CONSUMPTION OF AROMATIC MONOMERS RELEASED FROM POLY(TEREPHTHALATE ETHYLENE) (PET) DEPOLYMERIZATION BY *Yarrowia lipolytica*

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ABSTRACT

Plastic pollution has been a topic of great concern globally due to the major environmental, economic, health and social impacts generated by the massive amount of daily plastic waste. Poly(ethylene terephthalate) (PET), an essential synthetic polyester derived from petroleum, is widely used in plastic packaging, mainly bottles and trays. Unfortunately, no greener polymer alternative has large-scale production to meet global demand. Consequently, PET recycling is the most sustainable approach to managing the massive amounts of post-consumer PET materials by recovering its fundamental monomeric units: terephthalic acid (TPA) and ethylene glycol (EG). Biological recycling (using cells or free enzymes as catalysts) has been an emerging eco-friendly alternative to tackle PET waste. Recently, the yeast *Yarrowia lipolytica* (strain IMUFRJ 50682) was reported to be able to biodepolymerize PET and consume the released monomers. However, the enzymes and metabolic pathways of these processes remain unknown.

In this study, different cultivation conditions of *Y. lipolytica* were investigated in YP medium containing different concentrations of aromatic monomers (BHET and TPA: 0.5, 5 and 10 $g.L^{-1}$) and initial inoculum loading of 1 $g.L^{-1}$. BHET was consumed entirely in less than 24 hours in all concentrations tested, accumulating mostly MHET in the reaction after 72 h (> 90%). TPA was less consumed, reaching 9.4% after 72 hours at the highest concentration tested. The maximum non-inhibitory concentration of TPA was also tested, demonstrating that this yeast can tolerate up to 35 $g.L^{-1}$ of TPA.

Keywords: poly(terephthalate ethylene). *Yarrowia lipolytica*. biorecycling. bioupcycling. circular economy.

1 INTRODUCTION

Since the discovery of synthetic plastics and their massive global production over the years, the world has drastically changed in positive and negative ways. Circa 46 million tons of plastics were produced in 2019 alone, around 230 times greater than initial large-scale production during the 1950s[1]. Based on predictions of annual global demand and in conjunction with the increase in global pollution, production will triple in 2060, reaching 1.32 billion tons[2,3]. Although the advantages of using these polymeric materials are unquestionable (such as versatility, lightness, durability and low production cost), incorrect waste management has generated one of humanity's biggest crises: plastic pollution. According to data referring to all plastic waste generated in the world in 2019, only 9% was recycled and 15% incinerated (releasing polluting gases into the atmosphere if not treated), 49% being sent to landfills and 22% being discarded directly. in nature[1]. This pollution leads to the dumping of macro-, micro-, and nanoparticles of plastics made up of synthetic monomers, mostly from fossil raw materials, and which were previously valuable for producing plastics. Therefore, there are economic losses when plastic is discarded. The crisis is worsened by the persistence of waste in marine and terrestrial ecosystems due to their high recalcitrance to degradation by biotic and abiotic factors.

Single-use or short-term plastics, such as those used in beverage and food packaging, are the main polluting waste because they are consumed and quickly discarded by consumers. Packaging represents the largest sector of the plastics industry, comprising 31.2% of all plastic manufactured in 2022[4]. This reflects the importance of these materials: they are light reducing transportation costs, in addition to provide a barrier against contaminants, gases and humidity, extending the useful life of many products. However, most of these packaging is not designed to be recycled, generating a linear chain of production, use and disposal that results in a massive daily amount of waste[5]. Poly(ethylene terephthalate) (PET) is the third most used synthetic polymer in the plastics industry, with the packaging sector being the largest application segment. PET production is made from the polymerization of two initial monomers: terephthalic acid (TPA; aromatic portion) and ethylene glycol (EG; aliphatic portion). Both are mostly obtained from fossil raw materials, and are polycondensed to form a polyester chain[6]. This polymer has several desirable attributes, (e.g. bottles of water and carbonated drinks are well-known packaging because they are made from PET due to its transparency, lightness, and excellent gas barrier). However, these packages are generally consumed and discarded quickly, listing this polymer as one of the main contaminants found in urban solid waste [5]. Recycling is an important alternative to reduce the accumulation of plastic waste, and there are different technologies proposed for PET waste[7]. Mechanical recycling is the simple way to reprocess plastic packaging, without promoting the depolymerization of the polyester chain. However, this method does not solve the problem in the long term, as desirable properties are lost after the second reprocessing cycle (such as reduced molecular weight, rigidity and transparency)[8]. Therefore, the materials formed are eventually downcycled, that is, they have lower characteristics and qualities than the initial materials.

