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Isolation and identification of bacteria and fungi growing spontaneously on polyhydroxyalkanoate pellets recovered by a new biological process

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ABSTRACT

Aims: The study was carried out to isolate and identify the spontaneously growing populations of bacteria and fungi on the surface of biologically recovered polyhydroxyalkanoate (PHA) copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)].
Methodology and results: Large-scale PHA biosynthesis was carried out using 300 L fermenter and a biological methodology developed in our laboratory was utilized for PHA recovery. Using standard microbiological and molecular biology techniques the naturally growing microbial populations on the surface of biologically recovered PHA were identified. Scanning electron microscopy (SEM) analysis showed that the identified bacterial (*Bacillus cereus* and *Burkholderia cepacia*) and fungal isolates (*Aspergillus niger*, *Byssochlamys nivea*, *Penicillium citrinum* and *Penicillium griseofulvum*) were able to grow on and degrade the P(3HB-*co*-3HHx) copolymer.
Conclusion, significance and impact of study: This is the first report on biologically recovered PHA pellet addressing the occurrence of microorganisms that grew spontaneously on it during storage under laboratory conditions. Fungi appeared to be dominant over bacteria in their ability to colonize the biologically recovered PHA.

Keywords: Aspergillus, Bacillus, biological recovery, *Penicillium,* poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

INTRODUCTION

Polyhydroxyalkanoates (PHA) are naturally occurring polyesters of various 3-, 4- or 5-hydroxyalkanoates which are synthesized by a wide range of microbes as intracellular granules of carbon and energy reserve compounds under unfavourable growth conditions (Lee, 1996). PHAs are sometimes referred to as bioplastics because of their plastic-like properties and its biological origin. Bioplastics have enjoyed much attention over past several decades owing to their useful characteristics similar to petrochemical plastics like thermoprocessability and formability. Among the various PHA, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is known to exhibit superior industrial characteristics due to its useful physical and mechanical properties along with decreased crystallinity resulting from varying content of medium-chain-length 3-hydroxyhexanoate (3HHx) monomers (Doi *et al.*, 1995; Jeon *et al.*, 2014).

PHA being biopolyesters synthesized from a wide range of renewable resources are subjected to biological process of degradation. Unlike synthetic plastics, PHAs are capable of complete degradation in a wide range of natural environments such as soil, water and sewage (Brandl *et al.*, 1990; Swift, 1993; Steinbüchel and Valentin, 1995; Jendrossek and Handrick, 2002; Kamilah *et al.*, 2013; Kumar *et al.*, 2016). In this respect, identification of microbes associated with PHA degradation and their ability to metabolize PHA is of significant importance foreseeing the future growth of PHA as a potential substitute for conventional plastics. Among the PHA degrading microbes, fungi are the most predominant group owing to their rapid surface growth rate and high depolymerase activity (Sang *et al.*, 2000). Biodegradation of PHA has been reported in a large number of microorganisms including halophiles (Quillaguaman *et al.*, 2010), bacteria (*Alcaligenes*, *Comamonas*, *Pseudomonas*, *Streptomyces*) and fungi (Ascomycetes, Basidiomycetes, Deuteromycetes, Mastigiomycetes, Myxomycetes) (Mergaert and Swings, 1996; Tokiwa and Buenaventurada, 2004; Yew *et al.*, 2006; Boyandin *et al.*, 2012).

PHA biosynthesis coupled with downstream processing for PHA recovery from bacterial biomass has been developed over the past several decades. Solvent extraction, digestion (chemical and enzymatic), mechanical disruption and several modified methods have been reported for PHA recovery (Jacquel *et al.*, 2008; Kunasundari and Sudesh, 2011; Madkour *et al.*, 2013). Recently, a novel biological process for PHA recovery was reported from our laboratory yielding

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reasonable quality PHA without using any solvents or chemicals (Kunasundari *et al.*, 2013). Spontaneous microbial growth was observed on the surface of biologically recovered PHA upon storage under laboratory conditions after several months. The present research was focused on the isolation and identification of these microorganisms growing spontaneously on the surface of biologically recovered PHA.

