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Enhanced production of biobased, biodegradable, Poly(3-hydroxybutyrate) using an unexplored marine bacterium *Pseudohalocynthiibacter aestuariivivens*, isolated from highly polluted coastal environment

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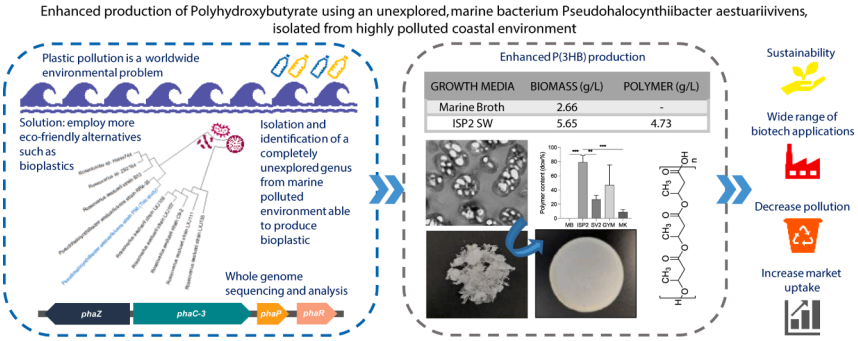
HIGHLIGHTS

- Pseudohalocynthiibacter aestuariivivens P96 is a novel producer of P(3HB) polymer.
- Genome analysis revealed the presence of genes involved in the PHB biosynthesis.
- SEM and TEM analysis clearly showed granule accumulation in the productive medium.
- The P(3HB) production is 4.73 g/L and the intracellular content 87 % dcw.
- P. aestuariivivens P96 displayed high industrial potential.

GRAPHICAL ABSTRACT



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ABSTRACT

The production and disposal of plastics from limited fossil reserves, has prompted research for greener and sustainable alternatives. Polyhydroxyalkanoates (PHAs) are biocompatible, biodegradable, and thermoprocessable polyester produced by microbes. PHAs found several applications but their use is limited due to high production cost and low yields. Herein, for the first time, the isolation and characterization of *Pseudohalocynthiibacter aestuariivivens* P96, a marine bacterium able to produce surprising amount of PHAs is reported. In the best growth condition P96 was able to reach a maximum production of 4.73 g/L, corresponding to the 87 % of total cell dry-weight. Using scanning and transmission microscopy, lab-scale fermentation, spectroscopic techniques, and genome analysis, the production of thermoprocessable polymer Polyhydroxybutyrate P(3HB), a PHAs class, endowed with mechanical and thermal properties comparable to that of petroleum-based plastics was confirmed. This study represents a milestone toward the use of this unexplored marine bacterium for P(3HB) production.

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1. Introduction

Global production of fossil-derived synthetic plastics has grown exponentially reaching a value of 367 million metric tons in 2020 (Obulisamy & Mehariya, 2021). The production and after-use phase of synthetic plastics contribute to 400 million tons of annual CO_2 emissions accounting for 4.5 % of Global Greenhouse Gas Emissions (Taguchi & Matsumoto, 2021). Furthermore, only 9 % of plastic is recycled, with the remaining escaping the collection system and ending up in landfills and oceans. A transition to a circular economy by providing environmentally sustainable alternatives to fossil-based plastics is required to tackle plastic pollution. Polyhydroxyalkanoates (PHAs) are a class of bio-based, biodegradable, biocompatible, and thermoprocessable polyester, which makes them expedient for several applications including packaging, agriculture, and biomedicine, and generally for a sustainable environment (Dalton et al., 2022). PHAs are synthesized as granules intracellularly by bacteria (granular size ~0.2–0.5 μm) through fermentation of renewable resources, such as carbohydrates and lipids, under unbalanced growing conditions (Anjum et al., 2016). Such conditions are achieved by exposing bacteria to a surplus amount of carbon source coupled with a limited amount of other essential nutrients, for example, nitrogen, or by other exogenous stresses, such as oxidative damage, high temperature, hypersalinity, and other hazardous environmental conditions (Koller & Braunegg, 2018). Among PHAs, Poly(3-hydroxybutyrate) or P(3HB) is a widely reported type of PHAs. It was initially discovered by Lemoigne in *Bacillus megaterium* (Lemoigne, 1926). The thermal and mechanical properties of P(3HB) make this isotactic crystalline thermoplastic polyester an attractive biodegradable alternative to petroleum-based plastics. The P(3HB) market is predicted to reach $98 million by 2024 (Sirohi et al., 2021). However, the cost of P(3HB) of microbial origin is still high at around 30 USD/kg, as sold by Goodfellow®, which is 20–80 % higher than fossil based synthetic plastics (Fernández-Dacosta et al., 2015; Manikandan et al., 2021). Several Gram-negative and Gram-positive bacteria employed for the production of PHAs, such as *Cupriavidus necator*, *Bacillus* sp., *Pseudomonas* sp., and *Halomonas* sp., among others, are considered to be a model system for PHAs production (Sehgal & Gupta, 2020). However, one of the issues hindering microbial bioplastic production at commercial level is the low yield of products and the high costs of downstream processing (Koller & Braunegg, 2018). To circumvent this, it is essential to explore novel bacterial species that can achieve high yields of PHAs in a more cost-effective and sustainable way. In this study, the isolation and identification of an unexplored marine bacterium, Pseudohalocynthiibacter aestuariivivens from highly polluted shallow water sediments (in the Gulf of Naples) was performed. After the investigation of its potential to produce PHAs, the presence of intracellular PHA content was confirmed using scanning and transmission microscopy, lab-scale fermentation experiments, spectroscopic techniques, and genome analysis. Resultant PHA was characterised and processed into 2D films and 3D constructs to explore their potential for industrial applications. This work highlights the importance of the exploration of marine and aquatic biodiversity for biotechnological purposes and provides a novel biological tool for the production of bio-based polymers.

2. Materials and methods

2.1. Isolation and identification of P96 strain

Strain P96 was isolated, among other microorganisms, from shallow water sediments collected from the mouth of the river Sarno, Gulf of Naples (Italy). Sediments were incubated with filtered sea water at 20 °C over 45 days simulating a natural light cycle of 12 h light/night. Then 1 g of sediment was dissolved in sterile natural sea water, homogenised using a vortex and serially diluted. Finally, 150 μL were spread on Marine agar (Condalab, Madrid, Spain) plates and incubated for 10 days at 20 °C. The strain was identified by the amplification of 16S rDNA gene. PCR was performed by DreamTaq PCR Master Mix (final volume 50 μL) using 0.2 μM of primer 27F and Univ1492R. The amplified DNA fragments were purified by using GenElute™ PCR Clean-UP kit, sequenced by Eurofins Genomics and submitted to Prabi CAP3 and to BLAST for preliminary identification. The sequences have been aligned with MUSCLE by MEGAX software and used to build the phylogenetic tree.

