Depolymerization and Assimilation of Poly (Ethylene Terephthalate) By Whole-Cell Bioprocess

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Abstract. Now, biofunctionalization of synthetic polymers can be achieved through biocatalysts. Uniquely among the great enzyme, whole-cell biocatalysts provide a promising alternative for bioprocess. The bioprocess of depolymerization and assimilation of polyethylene terephthalate (PET) was investigated with whole-cell from aeromonas as biocatalyst in this investigation. The engineered aeromonas strain, which can grow with PET particle as sole carbon source and major energy, was employed as biocatalysts. The main products, such as terephtalic acid (TA), bis (2-hydroxyethyl terephthalate) (BHET) and benzoic acid (BA), muconic acid (MA), were recognized in whole-cell bioprocess of PET depolymerization. It showed that variation of these products was more complex than that in case of enzyme treatment, for the interaction of solid insoluble substrate, microbe cell and enzymes secreted by cells in this heterogeneous process. The result demonstrated that not only the ester bonds can be cleaved, but also the benzene ring can be decomposed in the process of whole-cell catalysis. And some of the intermediate products, which have inhibiting effect in enzyme catalysis process, can be utilized and further degraded in whole-cell bioprocess. Furthermore, assays by DSC showed that as degradation of PET progressed, crystallinity of PET granular increased by 1.81% due to preferential degradation of amorphous regions.

1. Introduction
The functionalization of synthetic polymers such as PET to improve their hydrophilicity can be achieved biocatalytically using hydrolytic enzymes [1, 2]. Moreover, numerous enzymes which are active on PET have been identified and characterized [3-6], such as cutinases, lipases, and esterases. Thus, enzymes are considered to be more satisfactory catalysts than conventional chemical agents for the further biofunctionalization processes of polymers in textile finishing, technical material, and biomedical applications [7, 8]. Recently, there are reported that Yoshida and co-workers [9] isolated a novel bacterium, Ideonella sakaiensis 201-F6.that is able to use PET as its major energy and carbon source, and enzymatically convert PET into its two environmentally benign monomers.

Although both enzymes and whole cells have been used industrially as catalysts, microbial cells play a leading role in bioprocess because of their unique advantages. Firstly, whole cell catalysts can
be much more readily and inexpensively prepared for industrial purposes due to their diversity and ease of handling [10]. Secondly, enzymes in cells are generally more stable in the long-term bioprocess than free enzymes which are protected from the external environment [11]. What’s more, the presence of the microbe itself was able to enhance the hydrolysis rate when compared to the enzyme [12]. Several microbes with unique catalytic abilities have been found through intensive screening and put into industrial application [13]. Therefore, whole-cell biocatalysts provide a promising alternative for biocatalysts.

Several kinds of microorganism have been studied in the purpose of preparation of enzymes for biotreatment of PET, such as Fusarium solani [14, 15], T. lanuginosus [16, 17], Aspergillus oryzae [18], Pseudomonas mendocina [19], and Thermobifida fusca [20-22]. Although these strains can produce PET hydrolases, they were screened and cultivated with PET model compound, which is monomer or oligomer of PET, such as Diethyl terephthalate (DET) and bis(benzoyloxyethyl) terephthalate (PET trimer).

In our previous study, a kind of aeromonas strain, Comamonas testosterone F4, was isolated from the waste water of PET production factory and applied in biodegradation of PET fiber [23]. Then this strain was improved and the engineered strain was obtained, which can grow with PET particles as sole carbon source. This provides a possibility for investigation of PET bioprocess with microbial whole-cell catalysts. In the present work, bioprocess of depolymerization and utilization of PET was investigated with whole-cell from aeromonas as biocatalyst.

2. Material and methods

2.1. Microorganism

A kind of aeromonas strain, Comamonas testosterone F4, was isolated from the waste water of PET production factory. After an extended series of experiment, microorganisms with the ability to metabolize PET as their sole carbon source were screened through the method of evolutionary engineering, which can generate strains with improved phenotypes using new circuits [24].

2.2. Materials

PET granular obtained from Sigma-Aldrich Chemie Gmbh (China) were grinded through a high speed grinder (WND-100, China) until ultrafine powders. TA, BHET, BA, which were used as standard samples for HPLC analysis, were purchased from Heowns (Tianjin) and MA from Alfa Aesar (Tianjin). Methanol and phosphoric acid were HPLC grade and obtained from Kemiou (Tianjin). All the other chemicals used were laboratory grade reagents.

2.3. Degradation experiment

The strains were incubated in a mineral salt medium according to a previous prescription [25] which consist of NH_4Cl(1.0gL^{-1}), KH_2PO_4(3.0gL^{-1}), Na_2HPO_4(7.0gL^{-1}), NaCl(0.5gL^{-1}), MgSO_4·7H_2O(0.25gL^{-1}), H_3BO_3(0.5μgL^{-1}), FeCl_3·6H_2O(0.2μgL^{-1}), MnSO_4·5H_2O(0.4μgL^{-1}), ZnCl_2(0.4μgL^{-1}), (NH_4)_6Mo_7O_24·9H_2O (0.2μgL^{-1}), CuSO_4·5H_2O(40μgL^{-1}) in tap water. PET powder and small molecular intermediates were added to the medium as the only carbon source, respectively. Following inoculation, flasks (500mL) containing 120 mL medium were incubated at 37°C, 140rpm min^{-1} on a reciprocal shaker, and samples were taken for analysis at different time intervals.

