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MALDI-Biotyper as a tool to identify polymer producer bacteria

Bruno Karolski[a,b,*], Letícia O.B. Cardoso[a], Louise H. Gracioso[a,b], Cláudio A.O. Nascimento[b], Elen A. Perpetuo[c]

[a] Environmental Research and Education Center, University of São Paulo, CEPEMA-POLI-USP, Conego Domenico Rangoni Rd, 270 km, Cubatão, SP, Brazil
[b] Chemical Engineering Department, University of São Paulo, POLI-USP, Lineu Prestes Ave, 580, São Paulo, SP, Brazil
[c] Department of Marine Sciences, Federal University of São Paulo, Imar-UNIFESP, Carvalho de Mendonça Ave, 144, Santos, SP, Brazil

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ABSTRACT

The methylotrophs bacteria can use methane and methanol as carbon sources to produce biopolymers including the polyhydroxybutyrate (PHB) a very promised substitute for the environment contaminant oil-derived polypropylene. This kind of bacteria can be very effective to help to decrease PHB price production and promote its use in substitution of several environment contaminant plastics. The search for methylotroph bacteria able to produce PHB is a very arduous job being necessary to grow all isolates and submit all of them to extraction processes and product characterization. Looking for time reducing and optimization of resources, we tested the Matrix Assisted Laser Desorption/Ionization technique (MALDI-Biotyper) to identify polymer producer bacteria based on a single isolated colony with success. The results showed here will contribute to speed-up and increase the discoveries of new bacteria strains able to produce PHB and other biopolymers.

1. Introduction

The environmental microbial diversity continues to be a challenge for microbiologists. Culture-dependent and non-dependent techniques focusing on different biomolecules as DNA, RNA, proteins, and metabolites are been used together attempting to increase data quality and depth of environmental microbiology. The use of Matrix-Assisted Laser Desorption/Ionization (MALDI) technique is being used as a rapid and trustable method for bacterial identification using ribosomal proteins mass/charge spectrum comparison. Many studies have shown the identification accuracy for isolated strains of clinical importance, the main use of MALDI-Biotyper, contributing for fast disease diagnosis reducing time in microbial identifications (Chen et al., 2017; Harris et al., 2010). The accuracy and fast microbial identification expanded the use of MALDI-Biotyper comparison method to environmental samples. Based on the spectrum database comparison, the MALDI-Biotyper present a small database compared to rDNA 16S database, the most used method for microbial identification based on genetic analysis. Although the disadvantage of a small database and most of them towards clinically important bacteria, the use of MALDI-Biotyper to identify environmental isolated strains are growing. Isolated bacteria from aquatic environment for monitoring purposes (Popović et al., 2017) and even extremophiles microorganism from Arctic sea water (Timperio et al., 2017), and from metal contaminated soil (Avanzi et al., 2017) have been identified with MALDI-Biotyper achieving high accuracy for genus and even for species.

The MALDI-TOF (Matrix Assisted Laser Desorption/Ionization - Time of Flight) technology is the base of MALDI-Biotyper microbial identification. This technology can measure the mass/charge of peptides detached from whole protein when a high energy source (in this case a high voltage laser) is applied to a fixed protein in a matrix bed (De Hoffmann and Stroobant, 2007). Each peptide will reach the detector in a specific time according to the acquired charge and its specific molecular mass give to the protein a specific “fingerprinting” spectrum. Polymers are molecules that can be analyzed by MALDI-TOF technology (Adamus et al., 2006). Break of a polymer molecule generates a repetitive spectrum in MALDI-TOF with exactly mass/charge ratio between adjacent peaks. This exactly mass/charge ratio difference represents the monomer that constitutes the polymer. This is a very particular mass/charge spectrum.

Some bacteria can produce polymers from different carbon sources. One already known polymer from bacterial origin is the PHB (polyhydroxybutyrate). The PHB is a homopolymer composed of 3-hydroxybutyrate monomers produced and accumulated in some microorganisms as a reserve of energy inside the cells (Akaraonye et al., 2010). Due to its physical and chemical characteristics, PHB is a

* Corresponding author at: Environmental Research and Education Center, University of São Paulo, CEPEMA-POLI-USP, Conego Domenico Rangoni Rd, 270 km, Cubatão, SP, Brazil.
E-mail address: bruka@usp.br (B. Karolski).

