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ORIGINAL PAPER

Investigation of the poly-β-hydroxybutyrate (PHB) producing in mountain bacterial strains by transmission electron microscopy

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Abstract

The study focused on the producing of poly-β-hydroxybutyrate (PHB) granules by Azotobacter strains isolated from five altitudinal vegetation zones of Parâng Massif, Romania (alpine, subalpine, coniferous and beech zones and the Maleia flood plain). The poly-β-hydroxybutyrate (PHB) granules from cysts were highlighted in transmission electron microscopy (TEM) by negative staining with 2% potassium phospho-tungstate (KPT). The bacteria were grown on sucrose medium and were analysed by transmission electron microscopy (TEM) after 24 hours and after 5 days of incubation in order to assess the presence of poly-β-hydroxybutyrate granules. It was noticed that all the mountain strains produce these biopolymers but the maximum density of granules was recorded at those from subalpine, alpine and beech zones.

Keywords: poly-β-hydroxybutyrates (PHB), *Azotobacter* sp. transmission electron microscopy (TEM)

Introduction

Poly-β-hydroxybutyrates (PHB) are substances accumulated intracellularly as reserve granules by many bacteria in harsh environmental conditions (B.S. KIM [1]). PHB belong to the class of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs). PHAs have properties similar to polypropylene and are important due to their complete biodegradability, with recognised potential applications in reducing disposable waste problems and in certain medical applications (S. HERMAWAN & D. JENDROSSEK [2]). These biodegradable thermoplastics can be used both as packaging material and as drug delivery systems, since these polymers are immunologically inert. Biodegradable polymers would help to reduce solid waste disposal problems associated with most plastics. The polymer which provides a reserve of carbon and energy accumulates as intracellular granules.

In the cell, poly-β-hydroxybutyrate (PHB) is an intracellular storage material synthesized and accumulated during unbalanced growth. It accumulates as distinct white granules that are clearly visible in the cytoplasm of the cell. Under conditions of nutrient starvation, PHB is used by the cell as an internal reserve of carbon and energy. Many bacteria including those in the soil, are capable of PHB production and breakdown (A.J. ANDERSON & E.A. DAWES [3]). A series of enzymes, synthetases or depolymerases, are implied in the biosynthesis and biodegradation of poly-β-hydroxybutyrates and also of other polyhydroxyalkanoates (PHA) (T. ABE & al. [4]).

Many studies on PHB metabolism have presented microscopical images of bacteria cells full of randomly localized PHB granules. The number of such studies is too high to

justify a selection of special references. These biodegradable polyesters display a special interest due to their possible use as substitutes of common plastics (S. KHANNA & A.K. SRIVASTAVA [5]) because they are completely degraded by the microoganisms present in the environment and they can be produced from regenerable carbon sources (M.J. PETTINARI & al. [6]). They can be extracted and exposed alongside other synthetic polymers in films of heteropolymers with biotechnological applications (S. PAL & al. [7]). They can also be used as viscosity and gelification agents in pharmaceutic industry (G.R. VELA & al. [8]) and also as a model for developing fermenting strategies (A. ALMEIDA & al. [9]).

Many nitrogen-fixing microorganisms synthesize PHB. The *Azotobacter* species fix the molecular nitrogen and have the capacity to accumulate polyhy-β-droxybutyrates when they are grown on different carbon sources, including sucrose media (J.C. QUAGLIANO & al. [10]). *Azotobacter* sp. are Gram negative bacteria, polymorphic, of different sizes and shapes. Old population of bacteria includes encapsulated forms and have enhanced resistance to heat, desication and other adverse conditions. The cysts germinate under favourable conditions to give vegetative cells. They also produce polysachharides. These are free living bacteria which grow well on a nitrogen free medium. These bacteria utilize atmospheric nitrogen (N_2) for their cell protein synthesis.

The strains used in this study were obtained from different mountain soils, where the environmental conditions are harsh (R. CARPA & al. [11]). We supposed that these harsh climatic conditions, with long periods of cold, high humidity and extreme weather would determine the bacteria to produce higher amounts of reserve carbon and energy deposits. So we focused here on microscopical investigation of the capacity that these bacterial strains have to accumulate PHB, amplified by the mountain environments. This is a simple and efficient method for assessing the existence of such deposits.

