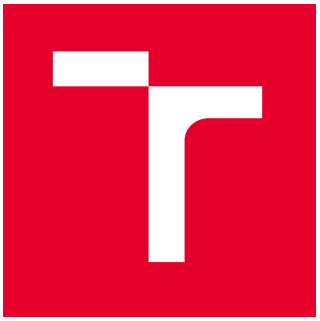

BRNO UNIVERSITY OF TECHNOLOGY

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY

FAKULTA CHEMICKÁ

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

STUDY ON PRODUCTION OF POLYHYDROXYALKANOATES BY PSYCHROPHILIC BACTERIA

STUDIUM PRODUKCE POLYHYDROXYALKANOÁTŮ U PSYCHROFILNÍCH BAKTERIÍ

MASTER'S THESIS

DIPLOMOVÁ PRÁCE

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Study on production of polyhydroxyalkanoates by psychrophilic bacteria

Master's Thesis:

1. Review on the topic
2. Detection of presence of phaC genes in selected psychrophiles employing PCR
3. Evaluation of PHA accumulation using phenotyping methods
4. Optimization of cultivation and study on PHA production in selected strains

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Bc. Katarína Šlosárová student	Ing. Iva Pernicová, Ph.D. Head of thesis	prof. RNDr. Ivana Márová, CSc. Head of department
		prof. Ing. Michal Veselý, CSc. Dean

In Brno dated 1.2.2023

Abstract

Bacteria produce polyhydroxyalkanoates (PHA) as intracellular storage of carbon and energy. From a technological point of view, they have great potential as biodegradable versions of conventional plastics. However, their main disadvantage compared to them is the high cost of production. Research is therefore focusing on ways to overcome this obstacle, one of which is the use of strains of extremophilic micro-organisms. However, the amount of research on psychrophiles is lower compared to other types of extremophiles. Here, the results of the first screening involving 78 cold-adapted bacterial strains isolated from East Antarctica are presented. Both genotypic and phenotypic methods were used for the selection of potential PHA producers. The polymerase chain reaction mainly demonstrated the presence of genes encoding PHA synthases, namely PHA synthases class I and II. Staining with Nile Red and Sudan Black B, as a typical initial screening method from the phenotypic level, did not provide indicative results for psychrophiles and/or psychrotolerants. In this work, FTIR spectroscopy in combination with a high-throughput microculture system (Duetz-MTPS) was used to explore the presence of PHA, which was later confirmed by gas chromatography. Cultivation was conducted on five different media. Based on all the results the highest biomass production and PHA accumulation showed *Pseudomonas sp.* belonging to the green snow isolates. Interestingly, a high synthesis of PHA, specifically P(3HB), was observed on complex BHI medium with extra glucose addition, which did not provide the nitrogen limitation that is typical for the stimulation of the PHA synthesis and accumulation. It can be assessed that the presence of promising producers among polar bacteria was confirmed and the yield of PHA can be further increased by optimizing the media composition and growth conditions.

Keywords

Polyhydroxyalkanoates, PhaC synthase, extremophiles, psychrophilic and psychrotolerant bacteria

Abstrakt

Baktérie produkujú polyhydroxyalkanoáty (PHA) za účelom vnútrobunkových zásob uhlíka a energie. Z technologického hľadiska majú veľký potenciál ako biologicky odbúrateľné verzie bežných plastov. Ich hlavnou nevýhodou v porovnaní s nimi sú však vysoké výrobné náklady. Výskum sa preto zameriava na spôsoby, ako túto prekážku prekonať, pričom jedným z nich je použitie kmeňov extrémofilných mikroorganizmov. Množstvo výskumov so psychrofilami je však v porovnaní s inými typmi extrémofilov menšie. V tejto práci sú uvedené výsledky prvého skríningu zahŕňajúceho 78 bakteriálnych kmeňov adaptovaných na chlad izolovaných z východnej Antarktídy. Na výber potenciálnych producentov PHA sa použili genotypové aj fenotypové metódy. Polymerázová reťazová reakcia predovšetkým preukázala prítomnosť génov kódujúcich PHA syntázy, konkrétne PHA syntázy triedy I a II. Farbenie Nílskou červenou a Sudánskou čerňou B, ako typická metóda prvotného skríningu z fenotypovej úrovne, neposkytlo smerodajné výsledky u psychrofilov a/alebo psychrotolerantov. V tejto práci sa na zisťovanie prítomnosti PHA, ktorá sa neskôr potvrdila plynovou chromatografiou, použila FTIR spektroskopia v kombinácii s vysokovýkonným mikrokultivačným systémom (Duetz-MTPS). Kultivácia sa uskutočnila na piatich rôznych médiách. Na základe všetkých výsledkov bola najvyššia produkcia biomasy a zároveň akumulácia PHA zaznamenaná u *Pseudomonas sp.* patriaceho k izolátom zo zeleného snehu. Zaujímavé je, že zvýšená syntéza PHA, konkrétne P(3HB), bola pozorovaná na komplexnom BHI médiu s dodatočným prídavkom glukózy nepredstavujúcim limitáciu dusíkom, ktorá je typická pri stimulácii syntézy a akumulácii PHA. Možno zhodnotiť, že prítomnosť sľubných producentov medzi polárnymi baktériami bola potvrdená a výťažok PHA je naďalej možné navýšiť optimalizáciou zloženia média a rastových podmienok.

Kľúčové slová

Polyhydroxyalkanoáty, PhaC syntáza, extrémofily, psychrofilné a psychrotolerantné baktérie

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Prehlásenie

Prehlasujem, že som diplomovú prácu vypracovala samostatne a všetky použité literárne zdroje som správne a úplne citovala. Diplomová práca je majetkom Fakulty chemickej VUT v Brne a môže byť byť použitá na komerčné účely len so súhlasom vedúceho práce a dekana Fakulty chemickej VUT v Brne.

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Poďakovanie

Moje poďakovanie patrí predovšetkým vedúcej diplomovej práce Ing. Ive Pernicovej, Ph.D. za jej ochotu, ľudský prístup a cenné rady pri tvorbe práce. Taktiež by som chcela poďakovať prof. Ing. Stanislavovi Obručovi, Ph.D., vďaka ktorému mi bolo umožnené absolvovať zahraničnú stáž. Touto cestou by som sa ďalej chcela poďakovať aj členom skupiny BioSpec na NMBU, hlavne konzultantke Ing. Dane Byrtusovej, Ph.D. za pomoc pri experimentoch a zhotovovaní práce. V neposlednom rade patrí ďakujem aj mojej rodine a priateľom, ktorí ma podporovali počas celého štúdia.

CONTENT

1 INTRODUCTION

Plastics are an essential part of our daily lives and almost everything that surrounds us. This is due to their excellent properties, such as chemical inertness, resistance to corrosion, lightness, and impermeability to water, but at the same time, their properties can be modified in various ways to suit the needs during the production process, which is very inexpensive due to their high usability. However, ever-increasing production brings many negatives, especially in the form of plastic pollution, which has become one of the main environmental issues of our time and is outstripping the world's ability to deal with it. Recycling does not solve the problem in this case either, as most plastics are made of composites and are therefore not suitable for recycling.

One approach is to try to replace petrochemical plastics (in certain areas such as single-use plastics) with biobased and/or biodegradable plastics, both of which are met by polyhydroxyalkanoates. Polyhydroxyalkanoates are naturally produced and accumulated by microorganisms as a storage of carbon and energy. However, they face the major problem of high production costs (especially carbon substrate for the growth of microorganisms, sterility of the biotechnological process, and purification of the product), which puts polyhydroxyalkanoates at a significant disadvantage as a possible substitute for petrochemical plastics. More economically sensible approaches are being searched for, in particular, the use of waste substrates or the use of extremophilic microorganisms as producers.

The advantage that extremophiles bring from the perspective of biotechnology (among other attributes) is a reduction in the cost of sterility of the work, which is precisely what benefits in conjunction with polyhydroxyalkanoates. In this context, research has so far concentrated mostly on thermophilic and halophilic producers, while the category of psychrophiles has not received much attention. Even though most of the earth's biosphere is permanently cold, the polar regions have been very little studied so far. This is probably due to the low availability of sampling material and the unusual cultivation regime. Psychrophiles have had to adapt in various ways to the extreme conditions in which they survive, allowing them to open up many new areas of biotechnology – they could be for example a source of cold-active enzymes, exopolysaccharides, polyunsaturated fatty acids and antifreeze proteins with the applications in many fields – including the possibility of significant production of the aforementioned polyhydroxyalkanoates as green plastics.

2 THEORETICAL PART

2.1 Polyhydroxyalkanoates

Many bacteria produce and accumulate a family of biopolyesters known as polyhydroxyalkanoates (PHA) to store energy and carbon [1]. PHA occurs naturally in cyanobacteria, and this has been known at least since 1966 [2]. As soon as PHA are extracted from bacterial cells, these polymers exhibit elastic, crystalline, flexible, and thermoplastic properties. They also don't deplete finite resources because they are made from renewable carbon resources, such as agricultural wastes, industrial wastes, or fermentation feedstocks. Bacterial PHA have drawn a lot of attention since they are entirely biodegradable, non-toxic, biocompatible, and useful as sources of chiral monomers [3]. Due to these properties, polyhydroxyalkanoates have found wide applications in various industrial applications, primarily because they may be used as an alternative to petrochemical plastics. In the interests of the research, it is necessary to search for alternatives to reduce the costs of their production and thus bring them closer to the petrochemical plastics in terms of economics as well [4].

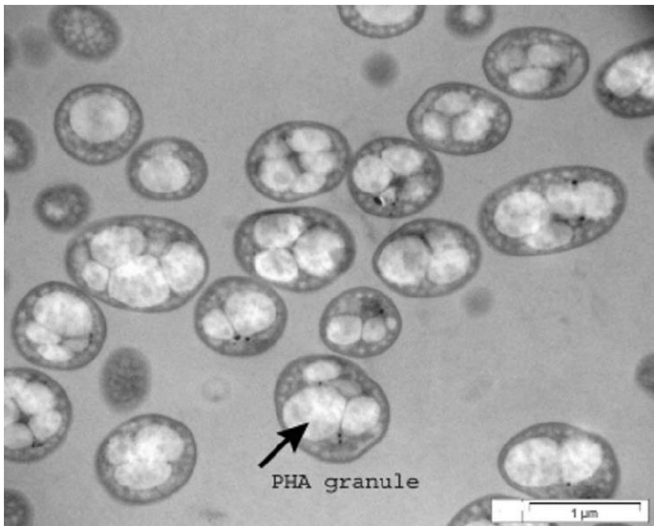

Figure 1 TEM image of Pseudomonas putida CA-3 cells containing PHA granules [5]

This intracytoplasmic reserve polymer could enable cell survival under stress conditions when the cells are exposed to carbon starvation or when they are frozen. It is thought to be due to an increase in the levels of ATP, ppGpp and RpoS molecules, which could be essential for bacterial cells to respond to stress [6]. As for cold conditions, microorganisms can adapt to them by producing and accumulating cryoprotectants (substances that can protect cells from the harmful effects of freezing) [7]. Thus, many bacteria appear to use the accumulation of PHA, specifically poly(3-hydroxybutyrate) P(3HB), as a common metabolic mechanism for low-temperature conditions. Analysis showed that P(3HB)-containing cells have a higher rate of transmembrane water transport, protecting them from the development of intracellular ice [8]. In a different study, scientists looked at how PHA accumulation affected *Pseudomonas* species' tolerance to low temperatures. A direct comparison was made between the parent strain and the PHA synthase-minus mutant (obtained by removing the *phaC* gene encoding for the PHA synthase). The PHA mutant did not grow at 10 °C and was more prone to freezing [9].

2.1.1 Structure and classification

From a chemical structural point of view, PHA are linear polyesters of hydroxyalkanoic acids, specifically (R)-3-hydroxy fatty acids [3]. The carboxyl group of the monomer is bonded to another monomer unit by an ester bond [10]. More than 150 types of monomeric units are known. This number is continuously increasing with the introduction of new types of PHA through chemical or physical modification of naturally occurring PHA or through the creation of genetically modified organisms. According to different types of monomer units, PHA can be divided into homopolymers containing one, identical type of monomer and heteropolymers containing at least two different types. If exactly two types are involved, we speak of copolymers [4; 11]. The molecular weight of PHA is in the range of 2×10^5 to 3×10^6 Da, depending on the number of carbon atoms forming the monomer units, the type of microorganism, and the growth conditions [12].

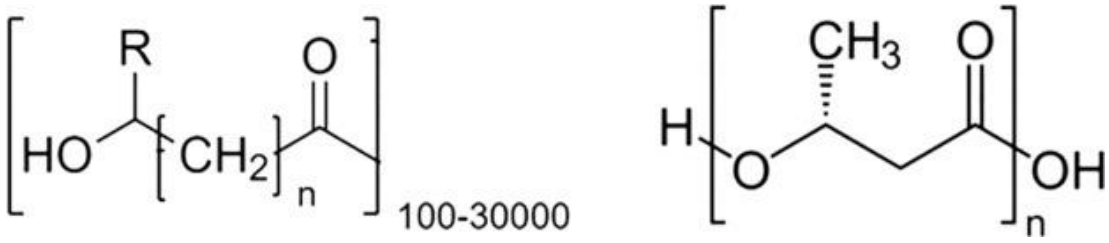

Figure 2 (a) The general molecular structure of polyhydroxyalkanoates; (b) P(3HB) molecule [13]

According to the length of the monomer chain and the number of carbons, PHA can be divided into three classes – short chain length scl-PHA (most widely), medium chain length mcl-PHA and long chain length lcl-PHA [14]. There are also clearly defined limits between the classes based on the number of carbon atoms in the monomer units. Scl-PHA consist of a maximum of five carbons, mcl-PHA of six to fourteen carbons, and a monomeric unit with more than fourteen carbons belonging to the lcl-PHA group. This group is uncommon and less studied [15]. In numerous bacteria, PHA with both scl- and mcl-monomer units have been found [3].

