indicates that the tested microcosms might exhibit different microbial functions, since the microbial communities are different among the soil microcosms.

Variation in functional diversity among different soil microcosms

Ecosystem represents a complex interaction between biotic and abiotic components. Subtle changes in either of the two components bring substantial changes in the overall ecosystem dynamics (Goswami et al. 2017). The existing literature reveals that it is the functional traits of the organisms and not their taxonomic identification that affects the ecosystem linked processes considerably (Diaz and Cabido 2001). Thus, it is the functional diversity that happens to be the most vital index for addressing the efficiency of an ecosystem (Sarkar et al. 2017). Ecosystem with higher functional diversity represents healthy ecosystem and ecosystem with lower functional diversity represents less productive ecosystem (Marinari et al. 2013; Goswami et al. 2017). Thus, it can be considered that an ecosystem exhibiting higher functional diversity is capable of executing various biological functions right from healthy agriculture to ecosystem bioremediation. Microbial functional diversity depends on functional richness and evenness. Functional richness represents the amount of an ecological niche covered by the species within a community (Goswami et al. 2017). Higher functional richness showed better ecosystem productivity, wherein lower functional richness revealed that some of the existing resources are left unused as the microbial species living in that niche is unable to utilize all the available resources (Goswami et al. 2017). In the current study, we determined Shannon diversity index of the soil samples to measure the functional richness of soil microcosms as it has been used as a popular index of measuring microbial functional richness in the literature (Tribedi and Sil 2013). The result showed that the Shannon diversity index varied significantly among the soil microcosms tested, wherein the highest and lowest values of Shannon diversity index were observed in microcosm T2 and T5, respectively (Fig. 4a). Functional evenness defines the pattern of distribution of microorganisms in a particular ecological niche. To verify the evenness, Gini coefficient was calculated (Sarkar et al. 2017). Gini coefficient is a measure of the inequality of distribution in a population and it varies from zero when all individuals are equal and most evenly distributed, to a maximum of one in which the population exhibits highest inequality (Liu et al. 2011). The result showed a significant variation in Gini coefficient among all the tested soil microcosms, wherein the lowest level of Gini coefficient was observed in T2 and highest level of Gini coefficient was

Fig. 4 Diversity and evenness profile for microbial populations in different soil microcosms. **a** Functional diversity profile of soil microcosms was determined by measuring Shannon–Weaver index. Error bars indicated standard deviation ($\pm$ SD). Mean values with different letters represent that there is a significant difference among the experimentally used samples. **b** Variations of Gini coefficients among microbial populations of different microcosms were determined by plotting Lorenz curve. Error bars indicated standard deviation ($\pm$ SD). Mean values with different letters are significantly different among the experimentally used samples

observed in T5 (Fig. 4b). Since evenness varies inversely with Gini coefficient of a system, these results indicate that the microbial community in microcosm T2 is the most diverse as well as most evenly distributed with respect to their ability to utilize different C-sources in BiOLOG ECO plates. In contrast, the microbial community in T5 is the least diverse and has maximum uneven distribution. Thus, the results indicate that microcosm T2 and T5 exhibits the highest and lowest levels of functional diversity, respectively, among the entire tested microcosm. Taken together, considering the results, it can be stated that the microbial functional diversity depends on both richness and evenness of microorganisms. Higher value of Shannon diversity index (functional richness) and lower value of Gini coefficient (functional evenness) develop an ecosystem of

higher functional diversity that leads to the development of a healthy productive ecosystem.

Variation in PHB degradation among different microcosms

Soil has been found to be the most abundant and comfortable habitat for accumulating PHB degrading microorganisms. Microorganisms like *Pseudomonas*, *Bacillus* sp., *Streptomyces*, *Microbispora*, *Thermoactinomyces*, *Actinomadura*, *Aspergillus* sp., *Penicillium*, and *Saccharomonospora* have been found to degrade PHB (Altaee et al. 2016). It was reported that the microbial PHB depolymerase can degrade PHB to 3-hydroxybutyric acid (Altaee et al. 2016). The dehydrogenase enzyme then oxidizes 3-hydroxybutyric acid to acetyl acetate. Then, β -ketothiolase converts acetyl acetate to acetyl coenzyme A which can be used for cell regeneration (Kobayashi et al. 2005; Doi and Fukuda 2014). To test the PHB degradation among different tested microcosms, we had collected soil from five different locations of landfill wherein we measured functional diversity and PHB degradation in all the tested microcosms. The results showed that PHB degradation varied considerably among the microcosms wherein microcosm T2 and T5 exhibited highest and lowest levels of PHB degradation, respectively (Fig. 5). Interestingly, earlier, we also noticed that microcosms T2 and T5 exhibited highest and lowest levels of functional diversity, respectively (compare Fig. 4a with Fig. 5). The result showed that all the microcosms exhibited PHB degradation patterns similar to their functional diversity profile (compare Fig. 4a with Fig. 5). To gain further confidence, we determined the correlation coefficient (r) between these two variables (functional diversity and PHB degradation) and that was found to be 0.95 ($p < 0.05$, $n = 5$).

Fig. 5 Variation in PHB degradation among different microcosms. PHB degradation in different soil microcosm was assessed by measuring the weight loss of the PHB films as described in Materials and methods. Error bars indicated standard deviation ($\pm$ SD). Mean values with different letters are significantly different among the experimentally used samples

This r value of 0.95 establishes a strong positive correlation between PHB degradation by landfill soil and their microbial functional diversity.

Conclusion

The irresponsible littering and uncontrolled accumulation of PHB poses a great threat to the environment. To get rid of this problem, bioremediation is considered to be the most efficient one. For effective bioremediation, the isolation of desired microbial strain having the proper function is the big challenge. In this context, functional diversity appears to be a guiding tool to screen soil samples having the desired function of polymer remediation. The present study demonstrates a strong positive correlation between functional diversity of landfill soil and its ability to degrade PHB. Moreover, since microbial function is not confined to polymer degradation alone, functional diversity can be exploited in wide range of applications where microbial activities are involved.

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Compliance with ethical standards

Conflict of interest Authors declare that they do not have any conflict of interest.

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