Material and methods

The bacterial strains and the growth conditions. Five *Azotobacter* strains originating from five altitudinal vegetation zones of Parâng Massif, Romania (alpine altitude = 2216 m, subalpine altitude = 1871 m, coniferous altitude = 1646 m, beech altitude = 1286 m and Maleia flood plain altitude = 805 m) and the standard strain *Azotobacter chroococum* 2286^T – DSMZ were used in order to highlight the synthesis of poly-β-hydroxybutyrates (PHB). The growth medium used to isolate and to produce PHB was a solid sucrose medium (R.M. ATLAS [12]). Because the strains are from acid soils, the pH of the culture medium was ajusted to 5.8. In order to observe the formation of *Azotobacter* sp. cysts, cultures incubated for 4-5 days on medium at 30 ºC were used. The granules of poly-β-hydroxybutyrates (PHB) from these cysts were microscopically highlighted.

Transmission electron microscopy (TEM). Poly-β-hydroxybutyrates (PHB) granules were assessed by transmission electron microscopy using negative staining method. The stain (potassium phospho-tungstate (KPT) 2%) does not chemically interact with the preparation but creates a dark background, while the preparation remains transparent (J. PARSHAD & al. [13]). The preparations were made on 400 mesh cooper grids with carbon film obtained by vacuum evaporation on freshly cleaved mica, as drops of aqueous solution. The laid-down material was fixed with 2-3% glutaraldehyde. The grids on which the fixed material laid were left aside to allow the sedimentation of suspended particles. The extra liquid was removed with filter paper. Then, the grid was laid down on a drop of negative stain, so that the specimens deposed on the pelicule reach the stain. It was washed with 2-3 drops of the same stain. After a short time interval the grids were taken from the stain and the exces of it was

removed with filter paper. After a short dry in vacuum, the grids were examined by TEM. The image of the specimen appears more electron-transparent on a background more electron-dense (dark).

Results and discussions

It is well recognized that PHB are accumulated in Gram negative and Gram positive bacteria as well as in archaea. They are produced under an excess carbon source and nutrient-limiting conditions such as the absence of nitrogen, phosphorus or sulfur. *Azotobacter* strains were isolated from all the mountain soil samples. In order to do this a solid selective culture medium was used, on which soil granules were placed. While incubating, mucilaginous colonies specific to *Azotobacter* genus have appeared around the soil grains. Using subcultivation techniques pure cultures were obtained, out of which *Azotobacter* cells were microscopically highlighted (Fig. 1).

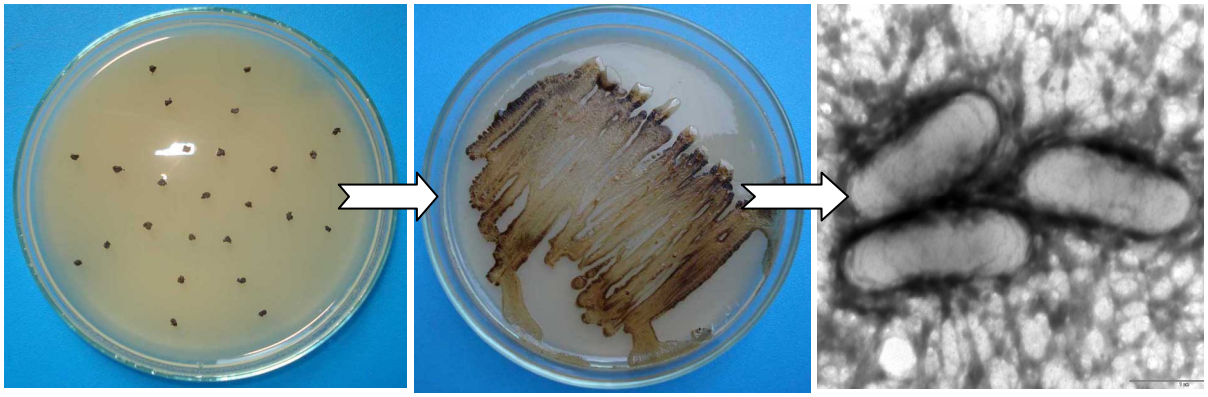

Fig. 1. The *Azotobacter* cells isolation from mountain soils

For marking out the formation of cysts and production of poly-β-hydroxybutyrates granules as reserve energetic material in *Azotobacter* cells, the negative staining method from transmission electron microscopy (TEM) was performed (M.A. HAYAT [14]). The experiments of PHB highlighting were performed at the Electron Microscopy Center, Babeş-Bolyai University, Cluj-Napoca.

