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BACTERIA FROM WASTEWATER: POTENTIAL PRODUCERS OF POLYHYDROXYALKANOATES IN VILNIUS, LITHUANIA

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Abstract. Polymers are currently used as a major raw material in industry, but they are quickly discharged into the environment and cause significant pollution. To tackle this environmental pollution problem, particular attention is being funded to biodegradable polymers, namely polyhydroxyalkanoates (PHAs) produced by microorganisms. This research detects PHA-producing bacteria from the Vilnius City municipal wastewater treatment plant. We confirmed 5 PHA-positive strains belonging to the following genera: *Brachymonas, Aeromonas*, *Enterobacter*, *Pseudomonas*. One of the isolates, *Aeromonas media*, is a promising strain to produce PHAs with production values ranging up to 0.544 g/L. Bacteria producing more than 0.300 g/L are considered useful for the industrial production of bioplastics. We recommend large-scale studies on this strain to assess their use in the industrial production of biopolymers to develop high-impact bioconversion processes of industrial relevance.

Keywords: wastewater, recycling technology, polyhydroxyalkanoates, *Aeromonas*, circular economy.

Introduction

With the growth of the human population, the planet has seen a huge increase in non-degradable waste, of which plastic waste is the most harmful to the environment, as it decomposes slowly and emits very harmful toxins (Gross & Kalra, 2002, pp. 803–807). At the same time, increasing urbanisation and industrialisation are coming close to filling up the system, generating uncontrollable amounts of wastewater, putting our society under severe pressure (Ajibade et al., 2021, pp. 321–354). Municipal wastewater can accumulate residues of certain substances used by microorganisms (Koul et al., 2022), making it an attractive medium for the growth of certain microbial populations (Monroy & Buitrón, 2020, pp. 39–47). These microorganisms can work in synergy with each other to carry out metabolic reactions that degrade organic matter and remove excess nutrients from the wastewater (Sial et al., 2021, pp. 723–738).

The general population uses wastewater treatment plants to manage organic and solid organic waste through aerobic and anaerobic processes (Vinardell et al., 2021). These biological processes form mixed microbial consortia (MMCs), which contain microorganisms such as bacteria, yeasts and fungi that can potentially synthesize biopolymers in the presence of an excess of carbon and limited nitrogen or phosphorus in the growth medium (Valentino et al., 2017, pp. 9–23).

Polyhydroxyalkanoates (PHAs) are a group of biodegradable and biocompatible polyesters of natural origin, synthesised as carbon stores by many bacteria (Niaounakis, 2015). PHA is typically produced as a polymer of 103-104 monomers, which aggregate as inclusions of 0.2–0.5 in diameter (Brandl et al., 1990, pp. 77–93). PHA has been extensively researched and thoroughly characterised – it resembles traditional petroleum plastics such as polyethylene and polypropylene. The composition, physico-chemical properties, size, and structure of the monomers depend on the type of PHA-producing bacterium (Ha & Cho, 2002, pp. 759–809).

As bacteria can be easily grown in a variety of materials depending on their availability (Jiang et al., 2008, pp. 167–172), and as these bioplastics are biodegradable and can be produced from renewable resources, they have an advantage over conventional plastics, and researchers are exploring various renewable carbon sources that can be used for PHA production. The use of various wastes for the biosynthesis of PHA is a good strategy as the production is cost-effective and it helps to overcome waste disposal problems (Koller et al., 2005, pp. 561–565). PHA polymers are synthesised from inexpensive carbon sources with different compositions, which can lead to new biopolymers with unique properties. Some of these bacteria and inexpensive carbon sources are of interest to industry due to their cost-effective substrate

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transformation process and their ability to ultimately concentrate biopolymers in cells.

PHA-producing bacteria, due to their lipidic nature, can be studied and isolated using lipophilic spots such as Sudan Black (Schlegel et al., 1970, pp. 283–294), Nile Blue (Ostle & Holt, 1982, pp. 238–241), Fluorescent Oxazone and Nile Red (Spiekermann et al., 1999, pp. 73–80). For the identification of PHA-producing bacteria, it is important to perform PCR-based molecular screening to determine the bacterial genus and species (Solaiman et al., 2000, pp. 690–694). The 16S rDNA gene is the most commonly used gene in the assays and provides high resolution for taxonomic identification (Rosselló-Mora & Amann, 2001, pp. 39–67).

The aim of this study is to identify PHA-producing bacteria in municipal wastewater by microbiological and molecular methods, searching for indigenous and non genetically modified strains that can be used for industrial biopolymer production. Identifying these bacteria may facilitate using cost-effective substrates from municipal wastewater treatment plants, which are the main source of activated sludge-containing feedstocks to produce bioplastics. The identified microorganisms can also serve as a complementary alternative to conventional biological wastewater treatment, as municipal wastewater can be a suitable medium for biodegradation and bioplastic production, thus promoting the development of biotechnology in the region and the use of biowaste cascading.