2.2. Genome sequencing and annotation

P96 was cultivated in 2 mL of MB for two days. The genomic DNA was extracted by GeneElute Bacterial genomic DNA kit, and sequenced by Macrogen using Illumina sequencing with a 2 × 151 nt read length. Trimmomatic (v0.36) was applied to trimm the sequences, then quality control was assessed with the FastQC (v0.11.9). The assembly was performed using MEGAHIT (v1.2.9) and the obtained contigs were further assembled in a scaffold by the MeDuSa web-server. Finally, the presence of plasmid was investigated with PlasFlow tools. The whole genome was subjected to ANI Calculator by EZBiocloud for the bacterial identification and to RASTtk (v1.073) for the annotation. Manual curation allowed to search for specific genes. Multiple alignments of conserved sequences were carried out by MEGAX and Jalview software.

2.3. Biosynthesis of PHA using P. aestuariivivens (P96) as the producer organism

4 mL of sterile marine broth was inoculated with *P. aestuariivivens* and cultured for 48 h at 20 °C at 180 rpm. The seed culture was transferred into 2L shaken flasks containing 400 mL of the production media and incubated for 5 days with an agitation of 180 rpm and a temperature of 20 °C. Five different media compositions that were screened for the production of PHA are listed as follows 1) Marine Broth 2216; 2) ISP2: Glucose 4 g/L, Yeast Extract 4 g/L, Malt Extract 10 g/L; 3) SV2: Glucose 15 g/L, Peptone 15 g/L, Glycerol 15 g/L, CaCO3 1 g/L; 4) GYM modified: Glucose 4 g/L, Yeast Extract 4 g/L, Malt Extract 4 g/L, CaCO3 2 g/L; 5) Modified Kannan and Rehacek medium: Glucose 35 g/L, Yeast Extract 2.5 g/L, Ammonium sulphate 5 g/L, Potassium chloride 3 g/L. Artificial seawater (ASW) containing 30 g/L of sea salt was used for all conditions (except for MB) after the salinity of seawater was determined using a refractometer. After 5 days, the biomass was lyophilized and the PHA was recovered using dispersion method (Rai et al., 2011).

2.4. Scanning and transmission electron microscopy

The bacterium was cultivated in MB and ISP2 for three days, then the cultures were centrifuged for 10 min at 3000 × g, and the obtained pellet was cleaned in filtered seawater (FSW). Scanning Electron Microscopy (SEM) was performed fixing the cells overnight at 4 °C in 2 % glutaraldehyde in FSW, resuspended in FSW and poured on a 0.4 μm filter. The cells were then washed in FSW and post-fixed in the dark for 1 h at 4–8 °C in 1 % OsO_4 + 0.8 % potassium ferrocyanide in FSW. The filters were washed in FSW, successively in MilliQ water, and then dehydrated in graded ethanol series. Finally, the samples were vacuum coated with gold. Observations were made on JEOL 6700 FE SEM (JEOL, Tokyo, Japan). The cells treated for transmission electron microscopy (TEM) were prepared with the same protocol for SEM analysis until dehydration in graded ethanol series. After that, the samples were enclosed with EMbed 812 resin for 48 h at 60 °C. Finally, they were observed with a ZEISS LEO 912 AB TEM (ZEISS, Oberkochen, Germany).

2.5. Fourier transform infrared spectroscopy (FT-IR)

The PHA produced was characterised using FT-IR spectrometer Spectrum Two (PerkinElmer Inc, USA) (4000 to 400 cm^{-1}, 4 cm^{-1} – resolution and CsI: 5 scans).

2.6. Gas Chromatography-Mass spectrometry (GC–MS)

Monomer identification of the PHA produced was determined using GC–MS Varian system with Chrompack CP-3800, Elite-5MS capillary column and Saturn 200 MS/MS block. Methyl benzoate was used as the internal standard (STD). 1 mL/min of methanolysed sample in the organic phase was injected with helium gas, with a temperature increment of 18°C/min from 40 to 240 °C. The temperature of the injector was 225 °C.

2.7. Nuclear magnetic resonance (NMR)

^{13}C and ^{1}H-NMR (Bruker Avance III 600 Cryo) was used to elucidate the structure of the PHA produced. Samples concentration was 20 mg of the PHAs per mL of deuterated chloroform ($CDCl_3$).

2.8. Thermal and mechanical characterisation of the solvent cast PHA films

2.8.1. Preparation of solvent cast PHA films

To investigate the mechanical and thermal characterisation of the PHA produced, 5 wt% solvent cast PHA films were prepared. 0.5 g of PHA was dissolved in 10 mL of chloroform and poured into glass petri dishes. Films were dried at room temperature for five weeks prior to analysis.

2.8.2. Differential scanning calorimetry (DSC)

DSC Polyma (Netzsch, Germany) with Intracooler IC70 cooling system was used to measure the melting temperature (T_m), the enthalpy of fusion (ΔHm) and glass transition temperature (T_g) of the PHA produced. Proteus 7.0 Software (Netzsch, Germany) was used to analyse the thermograms. For the analysis, 5 mg of the polymer was exposed to two cycles of heating and cooling (−70 °C and 200 °C) at a heating rate of 20 °C min^{-1}.

2.8.3. Tensile testing

The mechanical properties, elastic modulus (E), ultimate tensile stress (u), and elongation at break (b) of the PHA produced were studied using Instron 5940 testing system with a maximum load of 500 N. Each 5 wt% PHA film samples (5 mm width and 3.5–5.0 mm) was deformed at a rate of 5 mm/minute. The raw data obtained was analysed using BlueHill 3 software.

2.9. Contact angle study

The wettability of the film specimen was measured using KSV Cam 200 optical contact angle meter (KSV Instruments Ltd). Using a gas-tight microsyringe, 200 μL of deionised water was added onto the PHA film samples. Ten photographs were shot at one-second intervals as the water droplet impacted the surface of the PHA film. The photographs were analysed using the KSV Cam software.

2.10. Processing of PHA

Resultant PHA was processed into 3D constructs using the particulate leaching technique (Tomar et al., 2022). Dried constructs were placed in sterile water for the porogen leaching process. To ensure the removal of porogen, the pH of the water was measured. Post leaching, dried 3D constructs were viewed under the SEM.

2.11. Statistical analysis

The measurements were performed out in triplicate, and the results are presented as mean values with standard deviation. ANOVA single factor was used to assess statistical significance. GraphPad Prism 8 (GraphPad Software Inc., USA) was used to create the original data. The differences were considered statistically significant when the p-value resulted below than 0.03 (*), and $p < 0.05$ (**), very significant $p < 0.01$ (***), and highly significant when $p < 0.001$ (****).