The vegetative cells were photographed after 24 incubation hours, at 30 ºC, on elective solid medium. Convex, mucilaginous, slightly transparent colonies appear after 24 hours of incubation on solid sucrose medium. After more incubation days the colonies presented dirty yellow to dark brown pigments, which did not propagate in the culture medium. After 5 incubation days out of the same cultures preparations were made in order to highlight the formation of cysts with poly-β-hydroxybutyrate (PHB) granules.

At the standard *Azotobacter chroococcum* 2286^T – DSMZ strain rod shaped vegetative cells were highlighted by TEM after one incubation day. Their dimensions are 1.5 x 0.7 µm (Fig. 2a).

After five days of incubation on solid medium specific for de *Azotobacter chroococcum* 2286^T – DSMZ strains cysts of 2.5 x 2 µm were obtained (Fig. 2b). After five days on solid medium many cysts appeared, most of them round, with a relatively high number of well individualized PHB granules (9 to 15 granules/cyst).

Ultrastructurally, the cysts display a central body containing poly-β-hydroxybutyrate granules (PHB) surrounded by exine and intine.

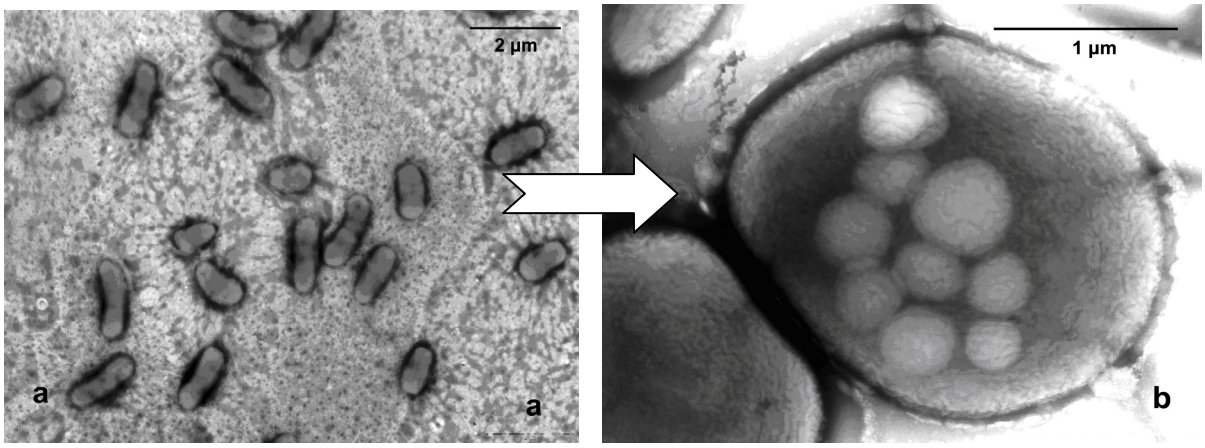

Fig. 2. *Azotobacter chroococcum* 2286^T – DSMZ cells on sucrose medium **a)** after 24 incubation hours **b).** cyst with poly-β-hydroxybutyrate granules after 5 incubation days.

At the strains originating from the alpine zone vegetative *Azotobacter* cells of 1.5 x 0.6 µm (Fig. 3a) were obtained after 24 incubation hours. Some rods were grouped, even in palisade.

After five incubation days on solid medium specific for *Azotobacter*, at the strains from the alpine zone 6.5 x 4 µm (Fig. 3b) cysts were obtained. These were egg-shaped, with a relatively high number of PHB granules (7-15 granules/cyst), well individualized.

Poly-β-hydroxybutyrates are structurally simple macromolecules that accumulate as discrete granules to a level as high as 90% of the cell dry weight (D. ABD-EL-HALEEM & al. [15]; D.S. KADOURI & al. [16]).