2.4. Turbidimetric determination of microbial biomass

The fermentation liquid taken at a certain time was measured at 600 nm (OD_{600mm}) using a V-1200 spectrophotometer (Mapada). For the blank test, carbon source was placed into the culture medium without strain addition and incubated under the same conditions. Experiments were run in triplicate if not stated otherwise.
2.5. High Performance Liquid Chromatography (HPLC) analysis
HPLC analysis was applied to determine the degree of PET degradation. The equipment used was a Essentia LC-15C pump (Shimadzu Corporation, Japan) equipped with an RP-C18/C8 column (250mm×4.6mm), a manual sample injector and SPD-15C UV visible dual wavelength detector. Separation was achieved with 60% methanol and 40% water (50mmolL⁻¹ KH₂PO₄, H₃PO₄, pH=2) (v/v) as eluent at the wavelength of 240nm. The flow rate was set to 1 mL min⁻¹ and the column was maintained at a temperature of 40°C. The injection volume of 1μL was performed. Some linear calibration curves were used for the conversion of standard products peak area to concentration.

2.6. Differential Scanning Calorimetry (DSC)
PET granular were analyzed by differential scanning calorimetry (DSC, DSC200F3, Netzsch, Germany). DSC scans were run in the temperature range from 0 to 300°C under a dry nitrogen atmosphere. All runs were carried out at a heating rate and cooling rate of 10°C/min, and PET sample weights were about 10±2mg.

3. Results and discussion

3.1. DSC studies of the PET granular
The samples were characterized for their thermal properties by differential scanning calorimetry (DSC). The first heating scan of untreated PET, Figure 1a, show a clear glass transition temperature (Tg) at 82.2°C, a cold crystallization peak at 122.7°C, and a melting temperature (Tm) at 255°C. The cold crystallization peak originates from crystallization of the amorphous regions. In contrast, the thermogram of treatment PET (Figure 1b) had the same trend as Figure 1a, but glass transition temperature (Tg) at 81.8°C, a cold crystallization peak at 122.5°C, and a melting temperature (Tm) at 254.8°C. Obviously, the curves of the two samples tend to be the same, just different values. It was indicates that the thermal properties of PET does not change in whole-cell bioprocess.

According to earlier research, lipases should not be able to degrade semicrystalline aromatic polyesters [22]. Therefore, a single enzyme catalytic hydrolysis polymer has some defects. However, the whole-cell bioprocess can form a community of bacterial strains and enzymes in the heterogeneous degradation of aromatic polyesters. From figure 1 it can be concluded that the PET particle used in this study clearly prove that PET can be attacked by whole-cell hydrolyses, which is a significance step of the biodegradation process. Furthermore, from analysis of the melting heat of fusion, the degree of crystallinity of the untreated PET and treatment PET calculated to above, be 22.71±0.5% and

![Fig 1. DSC thermograms of (a) untreated PET and (b) treated PET, recorded during first heating scans with a heating rate of 10°C/min.](attachment://image1.png)
24.52±0.5%. Heat of melting of a 100% crystalline PET was accounted for 140J/g [19]. Therefore, it results that increase in crystallinity is 1.81%, and there are sufficient amorphous regions available to be degraded.

3.2. Main products of PET in whole-cell bioprocesses

In this investigation, the evolved aeromonas strain was employed as biocatalysts for the depolymerization of PET. The extent of PET hydrolysis was detected by measuring the amount of soluble deterioration products in solution using HPLC (Fig. 2).

![Fig 2. HPLC Chromatogram of soluble deterioration products in culture solution of PET in whole cell catalyzed processes](image)

In this study, PET was employed directly as substrate and was the sole carbon source and major energy. Figure 2 shows an example of the obtained chromatogram of the culture medium of PET-fermenting aeromonas strain at the third hour. There are more peaks than hydrolysis of PET with polyesterase, such as cutinase or lipase in literatures [26]. The result demonstrates that biotransformation of PET in whole cell catalyzed process is more complicated than that of enzymatic treatment.

Several water soluble products of PET in the biocatalysis process were identified by comparison with the retention time (rt) of standards following their separation by HPLC were TA, MA, BHET and BA. Although several kinds of soluble hydrolysis products of PET films, fibers or oligomeric model compounds have been reported as hydrolysis products in previous literatures, such as TA, BHET, BA, mono (2-hydroxyethyl) terephthalate (MHET) and 2-hydroxyethyl-benzoate (HEB) [5, 27]. It is the first time that MA was detected and identified in the hydrolysis of PET. Among the products detected in the PET fermentation culture, it can be seen that MA, TA and BHET are the main products. It has been reported that an intermediate product, which is not hydrolyzed at lower enzyme concentrations, is hydrolyzed at higher concentrations due to the excess of enzyme [28] In this investigation, the amount of exoenzyme increased in culture solution with the growth of microorganism. This may be attributed to the diversification of released intermediates. The varieties of enzymes in whole cell catalysis may also be a reason for the multiplicity of products in PET bioprocess.