3. Results and discussion

3.1. Bacterial isolation and Genome-Based identification

Marine bioprospecting has demonstrated its effectiveness in providing novel tools for biotechnological applications. Research has shown that exploring the biodiversity of extreme environments can lead to the discovery of novel species with distinctive properties. In this study, microorganisms were isolated from the Sarno river mouth (Naples, Italy) a heavily polluted river characterised by a high concentration of heavy metals (Montuori et al., 2013), hydrocarbons (Montuori & Triassi, 2012), plastics and microplastics (De Falco et al., 2020).

A strain showing a similarity of 99.48 % with *Pseudohalocynthiibacter aestuariivivens* RR4-35 (see supplementary materials) was identified and designated as *Pseudohalocynthiibacter aestuariivivens* P96. The only other report on *P. aestuariivivens* was published by Won et al., 2015 where the authors described the isolation *of P. aestuariivivens* from a tidal flat in South Korea and its taxonomic characterisation. However, there have been no reports on the use of these strains for biotechnological applications (Won et al., 2015).

The whole genome of the newly isolated *P. aestuariivivens* P96 identified by average nucleotide identity (ANI) displayed around 98 % similarity towards *Pseudohalocynthiibacter aestuariivivens* (Genbank CP049037.1) and < 70 % with *Roseovarius litorisediminis* formerly known as *Pelagicola litorisediminis* (Genbank NZ_FWFL00000000.1) confirming the correct phylogeny of P96.

3.2. P. aestuariivivens P96 genome sequencing, annotation and assessment of PHA genes

Genome sequencing and assembly resulted in a draft genome of 4.302.922 bp in size, with 30 contigs and 59.3 % of GC content, 47 RNA genes (tRNA + rRNA) and 4,362 coding sequences. These contigs were further assembled into 8 scaffolds in which numbers 1 and 3 corresponded to the bacterial chromosome while the remaining scaffolds belonged to plasmids. The overall genome annotation carried out by using RAST revealed a high potential for adaptability to extreme environmental conditions and stresses. This was reflected by a number of genes involved in membrane transport (123), Virulence, Disease and Defense (61) especially characterised by the capability to cope with toxic compounds such as heavy metals (in this case Copper, Cobalt, Zinc, Cadmium, Mercury), and in stress response (75) confirming a predominant, well-developed antioxidant defense system (Sanders, 2012). The presence of a relatively high number (44) of genes involved in polyhydroxyalkanoate (PHA) metabolism indicated that P. aestuariivivens P96 could potentially produce bioplastics such as PHAs. Previous studies have shown that some strains inhabiting extreme environments accumulate energy in the form of PHA granules intracellularly (Obruca et al., 2018). Herein, the biotechnological potential of P. aestuariivivens P96 as a producer of PHAs was evaluated. The general known mechanisms for PHA/B production involves a ketothiolase (PhaA) for the condensation of two molecules of acetyl-coenzyme A to form acetoacetyl-CoA, followed by an acetoacetyl-CoA reductase (PhaB) to generate hydroxybutyryl-CoA and a final polymerization by PHA synthase (PhaC) to produce the PHA chain (Tan et al., 2014). An in-depth analysis of the *P. aestuariivivens* P96 genome resulted in the detection and annotation of the relevant genes involved in the P(3HB) biosynthesis (see supplementary materials).

Unlike C. necator, where the most important genes for P(3HB) production are all organized within the PhbCAB operon, in *P. aestuariivivens* P96, putative genes were found to be scattered throughout the genome (Tan et al., 2014). Two putative genes encoding for PHA synthases, on

scaffold 1 (*phaC-1*) and 3 (*phaC-3*), were detected. PHA synthase (PhaC) is the necessary enzyme for the polymerisation of PHAs. It belongs to the α/β-hydrolase superfamily constituted by an *N*-terminal domain and a catalytic C-terminal domain (Chek et al., 2017). Four classes of PhaC are known, each with their distinct characteristics and substrate specificity which determines the types of monomers to be included into the PHA polymer. They are characterised by a conserved lipase-like box (G-X-S-X-G), and by three conserved aminoacids cysteine, aspartic acid and histidine (CDH) which constitute the PhaC catalytic triad. Although no similarity was detected between the two putative genes, the aminoacid sequences showed 34.82 % identity (query cover 96 %). Both catalytic triad residues and the lipase-like box were detected, however, there were differences in the traditional amino acid sequence (GXCXG) (Fig. 1a); PhaC-1 (576 aa) holds the SXCXG, while PhaC-3 (596 aa) possesses GXCXA sequence. The latter is conserved in bacteria belonging to the Rhodobacteraceae family (for eg - *P. aestuariivivens* P96) (Nambu et al., 2020), whereas the sequence within PhaC-1, where the first glycine (G) of lipase box is replaced with S, has been detected in several other Rhodobacteraceae, but in depth characterized in halophilic bacteria, such as *Halomonas* spp. (Cai et al., 2011). The presence of more copies of the *phaC* gene displaying low similarity was also observed in the genome of other PHB producers such as *Cupriavidus necator, Bradyrhizobium japonicum, Rhodospirillum rubrum* (Jin & Nikolau, 2012; Pohlmann et al., 2006; Quelas et al., 2013) as a result of divergent evolution (Peplinski et al., 2010). However, this factor is not correlated with increasing PHA production. For example, *B.japonicum* contains five copies of *phaC* but only two of them were significantly expressed and even in that case only the *phaC-1* was responsible for the high production indicating that a multiple number of *phaC* is not essential for the production (Quelas et al., 2013). In P96, a short portion of the *phaC-1* nucleotide sequence matched with the *phaC-1* sequence found in *Cupriavidus nantongensis* (similarity 84.62 %, query cover 4 %), which is related to the well-known *C. necator* H16, a model bacterium for PHAs production. The amino acid sequence retrieved from *P. aestuariivivens* (98.61 %, query cover 100 %) have been recognised to be α/β fold hydrolase and showed similarity with other genera (belonging to Rhodobacteraceae family) including *Sedimentitalea* (80.24 %), *Pseudorhodobacter* (65.80 %), *Pelagivirga* (63.15 %). Furthermore, the sequence PhaC-1 is also classified as class I poly(R)-hydroxyalkanoic acid synthase of *Thioflexithrix, Neptuniibacter*, *Marinobacterium* spp. below 52 % identity (see supplementary materials). The latter has been demonstrated to be a promising genus for bioplastic production (Wang et al., 2022) while proteomic and metabolic studies indicate that *Neptuniibacter* is able to produce P(3HB) (Muthusamy et al., 2017). The analysis of PhaC-3 showed 98.99 % identity (query cover 100 %) with a class I poly(R)-hydroxyalkanoic acid synthase from *P. aestuariivivens* RR4-35 and<80 % similarity with PhaC from *Roseovarius* and other Rhodobacteraceae strains (see supplementary materials). However, these closely related strains (detected by aligning both PhaC-1 and PhaC-3) are still poorly investigated for PHAs production. Herein interestingly, it was observed that while *phaC-1* was "isolated" within the genome, *PhaC-3* was found to be surrounded by other functional genes involved in the PHA biosynthesis (Fig. 1b). More specifically, *phaC-3* was preceded by a *phaZ* gene encoding for polyhydroxyalkanoate depolymerase (86.62 % similarity with PhaZ of *Roseovarius spongiae*), the fundamental enzyme for the PHA degradation, followed by *phaP* encoding for granule-associated protein phasin (the aminoacid sequence showed 93.70 % similarity with PhaP of *Roseovarius spongiae*) which is known to play various roles in the P(3HB) formation (Mezzina & Pettinari, 2016). This was followed by polyhydroxyalkanoate synthesis repressor *phaR* (the aminoacid sequence showed 83.16 % similarity with PhaR of *Roseovarius lutimaris*) suggesting a strong regulation of PHA biosynthesis. The same genes configuration was observed in *Rhodobacter sphaeroides* FJ1, a purple non-sulphur bacterium belonging to Rhodobacteraceae, isolated from wastewaters in Taiwan (Chou et al., 2009). In this bacterium, two different *phaC* genes were retrieved with one preceded by a PHA depolymerase (*phaZ*) and followed by genes encoding for phasin and a repressor as the case of P96, and a second one in another portion of the genome. The regulatory system in *R. sphaeroides* FJ1 was elucidated and it was observed that the *phaC-1* is constitutively expressed and *phaZ* and