2.1.2 Physical properties

All bacterial PHA are biocompatible, sustainable, versatile, and bioresorbable polymers suitable for biomedical applications [16]. In addition, they are water insoluble and resistant to hydrolytic degradation, show a relatively high degree of polymerization, are optically active and isotactic (all repeating units have the same configuration – they consist only of R-stereoisomers due to the high stereospecificity of PHA biosynthetic enzymes) [3].

The mechanical properties of PHA are different depending on the length of the monomers or the distance between the ester bonds in the polymer chains [3]. Typically, mcl-PHA are amorphous and elastomeric materials with low crystallinity and low melting temperatures, whereas scl-PHA are extremely crystalline and have poor tensile strength. The most encountered scl-PHA in nature is P(3HB) (poly-3-hydroxybutyrate) [17]. The mechanical properties are very similar to isotactic polypropylene; however, the extensibility is considerably lower. Studies demonstrate that the applicability of P(3HB) is constrained by its brittleness, low extension-to-break, and lack of flexibility [18]. As a result, P(3HB) and most PHA need to be modified (also because of thermal instability and the deterioration of mechanical properties over time) [19]. Production of PHA copolymers is currently of study attention as a means of overcoming difficulties. The incorporation of other monomer units into the P(3HB) chain

significantly improves its mechanical properties. This makes it possible to obtain a wide range of polyesters with different properties, from highly crystalline plastics to elastic rubbers. For example, the copolymer P(3HB-*co*-3HV), i.e. poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), offers two significant advantages over P(3HB): a lower melting point and lower level of crystallinity [18].

2.2 PHA production

PHA can generally be made in three main ways: by enzymatic catalysis, through the synthesis of genetically modified plants, and through microbial fermentation. Among them, microbial fermentation was discovered to be an effective method for the production of PHA [20]. Biosynthesis leads to polymers with higher molecular weights, making them more preferred [21]. Microorganisms that are being investigated for the production of PHA using different carbon sources include mainly the genera *Cupriavidus, Burkholderia, Methylobacterium, Allochromatium, Pseudomonas,* and *Bacillus* [20].

Production may be growth associated or non-growth-associated. In the first case, it is a one-step process in which a certain group of microorganisms is able to produce PHA with a high yield (*Bacillus mycoides, Azohydromonas lata*). However, due to the higher productivity in most cases of microorganisms, a process unrelated to their growth is chosen. By using pure culture, productivity increases even further. In this case, the first is the growth of biomass under favourable conditions, followed by the accumulation of PHA under nutrient-limiting conditions [22] – biosynthesis takes place under low concentrations of nitrogen, phosphorus, oxygen, and possibly magnesium, and under excess carbon [23].

In addition to common agricultural raw materials with a high glucose/cellulose content, various types of industrial wastes are used as a carbon source for the metabolism of microorganisms [24]. By choosing them as materials for manufacturing, the production costs can be largely reduced, which is the main advantage [25]. For example, wastewater from industrial production (paper, confectionery, fruit processing industries), whey, waste glycerol from biodiesel or municipal solid waste can be used to produce PHA [24]. Because these waste fluxes are valorised, the process is both affordable and feasible [20].

The structure of the PHA depends on the carbon source used by the microorganism. An example can be given for *Pseudomonas pseudoflava*, which produced P(3HB) when using acetate as the sole carbon source, but P(3HB-*co*-3HV) when propionate was added as a co-substrate [24].

2.2.1 Metabolic pathways of biosynthesis

It is possible to define three main metabolic pathways, through which microorganisms can produce polyhydroxyalkanoates (Figure 3). The biosynthesis of PHA can be achieved by several enzymatic reactions. There are several types of stereospecific enzymes involved in PHA metabolism. They are localized in the cytosol of the cell, where the accumulation of PHA granules subsequently takes place [3].

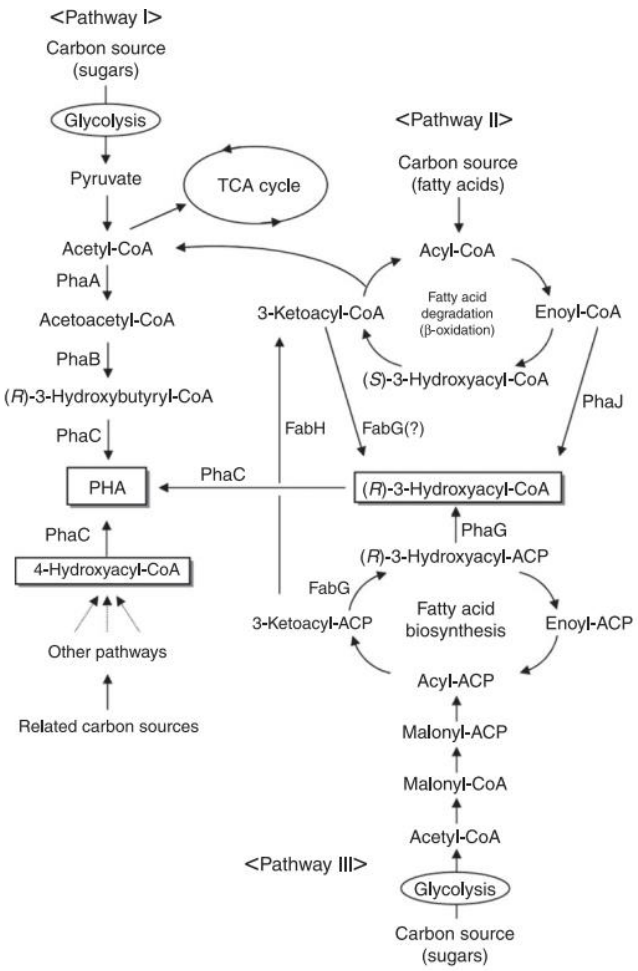

Figure 3 Three major metabolic pathways involving PHA biosynthesis [26]

The first pathway metabolizes scl-PHA, specifically P(3HB). This pathway can be described as the most common since the mechanism of formation of the aforementioned P(3HB) has been found in a wide range of bacteria [26]. The P(3HB) biosynthetic pathway consists of three enzymatic reactions catalysed by three different enzymes using fermentable sugars [27]. In the first reaction, two starting molecules of acetyl-CoA are condensed to form acetoacetyl-CoA by the enzyme 3-ketoacetyl-CoA thiolase (PhaA). The resulting acetoacetyl-CoA is subsequently reduced to (R)-3HB-CoA in the second reaction by acetoacetyl-CoA reductase (PhaB). Finally, (R)-3-HB-CoA monomers, which are the direct precursors of P(3HB), are polymerized to P(3HB) by PHA synthase (PhaC) [26; 28; 29]. By including either the propionic acid or valeric acid, the odd-numbered fatty acid precursors, Pathway I can also be used to produce the copolymer P(3HB-*co*-3HV). These inputs to the fermentation media produce concentrations of propionate inside the cells, either directly or as a product of β-oxidation, which can then be changed into propionyl-CoA by a propionyl-CoA synthetase. Acetopropionyl-CoA is created by PhaA's condensation of propionyl-CoA and acetyl-CoA, which can subsequently be processed in a series of steps by PhaB and PhaC to create the polymer PHV [30].

The second pathway draws on the degradation of fatty acids by the β-oxidative mechanism. While the first pathway used fermentable sugars as a carbon source, the second pathway uses fatty acids for growth and PHA production [20]. The intermediates from β-oxidation of fatty acids are efficiently converted by specialized enzymes to generate (R)-3-HA-CoA [26]. By using the hydratases (PhaJ), epimerases, and reductases (PhaB), it is possible to produce the (R)-3-HA-CoA monomer from fatty acids through three dissimilar kinds of intermediates in the β-oxidation cycle, namely trans-2-enoyl-

CoA, (S)-3-HA-CoA, and 3-ketoacyl-CoA [28]. Using this pathway, many microorganisms, typically *Pseudomonas putida*, *P. oleovorans*, and *P. aeruginosa* synthesize mcl-PHA [29; 31].

Pathway III depends upon the uptake of acetyl-CoA by fatty acid biosynthesis, where it is formed into acetyl-ACP and further elongated into acyl-ACP of varying sizes [30]. Synthesis requires two key enzymes, 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonyl-CoA-ACP transacylase (FabD). These enzymes supply the precursor (3-hydroxyacyl-ACP), which is further converted into 3-hydroxyacyl-CoA. The next step is catalysed by PHA synthase to synthesize PHA [29].

2.2.2 PHA synthase

Among all enzymes, PHA synthase (PhaC) is considered to be the key enzyme in PHA biosynthesis, its substrate specificity and activity are major factors in determining the properties and productivity of the PHA [32]. Some PhaCs only incorporate scl-PHA monomers, such as the PhaC from *Cupriavidus necator* H16, while other PhaCs, for example typically from *Pseudomonas oleovorans*, polymerize only mcl-PHA monomers [33]. It has been reported that very few synthases can be classified in the group of so-called low-specificity enzymes – enzymes that can polymerize both scl- and mcl-PHA monomers [31]. Based on the different substrate specificities and also the types of enzyme subunits, PHA synthases can be categorized into four groups – class I, II, III and IV [34].

The CoA-thioester of hydroxyalkanoates with 3 – 5 carbon atoms can be used as a substrate by Class I PhaCs. Single-unit enzymes of the Class I PhaC family are divided into the N- and C-terminal catalytic domains. While the N-terminal domain's purpose is still unclear, the catalytic site created by the triad of Cys, Asp, and His is located in the C-terminal catalytic domain. These single-unit enzymes prefer scl-PHA monomers and have molecular weights between 63 and 73 kDa. The model enzyme for Class I synthases is the PHA synthase from *C. necator* H16, formerly known as *Alcaligenes eutrophus*, *Wautersia eutropha*, and *Ralstonia eutropha* [33; 35].

Class II PhaCs encompass single-unit enzymes, in general, are smaller than class I PhaCs. Class II PhaCs are capable of using the CoA-thioesters of hydroxyalkanoates from 6 to 14 carbon atoms as a substrate. They are also suggested to have two-domain structures with molecular masses of about 62 kDa. The catalytic domain shares several conserved regions compared with class I [33]. These single-subunit enzymes in class II are encoded by the *phaC1* and *phaC2* genes and are characteristic of *Pseudomonas* species by occurrence [34].

PHA class III and IV synthases use scl-HA-CoA as substrates, but this is a subclass of polyestersynthases consisting of more than one subunit. Type III synthases include enzymes composed of two particular types of subunits with a molecular weight of approximately 40 kDa [36]: a PhaC subunit showing 21% to 28% amino acid sequence similarity with PHA class I and II synthases, and a PhaE subunit with no similarity to the first two classes [37]. The PhaC subunit is capable of *in vitro* polymerization of PHA in the absence of PhaE, albeit inefficiently. For example, in cyanobacteria, only class III have been observed [36]. Similarly, PHA IV synthases, which are generally found in certain *Bacillus* species, consist of two different subunits, in this case, encoded by the *phaC* and *phaR* genes [34]. Such Class IV synthases display a distinctive property – the PhaR subunit catalyses alcohol cleavage of the PHA chain in the presence of alcohol. Incorporating active groups like benzyl, thiol, or hydroxy groups into the carboxy terminus of the PHA chain by this process may be useful for further functionalizing and altering the polymer [35].

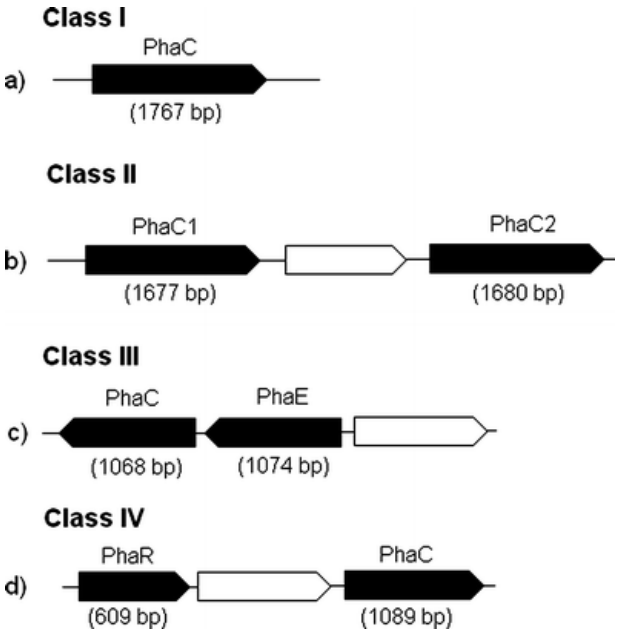

Figure 4 Classification of PHA synthases; genes are typically found in microorganisms: a) Cupriavidus necator; b) Pseudomonas aeruginosa; c) Allochromatium vinosum; and d) Bacillus megaterium [38]

However, this long-standing division of PHA synthases into four classes is currently undergoing adaptation as new synthases that do not fit into any of the four described classes have been discovered. One such potential new class of PHA synthase was recently discovered in Antarctic bacterial isolates [35; 39].

2.2.3 Other enzymes involved in the synthesis

Nowadays, we know many enzymes involved in the biosynthesis or biodegradation of polyhydroxyalkanoates [34].

One of these enzymes, β-ketothiolase, is the entry point of the scl-PHA biosynthesis cycle, specifically P(3HB). It is encoded by the *phaA* gene and catalyses the reversible condensation of two molecules of acetyl coenzyme A (CoA) to acetoacetyl-CoA [34; 40]. It has been found to consist of two constitutive isoenzymes, each with its substrate specificity. The first of these, β-ketothiolase A, shows activity only at four-carbon or five-carbon β-ketoacyl-CoA. The specificity of β-ketothiolase B is slightly greater (four- to ten-carbon β-ketoacyl-CoA). The condensation is inhibited by coenzyme A, and the action of β-ketothiolase also limits the use of pentanoic acid as a 3HV precursor [41].