MATERIALS AND METHODS

Biosynthesis and biological recovery of PHA

Biosynthesis of PHA was carried out using recombinant *Cupriavidus necator* strain Re2058/pCB11, which was kindly provided by Prof. Anthony Sinskey of Massachusetts Institute of Technology, USA. Fermentation was carried out at Standards and Industrial Research Institute of Malaysia (SIRIM, Bioplastic Pilot Plant Facility, Selangor, Malaysia) using a 300 L fermenter following single stage fed-batch fermentation process according to the method published by Budde *et al.* (2011). Crude palm kernel oil (CPKO) was used as the carbon source along with urea (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.23 g/L) and 1 mL/L of trace elements (Kahar *et al.*, 2004) and conditions of fermentation were in accordance with the recommendations of Kunasundari *et al.* (2013). The PHA containing cells were harvested after 54 h by continuous centrifugation using Alfa Laval centrifuge at 5000 × *g* for 10 min at 4 °C. Harvested PHA containing cells were freeze-dried and subjected to biological recovery process using Sprague Dawley (SD) rat system following the methodology reported by Kunasundari *et al.* (2013). PHA recovered in the form of rat fecal pellets was further cleaned with 0.5% of the commercial mild detergent (Fab, Unilever, Malaysia) followed by 4 rounds of washing in tap water at room temperature. These steps helped to remove water soluble components such as pigments and proteins associated with the fecal pellet.The resultant rat fecal pellets containing the recovered PHA granules were dried under sunlight for 3 consecutive days and again it was dried in hot air oven (Memmert 824, Schwabach) for two days at 60 °C till constant weights were obtained and was characterized to be P(3HB-*co*-3HHx) copolymer containing 65 mol% of 3HB and 35 mol% of 3HHx, hereafter referred to as P(3HB-*co*-3HHx). The PHA composition was analyzed by using a Gas Chromatography machine (Shimadzu GC-2010, Japan) equipped with flame ionization detector**.** 2.0 µL of sample prepared by methanolysis was injected by AOC-20i Auto Injector for analysis. Nitrogen gas injector was maintained at following specifications in temperature of 270 °C with a pressure of 84.2 kPa with flow rate of 14.0 mL/min. The flow rates of hydrogen gas and air was maintained at 40.0 mL/min and 400.0 mL/min, respectively. Column initial and final temperatures were set at 70.0 °C and 280.0 °C, respectively with flame ionization detector set at 280 °C.

Spontaneous growth of microbes on PHA pellets

The washed and dried PHA pellets (50 g) were placed in unplugged 250 mL sterile conical flasks under room temperature (25±3 °C) for 30 days and the microbial population that grew spontaneously on its surface was isolated and characterised. 50 g of PHA pellets were sterilized (autoclaved using Hiclave^{TM} Hve-50 Hirayama machine) and was used as control. PHA pellets harbouring microbes were cultured on tryptic soy agar (TSA) and potato dextrose agar (PDA) to grow bacteria and fungi, respectively. Plates were incubated at 28-30 °C and regular sub-culturing was performed until pure cultures were obtained (five sub-culturing at 15 days interval). The morphological characteristics of fungal isolates were studied using light microscope (Olympus BX41) and scanning electron microscope (SEM) on 7 days old culture grown on PDA. Samples were coated with gold on 10 nm thickness using an automatic ion sputter. The morphology and degradation of polymer were investigated at 10 kV by SEM (Leo Supra 50 VP Field Emission SEM, Model – Carl Zeiss SMT, Oberkochen, Germany).