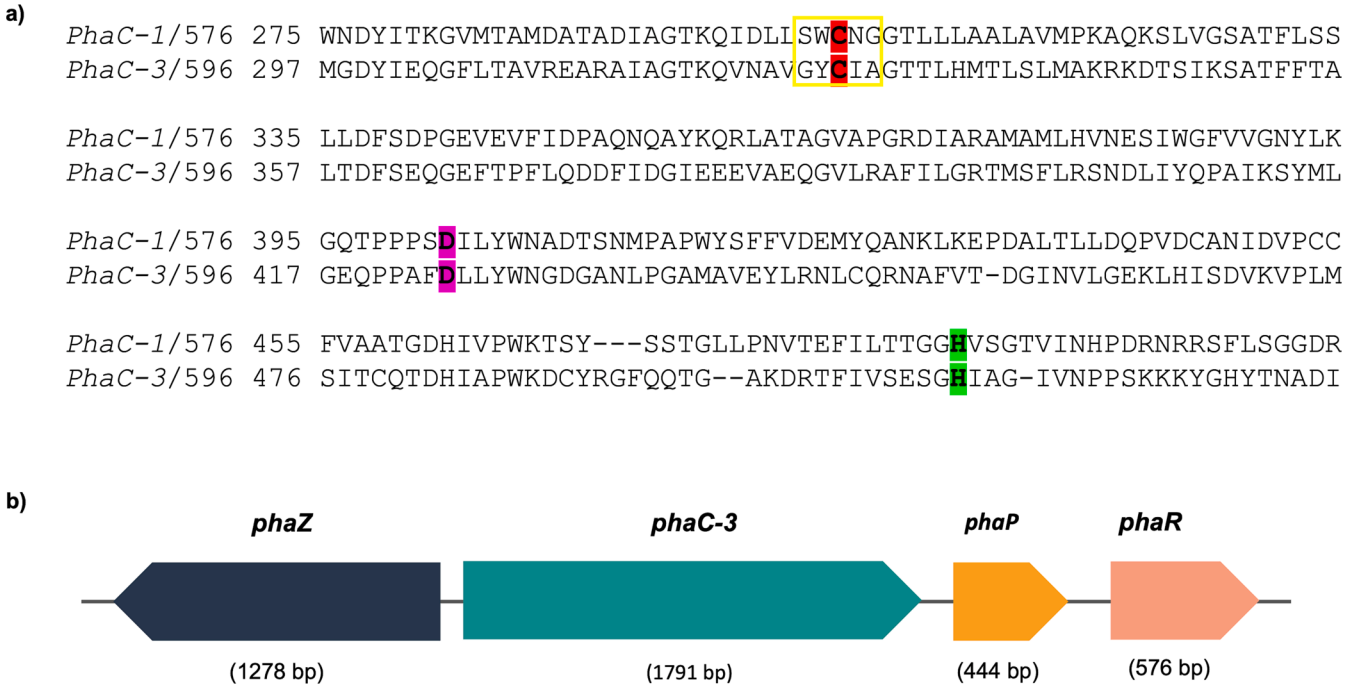

Fig. 1. Protein and cluster analysis of polyhydroxyalkanoate synthase of *P. aestuariivivens* P96. a) Sequence alignment of *PhaC-1* and *PhaC-3*, highlighting the lipase box (yellow line) and the conserved catalytic triad (CDH). The sequences were aligned and analysed by Jalview software. b) Annotation of P(3HB) genes clustered together on scaffold 3. *PhaZ*: polyhydroxyalkanoate depolymerase; *PhaC-3*: PHA synthase; *PhaP*: granule-associated protein phasin; *PhaR*: repressor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phaP are inhibited by the repressor encoded by *phaR*. Due to their correspondences in the organization of *phaC* cluster, P96 PHA production could be regulated in a similar way, however when aligned, P96 PhaCs showed<60 % similarity (data not shown). Moreover, in *R. sphaeroides* the levels of *phaC* expression changed based on the growth media, highlighting the importance of exploring different conditions in order to find the best productive strategy (Chou et al., 2009). It is important to note that the factors which could affect the PHA production are multiples, as reported by a recent review (Mitra et al., 2022) and involve control of enzymatic activity or transcriptional factors, stringent response, regulation by quorum sensing, among others.