The main role of the second enzyme in the sequence, acetoacetyl-CoA reductase (PhaB), is to reduce the natural substrate acetoacetyl-CoA with (R)-stereospecificity to the (R)-3-hydroxybutyryl-CoA form [42]. Two species of reductases have been found in *C. necator*, both with different substrates and also coenzyme (NADH or NADPH) specificity. Of greater importance in PHA biosynthesis is the NADPH-dependent acetoacetyl-CoA reductase, which mediates the aforementioned reversible reduction of four- to six-carbon β-ketoacetyl-CoAs to R isomers. NADH-dependent reductase in the reverse reduction reaction produces only S-3-hydroxybutyryl-CoA. It is suggested that the reduction of β-ketoacyl-CoA by NADPH-dependent reductase may be the rate-determining step in the synthesis of PHA [41].

Other enzymes (besides PHA synthase mentioned in the previous chapter) are (R)3-hydroxyacyl-ACP-CoA transferase (PhaG), whose role is to transform 3-hydroxyacyl-ACP into 3-hydroxyacyl-CoA; enoyl-CoA hydratase (PhaJ), which transforms enoyl-CoA into (R)3-hydroxyacyl-CoA or PHA

depolymerase (PhaZ) encoding intracellular PHA-depolymerizing enzymes [43]. In addition, several other granule-associated PHA proteins are known (PhaF, PhaI, PhaM and PhaP) [34].

Genes for PHA biosynthesis, as well as genes encoding other proteins related to PHA metabolism, are most commonly clustered in bacterial genomes. For example, in *C. necator*, genes for scl-PHA synthesis are located in the PhaCAB operon. In contrast, in some bacteria, the *phaC* gene (encoding PHA synthase) has been found to be dissociated from the others (*Z. ramigera, P. denitrificans, R. rubrum*) [37]. The PHA class III synthase subunits (PhaC and PhaE) are in turn typically encoded in a single operon [36].

2.3 Extremophilic microorganisms

An extremophilic microorganism can be defined as a microorganism that thrives in extreme environments. An extreme environment is an environment whose physical, chemical, and biological conditions in particular do not meet normal limits, i.e. are above or below a certain standard (an environment that is optimal for most microorganisms and organisms alike). This includes too high or too low pH, salinity, temperature, pressure, radiation, etc [44]. Depending on which of these extremes the microorganism lives in, extremophilic microorganisms can be divided into categories [45], which are shown in Table 1. These organisms are not only able to survive in that environment but on the contrary, they seek it for their life and die in a standard environment [46]. On the other hand, the definition of an extremotolerant microorganism is that it can tolerate such conditions [47]. We know many other categories, which include a smaller group of microorganisms, such as xerophilic microorganisms (adapted to extreme conditions – drought, the optimum growth is anhydrous), oligotrophic (growth in nutritionally limited conditions), endolithic (growth in rock or the pores of mineral grains) [48]. In terms of taxonomic range, extremophiles cover all three domains (mainly Bacteria and Archaea) [44]. However, the criteria for extremophiles in eukaryotes and prokaryotes differ markedly, extremophilic eukaryotes deal with less abnormal environmental conditions. Some organisms can also be termed polyextremophilic, meaning that the organism can survive and thrive in a combination of more extreme factors [45].

Table 1 Classification and examples of extremophiles [44; 45; 48; 49].

Extreme parameter	**Type**	**Growth optimum**	**Example**
Temperature	Hyperthermophile	> 80 °C	*Pyrolobus fumarii*
	Thermophile	60 – 80 °C	*Synechococcus lividis*
	Psychrophile	< 15 °C	*Psychrobacter*
Pressure	Piezophile	> 0,1 MPa	*Shewanella violacea*
pH	Alkaliphile	> 9	*Spirulina sp.*
	Acidophile	< 2 – 3	*Cyanidium caldarium*
Salinity	Halophile	2 – 5 M NaCl	*Halobacteriaceae*
Chemical extremes	Metalophile	Zn, Co, Cd, Hg, Pb	*Ralstonia sp.*
	Capnophile	High conc. of CO_2	*Cyanidium caldarium*
Radiation	Radiophile	Radiation	*Deinococcus radiodurans*

2.3.1 Advantages in biotechnology

Extremophilic microorganisms are of great biotechnological interest, concerning the abnormal conditions in which they survive, their metabolism has been adapted by the presence of extremozymes – enzymes that are functional under extreme conditions. Extremozymes are useful in industrial manufacturing processes and research applications because they can remain active under the harsh conditions typically used in these processes [48]. They are not only large-scale biotechnological applications (biofuel production process), but they are also applications affecting our daily life, such as food (lactose-free milk, bioinsecticides), medicines, clothing production, washing, etc. Many of the production processes related to the above-mentioned samples are often not perfectly optimised, as mesophilic enzymes are used at high temperatures, salinity, pressure, etc. This leads to improving the efficiency of these enzymes by chemical and genetic modification, which is time-consuming and, above all, economically demanding. Therefore, the use of enzymes produced by naturally extremophilic microorganisms, which are already adapted to work in extreme conditions due to nature without further intervention, is a suitable solution [50]. The most famous example is the use of a thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*, which caused a major development of the polymerase chain reaction [48]. Some more selected examples are summarised in Table 2.

Table 2 Examples of the use of extremophilic microorganisms [44; 48]

Source organisms	Biomolecule	Representative applications
Thermophiles	Xylanases	Paper bleaching
	Lipases, esterases	Hydrolysis in food and feed
Psychrophiles	Polyunsaturated fatty acids	Mariculture, pharmaceuticals
	Alkaline phosphatase	Molecular biology
	Bioremediation	Reduction of oil spills
	Dehydrogenases	Biosensors
Halophiles	Carotene	Food colouring
	Bacteriorhodopsin	Optical switches
	Glycerol, compatible solutes	Pharmaceuticals
Acidophiles	Proteases, cellulases	Feed component
	Sulfur oxidation	Desulfurization of coal
Alkalophiles	Antibiotics	Antibiotic production
	Pectinases	Pulp bleaching

A major advantage of extremophiles is their low susceptibility to contamination (as opposed to mesophiles) [51]. Extremophilic microorganisms have thus become the key to Next Generation Industrial Biotechnology (NGIB). The main idea behind NGIB is to minimize costs, both financial and energy, in terms of increasing competitiveness in the production of chemicals, materials, and biofuels compared to the conventional chemical industry. Simply, NGIBs are microorganisms that resist contamination by other microbes and thus allow biotechnological processes to be carried out

in non-sterile (open) conditions, thus eliminating factors such as complicated sterilization, difficult and expensive separation procedures or the need for trained personnel [52; 53]. In addition, biotechnological processes can also be performed in an efficient semi-continuous or continuous mode [54].

2.3.1.1 Production of PHA by extremophiles

Biotechnological strategies have focused on the use of extremophilic microorganisms in order to reduce production costs and thus increase competitiveness also in the field of PHA synthesis [55].

In biotechnological applications, halophilic microbes have seen significant advantages, especially as PHA producers [56]. Since one of the attributes of PHA production in microorganisms is an increase in stress resistance, in the context of halophilic archaea and bacteria, PHA have been found to protect them against the deleterious effects of hypertonic conditions. This suggests that PHA production is positively correlated with increasing salt concentration. The high salinity of the culture media reduces the risk of microbial contamination. In addition, the acid-catalysed hydrolysis of cheap waste materials (straw, whey, spent coffee grounds) used as a carbon source in PHA production must be subsequently neutralised by the addition of NaOH, which produces significant amounts of NaCl. This naturally contributes to the salinity of the environment required by halophiles [9]. Halophilic PHA producers include e.g. bacteria of the genus *Halomonas* (*Halomonas bluephagenesis, Halomonas halophile* or *Halomonas hydrothermalis*) [54].

The use of thermophilic microorganisms in biotechnology has a major advantage – the metabolic heat generated in biotechnological processes, together with the energy dissipated during mixing, can be used as the actual heating of the bioreactor and, more importantly, the cooling requirements are minimized due to the preferences of the thermophilic microorganisms themselves [52; 55]. In addition, the use of thermophilic bacteria in the context of PHA synthesis is aided by the fact that various waste feedstocks can be used as a carbon source and, as previously mentioned, demands on process sterility are greatly minimized [54; 55]. Thermophilic PHA producers include e.g. *Schlegelella thermodepolymerans, Aneurinibacillus thermoaerophilus* or *Rubrobacter xylanophilus* [54; 57; 58]. It has also been shown that PHA monomers can protect enzymes (lipase and lysozyme) from denaturation caused by high temperatures and also from oxidative damage, thus confirming the prevention of protein denaturation in thermophilic PHA-producing microorganisms under extreme conditions – in this case, specifically at elevated temperature [59].

2.3.2 Psychrophiles and psychrotolerants

Most of our planet's biosphere is permanently cold (at temperatures below 5 °C). That includes polar region, marine, and also higher altitudes of mountain environments. Psychrophilic organisms have been able to survive in habitats that are largely or continually frozen, even though these environments are typically thought to be inhospitable to life [60; 61]. The ability of psychrophiles to tolerate low temperatures, thrive and reproduce in such conditions implies that they have overcome barriers inherent to cold environments. These challenges mainly include decreased membrane fluidity; reduced enzyme activity; decreased rates of transcription; translation and cell division; altered transport of nutrients and waste products; and protein cold-denaturation [62].

Psychrophiles are cold-loving bacteria, whereas psychrotrophs are cold-tolerant bacteria. To describe organisms that can grow at low temperatures but whose optimal and maximal growth temperatures are above 15 and 20 °C, the term psychrotroph (also known as psychrotolerant) was preserved. Psychrophile was defined (Morita, 1975) as an organism that has the most ideal growth temperature

at about 15 °C or lower, maximal growth temperature at 20 °C, and minimum growth temperature at or below 0 °C [60; 63]. These microorganisms occupy a wide range of species, they include representatives from bacteria, fungi, archaea and also yeasts [48]. Given the ubiquity of psychrophilic bacteria, representatives can be isolated from a variety of sources, meaning in water and on land, in a large variety of plants and animals, and in foods. Psychrophilic bacteria can be long or short rods, cocci or vibrios, gram-positive or gram-negative, spore formers or non, and aerobic, facultative, or anaerobic [64].

They are a reliable source of industrial catalysts. Enzymes from the psychrophilic group were chosen primarily because of their great stability and lower risk of contamination at low temperatures [64]. In addition to the examples given in Table 2, enzymes such as proteases, lipases, α-amylases and cellulases isolated from psychrophiles are used as additives in detergents. The main advantage of their use in this sector is the possibility of washing at lower temperatures, thus reducing energy consumption. Cold-adapted cellulase has found its application in the textile industry. It makes it possible to reduce the concentration of this enzyme and also the temperature of the process in which the ends of the cotton fibres protruding from the main fibres are enzymatically removed [65]. The availability of low-temperature enzymes would also be advantageous for a number of food processing applications [66]. They are used widely in the dairy sector. In comparison to techniques using the enzyme from mesophilic microorganisms, β-galactosidase, which is derived from psychrophilic bacteria, can produce 70 – 80 % of the product yields. Psychrophilic microorganisms are capable of degrading a variety of substances in the environment. At low temperatures, they may offer the possibility of the bioremediation of several chemicals – Dodecane, Hexadecane, Naphthalene, and Toluene [64]. However, these enzymes do not only have advantages, most of the cold-adapted enzymes characterized so far show a thermolability that is significantly higher than their mesophilic counterparts [65; 67].

2.4 Analytical methods for the identification of PHA-producing bacteria

2.4.1 Fourier transform infrared spectroscopy (FTIR)

Although FTIR is a common method for analysing molecular structure, when it is performed on a sizable group of whole microbial cells, the spectra that are obtained show the total biochemical composition of the cells [68]. A certain chemical or functional group's specific pattern of infrared absorption results in characteristic bands in their FTIR spectra. The most commonly used region for biological applications is the mid-infrared (4,000 – 400 cm^{-1}) region [69]. Because FTIR spectra are comparable to chemical profiles of samples, they can be used to predict an analyte's concentration – the concentration of P(3HB), for example [68]. Even though several studies have developed models for measuring PHA production using FTIR, this method is typically only regarded as semi-quantitative and only provides relative quantitative information concerning PHA production [70].

The major spectral features due to the organism itself are protein absorbances. These are the amide I band at 1,654 cm^{-1}, which is due primarily to the amide carbonyl stretching vibration, and the amide II band at 1,540 cm^{-1}, which is due mostly to N-H bending vibrations [68]. PHA show strong characteristic bands between 1,724 cm^{-1} and 1,745 cm^{-1} region due to vibrations of the ester carbonyl bond (C=O). Other characteristic bands were visible near 1,282 cm^{-1} and 1,165 cm^{-1} respectively. The band shift in intact cells might be explained by the PHA's surrounding chemical environment, which is more complicated than it is for purified PHA. It should therefore be noted that the carbonyl ester peak is not specific to PHA alone, as other lipid types such as acylglycerides, wax esters and rhamnolipids also show signals in this band region. For this reason, false-positive signals considered as PHA can be expected in FTIR-based PHA screening by analogy with the use of lipophilic dyes [70; 71].

Figure 5 shows the characteristic spectrum for the microbial cell together with the peak assignments (functional groups or regions characteristic of the substance groups) [68; 69; 72].

