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PHB Produced by Bacteria Present in the Argan Field Soil: A New Perspective for the Synthesis of the Bio-Based Polymer †

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Abstract: Bio-based plastics, i.e., non-synthetic polymers produced starting from renewable resources, are gaining special attention as a feasible solution to the environmental issues caused by the concerns due to the impact of waste plastics. Such materials, furthermore, can also represent an alternative to petroleum-derived polymers, due to the scarcity of this raw material in the close future. In the polyhydroxyalkanoates (PHA) family, polyhydroxybutyrate (PHB) has been the first to be synthesized and characterized. PHB soon gained a great attention from industrial and academic researchers since it can be synthesized from a wide variety of available carbon sources, such as agro-industrial and domestic wastes. The aim of this original research has been the identification of the presence of PHB synthetizing bacteria in some soils in Morocco region and the production of the bio-based PHB. In particular, the soils of the argan fields in Taroudant were considered. Taroudant is a southwestern region of Morocco where the argan oil tree Aragania spinosa is an endemic and preserved species. Starting from rizhospheric soil samples of an argan crop area, we isolated heat-resistant bacteria and obtained pure cultures of it. These bacteria present intracellular endospores stained by Schaeffer-Fulton methods. The presence of intracellular endospores is a very important starting point to verify the effective production of PHB as compartmentalised material. Further analyses are currently ongoing to try to extract and characterize PHB granules.

Keywords: bio-based polymers; polyhydroxyalkanoates (PHA), polyhydroxybutyrate (PHB), polyhydroxybutyrate producing bacteria

1. Introduction

The increasing worldwide consumption of coal, oil and natural gases is inducing a rise of these fossil prices, and an increase of the carbon dioxide emissions that cause the world's climate change. In addition, in the following decades, because of the continued need for fossil fuels, petroleum resources will be depleted in a short time. It is necessary, therefore, to reduce and replace these non-renewable resources with alternative "green" resources for a sustainable development of the next

generations. As an example, biomass represents an energetic resource to solve economic, political, and environmental issues [1].

Biomass means non fossilized and biodegradable organic material originating from plants, animals and microorganisms. Biomass is considered a renewable resource as long as its exploitation rate does not exceed its replenishment by natural processes; it can be, therefore, conveniently used to synthesize bio-based polymers [1].

Among bio-based polymers, polyhydroxyalkanoates (PHA) are a group of polyesters synthesized by prokaryotic Gram positive and Gram negative enterobacteria [2,3], cyanobacteria [4,5] and archaea organisms living in extreme environmental conditions [6,7]. PHAs are thermoplastic, biodegradable, biocompatible and non-toxic bio-based polymers. They have a high degree of polymerization, are highly crystalline and isotactic, possess optical properties, and are insoluble in water.

Microbial-derived polymers belonging to the PHA family are entirely synthetized by bacteria; at the same time, they can be completely bio-degraded by living organisms, which means that they can undergo a decomposition process that leads to small compounds, such as methane, carbon dioxide, and water, due to the microorganism's activity in particular composting conditions. The microbial species able to synthesize PHA act in the presence of renewable feedstock of pentoses, hexoses, starch, cellulose, sucrose, lactose, CO_2 and CH_4 under unfavorable growth conditions due to unbalanced nutrients availability. Generally, these conditions include a sufficient carbon source and reduced number of other substrates such as nitrogen, phosphate, and oxygen. During starvation periods, in the absence of external sugars, the PHA producers can use the carbon source stored in the granules to provide the cell with the necessary amount of energy to survive in this uncomfortable condition, particularly for osmoregulation, motility and metabolic pathways [8].

The Poly-3-hydroxybutyrate (PHB) is the most studied and characterized member of the PHA family. It is a thermoplastic homo-polyester consisting of hydroxybutyrate monomers which give the polymer 80% crystallinity. PHB has an extremely regular structure: it is almost completely isotactic (like synthetic polypropylene), with the chains forming helical structures. The chains are able to closely pack to form crystals, obtaining a stiff polymer [9].