Ad infinitum recycling is a method in which the ester bonds of the polymer chain of PET waste are cleaved to recover the initial monomers, in order to use these molecules as raw materials (similar to virgin monomers from petroleum) to produce new PET packaging, maintaining the desirable characteristics of the material. This method is the most sustainable alternative to establish a circular economic model in the life cycle of PET packaging. There are currently different routes proposed for this type of recycling: pyrolysis, chemical and biological. Each has advantages and disadvantages that must be considered. However, these processes are still costly due to several factors (e.g. high energy demand, catalyst, monomer purification step). Therefore, it is still necessary to study and improve these routes to make implementation on a global scale economically viable.

In the last decade, the yeast *Yarrowia lipolytica* was reported to biodepolymerize PET[9–11]. In this case, a Brazilian strain was used (IMUFRJ 50682) isolated from an estuary in Guanabara Bay, Rio de Janeiro. Furthermore, this microorganism can also consume TPA and EG monomers. In the case of the last substrate, oxidation reactions occur to form glycolic acid as the final product[12]. In this sense, there is also the possibility of establishing a route for bio-upcycle PET by valorizing the recovered monomer, since the added value of glycolic acid is high (around US$ 40.kg^{-1}). These findings provide a new possibility for tackling plastic pollution. However, few bioprocess parameters were investigated so far, such as substrate concentration. Moreover, the molecular bases involving key enzymes and metabolic routes used by yeast in these reactions. Elucidating this process is essential to provide novel enzymes or improve this strain using biological techniques synthetic, as biodepolymerization and bio-upcycling reactions are still slow for large-scale implementation.

2 MATERIAL & METHODS

Composition of the YPD liquid culture medium was 1% yeast extract (w/v), 2% universal peptone (m/v), and 2% glucose (w/v). Alternatively, YP liquid medium was prepared without the addition of glucose. For preparation of solid culture medium, 2% agar (m/v) was added for gelation. All culture media were sterilized in autoclave at 121°C, 1 atm, for 15 minutes. The yeast *Y. lipolytica* strain IMUFRJ 50682, isolated from Guanabara Bay (Rio de Janeiro, Brazil)[13], was used in all tests. For strain maintenance, cells were initially cultivated in solid YPD medium at 28°C, and then stored at 4°C. A cell stock bank was created from the cultivation of a colony isolated in 200 mL of YPD liquid medium, contained in an Erlenmeyer flask of 500 mL, and incubated on an orbital mechanical shaker at 28°C, 160 rpm. After 24 hours of growth, 500 µL aliquots of the culture were reserved in cryovials, and 500 µL of a sterile 50% glycerol solution was added as cryopreservative agent. Cell stocks were stored in a ultrafreezer at -50°C.

Maximum non-inhibitory concentration (MNIC) of TPA-Na_2 for *Y. lipolytica* growth was determined as reference methodology M27-A2 determined by Clinical and Laboratory Standards Institute[14], adapted to 96-well microplates with flat bottom. Obtaining disodium terephthalate (TPA-Na_2) was carried out according to the methodology described by Kaduk[15]. YP liquid was used containing different concentrations of TPA-Na_2 (35 to 0.017 g.L^{-1}), obtained by serial dilutions, and each concentration was carried out in triplicate. Each well was inoculated with an aqueous suspension of *Y. lipolytica* to achieve initial concentration of 1 g.L^{-1} of cells. Additionally, they were carried out in quintuplicate positive controls (pure YP medium with cells) and negative controls (pure YP medium without cells). The plate was incubated at 28°C, without shaking, and visual identification of turbidity generated by cell growth was performed after 48h.

Y. lipolytica cultivation assays containing different substrates (PET_{CPR}, BHET, TPA and TPA-Na_2) were carried out in 500 mL Erlenmeyer flasks containing 200 mL of YPD liquid medium. Different concentrations of substrates and initial biomass of 1 g.L^{-1} were evaluated. All tested conditions were performed in experimental duplicates, and the flasks incubated in an orbital mechanical shaker at 28°C, 250 rpm, for different periods of time. Control tests were also carried out in experimental duplicates: (1) assays containing culture medium with different substrates without cells; or (2) assays containing pure culture medium with cells.