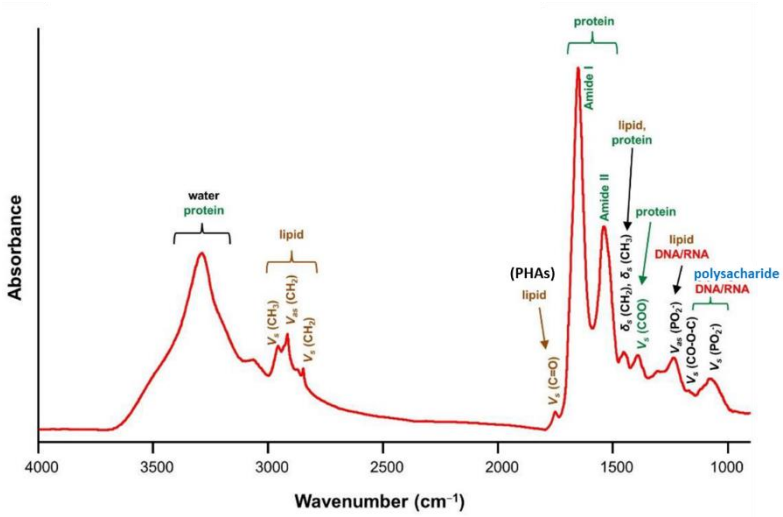

Figure 5 Common Fourier transform infrared (FTIR) bands for microbial cell; modified from [69]

2.4.2 Gas chromatography

Gas chromatography (GC) is the most popular technique currently available for analysing PHA in bacterial cells [68]. The GC approach uses a mild acid or alkaline methanolysis to determine poly-hydroxybutyric acid without previous extraction from the cells in the presence of sulfuric acid and chloroform because PHA must be converted into products that are stable and volatile at the temperature of the GC-column [73; 74]. The resulting methyl esters interact specifically with the solid phase thereby allowing the separation of different hydroxyalkanoate methyl esters in case co-polyesters of different hydroxyalkanoates have to be analysed. From the peaks on the chromatogram, it is possible to determine the qualitative and quantitative representation of individual PHA [74].

2.5 Prokaryotic taxonomy

The scientific field of taxonomy, which means "arrangement or rank" in Greek, is concerned with the classification of organisms. It is a difficult discipline that entails much more than classifying similar organisms or separating dissimilar ones. A taxonomic designation should ideally shed light on an organism's relationships, population structure, and evolutionary history [75]. The terms systematics and taxonomy are often used interchangeably, but systematics is not correct in this field [76]. Prokaryotic taxonomy has three main subtopics: classification, nomenclature, and identification. Classification is the process of grouping similar organisms into distinct groups; nomenclature is the process of giving these groups names using the bacterial code; and identification is the process of determining whether an unknown organism belongs to one of the groups mentioned in classification and named in nomenclature. Prokaryotic taxonomy uses hierarchical grouping as its primary method of classification. It is concerned with domain, phylum, class, order, family, genus, species, and subspecies, going from highest to lowest [77]. The basic unit of bacterial taxonomy is the species. According to the current phylogenetic definition, this unit in prokaryotes is defined as a well-defined group of interrelated strains comprising a type strain that shares 70% or higher DNA-DNA homology of complementary base pairs. In addition, it exhibits (with some exceptions) identical phenotypic traits, while at the same time possessing some distinct traits from other groups [76].

Prokaryotic taxonomy needs to be carefully curated and updated on time, this requires coordinated efforts of prokaryotic taxonomists, genomicists, and database curators [78]. There are currently 22,825

validly published bacterial species names on the International Code of Nomenclature of Prokaryotes (ICNP) at the time of this writing (November 2022) since the publication of the Approved List of Bacterial Names in 1980 [79; 80].

2.5.1 Methods

Genomic data and phenotypes must be thoroughly explored in order to properly classify organisms according to taxonomy. Genotyping methods are derived from the nucleic acids (DNA, RNA) present in the cell. They are directly focused on the study of these molecules and have a major impact on bacterial classification. The outward physical and biochemical expression of genotypes, which results from the interaction of genotype and environment, is known as phenotype. Although numerous methods have been developed for studying microorganisms, each has merits and drawbacks. A good technique would have strong taxonomic resolving power, be time and money efficient, and be automatable [76; 81].

2.5.1.1 Phenotypic typing methods

Bacterial phenotypic analysis entails identifying observable traits that permit species distinction. In addition to physical aspects, these characteristics also describe the cells' metabolic, physiological, and chemical properties. The phenotypic characteristics are the first traits that are determined when a strain is assayed, they have formed the base for microbial description, and they complement genotypic studies [82]. Conventional phenotypic typing method includes morphology of bacteria, biochemical characteristics of bacteria, serotyping of bacteria, phage typing of bacteria, and also many spectrophotometric methods that require specialized instrumentation, for example, FTIR, pyrolysis mass spectrophotometry, MALDI-TOF MS, and FAMEs analysis by GC [81].

2.5.1.2 Genotypic typing methods

These methods focus either on total DNA (DNA-DNA hybridization, Mol % G + C, RFLP analysis), only on a specific stretch of DNA (PCR methods, ribotyping), or alternatively on "fingerprinting" of plasmid DNA (less common, e.g., plasmid DNA polymorphism analysis). The advantage is that genotyping methods are universally applicable, reproducible and high-resolution [76].

The *16S rRNA* gene sequence is part of genotypic information – in the ribosome. There are two multimeric subunits, the large subunit (50S), which includes two rRNA molecules (5S and 23S), and the small subunit (30S), which contains just one rRNA molecule (16S) [83]. 16S rRNA classification is a highly useful tool for quickly classifying any isolate within the current classification system, at least down to the family and genus level [84]. Only a small alteration in the nucleotide sequence of rRNA can have a negative impact on translation. For the translation apparatus to be maintained with high efficiency, throughout evolution, rRNA is required to be conserved in its fundamental sequences. This may be the cause of the finding that rRNA gene sequences are better suited for phylogenetic analysis since they are less prone to spread horizontally among individuals of similar species [81]. For the purpose of complete species identification, DNA-DNA hybridization tests are the most accurate method [84].

2.5.2 Taxonomy of selected bacterial genera

2.5.2.1 Genus Pseudomonas

One of the most varied and widespread bacterial genera, *Pseudomonas* was first reported in 1894. Its species have been isolated from sediments, clinical samples, plant, fungi, and diseased animal specimens, as well as from water, soil, plant rhizosphere, sea, deserts, and other types of environments from the Antarctic to the Tropics. Many of the bacterial taxa that were originally included in the genus *Pseudomonas* have been reclassified into other genera or species from a different class of Proteobacteria over time as methods for characterizing and classifying microorganisms have advanced. Over 70 new species have been described in recent years [85; 86]. The genus *Pseudomonas* forms gram-negative straight or slightly curved rods. Typically, many species accumulate PHA. They are motile with one or a few flagella, aerobic with a strictly respiratory type of metabolism, and chemoorganotrophic, but some species are able to use H_2 or CO as an energy source [76]. The taxonomic classification of the genus *Pseudomonas* is shown in Table 3.

Table 3 Pseudomonas taxonomy [76]

Domain	Bacteria
Phylum	Pseudomonadota
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	*Pseudomonadaceae*
Genus	*Pseudomonas*

2.5.2.2 Genus Arthrobacter

The *Arthrobacter* genus is a member of the ecologically and industrially significant group of bacteria that can be found in fish, other animal species, sewage, seawater, glacier silts, and mainly soil. *Arthrobacter* is abundant in nature and easily contaminates raw food, milk and dairy products, and meat products [87]. A rod-coccus development cycle is the main characteristic that sets *Arthrobacter* apart from other bacteria. Although both the rod and coccoid forms are gram-positive, they may both be easily decoloured. Endospores are not produced by cells, which are obligate aerobes and either non-motile or motile by one subpolar or a few lateral flagella. They never have a fermentation-based metabolism [88]. Table 4 shows the taxonomic classification of the genus *Arthrobacter*.

Table 4 Arthrobacter taxonomy [89]

Domain	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Micrococcales
Family	*Micrococcaceae*
Genus	*Arthrobacter*

2.5.2.3 Genus Rhodococcus

The genus *Rhodococcus* is obligate aerobic, gram-positive, non-motile, catalase-positive, and non-endospore bacteria. They lack a flagellum, yet this genus occasionally has a few pili. The most common cause of foal pneumonia is *Rhodococcus equi*, and cases in immunocompromised patients have implications for human health [90]. The taxonomic classification of the genus *Rhodococcus* is shown in Table 5.

Table 5 Rhodococcus taxonomy [89]

Domain	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Corynebacteriales
Family	*Nocardiaceae*
Genus	*Rhodococcus*

The most distinctive elements of the cell wall are mycolic acids, which are 3-hydroxyl long fatty acids that have a relatively long aliphatic chain substituted at position 2. These acids also contribute as one of the chemotaxonomic indicators. Besides the genus *Mycobacterium*; *Rhodococcus*, *Gordonia*, and *Dietzia* are characterized as mycolic acid-containing genera [91; 92]. *Rhodococcal* mycolic acids typically contain 34 – 54 carbons, which is considered very long. The fully saturated alkyl branch typically has 10 – 14 carbons. The meromycolate side chain is longer and may carry up to four double bonds (Figure 6) [93].

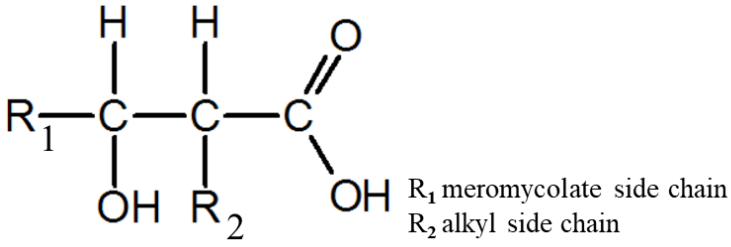

Figure 6 General structure of mycolic acids in Rhodococcus [93]

3 EXPERIMENTAL PART

3.1 Used instruments

- Autoclave 2870ELV, Tuttnauer
- Biosafety cabinet, Fatran LF
- Biosafety cabinet, NuAire
- Centrifuge EBA 200, Hettich
- Centrifuge, Sigma 1-14
- Deep-freezer, Artico
- ELFO source Enduro Power Supplies 300V, Labnet
- Freeze Dryer FreeZone 2.5, Labconco
- Gas chromatograph 820A System, Agilent Technologies
- Incubator shaker MaxQ4000, Thermo Scientific
- Incubator shaker KS4000i control, IKA
- Incubator shaker ZWYR-D2401, LABWIT
- Magnetic shaker MMS-3000, Biosan
- Scales EW 620-3NM, Kern
- Spectrophotometer Vertex 70 FTIR, Bruker Optik
- Termocycler CG1-96 PCR, Corbett Research
- Termocycler My Cycler^{TM}, BIO-RAD
- Termostat INCU-Line 68R, VWR
- Transilluminator TVR-312A, Spectroline
- Visualization system c200, Azure Biosystem
- Vortex BenchMixer, Benchmark
- Water bath, WSL

3.2 Used chemicals

3.2.1 Chemicals for cultivation

- Agar powder (HiMedia, IND)
- Brain Heart Infusion Broth (HiMedia, IND)
- $CaCl_2 \cdot 2H_2O$ (Lachema, CZE)
- $CoCl_2 \cdot 6H_2O$ (Lach-Ner, CZE)
- $CuCl_2 \cdot 2H_2O$ (Lach-Ner, CZE)
- D-fructose (Lach-Ner, CZE)
- D-glucose (Lach-Ner, CZE)
- EDTA (Lach-Ner, CZE)
- $FeCl_3$ (Lach-Ner, CZE)
- Fe^{III} NH_4 citrate (Fluka, CZE)
- H_3BO_3 (Lachema, CZE)
- KH_2PO_4 (Lach-ner, CZE)
- $MgSO_4 \cdot 7H_2O$ (Lach-ner, CZE)
- $MnCl_2 \cdot 6H_2O$ (Lach-Ner, CZE)
- $Na_2HPO_4 \cdot 12H_2O$ (Lach-ner, CZE)
- NH_4Cl (Lach-ner, CZE)
- Nutrient Broth w/ 1% Peptone (HiMedia, IND)
- Yeast extrakt (HIMEDIA, IND)
- $ZnCl_2$ (Lach-Ner, CZE)

3.2.2 Chemicals for molecular technologies

- Agarose (Sigma-Aldrich, DEU)
- DNA ladder 100 bp (Nippon Genetics Europe, DEU)
- Ethyl alcohol 99,8% (Lach-Ner, CZE)
- Kit NucleoSpin Tissue (Macherey-Nagel, DEU)
- Lysozime (Roche, DEU)
- Master mix One Taq Hot Start (2× concentrated) with standard buffer (New England BioLabs, GBR)
- $MgCl_2$ 25 mM (Thermo Scientific, USA)
- Midori Green Advance (Nippon Genetics Europe, DEU)
- PCR loading Yellow load (Top-Bio, CZE)
- PCR water (B. Braun Medical, DEU)
- Primers (Generi Biotech, CZE)
- Proteinase K (Macherey-Nagel, DEU)
- Tris(hydroxymethyl)aminomethane (Lach-ner, CZE)

3.2.2.1 Primers

The list of primers used in this work with selected key information is shown in Table 6.