The processing of PHB is usually carried out by extrusion in melt state for the production polymeric films or other items [10]. PHB displays a melting temperature of 175 °C–180 °C and thermal degradation temperature around 240 °C, which means a narrow temperature window for the processability of this material, that is, therefore, difficult to process [11]. Furthermore, unfavorable conditions during processing, e.g., too high levels of humidity/temperature and/or too long permanence in the machine, can cause the polymer degradation, corresponding to lower performance of the final products.

To overcome problems during processing, the bacterial production process can be modified to produce co-polymerized PHB. The crystallization of PHB can be, in fact, interrupted by adding different building blocks, such as the 4-hydroxybutyrate (4-HB), the 3-hydroxyvalerate (3-HV) [10], or the 3-hydroxyhexanoate (3-HH) [11]. Co-polymerization lowers the melting temperature even down to 75 °C, thus facilitating the processing and production of PHB; as a consequence, the degree of crystalline phase is reduced, depending on the percentage of PHB in the co-polymer.

The tensile strength of PHB is close to that measured on isotactic polypropylene (iPP); on the other hand, its elongation at break is significantly lower (6%) compared to that of iPP, being about 400%. This means that PHB is much more brittle compared to iPP. To overcome this weakness, PHB can be also blended with different synthetic and bio-based (such as starch, lignin-derivate) polymers with a similar melting temperature range or modified upon the addition of fillers or additives: in this way it is possible to improve the characteristics of PHB with the aim of widening its field of applications, possibly reducing also the costs of the final products [9].

The high costs of carbon substrates limit somehow the production of PHB on a large scale. To overcome this problem, alternative substrates rich in carbohydrates are proposed, like biomass from

green spaces, wastes, secondary products of industrial processes, such as glycerol, sugarcane bagasse, and lignocellulose from agricultural and forestry residues [12].

In the last years it is becoming interesting to study the possibility of blending the PHB with industrial biowastes for two reasons: first because the disposal of industrial bioproducts is an environmental issue, and second because it has been reported that this blending process can improve the mechanical properties of the natural polymer by creating a matrix with multiple applications [13].

The high costs of carbon substrates represent also an ethical problem for the PHB production, particularly in those regions where the nutritional situation is a difficult issue to be solved. With the biotechnology techniques it is possible to synthesize PHB from cheap renewable resources such as agricultural and industrial wastes [8,14,15], representing promising alternatives to produce PHB at competitive costs, without causing ethical conflicts [16].

The aim of this work was to isolate, for the first time, PHB synthesizing bacteria from the argan field soil and to identify the presence of the bio-based polymer granules by adopting the Schaeffer-Fulton endospore staining methods. The intracellular granules accumulation was first examined by adopting Malachite Green. Afterwards, a second identification test was implemented by adopting the variation of the Schaeffer-Fulton staining, where the Malachite Green dye was replaced by a Methylene Blue solution. The presence of endospores was observed at the compound light microscope using oil immersion. This experimental work represents the first step of the synthesis of PHB bio-based polymer starting from argan wastes, resulting from the fruits and the pressing process for the argan oil extraction.

2. Materials and Methods

2.1. Samples Collection

The soil samples for the isolation of the PHB producing bacteria were collected from rhizospheric soil in the agricultural crops of argan oil (*Aragania spinosa*) in Teroudant, a southwestern region of Morocco where the argan tree is endemic. All samples were collected from six different locations of the same area of the argan crop to screen the physical characteristics that best suit the PHB producing bacteria growth. The physical characteristics of the soil were the presence compared to the absence of water, the presence of domestic pollutants in the urban location compared to the absence of pollution in the rural area, and the proximity to either dead trees or healthy trees. Soil samples were collected in sterile conditions by using ethanol 70%, preserved into sterile vials, and stored at 22 °C temperature for 48 h. Afterwards, all samples were stored at 4 °C for 3 weeks for the bacterial isolation.