3.3. PHA biosynthesis by *P. aestuariivivens P96 via fermentation process*

In order to assess the ability of P96 to produce PHAs, the strain was cultivated in five different production media (400 mL) in 2L shaken flasks and incubated for 5 days at 20 °C as indicated in the MM section 2.3. Biomass obtained was lyophilised and used for PHA extraction (Fig. 2). In this study, P96 demonstrated the ability to accumulate a high amount of PHAs with a maximum PHA production of 4.73 g/L, intracellular content of 87 % dcw and volumetric productivity of 0.04 g/l/h when cultivated in an ISP2 medium. The global regulation of PHA metabolism is complex and still not fully characterized indicating that the reasons for the high production could be attributed to specific regulators or derive from a combination of factors (Mitra et al., 2022). The unbalanced nutrient condition C/N is a well-known factor which drives the accumulation of PHA in the bacterial cell. In this work, the best results in terms of PHA yield were observed in ISP2 (87 % dcw) composed of Glucose 4 g/L, Yeast Extract 4 g/L and Malt Extract 10 g/L in artificial sea water. This medium is very rich in carbon source because of the presence of glucose and malt extract. GYM medium (around 50 % dcw) has a similar composition but a lower concentration of Malt extract (4 g/L). Finally, Marine broth is constituted by an excess of nitrogen source (5 g/L of Peptone, 1 g/L Yeast Extract) an unfavourable condition for PHA biosynthesis. These results clearly show the inducible effect of the nutrient regime on the PHA accumulation. In addition to nutrients availability, other parameters affecting the PHA production are temperature, light, aeration, incubation time, among others. For example, an optimization strategy applied to *Rhodobacter sphaeroides* KCTC1434 identified the optimal PHA production at 30 °C (with succinic acid). This was correlated with an increased expression of *phaA* and *phaB* genes (Lee et al., 2020). Starting from these findings, an experimental design to optimize the production process, and incorporation of metabolic engineering tools for strain improvement will be crucial to make the PHA production more competitive. The biosynthetic and metabolic potential of P96 could be ascribed to its adaptation strategy to cope with environmental stress such as high salinity (Pernicova et al., 2020), high concentration of heavy metals (Montuori et al., 2013), and hydrocarbons (Montuori & Triassi, 2012). Several studies in the literature supported the findings of this study confirming the ability of the P96 strain to compete on a significant scale with other extremophilic PHA-producing bacteria, e.g. halophiles. As a fact, the intracellular storage of carbon source as PHA granules provides the integrity of cytoplasmic membrane from potentially damaging plasmolysis caused by hyperosmotic stress (Sedlacek et al., 2019). In a study conducted by Rodríguez-Contreras and co-workers, (Rodríguez-Contreras et al., 2016) the halotolerant *Bacillus megaterium* uyuni S29 accumulated P(3HB) content of 41 % dcw and volumetric productivity of 0.1 g/l/h using glucose as carbon source. *Halomonas* spp. has also been used to obtain varying levels of P(3HB) content ranging from 12 to 84 % dcw and with volumetric productivity ranging from 0.004 to 0.04 g/l/h (El-malek et al., 2020; Fu et al., 2014; Pernicova et al., 2019). Due to its ability to produce high amounts of PHAs, P96 could be considered a promising microbial chassis to develop Next-Generation Industrial Biotechnology (NGIB) strategies for producing PHA more competitively. Principally, NGIB processes employ extremophilic bacteria to address the issues associated with Current Industrial Biotechnology (CIB) strategies for PHA production (Chen & Jiang, 2018). The market success of CIB is limited due to complex contamination prevention procedures, high production and downstream processing costs, wastewater treatment costs, low productivity, unstable process and product quality which NGIB envisages addressing (Chen & Jiang, 2018). *C. necator*, *Pseudomonas* spp., and recombinant *E. coli*, on the other hand, are among the bacterial strains that have been widely investigated in CIB processes to produce PHA with an overall content of 51.4 to 80 % dcw and volumetric productivity ranging from 1.91 to 4.63 g/l/h (Choi et al., 1998; Lee et al., 2000; Ryu et al., 1997). Based on the available data, NGIB bacterial strains have the possibility for productivity improvement. Most PHA-producing companies such as Danimer Scientific in the United States, Biomer in Germany, Ecomann and GreenBio in China, among others use CIB for PHA synthesis. Recently, companies such as PhaBuilder, Medpha, COFCO, and Bluepha, have been established to investigate NGIB for low-cost PHA production. The introduction of NGIB PHA-producing bacteria is expected to reduce the cost to US$1.68/kg which is less than the US$2.3/kg cost of CIB when recombinant *E. coli* was employed (Li et al., 2014). The marine strain used in this work is thus a novel promising NGIB candidate for PHB production, as evidenced by its capacity to accumulate higher levels of PHB (87 % dcw) when compared not just to CIB but also to other NGIB bacteria. However, in order for the production to be competitively commercialised, the goal of increasing volumetric productivity must be achieved, as its value (0.04 g/L/h) is lower than that of CIB bacteria. Optimising the

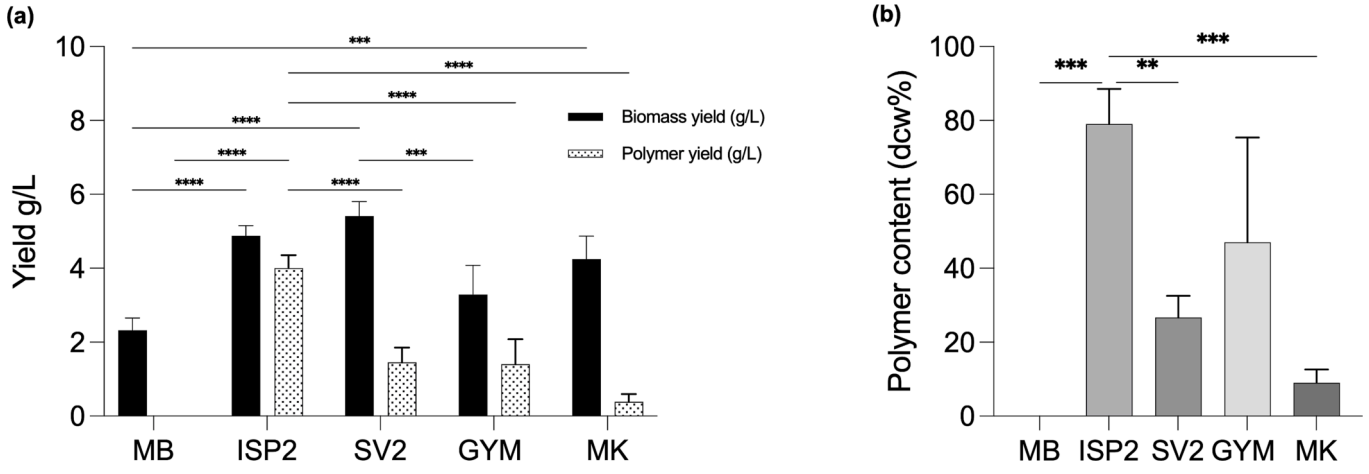

Fig. 2. (a) Biomass and polymer production (g/L) and (b), polymer content (dry cell weight dcw %) obtained by P96 strain in five different production media.

fermentation conditions will be therefore the focus of future strategy for industrial-scale application.