Table 6 List of used primers [94; 95]

Amplified gene	Designation	Primer sequence (5´ – 3´)	Annealing temperature [°C]	Amplicon size [bp]
16S rRNA	16S-F	AAG AGT TTG ATC CTG GCT CAG	55	1,500
	16S-R	GGT TAC CTT GTT ACG ACT T		
phaC	G-D	GTG CCG CCS YRS ATC AAC AAG T	55	551
	G-1R	GTT CCA GWA CAG SAK RTC GAA		
phaC1	E1-D	GGA GCG TCG TAG ATG AGT AAC AAG AA	62	1,965
	E1-R	AGG TTG GCG CCG ATG CCG TTG AA		

3.2.3 Other chemicals

- Benzoic acid (Sigma-Aldrich, DEU)
- Glycerol (Lachema, CZE)
- Chloroform p.a. (VWR, GBR)
- Methanol (Supelco, DEU)
- Nile Red (Sigma-Aldrich, DEU)
- Sodium hydroxide (VWR, GBR)
- Sudan Black B (Sigma-Aldrich, DEU)
- Sulfuric acid (VWR, GBR)

3.3 Used bacteria

Samples of psychrophilic and psychrotolerant bacteria were sent from Norway in two sets, in both cases in the form of slant agars. All of them were previously identified, taxonomically classified (*16S rRNA*) and characterized in certain areas by Akulava *et al.* and Smirnova *et al* [72; 96; 97]. It is important to note that these bacteria are still of research interest and have not yet been explored in all areas. All 78 samples are shown in Table 7 and Table 8 along with their numerical designations used in this work. The first set contained a total of 33 isolates (isolate 8 was missing). Samples number 1.1 – 1.30 represent fast-growing bacteria isolated from the sea-affected temporary meltwater ponds (Figure 7a), the last four samples (1.31 – 1.34) were isolated from soil in the form of core samples. All these samples were collected from East Antarctica (Figure 7c) during the 5th Belarusian Antarctic Expedition (2013) [96]. The second set contained 45 bacterial isolates that were collected during the 7th Belarussian Antarctic Expedition (2014 – 2015). The source for these samples was green snow (Figure 7b), also from the East Antarctic region (Figure 7c) [72]. All bacterial isolates were deposited at the Belarussian Collection of Non-pathogenic Microorganisms (Institute of Microbiology of the National Academy of Sciences of Belarus) – BIM [96; 97].

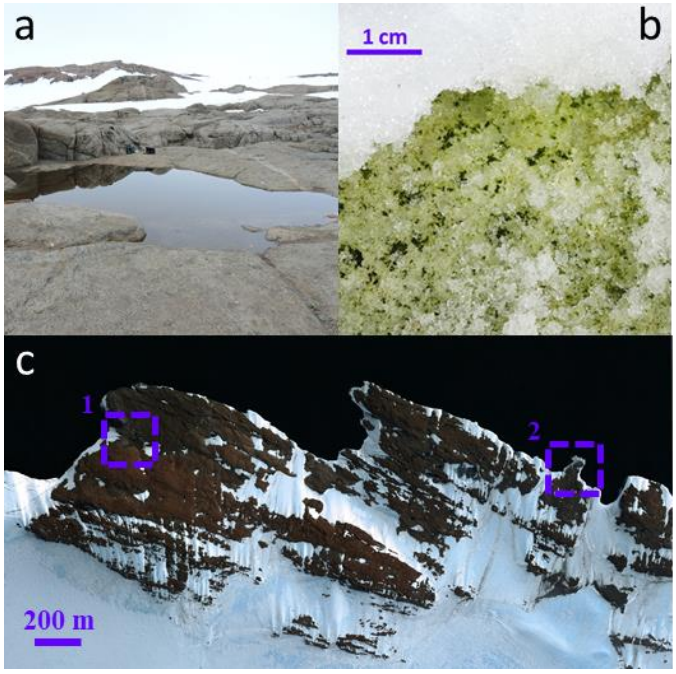

Figure 7 (a) meltwater pond; (b) green snow; (c) Thala Hills oasis of East Antarctica, area 1 represents the sampling site for the green snow samples, area 2 represents the sampling site for the green snow and meltwater ponds samples [72; 96]

*Table 7 List of isolates from the first set and their samples number used in this work; *isolates are considered psychrophilic, the rest psychrotolerant [96]*

Samples number	Collection number (BIM)	Bacterial isolate name from the first set
1.1	B-1565	*Shewanella baltica*
1.2	B-1554	*Pseudomonas lundensis*
1.3	B-1555	*Pseudomonas lundensis*
1.4	B-1556	*Pseudomonas lundensis*
1.5	B-1557	*Shewanella baltica*
1.6	B-1558	*Acinetobacter lwoffii*
1.7	B-1559	*Pseudomonas leptonychotis*
1.9	B-1560	*Pseudomonas peli*
1.10	B-1539	*Sporosarcina sp.*
1.11	B-1561	*Shewanella baltica*
1.12	B-1540	*Carnobacterium inhibens subsp. inhibens*
1.13	B-1562	*Flavobacterium degerlachei*
1.14	B-1563	*Shewanella sp.*
1.15	B-1549	*Arthrobacter sp.*
1.16	B-1571	*Brachybacterium paraconglomeratum*
1.17	B-1569	*Pseudomonas peli*
1.18	B-1568	*Pseudomonas leptonychotis*
1.19	B-1566	*Pseudomonas leptonychotis*
1.20	B-1546	*Pseudomonas peli*
1.21	B-1545	*Micrococcus luteus*
1.22	B-1552	*Pseudomonas peli*
1.23	B-1547	*Agrococcus citreus*
1.24	B-1543	*Arthrobacter agilis*
1.25	B-1542	*Pseudomonas peli*
1.26	B-1548	*Pseudomonas peli*
1.27	B-1541	*Carnobacterium funditum**
1.28	B-1544	*Carnobacterium iners**
1.29	B-1577	*Facklamia tabacinasalis*
1.30	B-1567	*Leifsonia sp.*
1.31	B-1564	*Pseudomonas libanensis*
1.32	B-1551	*Pseudomonas peli*
1.33	B-1553	*Pseudomonas peli*
1.34	B-1550	*Pseudomonas peli*

*Table 8 List of isolates from the second set and their samples number used in this work; *isolates are considered psychrophilic, the rest psychrotolerant [72]*

Samples number	Collection number (BIM)	Bacterial isolate name from the second set
2.1	B-1619	*Cryobacterium arcticum**
2.2	B-1620	*Cryobacterium arcticum**
2.3	B-1621	*Rhodococcus yunnanensis**
2.4	B-1622	*Leifsonia rubra**
2.5	B-1623	*Leifsonia rubra*
2.6	B-1624	*Arthrobacter sp.**
2.7	B-1625	*Arthrobacter sp.*
2.8	B-1626	*Arthrobacter sp.**
2.9	B-1627	*Arthrobacter cryoconiti**
2.10	B-1628	*Arthrobacter sp.**
2.11	B-1629	*Psychrobacter glacinicola**
2.12	B-1630	*Salinibacterium sp.*
2.13	B-1631	*Leifsonia antarctica**
2.14	B-1632	*Leifsonia antarctica**
2.15	B-1633	*Leifsonia kafniensis*
2.16	B-1634	*Leifsonia rubra**
2.17	B-1635	*Pseudomonas sp.*
2.18	B-1636	*Salinibacterium sp.*
2.19	B-1637	*Leifsonia antarctica*
2.20	B-1638	*Leifsonia antarctica*
2.21	B-1639	*Leifsonia antarctica*
2.22	B-1669	*Leifsonia antarctica*
2.23	B-1654	*Salinibacterium sp.*
2.24	B-1655	*Psychrobacter urativorans**
2.25	B-1662	*Psychrobacter urativorans**
2.26	B-1663	*Arthrobacter oryzae*
2.27	B-1671	*Leifsonia antarctica*
2.28	B-1670	*Rhodococcus yunnanensis*
2.29	B-1664	*Arthrobacter sp.*
2.30	B-1665	*Salinibacterium sp.*
2.31	B-1666	*Arthrobacter sp.**
2.32	B-1667	*Pseudomonas sp.*
2.33	B-1668	*Pseudomonas fluorescens*
2.34	B-1673	*Pseudomonas sp.*
2.35	B-1672	*Pseudomonas extremaustralis*
2.36	B-1674	*Pseudomonas versuta*
2.37	B-1656	*Arthrobacter sp.*
2.38	B-1657	*Paeniglutamicibacter antarcticus**
2.39	B-1658	*Cryobacterium soli*
2.40	B-1659	*Cryobacterium soli*
2.41	B-1677	*Cryobacterium soli*
2.42	B-1676	*Polaromonas sp.**
2.43	B-1675	*Cryobacterium soli*
2.44	B-1660	*Rhodococcus erythropolis*
2.45	B-1661	*Rhodococcus erythropolis*

An example of four selected isolates on Petri dishes is shown in Figure 8. As can be seen from this figure, the bacteria possess a diverse morphology, and also pigmentation (pigmentation studies are currently underway in the BioSpec group).

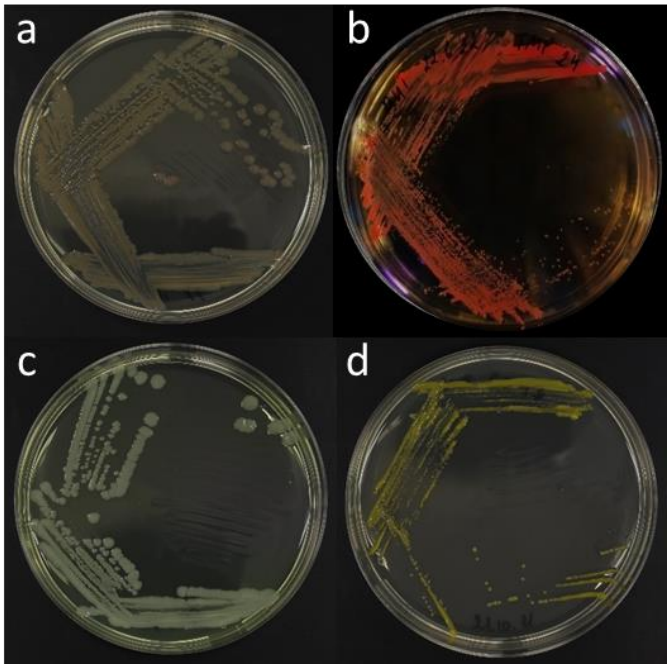

Figure 8 Bacterial cultures grown on Petri dishes; (a) 1.11 Shewanella baltica; (b) 1.24 Arthrobacter agilis; (c) 2.33 Pseudomonas fluorescens; (d) 1.43 Cryobacterium soli

In addition to the psychrophilic and psychrotolerant bacteria, bacterial strains representing positive/negative controls were also used in this work: *Cupriavidus necator* H16 DSM 428 as a positive control for the presence of the *phaC* and also *16S rRNA* genes (in PCR experiments), or as a positive control for phenotypic analyses (in staining, FTIR, and GC experiments); *Pseudomonas putida* KT2440 DSM 6125 as a positive control for the presence of the *phaC1* gene (in PCR experiments) [98]; or the *Cupriavidus necator* PHB^{-4} DSM 541 mutant as a negative control, since it is not able to accumulate PHA (in staining experiments) [99].

3.4 Storage of strains and cultivation media

All isolates (78) were inoculated from slant agars by streaking cultures in parallel on two different nutrient-rich media into Petri dishes – NB and BHI broth (Table 9) with an additional 17 g/L of agar. Growth was carried out for 5 days at 18 °C [72; 96]. Long-term storage of all bacterial isolates was performed by mixing 1 mL of 24/48h old bacterial culture (NB) with 0.5 mL 30% glycerol in cryovials and stored at -80 °C until use.

Inoculums grew on complex media, the compositions of which are shown in Table 9. In Table 10, there is the composition of the mineral medium, which was used for PHA production in MTPs. The composition of the media, that were used for the optimization of PHA production, are shown in Table 12. If the cultivation was carried out on a solid medium, an additional 17 g/L of agar was always added to the medium during its preparation.

Table 9 The list of used commercial complex media and their components

Medium name and designation	**Component**	**Amount [g/L]**
Nutrient Broth (NB)	Peptone	10
	NaCl	5
	Beef extract	10
Brain Heart Infusion Broth (BHI)	Calf brain (infusion from 200 g)	12.5
	Beef heart (infusion from 250 g)	5
	Protease peptone	10
	D-glucose	5
	NaCl	5
	Na_2HPO_4	2.5

*Table 10 Mineral medium and its components, components marked with * were added after sterilization*

Medium name and designation	**Component**	**Amount [g/L]**
Mineral salt medium (MSM)	$Na_2HPO_4 \cdot 12H_2O$	9
	KH_2PO_4	1.5
	NH_4Cl	1
	$MgSO_4 \cdot 7H_2O$	0.2
	$CaCl_2 \cdot 2H_2O$	0.02
	NH_4Fe^{III} citrate	0.0012
	Yeast extract	0.5
	Carbon source*	
	TES II*	1 mL/L

Table 11 The composition of the trace element solution designated TES II

Component	**Amount [g/L]**
EDTA	50
$FeCl_3$	8.3
$ZnCl_2$	0.84
$CuCl_2 \cdot 2H_2O$	0.13
$CoCl_2 \cdot 6H_2O$	0.1
$MnCl_2 \cdot 6H_2O$	0.016
H_3BO_3	0.1

Table 12 The media used for the optimization of PHA production

Medium 1 (M1)	BHI (Table 9) with 10 g/L of glucose
Medium 2 (M2)	MSM (Table 10) with 20 g/L of glucose
Medium 3 (M3)	MSM (Table 10) with 20 g/L of glycerol
Medium 4 (M4)	MSM (Table 10) with 20 g/L of glucose and ½ of phosphates
Medium 5 (M5)	MSM (Table 10) with 60 g/L of glucose

3.5 Genothype methods

3.5.1 DNA isolation

DNA isolation was performed from cultures grown on agar plates. For that purpose, the kit from NucleoSpin Tissue was used. The isolation took place according to the protocol provided by the producer (with little modification). The isolation started with culture added to 180 µL of TE with lysozyme (20 mg/mL) and the samples were incubated for 30 min at 37 °C. After that, 25 µl Proteinase K was added and incubated at 56 °C until the complete lysis was obtained. Samples were vortexed. Then 200 µl Buffer 3 was added to each sample. Afterward, all samples were incubated at 70 °C for 10 min. After vortexing, 210 µl ethanol (99,8%) was added to the samples and vortexed again. For each sample, one NucleoSpin Tissue Column was placed into a Collection Tube. The sample was applied to the column and centrifuged for 1 min at 11,000 × g. The flow-through was discarded, and the column was placed back into the Collection Tube. First, wash – 500 µl Buffer BW was added, and centrifuged for 1 min at 11,000 × g, the flow-through was discarded, and the column was placed back into the Collection Tube. Second wash – 600 µl Buffer B5 was added and centrifuged for 1 min at 11,000 × g, the flow-through was discarded and the column was placed back into the Collection Tube. To dry the silica membrane, each column needed to be centrifuged for 1 min at 11,000 × g. The NucleoSpin Tissue Column was placed into a 1.5 mL microcentrifuge tube and 50 µl Buffer BE was added to it. The samples were incubated at room temperature for 1 min and then centrifuged for 1 min at 11,000 × g. This last step was repeated twice (from the point of adding 50 µl Buffer BE). Finally, the supernatant containing DNA was transferred to a clean sterile microtube and stored at -30 °C for further use.