2.2. Pretreatment: Isolation of Microorganisms

One gram of each soil sample was dispersed in 10mL of sterile water. These samples were homogenized at 200 rpm fro 3 h to ensure homogeneity of bacterial organisms in a culture, and after heated at 80 °C for 10 min to isolate endospore forming bacteria. All samples were serially diluted to 10–8 using sterile water and plated by spreading 100 µL of the dilutions −5, −6, −7, −8 on sterile nutrient agar plates, composed as follow: Peptone 5 g/L, Yeast extract 3 g/L, Sodium chloride 5 g/L, Glucose 1 g/L, Agar 18 g/L, in 1L of distilled water, at pH 7.0. Thereafter the plates were incubated at 30 °C for 48 h. All grown bacteria were isolated on modified agar plates consisting of: Beef extract (0.3%), Peptone (0.5%), Sodium Chloride (0.8%), Glucose (1%), and Agar (1.5%) [17].

2.3. Screening for PHB-Producing Bacteria

2.3.1. Schaeffer-Fulton Endospore Staining Using Malachite Green and Safranin

The detection for PHB-producing bacteria was performed by using the Schaeffer-Fulton method for staining endospores. The staining was prepared by dissolving 0.5 gr of Malachite Green in 100

mL of distilled water. The Safranin counterstain was prepared by dissolving 2.5 gr of Safranin powder in 100 mL of 95% ethanol. After fixing the microorganisms on the glass slide, the specimen was covered with a square of blotting paper and saturated with Malachite Green stain solution for 5 min by steaming over boiling water and adding more dye when it dried off. After washing the slide with distilled water, the specimens were counterstained with safranin for 30 s, and once again washed with distilled water. The slides were examined under oil immersion at 1000× for the presence of endospores. The vegetative cells appear red to pink, while the endospores are bright green [18].

2.3.2. Schaeffer-Fulton Endospore Staining Using Methylene Blue Solutions

The newly alternative Schaeffer-Fulton endospore staining using Methylene Blue solutions was implemented to confirm the presence of endospores already identified in the staining with Malachite Green. In this proposed study, the alternative staining method resulted in coloring endospores of the species *Bacillus subtilis* and *Clostridium tetani.* A solution of Methylene Blue stain 0.5% at pH 12 was prepared by diluting 0.1gr of the dye with 20 mL of a buffer solution at pH 12. A bacterial smear of the positive endospore producing bacteria, already identified using Malachite Green staining, was prepared and fixed over low heat. The Methylene Blue stain 0.5% at pH 12 was added to the specimens and heated over a steam bath for 5 min. The slide was afterwards washed with distilled water and counterstained with Safranin for 30 s. Before proceeding with the microscopic observations, the slides were again washed with distilled water to remove the counter staining dye. According to this work., the warming process allows the Methylene Blue solution to penetrate the endospores wall, and the alkaline pH of the staining solution allows the dye to penetrate the alkaline bacterial cytosol [19].