1. Materials and methods

1.1. Study area

The study area consisted of the Vilnius City Wastewater Treatment Plant (WWTP) (54.676512, 25.153217) located in Vilnius, Lithuania. The selected site for the observation was the active primary sludge chamber after the primary radial settling unit (Figure 1).

1.2. Sample collection and selection of isolates with PHA-producing capacity

Mixture of activated sludge and wastewater were collected from Vilnius WWTP after primary treatment. Vilnius WWTP wastewater was collected in 3×3 L glass containers previously cleaned by washing in non-ionic detergent, rinsed with tap water, and later soaked in 10% for 24 hours, and finally rinsed with deionized water before usage. The grab sampling was performed according to Standard Methods (American Public Health Association [APHA], 2005). The samples were labelled and transported to the laboratory, stored in the refrigerator at about 4 before analysis.

For isolation from the wastewater sample was performed according to Liu et al. (1998).

The rapid detection and isolation of PHA producing bacteria with 0.02% alcoholic solution of Sudan black B (ROTH) was performed according to Schlegel et al., (1970).

Sudan black B positive isolates were checked for PHA production by Nile blue A (ROTH) staining and it was performed by Spiekermann et al. (1999) proposed method. Using a UV transilluminator (ULTRA-LŪM) the bright orange fluorescence bacteria were selected as PHA accumulators.

1.3. Molecular identification of PHA-producing strains

The isolates were stored in cryovials with 15% glycerol at – 80 °C. For bacterial genomic DNA isolation,

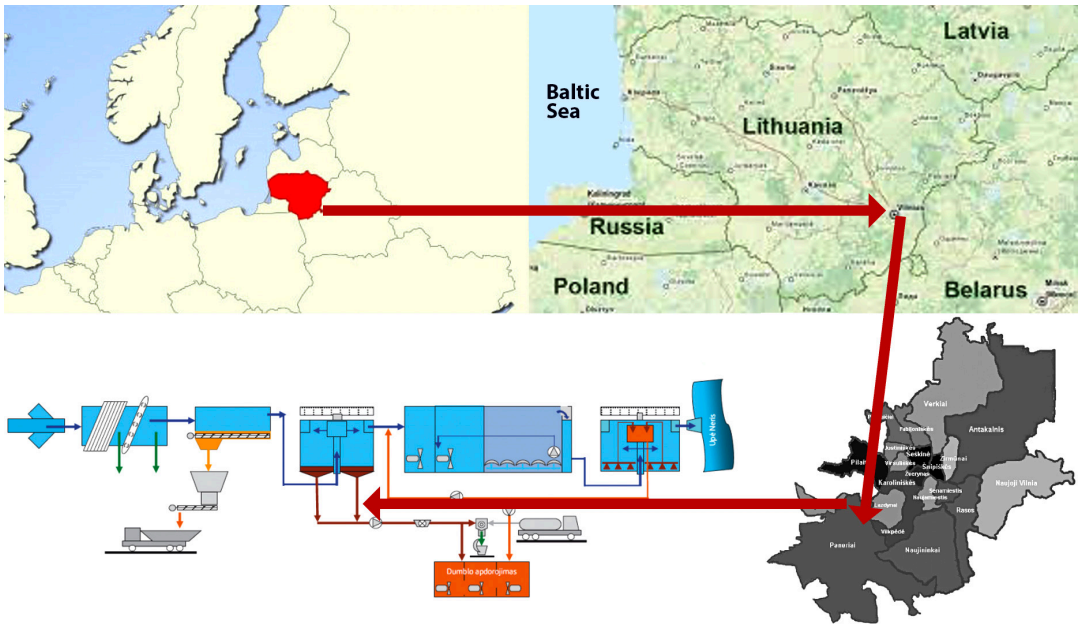


Figure 1. Study area and monitoring site at the wastewater treatment plant

amplification, purification, and 16S rDNA gene sequencing, the bacteria were plated on microbiological medium (peptone; beef extract; NaCl; agar) Petri dish and submitted to the Molecular Ecology Laboratory of the Center for Natural Research for analysis. DNA sequencing reactions were prepared using the BigDye Terminator v3.1 Cycle Sequencing Kit. The analysis was performed on an automated 16-capillary genetic analyzer 3130×1 Applied Biosystems, which performs electrophoresis, laser detection, and computer analysis of fluorescently labelled DNA fragments.

The sequenced sequence was compared to the 16S rDNA sequences in the NCBI database using the BLAST computer program.