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potential substitute of polypropylene from petroleum and is already used in films, containers, plastic bags and even in medicine as part of sutures and bandages (Akaraonye et al., 2010; Khanna and Srivastava, 2005). In addition to polypropylene characteristics, PHB is highly biodegradable into carbon dioxide (CO_2) and water (H_2O) by aerobic degradation and into methane (CH_4) and H_2O by anaerobic degradation (Khanna and Srivastava, 2005). The Large-scale microbial PHB production is based on starch and sugar which represent 30–40% of the product's final price. Because of the relatively high price of the raw material for PHB production, the final product is currently more expensive than oil-derived plastics and this is the major reason because the PHB is not widespread all over the world (Chanprateep, 2010). One way to decrease the price of PHB and give competitivity facing the conventional polypropylene is the searching of new bacteria that can use different and cheaper substrates to produce PHB. Some bacteria, the methylotrophs, can use small carbon molecules as methane and methanol to grow and produce biopolymers including PHB (Strong et al., 2015).

The search of new PHB producer bacteria involves several steps from sampling to PHB production confirmation. In general, the process of isolation after sampling includes enrichment of bacteria that can use the substrate of interest, isolation of colonies in plates, culture of an isolated colony in liquid medium for production, cell harvest, PHB extraction and purification and analysis of the extracted polymer. All the process can take time and resources to test each isolated colony for PHB production, and the number of isolated colonies can be restricted due to laboratory capacity. To optimize the search for bacteria able to produce polymers, we tested 105 colonies isolated from mangrove region using the MALDI-Biotyper. All colonies were directly submitted to the MALDI-Biotyper and all spectra were analyzed for peak patterns (i.e. spectrum presenting same mass/charge variations between peaks). The bacteria presenting the peak pattern spectrum were tested for polymer production with positive results. This method described here could reduce laboratory resources and increase the number of study colonies concurrently processed and speed up the search for new and promising biopolymer producer bacteria from the environment.

2. Materials and methods

2.1. Sampling

Samples were collected from mangrove located in São Paulo's coast in the cities of Guarujá (GPS: −23.91358, −46.20936) and Cubatão (GPS: −23.89623, −46.42034). Soil and water were collected from two locals in sterile 50 mL polypropylene flasks in triplicate. The samples were processed for bacterial enrichment in less than 24 h and spare material was frozen at −80 °C.

2.2. Bacterial cultures

For methylotrophs bacterial enrichment, 1 g of sampled soil or 1 mL of sampled water from each polypropylene tube collected were inoculated into 50 mL of M4 medium adapted from Bourque and coworkers (Bourque et al., 1995) containing $(NH_4)_2SO_4$ 1.5 $g{\cdot}L^{-1}$; KH_2PO_4 1.305 $g{\cdot}L^{-1}$; $Na_2HPO_4 \times 12H_2O$ 4.02 $g{\cdot}L^{-1}$; H_3BO_3 1.8 m $g{\cdot}L^{-1}$; $CoCl_2 \times 6H_2O$ 2.4 m $g{\cdot}L^{-1}$; $CuSO_4 \times 5H_2O$ 2.4 $mg{\cdot}L^{-1}$; $CaCl_2 \times 2H_2O$ 60 $mg{\cdot}L^{-1}$; $MgSO_4 \times 7H2O$ 1.35 $g{\cdot}L^{-1}$; $FeSO_4 \times 7H_2O$ 60 $mg{\cdot}L^{-1}$; $Na_2MoO_4 \times 2H_2O$ 2.4 $g{\cdot}L^{-1}$; $ZnSO_4 \times 7H_2O$ 7.8 $g{\cdot}L^{-1}$. For bacteria enrichment, 0.5% of methanol was added to the M4 medium using 125 mL Erlenmeyer flasks. The flasks were incubated in a shaker (Infors HT, Switzerland) at 28 °C and 180 rpm for 7 days. After the 7 days incubation, 1 mL of each flask was inoculated in new 250 mL Erlenmeyer flasks containing 50 mL of M4 medium with 0.5% of methanol to select only bacteria able to use methanol as sole carbon source. The cultures were incubated in shakers with the same previous setup for 7 days. After the second-time incubation, 50 µL of enriched culture was inoculated in triplicate in Petri dishes containing solid minimal mineral medium with 0.5% methanol. Plates were incubated at 28 °C until colonies appeared (from 3 to 5 days). These bacterial colonies were analyzed in MALDI-TOF Biotyper directed for polymer patterns identifications.