3.5.2 Polymerase chain reaction (PCR)

Since multiplex PCR was performed with two pairs of primers, a more correct name for it is duplex PCR. Duplexing in PCR is the simultaneous amplification of two target sequences in a single PCR assay, which makes it more economical and rapid compared to single (monoplex) PCR [100; 101]. Primers 16S-F and 16S-R were used to detect the *16S rRNA* gene, which is typical for bacterial DNA. G-D and G-1R are primers delimiting the stretch of the DNA molecule to which the *phaC* gene corresponds [94; 95]. It encodes the class I PHA synthase [102]. The sequences and the most important information concerning these primers are shown in Table 6. As can be seen, the annealing temperature is identical for both primers pairs and the size of the amplicons is at the same time markedly different, which allowed the following seamless separation and analysis by agarose gel electrophoresis.

The duplex PCR mixture contained the components shown in Table 13, including the quantities used. The resulting volume of each was 25 µL. In addition to the samples, a negative control (NC), which contained no DNA, was also prepared for each run, as well as a positive control (PC), which in this case

contained DNA from the *C. necator* H16, which had previously been shown to contain the *phaC* gene [98]. The temperature profile of duplex PCR is shown in Table 14.

Table 13 Duplex PCR components and their quantity

Component	**Default ratio [µL]**
Master Mix	12.5
Mg^{2+}	2.6
Primer	4 × 1
PCR H_2O	4.9
DNA	1

Table 14 Temperature profile of duplex PCR (fragments 16S rRNA and phaC) [94; 95]

Step	**Conditions**		
	Number of cycles	**Temperature [°C]**	**Duration [s]**
Initial denaturation	1	94	30
Denaturation	30	94	30
Annealing		55	30
Extension		68	90
Elongation	1	68	300
Cooling		4	60

PCR with primers E1D and E1R (Table 6) was used to confirm the *phaC1* gene, which encodes a class II PHA synthase [34]. This reaction was performed separately, as a single PCR. Table 15 and Table 16 are showing the component amounts and temperature program, which differ from duplex PCR. Also, in this case, negative and positive controls were analysed alongside the samples. The NC did not contain any DNA matrix and the DNA of *Pseudomonas putida* KT2440 was used as PC, as it is representative of bacteria producing mcl-PHA and thus containing class II PCR synthase [98].

The work involving DNA and preparation of PCR mixtures was always carried out in boxes with the utmost attention to sterility.

Table 15 Single PCR components and their quantity

Component	Default ratio [µL]
Master Mix	12.5
Mg^{2+}	1.8
Primer	2 × 1
PCR H_2O	7.7
DNA	1

Table 16 Temperature profile of single PCR (fragment phaC1) [95]

Step	Conditions		
	Number of cycles	Temperature [°C]	Duration [s]
Initial denaturation	1	94	30
Denaturation	30	94	30
Annealing		62	30
Extension		68	90
Elongation	1	68	300
Cooling		30	60

3.5.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed after each PCR. The gel consisted of TBE buffer (Tris base, 54 g/L; boric acid, 27.5 g/L; EDTA, 4.65 g/L), which had to be diluted ten times before gel preparation. The 2.25 g of agarose was mixed with 150 mL of TBE buffer (1.5% agarose gel) in an Erlenmeyer flask. Using a microwave, the solution was brought to a boil 4 to 5 times until the agarose was completely dissolved. After cooling slightly, Midori Green intercalation dye (7 µL) was added. The solution was poured into a mould with a comb and left for a minimum of 30 minutes for the gel to set. The wells were loaded with 10 µL of PCR products that had been previously mixed with the loading buffer. One of the wells was spiked with 5 µL of length standard. The gel mould thus prepared was placed in a tray, poured with TBE buffer up to the crease and connected to a voltage source. A voltage of 90 V was selected and electrophoresis was run for 40 min. After electrophoresis, the gel was photographed in a UV transilluminator at a wavelength of 302 nm.

3.6 Phenotype methods

3.6.1 Staining of cultures

3.6.1.1 Nile Red staining

In this work, the dye was first dissolved in ethanol (99.8%) to a concentration of 0.5 mg/mL. The staining with Nile Red consists of adding 1 mL/L to the medium (in our case MSM with 20 g/L

of glucose – Table 10) after sterilization before pouring it into the Petri dishes. The utilization of sugars for positive control – *C. necator* H16 and negative control – *C. necator* PHB^{-4} (non-producing PHA) is restricted to fructose and *N*-acetylglucosamine [99], so a separate Petri dish was prepared with the addition of 20 g/L fructose instead of glucose. The cultivation/production also ran in parallel on Nutrient Broth (Table 9) with the addition of 1 mL/L Nile Red to the medium. In this experiment, one Petri dish was divided into 6 parts and inoculated with six different samples (Figure 9). The cultivation was carried out at 18 °C for 48 h in the dark. The positive and negative control dish was also cultivated in the dark for 48 h but at laboratory temperature. Finally, the plates were illuminated with UV light and red fluorescent colonies were detected.

3.6.1.2 Sudan Black B staining

Plates with MSM (Table 10) were prepared with 20 g/L of glucose. The positive and negative controls were the same representatives as in the previous case (*C. necator* H16; *C. necator* PHB^{-4}) and therefore it was necessary to prepare one Petri dish with fructose as a carbon source. NB medium was also inoculated with the cultures, the inoculation and cultivation process were carried out similarly as with Nile Red staining (18 °C for 48 h, PC and NC 48 h at laboratory temperature). A solution was prepared using 15 mg of Sudan Black B (SBB), which was dissolved in 30 mL of 99.8% ethanol. Each Petri dish was covered with 0.9 mL of SBB dye solution, the dishes were left for 30 min and then washed with ethanol (70%). A difference in staining was observed. Figure 9 shows an example of the division of a Petri dish.

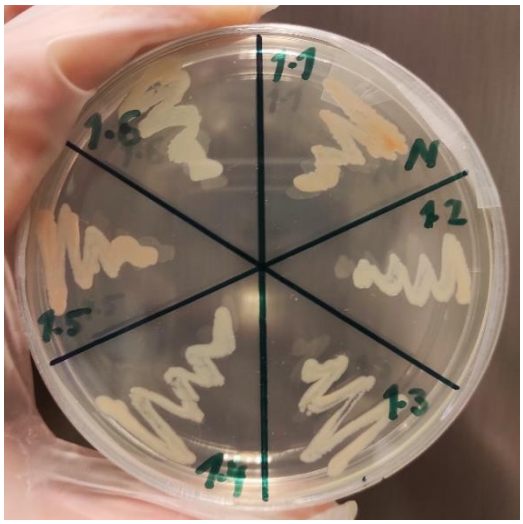

Figure 9 Demonstration of the distribution of bacteria on the Petri dish (same for Nile Red staining)

3.6.2 Cultivation in the Duetz-MTPS and biomass production

Cultivations were performed in the Duetz-MTPS (Duetz-Microtiter Plate System), consisting of 24-square, polypropylene extra deep well microplates (25 mL total volume and 7 mL working volume), low-evaporation version sandwich covers and extra high cover clamps for mounting MTPs into the incubator shaking platform (Figure 10). This system allows thousands of strains to be grown simultaneously in microplates without excessive repetitive handling while being easy to use and cost-effective [103].

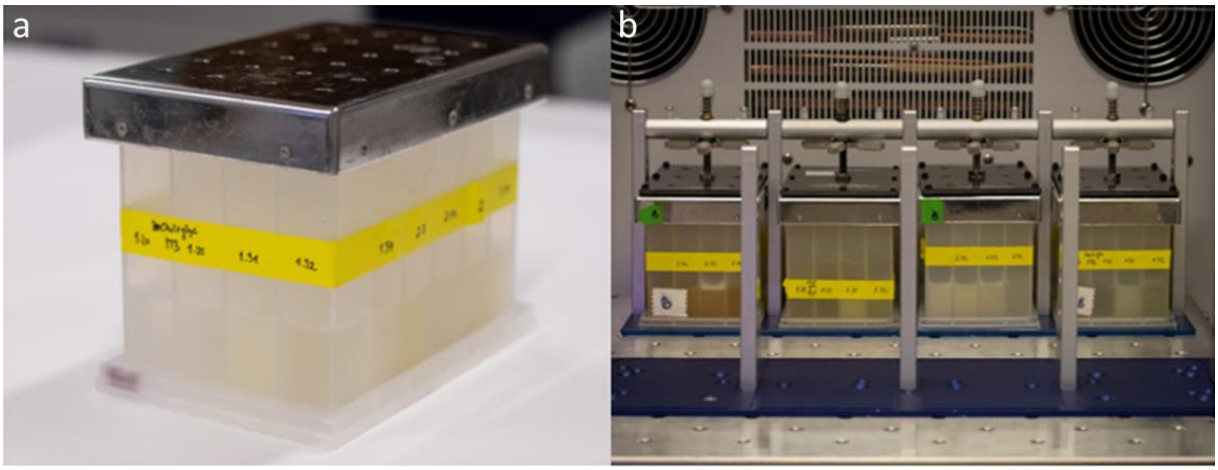

Figure 10 Duetz-Microtiter Plate System; (a) bacteria grown in 24 deepwell MTP shown with sandwich cover layer; (b) Duetz-MTPs stacked on a laboratory shaker with the universal clamp

12 selected strains together with *C. necator* H16 (positive control) were cultured in three steps: (1) growth on an agar plate for preparing biomass from the frozen stock, (2) growth in liquid media in Duetz-MTPS to prepare inoculum and (3) growth in Duetz-MTPS in five different media (Table 12). All cultivations were done in two independent biological replicates.

To prepare inocula, bacteria were cultivated by streaking cultures on BHI agar-based media (Table 9) from frozen cultures and grew for 5 days at 18 °C. Single colonies were transferred to sterile BHI broth in MTPs and incubated in a shaker at 18 °C and 400 rpm for 24 h.

After 24 h, inocula were transferred to 15mL Falcon tubes and centrifuged (11,510 × g, 15 °C, 10 min), the residual medium was removed, and cells were resuspended with fresh production medium (Table 12) to the original volume of 3 mL. The inocula were added in the volume ratio of 1:10 to the different production media. The cultivation in production media was performed in a Duetz-MTPS with 7 mL of culture volume at 18 °C, 400 rpm for 5 days. In this case, 2 wells were used for each biological replicate.

After cultivation, the biomass was washed as follows: cultures were transferred from MTPs into 15 mL Falcon tubes (two wells were merged together resulting in 14 mL of total volume). Samples were centrifuged (11,510 × g, 15 °C, 10 min) and washed three times with distilled water (vortexed in between, centrifuged under the same conditions as in the previous case). After the last (third) cycle, the biomass was stripped of water, frozen and, finally, freeze-dried. The biomass production was represented in g/L by weighing the dried samples.

3.6.3 FT-infrared spectroscopy analysis

Washed bacterial suspension from MTPs (7 µL) was transferred to an IR-light-transparent silicon 384-well microplate (Figure 11) in three technical replicates. Microplates with samples were dried at room temperature for at least 2 h to form completely dried films suitable for FTIR analysis. FTIR-HTS analysis of bacterial biomass was performed using the High Throughput Screening eXTension (HTS-XT) unit coupled to the Vertex 70 FTIR spectrophotometer in transmission mode. The spectra were collected in the 4,000 – 5,000 cm^{-1} spectral range, with 64 scans for both background and sample spectra, and using an aperture of 5.0 mm. Background spectra of the Si microplate were collected prior to each sample measurement. Measurements were controlled by the OPUS 7.5 software.

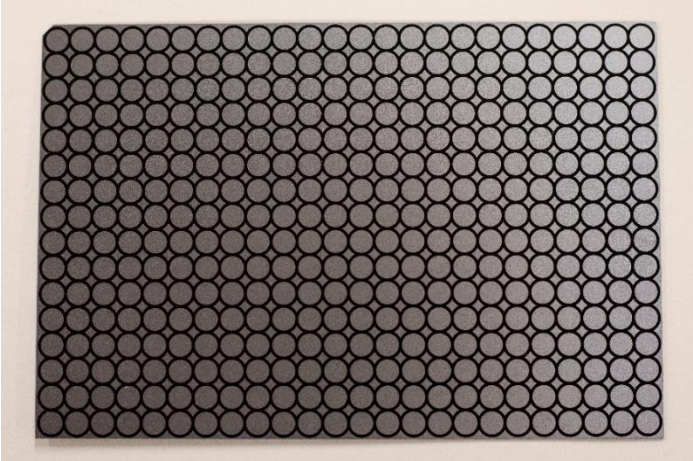

Figure 11 IR-light-transparent silicon 384-well microplate called "HTS plate"

For ratio analysis, FTIR-HTS spectra were pre-processed in the following way: (1) applying the Savitzky-Golay algorithm using a polynomial order of degree 2 and window size 11, (2) using the Extended Multiplicative Signal Correction (EMSC) to minimize variability due to the light scattering or sample thickness [104; 105]. The Orange3 version 3.31.1 software (University of Ljubljana) was used to perform the pre-processing of the spectra. An ester C=O stretching peak at 1,737 cm^{-1} was used for the estimation of the relative PHA content (lipid to protein ratio; L/P; 1,737 cm^{-1}/1,655 cm^{-1}).