Molecular identification of bacterial and fungal isolates that grew spontaneously on biologically recovered PHA

Pre-culture of bacteria and fungi for the molecular analysis were grown on TSA and PDA, respectively. Using i-genomic BYF DNA extraction kit (iNtRON Biotechnology, Korea) total DNA was extracted from the respective bacterial (type A protocol) and fungal (type D protocol) by strictly following manufacturer's instructions. Quality checked DNA was subjected to PCR analysis for molecular identification of the microbial strains. PCR analysis was performed in a 50 µL reaction using quality checked 10 ng of genomic DNA isolated from pure bacterial cultures using 1492r primer (5′-TACCTTGTTACGACTT) and two fold degenerate primer 27f-CM (5′-AGAGTTTGATCMTGGCTCAG, where M is A or C) to amplify partial sequence of 16S rRNA genes (Frank *et al*., 2008). Similarly, PCR was done using respective DNA for each fungal isolates targeting the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. Using ITS primer set ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) and primer set ITS1 along with ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), respective ITS regions were amplified by PCR in a 50 µL reaction comprising 10 ng genomic DNA from respective isolates (White *et al.*, 1990; Johnson *et al.*, 2003). Amplification products were eluted from the agarose gel (QIAquick gel extraction kit, QIAGEN, Netherlands) and was sequenced using the respective primer sets for 16S rRNA genes and ITS regions mentioned above.

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Sequence Analysis

16S rRNA and ITS sequences obtained from bacterial and fungal isolates were compared with the highest homology sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) at National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) using the BLASTN search (Altschul *et al.*, 1997).

RESULTS AND DISCUSSION

PHAs are subjected to natural process of degradation due to their biological origin from renewable resources. The ability to degrade PHA by bacteria and fungi is known with specific extracellular PHA depolymerases in natural environment (Jendrossek and Handrick, 2002). The development and implementation of biodegradable and biocompatible PHAs under natural conditions are needed for a wide range of industrial applications (Page, 1995; Kalia *et al.*, 2000; Mucha and Tylman, 2012; Madbouly *et al.*, 2014). Poly(3-hydroxybutyrate) [P(3HB)] has been reported to be degraded by several groups of microorganisms (Mergaert *et al.*, 1994; Lee *et al.*, 2005). Life span of PHAs and PHA-composites depend on the immediate environmental conditions and the associated microbial load. PHA degrading microorganisms have been much studied and microbes involved in the degradation of polymers of varying carbon chain lengths have been identified and characterized (Brandl and Puchner, 1992; Mergaert *et al.*, 1993; Briese *et al.*, 1994). Several PHA degrading microbes have been identified from various ecosystems, but the reports disclosing the mechanism of PHA degradation by these microorganisms in diverse environment is very much limited.

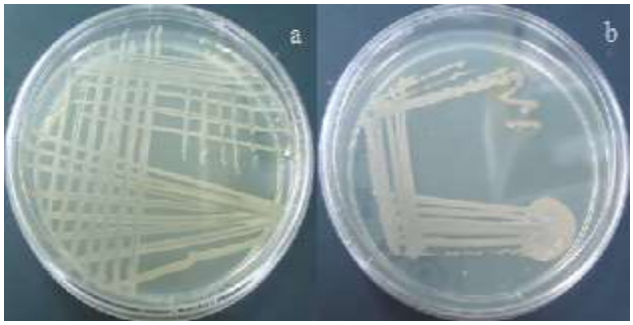

Figure 1: Pure cultures of bacteria isolated from surface of PHA pellet. a, *Bacillus* sp. and b, *Burkholderia* sp.