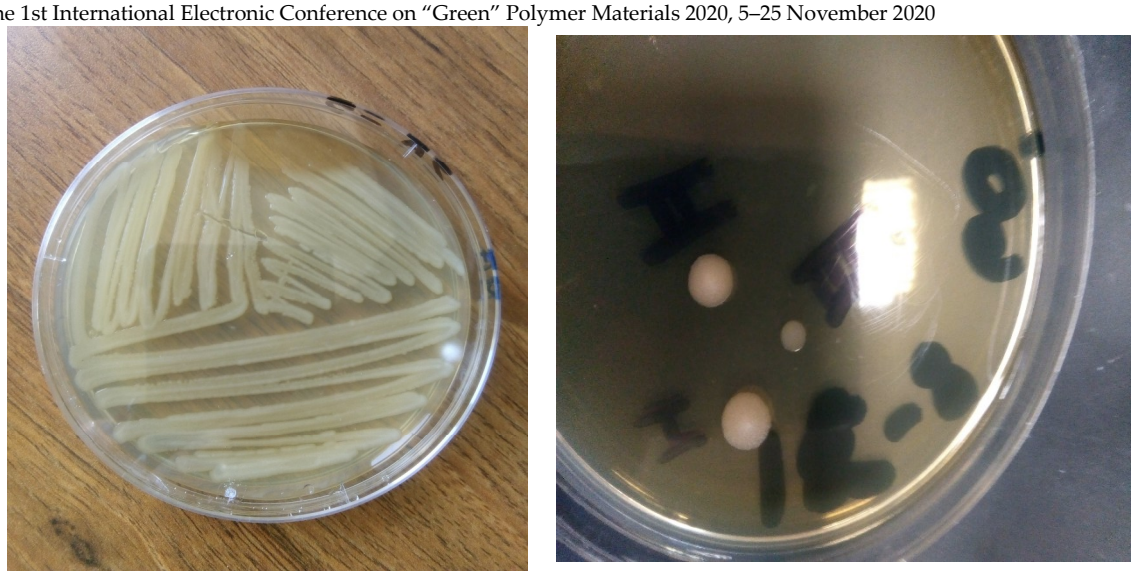
3. Results and Discussion

In this work, we investigate the potential presence of microorganisms able to synthesize the biopolymer PHB from *Argania spinosa* crop soil in a unique environmental area of Morocco where this species is endemic and preserved.

3.1. Isolation and Screening of PHB Producing Bacteria

Six soil samples were collected from the agricultural crops of argan oil (*Aragania spinosa*) in Teroudant, a southwestern region of Morocco. Four thermoresistant bacterial species were isolated from all the six soil samples. Only one different bacterial species, represented in Figure 1a,b, was isolated from a location in proximity of an urban area where wastewater and garbage contaminate the argan field. Recent works indicated the presence of six different bacterial strains PHB producers isolated from sewage samples. Among all the isolated species, one Strain 11 produced 45.9% of PHB by using glucose as sole carbon source [20]. In this perspective, the aim of this work is to isolate new bacterial strains able to synthesize the biopolymer PHB, to subsequently optimize the synthesis, modification and biodegradation processes of the polymer by using the argan waste biomass.

Morphological analysis of the pure isolated colonies of the PHB producing bacteria described the whole colony appearance as irregular to flat, with ondulate margins, with a white and opaque color, and a rough surface. Similar morphological characteristics were discussed by Kaliwal and colleagues. Of all the six PHB producing strains isolated from fodder field soil, the researchers identified a strain called BBKGBS6, classified as member of the genus *Bacillus*, that showed similar morphological characteristics and was able to accumulate 60% of intracellular PHB [21].



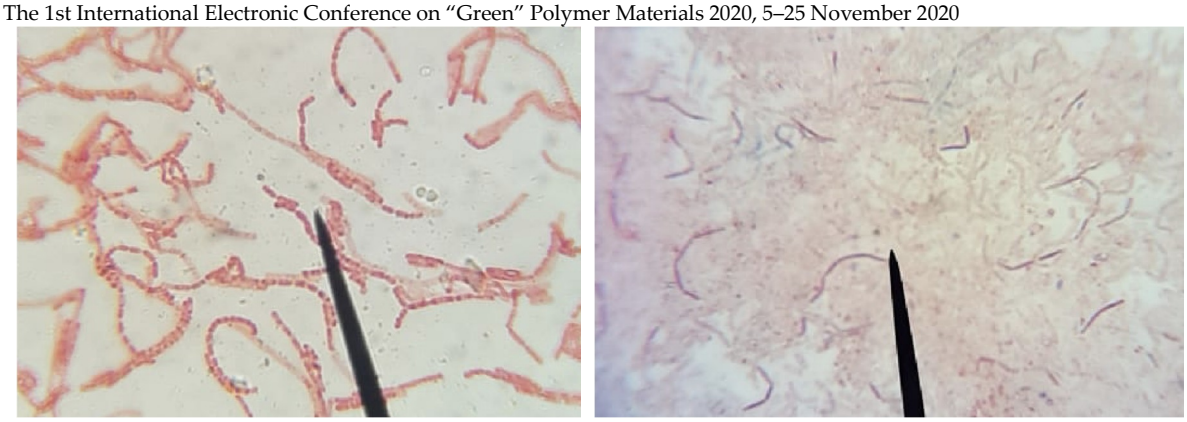
(a) (b)

Figure 1. (**a**) bacterial culture and (**b**) pure colonies of the isolated PHB producing bacteria.