3.6.4 Gas chromatography

The biomass for GC was obtained from the procedure outlined in 3.6.2. By using the acidic methanolysis method, the PHA content was determined as follows: dry biomass was weighed (8 to 11 mg) and suspended in 1 mL of chloroform in a 15mL screw cap reagent tube. In case the biomass in any of the replicates did not reach the required amount, two replicates were combined into one. Next, 0.8 mL of an esterification mixture (5 mg/mL benzoic acid and 15% H_2SO_4 in methanol) was added. The vials were sealed and left to react at 94 °C for 3 h. 0.5 mL of 50 mM NaOH was added to neutralize the mixture. The lower organic phase (200 µl) was then transferred to clean vials with 0.8 mL of chloroform for GC analysis. Determination of total PHA content (expressed as the wt% of sample dry weight) and PHA composition were performed by using gas chromatography 7820A System (Agilent Technologies, USA), equipped with an Agilent J&W 121–2323 DB-23 column, 20m × 180 µm × 0.20 µm and a flame ionization detector (FID). Helium was used as a carrier gas. For the identification and quantification of PHA, the P(3HB), P(3HB-*co*-3HV) and P(3HHx) were used as external standards, in addition to benzoic acid internal standards. Measurements were controlled by the Agilent OpenLAB software (Agilent Technologies, USA).

4 RESULTS AND DISCUSSION

At the Norwegian University of Life Sciences, the BioSpec group has been working with psychrophilic and psychrotolerant bacteria for the last few years. The 78 isolates from Antarctica were taxonomically classified, and they have been studied in terms of enzymatic activity, antibiotic resistance, or pigment production [72; 96; 97]. As already mentioned in this thesis, cold-adapted bacteria represent an interesting group of microorganisms that may also have the potential to produce PHA. In this respect, these bacteria have never been studied before, which became the unique subject of my experimental work.

The optimal strategy to analyse the ability to produce PHA is to combine both genomic and phenotypic approaches. All the procedures and the whole idea of this work were driven by the main goal – to try to find among these cold-loving bacteria a potential producer of PHA.

4.1 Polymerase chain reaction

The ability to accumulate PHA can be determined at the genotype level (e.g. by using the PCR method), specifically determining the presence of the gene for the key enzyme for polyhydroxyalkanoate synthesis – PHA synthase [33]. The advantage of genetic screening methods over metabolism-based ones is that they do not depend on the presence of granules. However, genetic screening methods can only reveal whether a strain has the genetic potential to produce PHA; they cannot reveal whether the gene is expressed or whether PHA is accumulated [106]. At the same time, detection by PCR is only possible for genes that are known and targeted by the primers used – failure to detect the presence of PHA synthase does not necessarily mean that the microorganism is not capable of PHA production, as it may contain PHA synthetic genes that are not amplified by a given PCR protocol.

The success of DNA isolation was verified by the presence of a *16S rRNA* gene that is specific to each bacterial cell – the 1,500 bp fragments by using 16S-F/R primers [94]. The 551 bp *phaC* gene fragments were amplified with the use of the primer pair G-D and G-1R (Table 6) [95]. The duplex and single PCR procedures are described in Chapter 3.5.2. While PhaC-negative samples only gave the amplicon corresponding to the *16S rRNA* general bacterial gene, PhaC-positive bacterial samples provided both amplicons as a result of the duplex PCR assay. In some cases, PHA-positive bacteria only have the amplicon corresponding to the *phaC* gene present due to preferential amplification of the amplicon with lower size, which is not considered a complication [107] – bacterial isolate 1.31 in Figure 12. The presence of the *16S rRNA* gene in bacterial isolate 1.18 was successfully confirmed in all subsequent PCR analyses and the *phaC* gene was also identified in this isolate, but unfortunately, this is not apparent from Figure 12. However, a different case is isolate 1.14 (*Shewanella sp*.), whose *16S rRNA* gene was not amplified (as the only one from both bacterial sets). As the first bacterial set contained three other isolates containing *Shewanella sp.* (1.1; 1.5; 1.11) and none of them was the gene for PhaC confirmed, it was not necessary to address this fact for the purposes of this work.

It should be mentioned that the results from agarose gel electrophoresis shown in the following figures were not indicative of the evaluation of the conclusions and in this case, they are presented only as a demonstration – both duplex and single PCR were performed several times for both bacterial sets as there were problems mainly with reproducibility of results during the work. The numerical designations of the bacteria contain only the second half (due to lack of space in the image), which set they are from is always recorded in the figure caption.

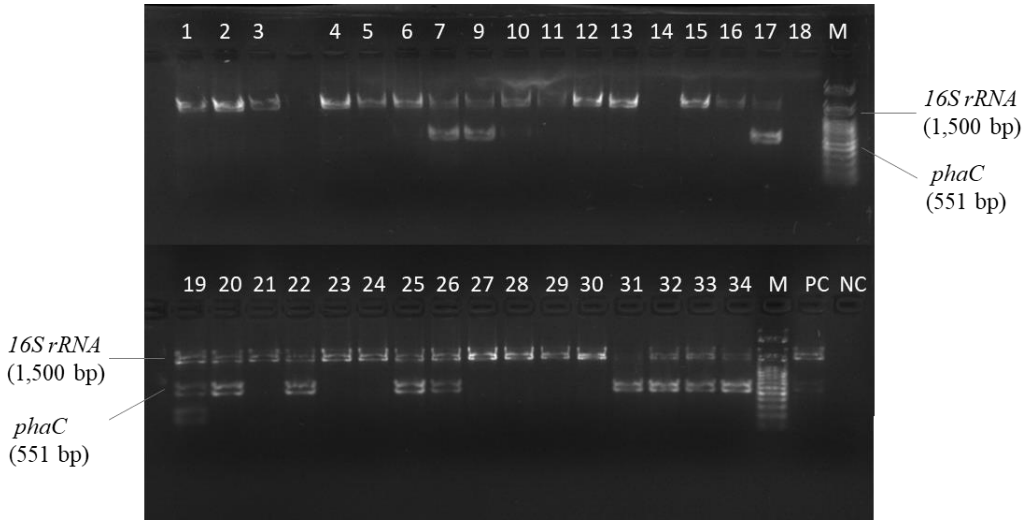

Figure 12 Agarose gel electrophoresis of duplex PCR products (first bacterial set) – detection of 16S rRNA and phaC genes; PC – positive control (C. necator H16); NC – negative control; M – DNA marker

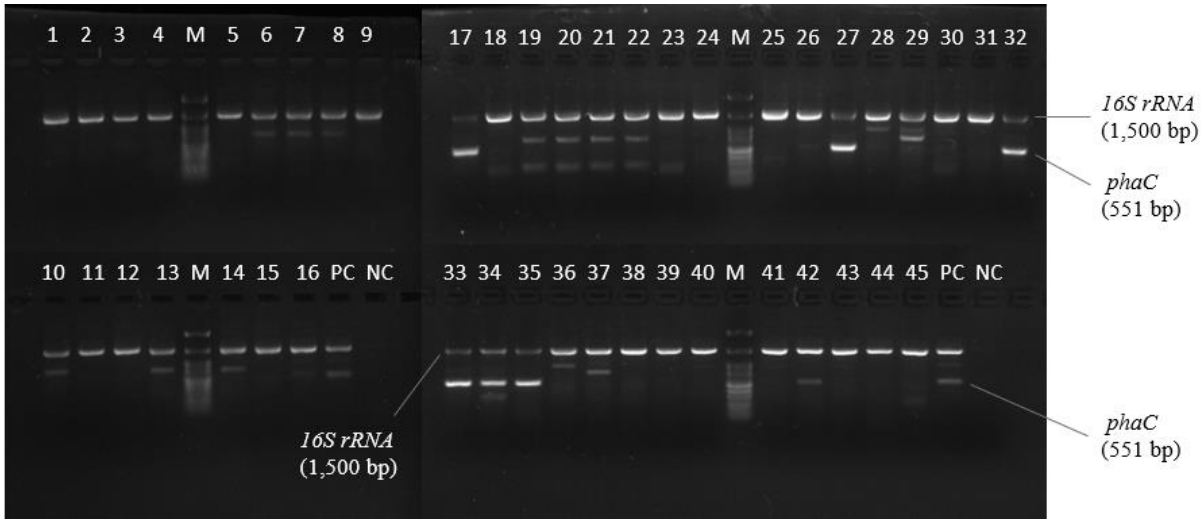

Figure 13 Agarose gel electrophoresis of duplex PCR products (second bacterial set) – detection of 16Sr RNA and phaC genes; PC – positive control (C. necator H16); NC – negative control; M – DNA marker

Figure 13 shows an example of a duplex PCR analysis involving bacteria from the second set. Since the appearance of other non-specific products can be observed, the analysis was repeated numerous times and was also performed as a single PCR using only primers G-D and G-1R. During all these measures, the presence of non-specific products was still recognised. The same issue was encountered by Rogala *et al*. who used an identical set of primers to detect PHA synthase class I in bacteria from polar regions. They reported that those primers frequently yielded multiple bands, either with or without the 551 bp-specific bands [106]. Therefore, PCR with elevated annealing temperature was also performed, specifically only with samples in which these products were observed after multiple repeats. The annealing temperature was increased to 58 °C and also 60 °C (Figure 14) from the original 55 °C (Table 14) [108]. However, even this did not lead to the disappearance of non-specific products; fragments with a different length than 551 bp, or up to two different products, were captured on the gel. Samples (9) for which this was the case are marked * in the final, resulting Table 18 shown at the end of this chapter.

One possible next step to avoid the formation of non-specific products using the same primers could be nested PCR, specifically, a semi-nested PCR using internal primers G-D and G-2R (5'GTAGTTCCA(GC)A(CT)CAGGTCGTT3'), with the G-D and G-1R acting as the external pair of primers. Analysis using this combination of primers has already been successfully performed and optimized in previous studies. However, these primers were designed from the genomes of gram-negative bacteria [95]. Formation of nonspecific products was observed in isolates that have in common that they all are assigned to gram-positive bacteria. Moreover, only two species are involved – *Arthrobacter sp*. and *Leifsonia antarctica*. Therefore, it would be more appropriate to propose other primers that might be more satisfactory. The fact that the DNA may have been contaminated, as only coarse lysates were worked with, did not contribute to the whole issue.

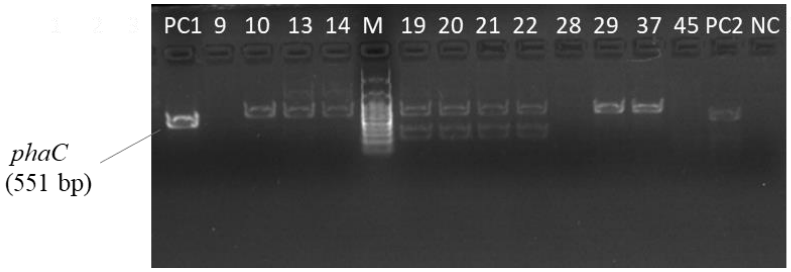

Figure 14 Agarose gel electrophoresis of PCR products (second bacterial set) – detection of phaC genes with increased annealing temperature to 60 °C; PC1 – positive control (isolate 2.34); PC2 – positive control (C. necator H16); NC – negative control; M – DNA marker

Since both class I and class II PHA synthases are formed by one subunit encoded by the *phaC* gene (class II PHA synthase specifically by the *phaC1* and *phaC2* genes) [34; 38], it is obvious that samples that were PhaC-negative should not be positive for class II PHA synthase, in other words, they should not contain the *phaC1* gene. Nevertheless, the analysis was performed with all bacterial isolates regardless of previous outcomes to overcome possible false-negative results. The primer pair E1-D and E1-R (Table 6), which delimits a 1,965 bp stretch of DNA and is specific for the *phaC1* gene, was used for simple PCR. Examples after the analysis are shown in Figure 15 and Figure 16.