Using standard microbial techniques we isolated two pure cultures of bacteria (Figure 1) and four cultures of fungi (Figure 2) naturally growing on the surface of biologically recovered PHA pellets. There was no microbial growth observed on the pellets which were autoclaved and kept as control. Total genomic DNA was extracted from the microbial cultures and was subjected to molecular analysis. By using 16S rRNA primers 27f-CM and 1492r, ~800 bp amplicon was obtained from bacterial isolates (Figure 3a). Similarly, ITS primer sets ITS1-ITS2 and ITS1-ITS4 generated ~550 to 620 bp amplicons (Figure 3b) from fungal isolates. The nucleotide analysis identified the bacterial isolates to be *B. cereus* and *B. cepacia* with 99% and 96% sequence homology for the respective accession numbers JQ311944.1 and AB162427.1 in NCBI. Nucleotide sequences of identified *B. cereus* and *B. cepacia* were deposited in NCBI, with GenBank accession numbers KT751533 and KT751534, respectively. The DNA sequences obtained for the four fungal isolates were compared to those available in the GenBank database and were identified to be *P. citrinum*, *P. griseofulvum*, *B. nivea* and *A. niger* (ITS1-ITS2) having respective sequence homology with accession numbers AM745115.1, KC110617.1, AY753338.1 and KF221088.1 to be 100%, 99%, 96% and 96%. GenBank accession numbers for the identified *P. citrinum*, *P. griseofulvum*, *B. nivea* and *A. niger* deposited in NCBI are KT751531, KT751530, KT751532 and KT751529, respectively. Several species of bacteria and fungi have been reported to degrade PHAs among which *Bacillus* sp., *Aspergillus* sp. and *Penicillium* sp. form the predominant group (Table 1). The present study revealed preliminary insights regarding the natural ability of these microbes to degrade PHA. It was observed that fungus grew more robustly than bacterial population on the biologically recovered PHA pellets (Figure 4), probably due to its enhanced activity of enzymes (Bhardwaj *et al.*, 2012). SEM analysis revealed that all the fungal isolates were able to grow and develop porous structures on the surface of PHA (Figure 5). Further studies at molecular and biochemical level, with emphasis on PHA depolymerases together with the evolutionary relationship of these cultures to the other reported organisms can provide vital information regarding their comparative effectiveness in PHA degradation.

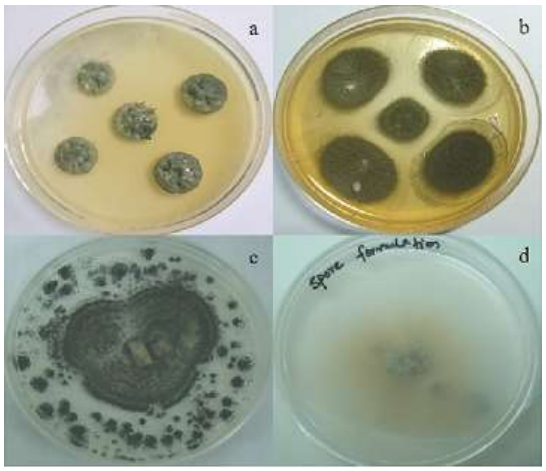

Figure 2: Pure cultures of fungi found growing on the surface of PHA pellets; a, *P. citrinum*; b, *P. griseofulvum*; c, *A. niger* and d, *B. nivea*.

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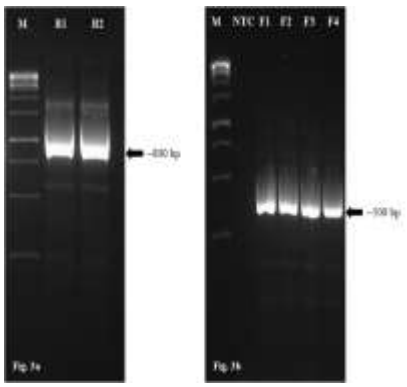

Figure 3: PCR analysis of bacterial and fungal DNA. a, PCR analysis using 16S rRNA primers. M -1 Kb ladder, B1 and B2 correspond to ~ 800 bp amplicons identified as *B. cereus* and *B. cepacia*, respectively. b, PCR analysis using 18S rRNA primers. M -1 kb ladder, NTC – Non template control, F1, F2, F3 and F4 refer to ~ 500 bp amplicons identified as *P. citrinum*, *P. griseofulvum*, *A. niger* and *B. nivea*, respectively.