3.4. Accumulation of PHA granules within P. aestuariivivens P96 cells observed by scanning and transmission electron microscopy

PHA granules were detected within *P. aestuariivivens* P96 cells cultured in different PHA production media (see supplementary materials) using microscopy analyses. The intracellular polymer content was assessed by both scanning and transmission electron microscopy (SEM and TEM) which are well-reported techniques for the visualization of PHAs within the cells (Tan et al., 2014). The images acquired by SEM revealed variations in the bacterial morphology when cultivated in MB (non-PHA -producing condition) or ISP2 (PHA-producing condition). P96 cells appeared to have an irregular shape when cultivated in MB media (see supplementary materials) whereas intact, rod-shaped morphology was visible when cultivated in ISP2 media (see supplementary materials). This was further investigated using TEM which confirmed the presence of PHA granules within P96 cells when cultivated in ISP2 media. There were no PHA granules within P96 cells cultivated in MB (see supplementary materials).

3.5. Chemical and mechanical characterization of PHA produced

The polymer produced by *P. aestuariivivens* P96 in ISP2 media was characterised to identify their chemical, structural and physical properties. The functional groups of the polymer produced were first characterised by ATR/FT-IR, confirming that the polymer produced in different production media was a polyester (Kann et al., 2014) (see supplementary materials). All the FT-IR spectra showed characteristic peaks at 1720–1723 cm^{-1} resulting from the carbonyl group stretching in the ester bond, indicating a higher crystallinity, and at 2900 cm-1 due to the aliphatic stretching bond of methyl and methylene groups (Kann et al., 2014). The monomeric composition was further investigated using the GC–MS. Using the NIST library, the peak at 4.072 min in the GC–MS spectrum (see supplementary materials) was recognised as the methyl ester of 3-hydroxybutyric acid. As a result, the polymer produced by P96 was identified as a 3-hydroxybutyrate homopolymer, Poly(3-hydroxybutyrate), or P(3HB). P(3HB) molecular structure was subsequently confirmed using ^{13}C and ^{1}H NMR spectroscopy (see supplementary materials). Three peaks were detected in 1H NMR (see supplementary materials). The predominant triplet peak at $\delta = 1.5$ ppm corresponds to the terminal methyl (–CH3) group of the hydroxybutyrate (HB) molecule. The two doublets around $\delta = 2.46$ ppm correspond to the methyl protons (–CH2), and the quadruplet at around 5.2 ppm corresponds to the –CH proton of the backbone of PHB. The ^{13}C NMR spectrum detected the presence of 4 peaks assigned to 4 carbons validating the P(3HB) molecular structure (see supplementary materials). The monomeric composition of the material has a great influence on its thermal and mechanical properties. Produced PHB was processed into 2D film and characterized (Fig. 3a). DSC thermograms indicated that the melting temperature was 168.4 °C, the glass transition temperature was 2.1 °C, the fusion enthalpy was 78.94 J/g, and the crystallinity was 54 % (Fig. 3b). The P(3HB) exhibited cold crystallisation (exothermic peak) during the first heating scan, with an average cold crystallisation temperature of 48 °C (Fig. 3b). The crystallinity of P(3HB) was estimated to be 54 % by comparing the measured enthalpy of fusion of the material to that of a 100 % crystalline sample of the same polymer, which is 146 J/g for P(3HB) (Ho et al., 2014). The presence of a rigid amorphous phase of the solvent cast films of P(3HB) is correlated to the physical ageing and secondary crystallisation of the material, occurring after five weeks of storage at room temperature. The glass transition temperature of P(3HB), lower than 25 °C, promoted the crystallisation that results from polymer chain mobility. These findings were consistent with previous research, since P(3HB) is reported as having a crystallinity more than 50 %, a glass transition temperature of 0 to 4 °C and a melting temperature range of 165 – 178 °C (Sudesh et al., 2000). The resulting P(3HB) was a stiff material with an ultimate tensile strength of 17 ± 0.3 MPa and a Young's modulus of 0.4 ± 0.1 GPa, but it was brittle due to its low average elongation at break (18 %) (Fig. 3c). Moreover, the surface of the produced P(3HB) films exhibited a hydrophobic nature with a water contact angle value of 72 ± 2.5° (Data not shown), which is consistent with previous research (Bonartsev et al., 2013). To summarise, thermoprocessable polymer P(3HB) produced had mechanical and thermal properties (high melting temperature and high tensile strength) comparable to that of petroleum-based plastics including polypropylene, polypropylene, and polyethylene terephthalate (Turco et al., 2020). However, in comparison to synthetic polymers, a number of limitations still prevent pure P(3HB) from being widely used. P(3HB) is less competitive than synthetic plastics as food packaging materials or biomaterials due to their brittleness, low ductility, and sensitivity to thermal degradation (Bucci et al., 2005). Additional approaches are required to enhance the behaviour of P(3HB), focusing on its primary deficiencies through the blending of natural raw materials or other biopolymers, along with specific additives like plasticizers and nucleation agents, lubricants, chain extenders, among others.

3.6. Production of P(3HB) based 3D construct

P(3HB) produced was processed into a porous, 3D construct (section 2.9) that were revealed by SEM analysis (see supplementary materials). Processing of P(3HB) into porous, 3D construct demonstrated its potential application in tissue engineering (TE) aimed at generating functional tissue using 3D porous constructs as substrates for cell attachment, proliferation and differentiation (altuntaş et al., 2017).

4. Conclusion

In this research we describe the isolation and characterization of of *Pseudohalocynthiibacter aestuariivivens* P96, a novel marine microbial strain able to produce high amount of P(3HB). Due to the high yield of product, P96 could represent a good candidate for a cost-effective bioplastic production. This will be possible by targeting growth conditions,

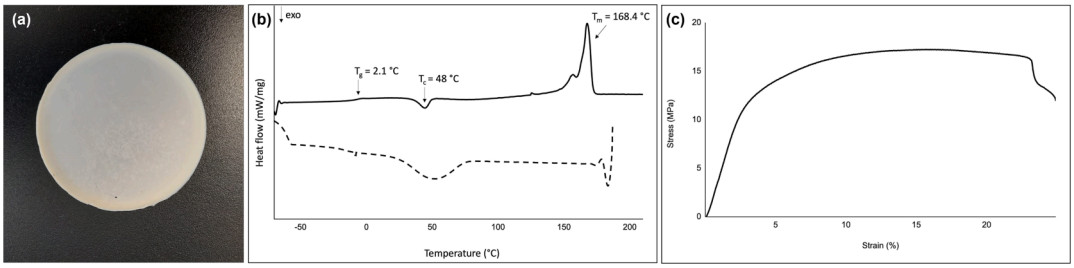

Fig. 3. (a) 5 %w/v P(3HB) film; (b) DSC thermogram of P(3HB); solid line represents the first heating (-) and dashed lines (–) second heating, (c) Stress–strain curve for P(3HB).

by screening low-cost feedstock, selecting the best temperature and incubation time. Moreover, a targeted metabolic engineering strategy targeting specific genes known to regulate PHB biosynthesis, will be pivotal to make this stain an efficient microbial cell factory for P(3HB) production.