2.3. Colony preparation and spectrum acquisition

Equipment calibration was set using Bruker's bacterial test standard (Bruker Daltonics, Bremen, Germany). Calibration and spectra acquisition was realized using 240 reads from 50 laser shots from different positions (automated mode) in positive linear mode and a mass/charge range of 800–11,000 *m/z*. The isolated grown colonies from sampled locals were transferred from agar plates to 96-wells polished steel plates with a sterile toothpick and spread inside the well. After transference, 1 µL of the matrix (saturated solution of α-cyano-4-hydroxycinnamic acid diluted in 50% (V/V) acetonitrile and 2.5% (V/V) trifluoroacetic acid in sterile water). Each spectrum was analyzed by BioTyper 3.0 software to spectra comparison between colonies and spectrum database. Spectrum with repeated patterns had the peaks measured to identify mass/charge values for comparison. Positive colonies for repeated pattern spectrum were identified and tested for biopolymer production.

2.4. Biopolymer production, extraction, and analysis

Each positive bacterium according to MALDI-Biotyper spectrum was cultivated in 20 mL modified M4 medium in 125 mL Erlenmeyer flasks and incubated under constant agitation of 180 rpm and 28 °C for 3 days. After incubation, 5 mL of bacterial culture were inoculated in a new 50 mL modified M4 medium in 125 mL Erlenmeyer flasks and incubated at the same conditions described above for 7 days. Grown cells were submitted to polymer extraction following Law and Slepecky (Law and Slepecky, 1961). Cells were harvest by centrifugation at 24,300 *g* for 10 min. The supernatant was discarded, and cells were resuspended in 5 mL commercial sodium hypochlorite for 1 h at 37 °C. The mixture was centrifuged at 14,400 *g* and washed with 5 mL of water, acetone, and ethanol subsequently, always discarding the previous supernatant. After last wash, the polymer was dissolved in 10 mL of chloroform and filtered with 0.22 µm PVDF syringe filter using a 50 mL glass syringe. The filtrate was transferred into a 20 mL glass beaker and incubated in a fume hood for at least 8 h for chloroform evaporation and pellet dry.

The product of extraction and standard polyhydroxybutyrate acquired from Sigma-Aldrich were analyzed by nuclear magnetic resonance (NMR). ^1H NMR spectra were recorded on a Bruker DPX 300 at 300 MHz, using deuterated chloroform ($CDCl_3$). Chemical shifts were acquired in ppm, referenced to the solvent signal of $CDCl_3$ as the internal reference.

2.5. Molecular identification

The molecular identification of selected bacteria was performed using primers 341F (5′ CCTACGGGNGGCNGCA 3′) and 826R (5´ GAC TACCAGGGTATCTAATCC 3′) (Soergel et al., 2012) using same amplification conditions and Ready Mix Taq PCR Reaction Mix (Sigma-Aldrich) as amplification reagents. To verify amplified fragments, the amplification products were analyzed in 0,8% agarose gel made with TAE buffer (Sambrook et al., 1989) and stained with Syber Safe (Invitrogen) according to manufacturer instructions and visualized in Imager 600 (GE, Sweden). Fragments were cleaned with DNA, RNA, and Protein purification kit (Macherey-Nagel, Germany) and sequenced in an ABI 3730 DNA Analyzer (Applied Biosystems) using BigDye Terminator V3.1 (Applied Biosystems) following manufacturer's instructions. After manual verification of sequence quality in Thermo Fisher Cloud (https://apps.thermofisher.com) with quality check module, sequences were deposited in NCBI database under

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