3.2 Identification of PHB-Producing Bacteria by Schaeffer-Fulton Endospore Staining

For a rapid detection of PHB producing bacteria Schaeffer-Fulton staining method was used. The pure colonies isolated from the modified agar medium were stained with Malachite Green, a preliminary screening agent for lipophilic molecules. Under microscopic observations at 1000x in oil immersion, the bacterial endospores showed a green coloration as reported in Figure 2a. This positive result is explained by the activity of the lipophilic endospore membranes that allow the Malachite Green to cross the membrane and to retain the green coloration [19] (Ruth, 2009).

The endospore using variation of Methylene Blue was used as a coloring alternative to Malachite Green in staining the bacterial species *Bacillus subtilis* and *Clostridium tetani* by Okatari et al. In this recent work, the endospore staining properties of Methylene Blue were investigated using different concentrated solutions of the dye at different pH levels. Their work demonstrated that the species *Bacillus subtilis* are well stained at an optimal stain concentration of 0.5% and pH 12, while in staining the species *Clostridium tetani* the optimal concentration was 0.5% at pH 11 [19]. In following this alternative, this study adopted a confirmatory staining that was performed by using the Schaeffer-Fulton staining method where the conventional Malachite Green dye was substituted by an alcoholic solution of Methylene Blue 0.5% at pH 12. The pH and concentration parameters selected for this work correspond to the optimal staining conditions of the bacterial species *Bacillus subtilis* since the morphological analysis previously conducted may suggest that the species isolated belongs to the genus *Bacillus.* After staining with Methylene Blues 0.5% at pH 12 the specimens were counter stained with Safranin. The colored specimens were observed at the compound light microscope at 1,000× in oil immersion. Figure 2b shows how the bacterial endospores retained the blue color of the Methylene dye, which passed through the endospore membranes and colored the internal granules.



(a) (b)

Figure 2. Endospore containing bacteria identified with (**a**) Malachite Green, and (**b**) Methylene Blue staning.

4. Conclusions

Top and rhizospheric soil samples collected from agricultural crops, industrial contaminated fields, and uncultivated fields have been selected as favorable environmental areas for the PHB producing bacteria growth. Notably, olive oil, vegetable oil, and sunflower oil crops have been identified as good reservoirs for the isolation of PHB synthesizers as well as for the optimization of the polymer synthesis and modification by using the corresponding biomass waste material like oil waste water and natural solid wastes.

In this work the soil samples were collected from the argan field in the southwestern region of Morocco, where the Argania spinosa species is endemic and preserved. Among all soil samples collected, five different bacterial thermoresistant were isolated and tested for the identification of intracellular endospores. Only one bacterial species was identified as positive. This selected species was isolated from an area of the argan field exposed to human contaminations, such as waste water and solid wastes. The positivity of this work was confirmed by the Schaeffer-Fulton staining methods for the identification of endospore producing bacteria. The conventional method by using Malachite Green evidenced the presence of intracellular material that retained the green dye in contrast with the red color of the vegetative compartments that retained the Safranine. Furthermore, the variation method that used the Methylene Blue stain instead of Malachite Green, confirmed the presence of intracellular compartments colored in blue because they retained the first colorant, while, also in this method, the rest of the bacterial cell appeared in red. After this first identification, further biochemical and biomolecular analysis are necessary to identify the bacterial species isolated. Moreover, further research is necessary to understand the role of the microorganism in the synthesis of the PHB and how the argan waste products can be valuable for the PHB synthesis, blending and biodegradation.

Conflicts of Interest The authors declare that they have no conflict of interest.

Abbreviations

PHB: polyhydroxybuthyrate
PHA: polyhydroxyalkanoates
4-HB: 4-hydroxybutyrate
3-HV: 3-hydroxyvalerate
3-HH: 3-hydroxyhexanoate
PP: polypropylene
P3-HB-*co*-3HHx: Poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate
Tg: Glass transition temperature

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