Concentrations of BHET, MHET, TPA and TPA-Na_2 were monitored during cultivation using reversed-phase high-performance liquid chromatography technique (RP-HPLC), according to the methodology previously described[16]. Monitoring of *Y. lipolytica* biomass was carried out based on a standard curve that correlates dry cell biomass and the optical density registered on spectrophotometer at 570 nm. Variation of pH during cultivation was monitored from the samples collected using a pHmeter.

3 RESULTS & DISCUSSION

Initially the cytotoxicity of PET aromatic monomers during cultivations with *Y. lipolytica* was investigated. Low solubility of BHET and TPA in aqueous media turned this process challenging for the standard methodologies available in the literature. In this sense, only TPA-Na_2 (TPA in form of salt) was tested due to its high solubility in water (131 g.L^{-1}, 25°C, 1 atm)[17]. *Y. lipolytica* was able to grow at all tested concentrations. However, it was observed lower turbidity in the plate wells containing the highest TPA-Na_2 concentration (35 g.L^{-1}), indicating interference in cell growth. Then *Y. lipolytica* was cultivated with different substrate concentrations (0.5, 5 and 10 g.L^{-1}). In reactions with TPA-Na_2, the yeast was capable of consuming the monomer up to 6.8% and 9.4% after 72h in cultivation with 5 and 10 g.L^{-1} of substrate, respectively. In terms of biomass, cell concentrations were similar between all concentrations tested. This could indicate that consumption of this monomer is not directed towards cell growth. Possibly yeast is carrying out biotransformation reactions to accumulate an intermediate molecule as product. In the case of bacteria that use TPA as a carbon source, this acid is first oxidized by hydroxylation of the aromatic ring (generating 3,4-dihydroxy-1,5-cyclohexadiene-1,4-dicarboxylic acid (DCD)) and then decarboxylated and dehydrogenated (generating protocatechuic acid (PCA)) by the action of two enzymes: TPA dioxygenase and DCD dehydrogenase. These aromatic molecules (DCD and PCA) can be evaluated by methodology of RP-HPLC very similar to the one used in this study[18,19]. However, no additional peaks were observed during analyzed samples.

In relation to BHET conditions [0.5 $g.L^{-1}$ (2 mM), 5 $g.L^{-1}$ (20 mM) and 10 $g.L^{-1}$ (40 mM)], this substrate was hydrolyzed to MHET in 24h of reactions, being slowly converted the TPA up to 72h. However, BHET conversion to TPA was reduced conforming higher BHET concentration. At the end of the assay, the conversion reduced by 40% and 60% in conditions containing 20 and 40 mM BHET, respectively, in comparison to 2 mM BHET. This could indicate a possible enzymatic inhibition by substrate concentration (i.e. MHET). It is worth mentioning that there was no longer BHET present in the reaction that could indicate inhibition by a competitor. This type of enzymatic inhibition by MHET has already been reported by Barth *et al*.[20] during the PET hydrolysis by a cutinase from *Thermobifida fusca* KW3 (*Tf*Cut2). This can be circumvented by protein engineering of the enzyme[21] or by continued removal of enzyme inhibitor's reaction by an ultrafiltration membrane reactor[22]. It would also be It is possible to use another biocatalyst in the process to produce TPA from MHET, if this is the desired product[16,23]. In relation to cell biomass (Figure 12), it was observed during all time course of the assays an increase in cell concentration with higher concentration of BHET, including at the beginning (0h). That may have occurred due to the presence of insoluble BHET particles that interfered with the spectrophotometric analysis, contributing to the increase in absorbance values. Therefore, another technique such as cell counting in Neubauer chamber can be a more assertive alternative to carry out the correct analysis of cell growth in this type of fermentation in the future. However, it is worth highlighting that *Y. lipolytica* presents dimorphism, which may also interfere with count values[24].

4 CONCLUSION

Based on *Y. lipolytica* cultivation assays, it was possible to reproduce behaviors similar utilization of substrates by the yeast as described by da Costa *et al*.[10], except for TPA, which showed less intense consumption. In the tests that evaluated the effect of higher concentrations of PET and TPA, consumption was not intensified. This may indicate a limitation in the yeast's metabolism in assimilating them in higher amounts. However, higher concentrations of BHET were completely consumed by the yeast, forming MHET, but its conversion to TPA was reduced when higher concentrations of this intermediate were accumulated in the medium.

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