CRediT authorship contribution statement

Fortunato Palma Esposito: Conceptualizing, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Vittoria Vecchiato:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Carmine Buonocore:** Investigation, Methodology, Writing – review & editing. **Pietro Tedesco:** Writing – review & editing. **Brendon Noble:** Investigation, Methodology, Writing – review & editing. **Pooja Basnett:** Conceptualizing, Investigation, Methodology, Resources, Writing – review & editing, Funding acquisition. **Donatella de Pascale:** Resources, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

altuntaş, E., Özkan, B., Yener, G. 2017. Porous scaffolds, pp. 27-59.
Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. Int. J. Biol. Macromol. 89, 161–174.
Bonartsev, A.P., Yakovlev, S.G., Zharkova, I.I., Boskhomdzhiev, A.P., Bagrov, D.V., Myshkina, V.L., Makhina, T.K., Kharitonova, E.P., Samsonova, O.V., Feofanov, A.V., Voinova, V.V., Zernov, A.L., Efremov, Y.M., Bonartseva, G.A., Shaitan, K.V., Kirpichnikov, M.P., 2013. Cell attachment on poly(3-hydroxybutyrate)-poly (ethylene glycol) copolymer produced by Azotobacter chroococcum 7B. BMC Biochem. 14 (1), 12.
Bucci, D.Z., Tavares, L.B.B., Sell, I., 2005. PHB packaging for the storage of food products. Polym. Test. 24 (5), 564–571.
Cai, L., Tan, D., Aibaidula, G., Dong, X.R., Chen, J.C., Tian, W.D., Chen, G.Q., 2011. Comparative genomics study of polyhydroxyalkanoates (PHA) and ectoine relevant genes from Halomonas sp. TD01 revealed extensive horizontal gene transfer events and co-evolutionary relationships. Microb. Cell Fact 10, 88.
Chek, M.F., Kim, S.-Y., Mori, T., Arsad, H., Samian, M.R., Sudesh, K., Hakoshima, T., 2017. Structure of polyhydroxyalkanoate (PHA) synthase PhaC from Chromobacterium sp. USM2, producing biodegradable plastics. Sci. Rep. 7 (1).
Chen, G.-Q., Jiang, X.-R., 2018. Next generation industrial biotechnology based on extremophilic bacteria. Curr. Opin. Biotechnol. 50, 94–100.
Choi, J.-i., Lee, S.Y., Han, K. 1998. Cloning of the *Alcaligenes latus* Polyhydroxyalkanoate Biosynthesis Genes and Use of These Genes for Enhanced Production of Poly(3-hydroxybutyrate) in *Escherichia coli*. **64**(12), 4897-4903.
Chou, M.E., Chang, W.T., Chang, Y.C., Yang, M.K., 2009. Expression of four pha genes involved in poly-beta-hydroxybutyrate production and accumulation in Rhodobacter sphaeroides FJ1. Mol Genet Genomics 282 (1), 97–106.
Dalton, B., Bhagabati, P., De Micco, J., Padamati, R.B., O'Connor, K. 2022. A Review on Biological Synthesis of the Biodegradable Polymers Polyhydroxyalkanoates and the Development of Multiple Applications. **12**(3), 319.
De Falco, F., Di Pace, E., Gentile, G., Castaldo, R., Avolio, R., Errico, M.E., Avella, M., Chiavazzo, G., Imaparato, M., Montuoro, F., Pucci, L., Di Vito, S., Cocca, M., 2020. Assessment of Microplastic Pollution in Sarno River. Springer International Publishing, Cham, pp. 183–186.
El-malek, F.A., Farag, A., Omar, S., Khairy, H., 2020. Polyhydroxyalkanoates (PHA) from halomonas pacifica ASL10 and Halomonas salifodiane ASL11 isolated from mariout salt lakes. Int. J. Biol. Macromol. 161, 1318–1328.
Fernández-Dacosta, C., Posada, J.A., Kleerebezem, R., Cuellar, M.C., Ramirez, A., 2015. Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment. Bioresour. Technol. 185, 368–377.
Fu, X.-Z., Tan, D., Aibaidula, G., Wu, Q., Chen, J.-C., Chen, G.-Q., 2014. Development of Halomonas TD01 as a host for open production of chemicals. Metab. Eng. 23, 78–91.
Ho, M.-H., Li, S.-Y., Ciou, C.-Y., Wu, T.-M. 2014. The morphology and degradation behavior of electrospun poly(3-hydroxybutyrate)/Magnetite and poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/Magnetite composites. **131**(22).
Jin, H., Nikolau, B.J., 2012. Role of genetic redundancy in polyhydroxyalkanoate (PHA) polymerases in PHA biosynthesis in Rhodospirillum rubrum. J Bacteriol 194 (20), 5522–5529.
Kann, Y., Shurgalin, M., Krishnaswamy, R.K., 2014. FTIR spectroscopy for analysis of crystallinity of poly(3-hydroxybutyrate-co-4 -hydroxybutyrate) polymers and its utilization in evaluation of aging, orientation and composition. Polym. Test. 40, 218–224.
Koller, M., Braunegg, G., 2018. Advanced approaches to produce polyhydroxyalkanoate (PHA) biopolyesters in a sustainable and economic fashion. The EuroBiotech Journal 2.
Lee, S.Y., Wong, H.H., Choi, J.-i., Lee, S.H., Lee, S.C., Han, C.S. 2000. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of Pseudomonas putida under phosphorus limitation. **68**(4), 466-470.
Lee, Y.R., Fitriana, H.N., Lee, S.Y., Kim, M.-S., Moon, M., Lee, W.-H., Lee, J.-S., Lee, S. 2020. Molecular Profiling and Optimization Studies for Growth and PHB Production Conditions in Rhodobacter sphaeroides. **13**(23), 6471.
Lemoigne, M., 1926. Produit de deshydratation et de polymerisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 8, 770–782.
Li, T., Chen, C., Chen, J.-C., Wu, Q., Chen, G.-Q., 2014. Open and continuous fermentation: products, conditions and bioprocess economy. Biotechnol. J. 9, 1503–1511.
Manikandan, N.A., Pakshirajan, K., Pugazhenthi, G., 2021. Techno-economic assessment of a sustainable and cost-effective bioprocess for large scale production of polyhydroxybutyrate. Chemosphere 284, 131371.
Mezzina, M.P., Pettinari, M.J., 2016. Phasins, multifaceted polyhydroxyalkanoate granule-associated proteins. Appl Environ Microbiol 82 (17), 5060–5067.
Mitra, R., Xu, T., Chen, G.-Q., Xiang, H., Han, J. 2022. An updated overview on the regulatory circuits of polyhydroxyalkanoates synthesis. **15**(5), 1446-1470.
Montuori, P., Lama, P., Aurino, S., Naviglio, D., Triassi, M., 2013. Metals loads into the Mediterranean Sea: estimate of Sarno River inputs and ecological risk. Ecotoxicology 22 (2), 295–307.
Montuori, P., Triassi, M., 2012. Polycyclic aromatic hydrocarbons loads into the Mediterranean Sea: estimate of Sarno River inputs. Mar Pollut Bull 64 (3), 512–520.
Muthusamy, S., Lundin, D., Mamede Branca, R.M., Baltar, F., González, J.M., Lehtiö, J., Pinhassi, J. 2017. Comparative proteomics reveals signature metabolisms of exponentially growing and stationary phase marine bacteria. **19**(6), 2301-2319.
Nambu, Y., Ishii-Hyakutake, M., Harada, K., Mizuno, S., Tsuge, T. 2020. Expanded amino acid sequence of the PhaC box in the active center of polyhydroxyalkanoate synthases. **594**(4), 710-716.
Obruca, S., Sedlacek, P., Koller, M., Kucera, D., Pernicova, I., 2018. Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: biotechnological consequences and applications. Biotechnol. Adv. 36 (3), 856–870.
Obulisamy, P.K., Mehariya, S., 2021. Polyhydroxyalkanoates from extremophiles: a review. Bioresour. Technol. 325, 124653.
Peplinski, K., Ehrenreich, A., Döring, C., Bömeke, M., Reinecke, F., Hutmacher, C., Steinbüchel, A. 2010. Genome-wide transcriptome analyses of the 'Knallgas' bacterium Ralstonia eutropha H16 with regard to polyhydroxyalkanoate metabolism. **156**(7), 2136-2152.
Pernicova, I., Kucera, D., Nebesarova, J., Kalina, M., Novackova, I., Koller, M., Obruca, S., 2019. Production of polyhydroxyalkanoates on waste frying oil employing selected Halomonas strains. Bioresour. Technol. 292, 122028.
Pernicova, I., Novackova, I., Sedlacek, P., Kourilova, X., Koller, M., Obruca, S., 2020. Application of osmotic challenge for enrichment of microbial consortia in polyhydroxyalkanoates producing thermophilic and thermotolerant bacteria and their subsequent isolation. Int. J. Biol. Macromol. 144, 698–704.