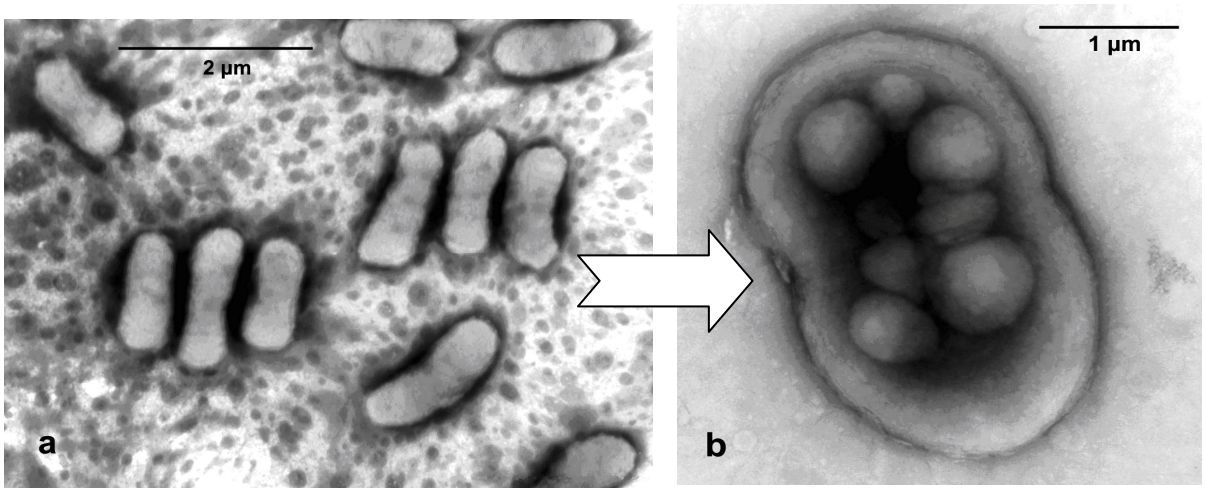

Fig. 3. *Azotobacter* sp. cells from the *alpine zone* on sucrose medium **a)** after 24 incubation hours **b)** cyst with poly-β-hydroxybutyrate granules after 5 days of incubation.

After 24 incubation hours at the strains from the subalpine zone *Azotobacter sp*. vegetative cells of different shapes were obtained (Fig. 4a). This is possible because the *Azotobacter* cells are pleomorphous. Their dimensions varied from 1.14 x 0.57 µm to 2.28 x 0.45 µm.

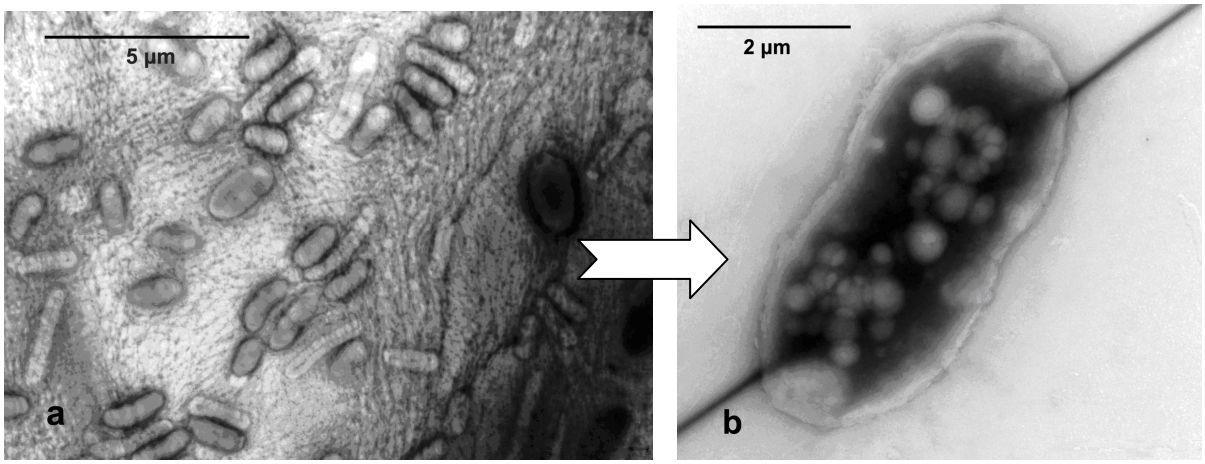

Fig. 4. *Azotobacter* sp. cells from the *subalpin zone* on sucrose medium **a)** after 24 incubation hours **b)** cell in division with poly-β-hydroxybutyrate deposits after 5 days of incubation.