1.4. Assessment of polyhydroxyalkanoate-producing strains

Seed culture media was prepared according to Munir and Jamil (2015), enriched with glucose and trace element solution. After growing, the cells are centrifuged at 5000 rpm for 10 min. After centrifugation, the supernatant is discarded, and the cells are transferred to 250 ml of sterilised wastewater of known composition. The inoculum was incubated at 37 °C for 24 h at 150 rpm (Munir & Jamil, 2015, pp. 1605–1611). The precipitate was recovered at 24 h of incubation. Together with 10 ml of 12% sodium hypochlorite, the biomass is incubated at 50 °C for 1 hour to lyse the cells. The resulting cell extract was centrifuged at 12 000 rpm for 30 minutes, followed by successive washing with distilled water, acetone, and absolute ethanol. After washing, the residual biomass was dissolved in 10 ml of chloroform (99%), incubated overnight at 50 °C, and then evaporated at room temperature. Quantitative analysis proposed by Bhuwal et al. (2013) was performed.

2. Results

2.1. Selection of the isolates with PHA-producing capacity

To determine that the available bacterial culture cells accumulate polymers, 100 single colonies were inoculated into the microbiological medium (beef extract 10, peptone 10, sodium chloride 5, and agar 18) in a Petri dish. Sudan Black B showed 24 affected colonies – 17 with dilution of 1000 times and 7 with dilution of 100 times. The confirmatory test (Nile blue A staining) allowed identifying 6 strains capable of producing PHAs (Table 1).

Table 1. PHA-producing strains isolated from wastewater of Vilnius WWTP

	Number of strains	Sudan Black B affected colonies	Positive Nile Blue A
Dilution 1000	50	17	3
Dilution 100	50	7	3

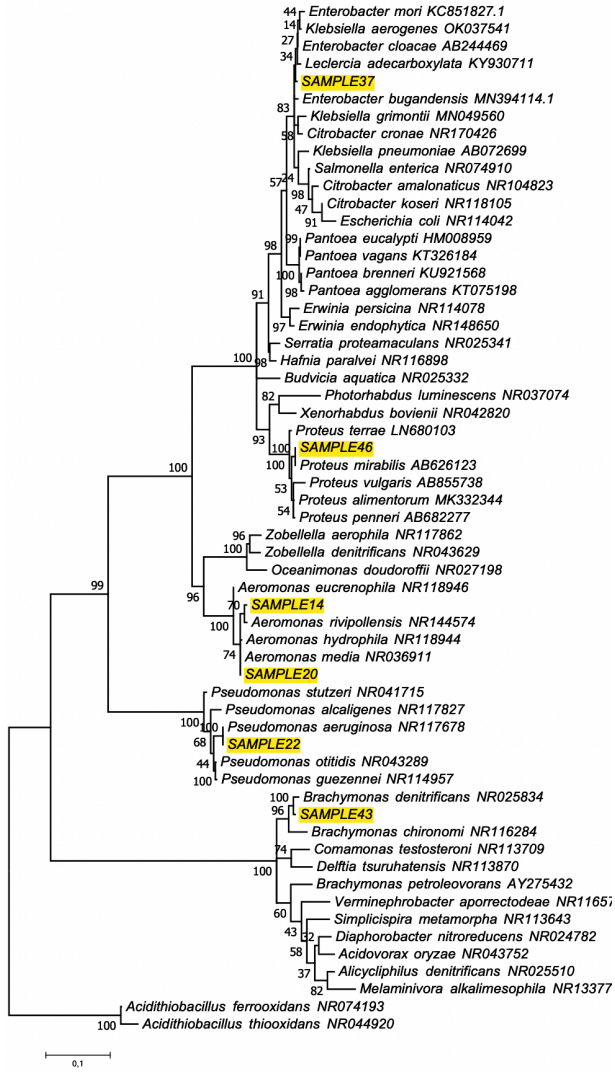


Figure 2. Neighbor-Joining tree of 16S rRNA gene sequences based on the Tamura-Nei model. Bootstrap values plotted on a scale of 1000 replicates on the branches of the tree. The scale at the bottom shows the genetic distance

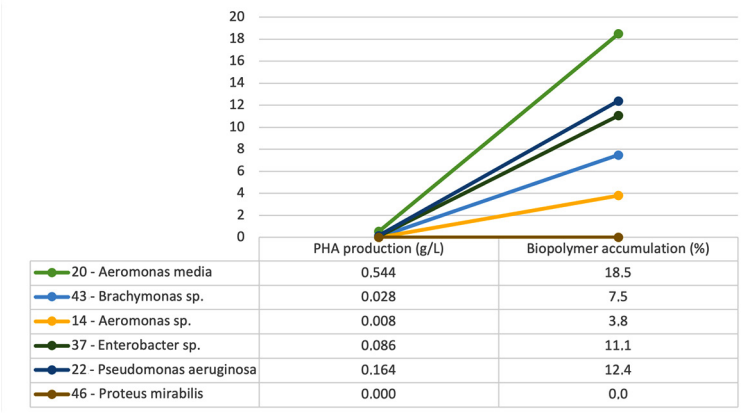
2.2. Molecular identification of PHA-producing strains