3.3. Relationship between PET main products and biomass

To investigate the bioprocess of PET with whole-cell catalyst in more detail, the amount of products and intermediates released from PET substrate in the culture were monitored through the growth cycle of the strain and the biomass of strain was measured in the culture (Fig. 3).
The result in Fig. 3 shows the variation of all intermediates is not a linear relationship but rather an oscillation in whole cell bioprocess. However, in previous investigations of enzymatic hydrolysis of PET model substrate, the amount of soluble hydrolysis products was accumulated and presented an almost linear increase at the process of enzymatic hydrolysis [5] and the released products of PET, such as TA, was used as indicator to assess the effect of biocatalysis of PET [6].

Therefore, the oscillation in the data points was presumably caused by the mechanism of biological decomposition of PET, which can be attributed to the interaction of microbe cell, exoenzymes and substrates. Biodeterioration of PET is usually a heterogeneous process. Due to water insolubility and large molecules, microorganisms are not able to pick up the PET directly into the cells where most of the biochemical processes take place [29]. Therefore, secretion of extracellular enzymes is the first step of the biodeterioration process. Then the PET will be attached by enzymes and the esters available will be cleaved. The small molecule segments are released from the PET and the water soluble intermediates can be decomposed further before transported into the cells for metabolism, which cannot happen in the situation of hydrolytic enzyme focused ester bond. As a final result of these processes, microbial metabolic end-products such as water, carbon dioxide, etc. are produced and new biomasses are generated. Thus, the amount of products in culture solution was determined by the release of PET and the intake of cells.

The results in Fig. 3 were processed with FFT filter in the data process software and the trend of these curves was obtained (Fig. 4)
The processing data shows a clear trend of biomass and the amount of products changing with time in the niche of microorganism cultivation condition. In the early stage of logarithmic phase, the concentration of all the three products in the culture increased at different extent. Then, the amount of TA and MA began to decline, while BHET remained a constant concentration level approximately. In the middle of stationary phase, the trend of MA appeared to ascend.

This result demonstrates that TA is more consumed than obtained after late logarithmic growth phase. In our previous study, TA was employed as the only carbon source for cultivation of the aeromonas strain and MA was monitored in the culture. This result indicated that TA can be further decomposed into MA. So, it's worth mentioning that in addition to cleaving ester bonds of dissolved materials, the strain of aeromonas has the ability to decompose the benzene ring. In a short, the variation of products in whole-cell catalyzed processes may be a response of the growth and metabolism of microbe.

3.4. Utilization of micromolecule intermediates in whole-cell bioprocess

The influence of intermediates on the degradation of PET by polyester hydrolase was analyzed and product inhibition was detected in the process of enzymatic treatment [30]. In this study, effect of intermediates on bioprocess by whole-cell based biocatalysts was investigated. The micromolecule intermediates, such as MA, TA and BHET, were employed as sole carbon source at the same molar concentration and the biomass was measured for estimating the efficiency of utilization of these PET breakdown products with the engineering strain (Fig. 5).

![Fig 5. Growth curve of aeromonas strain cultivated with MA, TA and BHET respectively](image)

As shown in Fig. 5, the growth rate and maximum biomass are diversity in growth curve of aeromonas strain which was cultivated with different PET breakdown products. The result demonstrates that TA can be utilized efficiently by cells as the growth rate is close to the sample which cultured with PET particles. The fact that TA can be acted as substrate to aeromonas strain was conformed to our previous study [23]. However, the result shows that the strain cannot grow with MA. This may be explained the fact that MA was accumulated in the process of PET biodeterioration.

The strains cultivated with BHET have preferably biomass which indicates that BHET can be applied efficiently by cells. However, BHET was identified as inhibitor in enzymatic hydrolysis of PET [30]. It has been assumed that the inhibition was caused by the ester bonds of MHET and BHET occupying the substrate binding site of the enzyme. On the contrary, the inhibitory of hydrolysis products can be avoid in whole-cell bioprocess, in which the soluble intermediate may be uptaken by cells and further hydrolysis in cellular metabolism.

4. Conclusion

This paper compares PET crystallinity changes before and after the whole-cell hydrolysis PET.
Consistent with reports by using enzyme-polymer systems, increased PET crystallinity. Furthermore, TA, MA, BHET and BA were recognized as the intermediate products in whole-cell bioprocess of PET biodeterioration. The result demonstrates that the variation of PET main products in whole-cell bioprocesses is more complex than enzymatic treatment. Biomass and the amount of products are all presented in an oscillation shape, which may attribute to the interaction of insoluble substrate, microbe cell and the enzymes in a microbial active environment. The relationship between biomass and micromolecule intermediates indicates that the strain can grow with the water soluble products. And this conclusion was further demonstrated with the result of assimilation of micromolecule intermediates in whole-cell bioprocesses.

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