An efficient and cost-effective recovery of PHA together with an understanding of its biological degradation is vital in industrial production and commercialization of bioplastics as potential alternative to conventional plastics for ecological stability. Efforts have been made to blend PHA with distiller's dried grains with solubles to form agricultural plastics having the potential to end their life cycle as organic content in soil (Madbouly *et al*., 2014). The present study is the first to report on the identification of microbes spontaneously growing on the surface of biologically recovered PHA pellets and more precisely on PHA copolymer P(3HB-*co*-3HHx). Further studies are in progress with regard to the substrate specificity of the identified microorganisms. A comparative study utilising different PHA polymers in diverse ecosystems can provide vital information regarding the choice of PHA to be used in agricultural application such as in the development of controlled release of fertilizers. The biologically recovered PHA may also be used as an inoculum to enrich the microbial populations in soil, compost and biological waste water treatment systems.

Table 1: List of bacteria and fungi with ability to degrade PHA.

Organism	Ecosystem	Group	Domain	References
Acidovorax facilis	Soil, compost	Gram negative	Prokaryote	Mergaert *et al.*,1992a
Aspergillus sp.	Soil, compost	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Aspergillus fumigates	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992b
Aspergillus pencilloides	Soil, compost	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Bacillus megaterium	Soil, compost	Gram positive	Prokaryote	Mergaert *et al.*, 1993
Bacillus polymyxa	Soil	Gram positive	Prokaryote	Mergaert *et al.*,1992a
Cephalosporium sp.	Soil	Deuteromycetes	Eukaryote	Matavulj and Molitoris, 1992
Cladosporium sp.	Soil	Deuteromycetes	Eukaryote	Matavulj and Molitoris, 1992
Mucor sp.	Soil	Zygomycetes	Eukaryote	Matavulj and Molitoris, 1992
Penicillium adametzii	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Penicillium chermisinum	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Penicillium daleae	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Penicillium funiculosum	Soil	Ascomycetes	Eukaryote	Brucato and Wong, 1991
Penicillium ochrochloron	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Penicillium restrictum	Soil	Ascomycetes	Eukaryote	Mergaert *et al.*,1992a
Penicillium simplicissimum	Soil, compost	Ascomycetes	Eukaryote	McLellan and Halling, 1988
Pseudomonas pickettii	Laboratory	Gram negative	Prokaryote	Mukai *et al.*, 1994
Pseudomonas stutzeri	Lake water	Gram negative	Prokaryote	Mukai *et al.*, 1994
Streptomyces sp.	Soil, compost	Actinomycetes	Prokaryote	Mergaert *et al.*,1992a

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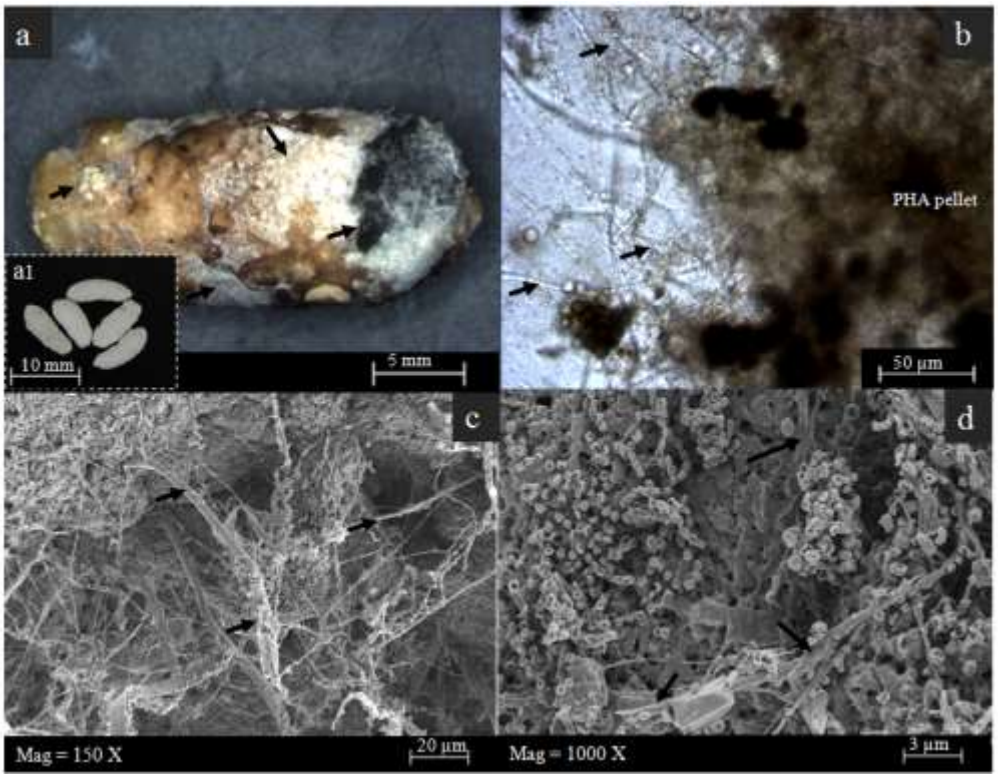