In the case of the strains from subalpine zone after five incubation days on solid medium dividing cells are displayed, presenting numerous poly-β-hydroxybutyrate granules (Fig. 4b). Although the cells with the most numerousPHB granules after five days are found in this zone (from 25 to 35 granules/cell), however the cysts are not entirely formed.

On the strains sampled from the coniferous zone after 24 incubation hours, by transmission electron microscopy (TEM), vegetative *Azotobacter* sp. cells of different size were observed (Fig. 5a). The size of the rods ranged from 1.3 x 0.8 µm to 3 x 0.66 µm.

No cyst was observed after five days of incubation in the cultures from the coniferous zone, only dividing cells which present a much smaller number of poly-β-hydroxybutyrate granules compared with the ones from the other altitudinal vegetation zones (5 - 10 granules/cell) (Fig. 5b).

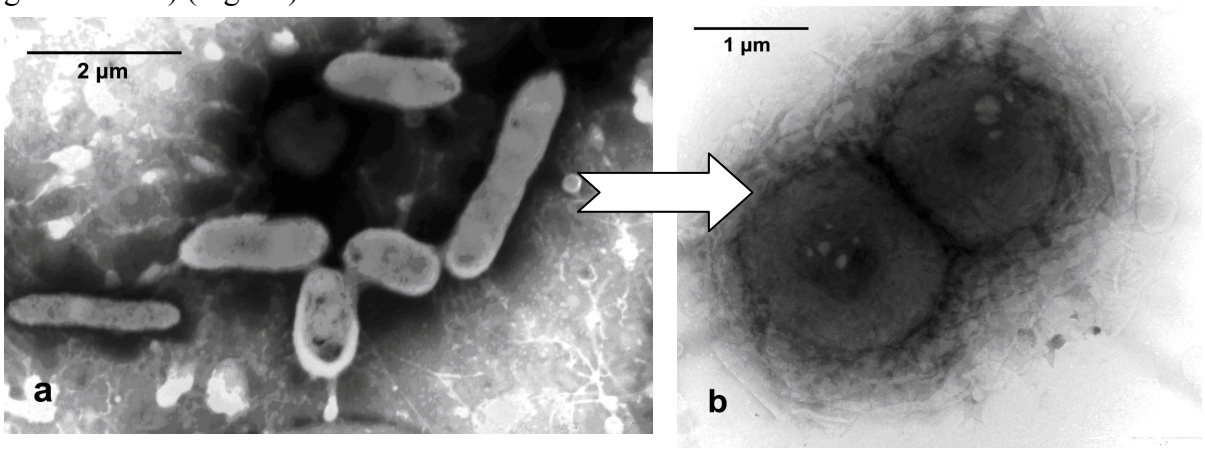

Fig. 5. *Azotobacter* sp. cells from the *coniferous zone* on sucrose medium **a)** after 24 incubation hours **b)** cell in division with poly-β-hydroxybutyrate granules after 5 days of incubation.

At the strains taken from the beech zone vegetative *Azotobacter* sp. cells were highlighted after 24 incubation hours with dimensions of 2 x 0.8 µm (Fig. 6a). Some rods were gruped.

After five incubation days in the cultures with strains from the beech zone there are cysts not very individualized but containing poly-β-hydroxybutyrate granules (10-16 granules/cyst) (Fig. 6b).