The amplification of the 16S rDNA gene confirmed the identity of 6 strains (Table 1). The 16S partial sequences showed 98.35% and 100% identity to reported sequences for *Proteus sp.*, *Brachymonas sp.*, *Aeromonas sp.*, *Enterobacter sp.*, *Pseudomonas sp.* (Figure 2).

2.3. Assessment of the PHA-producing strains

The identity of 5 PHA-positive bacteria were determined. *Aeromonas media* showed the highest PHA production (0.544 g/L), followed by *Pseudomonas aeruginosa* (0.164 g/L) (Figure 3) in sterilised wastewater of known composition (Table 2). Unfortunately, no PHA production from *P. mirabilis* 46 was detected.

Aeromonas media, Pseudomonas aeruginosa, and *Enterobacter sp.* showed the highest biopolymer accumulation rates, respectively – 18.5%, 12.4% and 11.1%.



	PHA production (g/L)	Biopolymer accumulation (%)
20 - Aeromonas media	0.544	18.5
43 - Brachymonas sp.	0.028	7.5
14 - Aeromonas sp.	0.008	3.8
37 - Enterobacter sp.	0.086	11.1
22 - Pseudomonas aeruginosa	0.164	12.4
46 - Proteus mirabilis	0.000	0.0

Figure 3. PHA production (g/L) and biopolymer accumulation (%)

Table 2. Wastewater from Vilnius WWTP of known composition after sterilisation

Measured parameters	After temperature sterilisation	Standards
mg/l	124	LAND 46-2007
mg/l	1004	ISO 15705:2002(E)
mg/l	615	LAND 47-1:2007
mg/l	8.62	LAND 58:2003
mg/l	45	LAND 84:2006, LAND 59:2003
mg/l	34	LAND 38:2000
mg/l	4.69	LAND 58:2003
Lipids mg/l	25	SVP-17

It is suggested that future studies on these PHA-positive bacteria should assess the purity of the PHAs and determine which wastewater parameters have the greatest influence on the accumulation of biopolymers.

Discussion

Identified PHA-producing bacterial strains isolated from Vilnius WWTP. The data provide new evidence that supports the PHA-producing bacterial isolates in wastewater treatment plant and cheap carbon source (wastewater) performance with those bacterial strains which shows that the use of these beneficial microorganisms for the industrial production of bioplastic in wastewater treatment plants.

The molecular identification of the 5 PHA-producing isolates showed that these belonged to the genera *Brachymonas*, *Aeromonas*, *Enterobacter* and *Pseudomonas*, which have been reported to produce PHAs (Koller et al., 2010, pp. 255–269). The genus *Enterobacter, Pseudomonas* and *Aeromonas* have been widely studied and isolated from diverse biowaste streams (Nighat et al., 2012, pp. 3321–3332; Kourmentza et al., 2015, pp. 202–210; Sangkharak & Prasertsan, 2012, pp. 173–182). *Brachymonas* genus have been studied as polyhydroxybutyrate (PHB) producer (Shi et al., 2007, pp. 625–632). PHB is a derivative of PHA that has been extensively studied and carefully described (McAdam et al., 2020).

The PHA production widely studied under feast–famine with municipal wastewater and mixed microbial consortium (Valentino et al., 2015, pp. 7281–7294; Morgan-Sagastume et al., 2014, pp. 177–184), no studies using single bacteria with municipal wastewater as carbon source were found after the literature search.

Fernández et al. (2005) report that bacteria capable of producing more than 0.300 g/L of PHAs are considered promising for the industrial manufacture of bioplastic. Of the 5 strains found here, 1 is promising for PHA production – *Aeromonas media*, showing value – 0.544 g/L.

Based on the findings of the study, recommended to carry out large-scale studies and evaluate the industrial production of biopolymers using these strains and mixture of activated sludge and wastewater after primary treatment. This would allow the development of high-impact bioconversion processes to extract industrially useful metabolites such as PHAs from contaminated by-products. Although solvent-based methods are cost-effective and show high yields of PHA, more environmentally friendly extraction methods, such as the use of physical cell disruption processes, need to be evaluated and implemented.

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Contribution

Author created the conception and design of the work; conducted analysis and interpretation of data and drafted the article.

Author revised it critically for important intellectual content.

Disclosure statement

Nothing to declare.

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