Figure 4: Images of biologically recovered PHA after spontaneous microbial growth: a, PHA pellets showing the spontaneous growth of microbes on its surface. a1, Inset shows freshly washed and dried PHA pellet before spontaneous microbial growth; b, PHA pellet observed under light microscope showing fungal mycelium; c, & d, SEM images of spontaneous fungal growth on PHA pellet under 150 × and 1000 × magnifications, respectively (arrows indicate fungal mycelium).

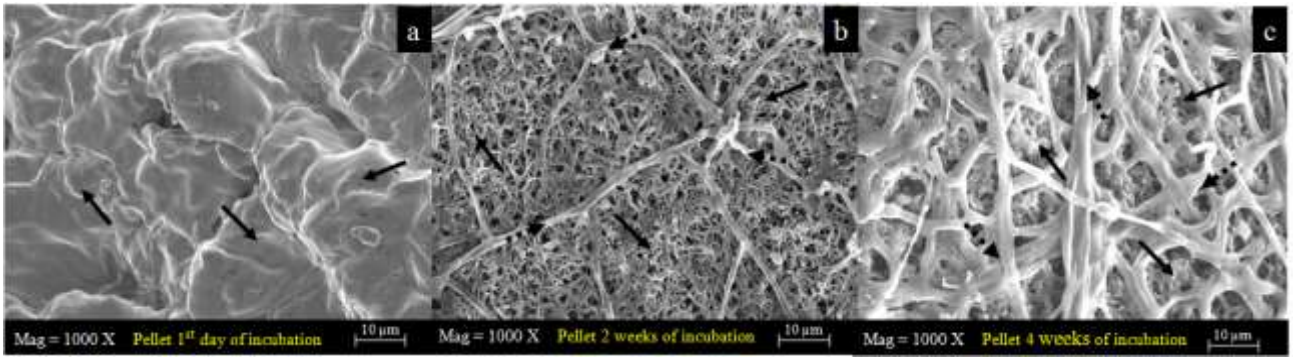

Figure 5: SEM analysis of biologically recovered PHA pellets showing microbial colonization and degradation: a, Surface structure of biologically recovered PHA pellets; b, PHA pellets after 2 weeks of incubation and c, PHA pellets colonized by fungal mycelium after 4 weeks of incubation. b and c show disintegration of surface layer of PHA pellet as porous and fibrous structures (solid arrows indicate surface layer of PHA and dotted arrows indicate fungal mycelium).

CONCLUSION

To summarise, the present study showed the ability of microbial population to grow and survive spontaneously on biologically recovered PHA copolymer of P(3HB-*co*-3HHx). SEM analysis clearly indicated that all isolates have the potential to degrade PHA copolymer. This study also revealed that fungal isolates showed better ability to grow and degrade PHA in comparison to the bacterial cultures. Further studies are in progress with emphasis on utilizing PHA copolymer in agriculture-based ecosystem to develop PHA-based controlled release agrochemicals.

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