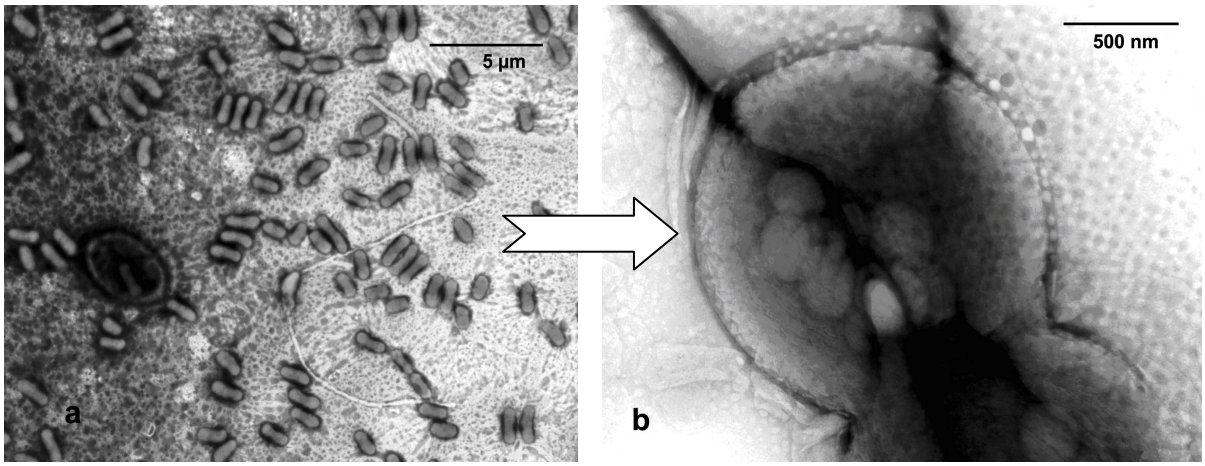

Fig. 6. *Azotobacter* sp. cells from the *beech zone* on sucrose medium **a)** after 24 incubation hours **b)** cyst with poly-β-hydroxybutyrate granules after 5 days of incubation.

Vegetative *Azotobacter* sp. cells of 1.33 x 0.53 µm and also other rods, much larger, of 5.33 x 1.33 µm (Fig. 7a), which demonstrate a high, even exceptional pleomorphism, were noticed using transmission electron microscopy (TEM) at the strains from Maleia flood plain, after 24 incubation hours.

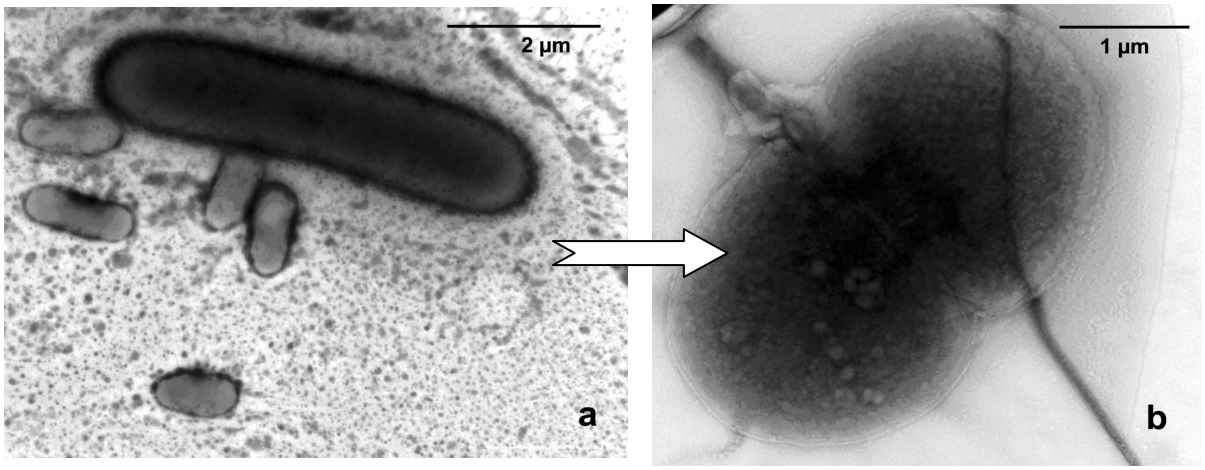

Fig. 7. *Azotobacter* sp. cells from the *Maleia flood plain zone* on sucrose medium **a)** after 24 incubation hours **b)** cell in division with poly-β-hydroxybutyrate granules after 5 days of incubation.

After five incubation days in the cultures from Maleia flood plain specific cysts can not be seen, but dividing cells presenting poly-β-hydroxybutyrate granules are visible (Fig. 7b). These granules are very small and reduced in numbers.

While encysting, all the metabolic activities cease. The formation of cysts has a special importance because these are much more resistant than the vegetative cells at harsh environmental conditions. The cysts can resist at the action of physico-chemical agents and when the conditions become favourable they germinate. Another important element is the formation of poly-β-hydroxybutyrates in these cysts. These polymers present a special interest in microbial biotechnologies.