Pohlmann, A., Fricke, W.F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M., Schwartz, E., Strittmatter, A., Voß, I., Gottschalk, G., Steinbüchel, A., Friedrich, B., Bowien, B., 2006. Genome sequence of the bioplastic-producing "Knallgas" bacterium Ralstonia eutropha H16. Nat. Biotechnol. 24 (10), 1257–1262.

Quelas, J.I., Mongiardini, E.J., Pérez-Giménez, J., Parisi, G., Lodeiro, A.R., 2013. Analysis of two polyhydroxyalkanoate synthases in Bradyrhizobium japonicum USDA 110. J Bacteriol 195 (14), 3145–3155.

Rai, R., Yunos, D.M., Boccaccini, A.R., Knowles, J.C., Barker, I.A., Howdle, S.M., Tredwell, G.D., Keshavarz, T., Roy, I., 2011. Poly-3-hydroxyoctanoate P(3HO), a Medium chain length polyhydroxyalkanoate homopolymer from pseudomonas mendocina. Biomacromolecules 12 (6), 2126–2136.

Rodríguez-Contreras, A., Koller, M., Braunegg, G., Marqués-Calvo, M.S., 2016. Poly[(R)-3-hydroxybutyrate] production under different salinity conditions by a novel Bacillus megaterium strain. New Biotechnol. 33 (1), 73–77.

Ryu, H.W., Hahn, S.K., Chang, Y.K., Chang, H.N. 1997. Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of Alcaligenes eutrophus with phospate limitation. **55**(1), 28-32.

Sanders, E.R., 2012. Aseptic laboratory techniques: plating methods. J VisExp 63, e3064.

Sedlacek, P., Slaninova, E., Koller, M., Nebesarova, J., Marova, I., Krzyzanek, V., Obruca, S., 2019. PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. New Biotechnol. 49, 129–136.

Sehgal, R., Gupta, R., 2020. Polyhydroxyalkanoate and its efficient production: an eco-friendly approach towards development. *3*. Biotech 10 (12), 549.

Sirohi, R., Kumar Gaur, V., Kumar Pandey, A., Jun Sim, S., Kumar, S., 2021. Harnessing fruit waste for poly-3-hydroxybutyrate production: a review. Bioresour. Technol. 326, 124734.

Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog. Polym. Sci. 25 (10), 1503–1555.

Taguchi, S., Matsumoto, K.I., 2021. Evolution of polyhydroxyalkanoate synthesizing systems toward a sustainable plastic industry. Polym. J. 53 (1), 67–79.

Tan, G.-Y.A., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I.M.N., Li, Y., Zhao, L., Mo, Y., Wang, J.-Y. 2014. Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review. **6**(3), 706-754.

Tomar, A., Uysal-Onganer, P., Basnett, P., Pati, U., Roy, I. 2022. 3D Disease Modelling of Hard and Soft Cancer Using PHA-Based Scaffolds. **14**(14), 3549.

Turco, R., Santagata, G., Corrado, I., Pezzella, C., Di Serio, M., 2020. In vivo and post-synthesis strategies to enhance the properties of PHB-based materials: a review. Front Bioeng Biotechnol 8, 619266.

Wang, M.-R., Li, H.-F., Yi, J.-J., Tao, S.-Y., Li, Z.-J., 2022. Production of polyhydroxyalkanoates by three novel species of Marinobacterium. Int. J. Biol. Macromol. 195, 255–263.

Won, S.M., Park, S., Park, J.M., Kim, B.C., Yoon, J.H. 2015. Pseudohalocynthiibacter aestuariivivens gen. nov., sp. nov., isolated from a tidal flat. *Int J Syst Evol Microbiol*, **65**(Pt 5), 1509-1514.