Conclusions

All the *Azotobacter* strains isolated from mountain soils produced biodegradable polymers of poly-β-hydroxybutyrate type, as it was shown by transmission electron microscopy.

The maximum poly-β-hydroxybutyrates density was obtained in the strains isolated from subalpine, alpine and beech zones.

Due to their high potential regarding the poly-β-hydroxybutyrates synthesis, the isolated strains should be further studied for their potential biotechnological use.

Acknowledgements

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References

1. B.S. KIM, Production of poly (3-hydroxybutyrate) from inexpensive substrates. *Enzyme Microb. Technol*., **27** (10), 774-777 (2000).
2. S. HERMAWAN, D. JENDROSSEK, Microscopical investigation of poly (3-hydroxybutyrate) granule formation in *Azotobacter vinelandii*, *FEMS Microbiol. Lett.* (**266**), 60–64 (2007).
3. A.J. ANDERSON, E.A. DAWES, Occurrence, metabolism, metabolic role, and industrial uses of bacterial PHA. *Microbiol. Rev*., (**54**), 450–472 (1990).
4. T. ABE, T. KOBAYASHI, T, SAITO**,** Properties of a novel intracellular poly (3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. *J. Bacteriol*., (**187**), 6982–6990 (2005).
5. S. KHANNA, A.K. SRIVASTAVA, Recent advances in microbial poly-hydroxyalkanoates. *Process Biochem*., **40,** 607–619 (2005).
6. M.J. PETTINARI, G.J. VASZQUEZ, D. SILBERSCHMIDT, B. REHM, A. STEINBUCHEL, B.S. MENDEZ, Poly (3-hydroxybutyrate) synthesis genes in *Azotobacter sp*. strain FA8. *Appl. Environ. Microbiol*., (**67),** 5331–5334 (2001).
7. S. PAL, A. MANNA, A.K., PAUL**,** Production of poly-β-hydroxybutyric acid and exopolysaccharide by *Azotobacter beijerinckii* VDN-01. *W. J. Microbiol., Biotechnol.,* **15**, 11-16 (1999).
8. G.R. VELA, G.D. CAGLE, P.R. HOLMGREN, Ultrastructure of *Azotobacter vinelandii*. *J. Bacteriol*., **104** (2), 933-939 (1970).
9. A. ALMEIDA, P.I. NIKEL, A.M.GIORDANO, M.J.PETTINARI, Effect of granule associated protein phaP on glycerol-dependent growth and polymer production in poly(3-hydroxybutyrate) producing *Escherichia coli*. *Appl. Environ. Microbiol*., **73,** 7912-7916 (2007).
10. J.C. QUAGLIANO, P. ALEGRE, S.S. MIYAZAKI, Isolation and characterization of *Azotobacter sp*. for the production of poly- β -hydroxyalkanoates. *Rev. Argent. Microbiol*., **26,** 21–27 (1994).
11. R. CARPA, A. BUTIUC-KEUL, C. DOBROTA, V. MUNTEAN, Molecular identification of diazotroph microbial consortia in mountain soil. *Cent.Eur. J. Biol*., **5** (5), 664-673 (2010).
12. R.M. ATLAS, Handbook of Microbiological Media. 3rd edition, CRC Press, New York (2004).
13. J. PARSHAD, S. SUNEJA, K. KUKREJA, K. LAKSHMINARAYANA, Poly-3-hydroxybutyrate production by *Azotobacter chroococcum. J. Biomedical and Life Science*, **46** (4), 315-320 (2001).
14. M.A. HAYAT, Principles and techniques of electron microscopy-Biological Applications, Cambridge University Press (2000).
15. ABD-EL-HALEEM, A. AMARA, S. ZAKI, A. ABULHAMD, G. ABULREESH, Biosynthesis of biodegradable polyhydroxyalkanotes biopolymers in genetically modified yeasts. *Int. J. Environ. Sci. Tech*., **4** (4), 513-520 (2007).
16. D. KADOURI, S. BURDMAN, E. JURKEVITCH, Y.OKON, Identification and isolation of genes involved in poly β hydroxybutyrate biosynthesis in *Azospirillum brasilense* and characterization of a *phbC* mutant. *Appl. Environ. Microbiol*., **68,** 2943–2949 (2002).