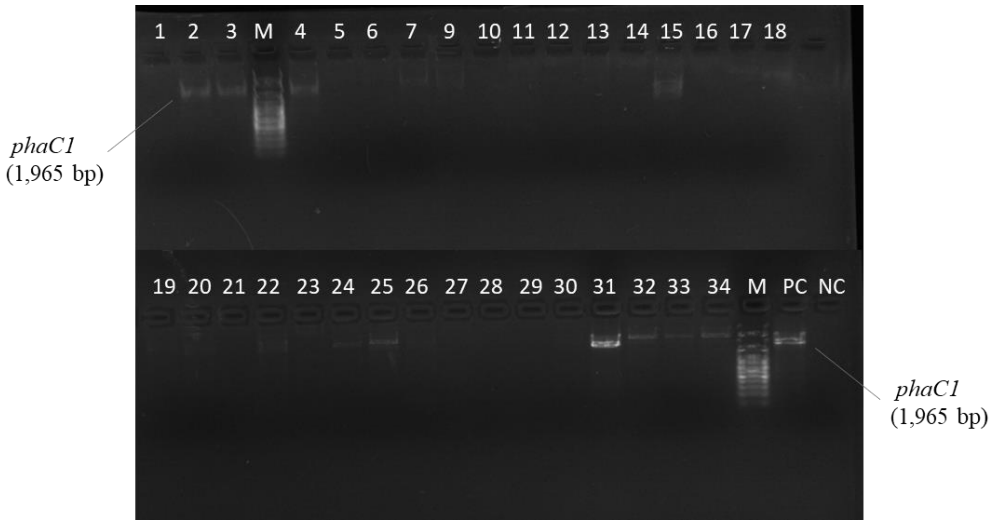

Figure 15 Agarose gel electrophoresis of PCR products (first bacterial set) – detection of phaC1 gene; PC – positive control (P. putida); NC – negative control; M – DNA marker

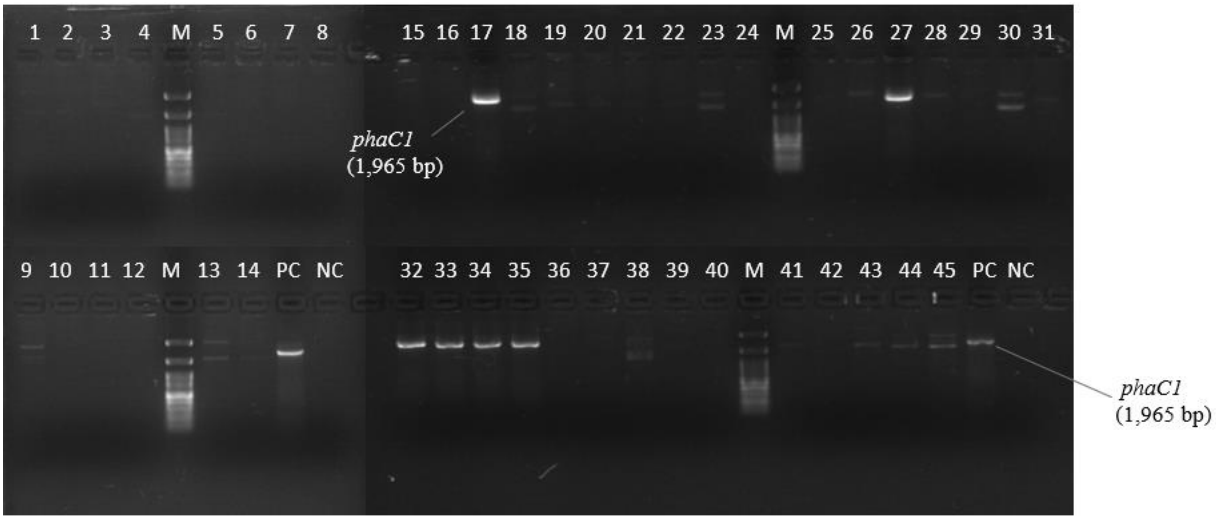

Figure 16 Agarose gel electrophoresis of PCR products (second bacterial set) – detection of phaC1 gene; PC – positive control (P. putida); NC – negative control; M – DNA marker

For the determination of PHA synthase class II, it was necessary to repeat the assay several times and to increase the annealing temperature, too. In this case, a higher success rate can be reported since the occurrence of false-positive results was avoided after increasing the annealing temperature from 62 to 65 °C (Table 16). However, the result for sample 2.45, in which the *phaC1* gene was detected even after increasing the annealing temperature, is interesting. Since the *phaC* gene was not confirmed in this bacterial isolate and it is questionable whether it also contains the *phaC2* gene, it is not appropriate to claim that this is a horizontal gene transfer related to bacterial evolution [109]. Most likely, this isolated bacterium from the polar regions acquired by bacterial conjugation stretch of DNA belonging to the *phaC1* gene, which encodes a class II PHA synthase [110]. The ability of this isolate to synthesize PHA will be verified at the phenotypic level in subsequent analyses.

The findings from all PCR analyses are summarized in the tables separately for the bacterial sets – Table 17 shows the results for the first set of bacteria, and Table 18 shows the results of the analyses of the bacteria from the second set also together with the marked exceptions.

Table 17 List of samples from the first bacterial set and its detection (product: + detected, – not detected)

Samples number	**Bacterial isolate name**	**Presence of the gene**		
		16S rRNA	*phaC*	*phaC1*
1.1	*Shewanella baltica*	+	–	–
1.2	*Pseudomonas lundensis*	+	+	+
1.3	*Pseudomonas lundensis*	+	+	+
1.4	*Pseudomonas lundensis*	+	+	+
1.5	*Shewanella baltica*	+	–	–
1.6	*Acinetobacter lwoffii*	+	–	–
1.7	*Pseudomonas leptonychotis*	+	+	+
1.9	*Pseudomonas peli*	+	+	+
1.10	*Sporosarcina sp.*	+	–	–
1.11	*Shewanella baltica*	+	–	–
1.12	*Carnobacterium inhibens subsp. Inhibens*	+	–	–
1.13	*Flavobacterium degerlachei*	+	–	–
1.14	*Shewanella sp.*	–	–	–
1.15	*Arthrobacter sp.*	+	–	–
1.16	*Brachybacterium paraconglomeratum*	+	–	–
1.17	*Pseudomonas peli*	+	+	+
1.18	*Pseudomonas leptonychotis*	+	+	+
1.19	*Pseudomonas leptonychotis*	+	+	–
1.20	*Pseudomonas peli*	+	+	–
1.21	*Micrococcus luteus*	+	–	–
1.22	*Pseudomonas peli*	+	+	+
1.23	*Agrococcus citreus*	+	–	–
1.24	*Arthrobacter agilis*	+	–	–
1.25	*Pseudomonas peli*	+	+	+
1.26	*Pseudomonas peli*	+	+	+
1.27	*Carnobacterium funditum*	+	–	–
1.28	*Carnobacterium iners*	+	–	–
1.29	*Facklamia tabacinasalis*	+	–	–
1.30	*Leifsonia sp.*	+	–	–
1.31	*Pseudomonas libanensis*	+	+	+
1.32	*Pseudomonas peli*	+	+	+
1.33	*Pseudomonas peli*	+	+	+
1.34	*Pseudomonas peli*	+	+	+

*Table 18 List of samples from the second bacterial set and its detection (product: + detected, – not detected); * products were detected, but they have a different length compared to PC*

Samples number	**Bacterial isolate name**	**Presence of the gene**		
		16S rRNA	*phaC*	*phaC1*
2.1	*Cryobacterium arcticum*	+	–	–
2.2	*Cryobacterium arcticum*	+	–	–
2.3	*Rhodococcus yunnanensis*	+	–	–
2.4	*Leifsonia rubra*	+	–	–
2.5	*Leifsonia rubra*	+	–	–
2.6	*Arthrobacter sp.*	+	+	–
2.7	*Arthrobacter sp.*	+	+	–
2.8	*Arthrobacter sp.*	+	+	–
2.9	*Arthrobacter cryoconiti*	+	–	–
2.10	*Arthrobacter sp.*	+	+*	–
2.11	*Psychrobacter glacinicola*	+	–	–
2.12	*Salinibacterium sp.*	+	–	–
2.13	*Leifsonia antarctica*	+	+*	–
2.14	*Leifsonia antarctica*	+	+*	–
2.15	*Leifsonia kafniensis*	+	–	–
2.16	*Leifsonia rubra*	+	–	–
2.17	*Pseudomonas sp.*	+	+	+
2.18	*Salinibacterium sp.*	+	–	–
2.19	*Leifsonia antarctica*	+	+*	–
2.20	*Leifsonia antarctica*	+	+*	–
2.21	*Leifsonia antarctica*	+	+*	–
2.22	*Leifsonia antarctica*	+	+*	–
2.23	*Salinibacterium sp.*	+	–	–
2.24	*Psychrobacter urativorans*	+	–	–
2.25	*Psychrobacter urativorans*	+	–	–
2.26	*Arthrobacter oryzae*	+	–	–
2.27	*Leifsonia antarctica*	+	+	+
2.28	*Rhodococcus yunnanensis*	+	–	–
2.29	*Arthrobacter sp.*	+	+*	–
2.30	*Salinibacterium sp.*	+	–	–
2.31	*Arthrobacter sp.*	+	–	–
2.32	*Pseudomonas sp.*	+	+	+
2.33	*Pseudomonas fluorescens*	+	+	+
2.34	*Pseudomonas sp.*	+	+	+
2.35	*Pseudomonas extremaustralis*	+	+	+
2.36	*Pseudomonas versuta*	+	–	–
2.37	*Arthrobacter sp.*	+	+*	–
2.38	*Paeniglutamicibacter antarcticus*	+	–	–
2.39	*Cryobacterium soli*	+	–	–
2.40	*Cryobacterium soli*	+	–	–
2.41	*Cryobacterium soli*	+	–	–
2.42	*Polaromonas sp.*	+	+	–
2.43	*Cryobacterium soli*	+	–	–
2.44	*Rhodococcus erythropolis*	+	–	–
2.45	*Rhodococcus erythropolis*	+	–	+

Pseudomonas sp. is a known producer of mcl-PHA, thus the presence of *phaC* and *phaC1* gene is characteristic of it [34]. In recent studies, both genes have been detected in Antarctic *Pseudomonas* isolates, too [111; 112]. The primers we used also confirmed these assumptions in (almost) all *Pseudomonas sp.* isolates studied; more precisely, out of a total of 22 isolates, both genes were confirmed in 19 of them, as shown in Table 17 and Table 18. Many of the isolates studied belong to the Actinobacteria phylum (*Cryobacterium sp., Arthrobacter sp., Leifsonia sp., Salinibacterium sp., Rhodococcus sp., Micrococcus sp., Paeniglutamicibacter sp.*) [72; 96]. Information on members of these genera as PHA producers are limited, but it is thought that isolates from the polar regions could represent an interesting source of new, bioavailable polyhydroxyalkanoates, as it has been reported that Actinobacteria tend in some cases to produce PHA of unusual chemical composition [106; 113]. PHA synthases have not been confirmed at the genotypic level in *Cryobacterium sp*. isolated from Antarctica in a recent study, nor has it been confirmed in most *Arthrobacter sp* [106]. The same trend was confirmed by our PCR analysis. In another study, researchers focused on investigating potential producers of PHA among bacteria isolated from the trans-Himalayan region (a higher altitude region whose environment can be also considered extreme). In this study, an identical pair of primers (G-D; G-1R) was used to detect PHA synthase class I, but the *phaC* gene was not detected in *Leifsonia sp* [114]. As noted in Table 18, and as previously mentioned, in genera *Leifsonia*, nonspecific products were detected in this work using these primers. The result for isolate 2.27, which represents *Leifsonia antarctica*, is interesting, as it was repeatedly observed to have a significant bend for the *phaC* gene (Figure 13) in addition to a significant bend for the *phaC1* gene (Figure 16). There is no mention in the literature that this genus possesses the *phaC* gene, let alone the *phaC1* gene. Therefore, the ability to form and accumulate PHA will certainly be tested at the phenotypic level in this isolate.

4.2 Staining of cultures

4.2.1 Nile Red

One of the lipophilic fluorescent dyes that can bind to the neutral hydrophobic cell structures and thus easily demonstrate their presence is Nile Red (also known as Nile Blue Oxazone). Straining with Nile Red is the fast and easy way, how to detect intracellular P(3HB) granules by fluorescence – when a polar solvent such as ethanol is used, Nile Red provides orange/red fluorescence [74; 115]. The dye is used in low concentrations that do not affect bacterial growth. Detection and quantification of lipid-like cell inclusions through Nile Red dye is also an ecological way compared to other conventional lipid extraction and estimation protocols [116].

All bacterial samples (78) were subjected to Nile Red staining according to the procedure in 3.6.1.1. In previous studies, it has been shown that the best results were observed with Nile Red staining in gram-negative PHA producers [117]. This method was chosen as the first pre-screening at the phenotype level since most of the bacteria belong to the gram-negative group [72; 96] and it is a fast and simple method for the detection of PHA.

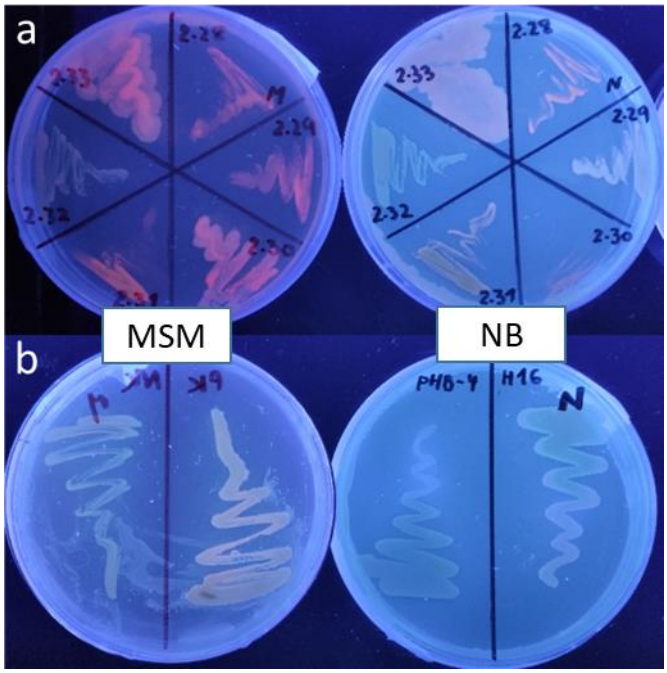

Figure 17 Fluorescence after 48 h; (a) demonstration of randomly selected bacterial strains from the second set; (b) negative control (C. necator PHB^{-4}) on the left side of the divided plate, positive control (C. necator H16) on the right side; MSM – Mineral salt medium; NB – Nutrient Broth

Figure 17 shows the result after staining with Nile Red and a comparison with the positive and negative control. As can be seen, the fluorescence in the producing strain *C. necator* H16 is not evident on the complex medium (NB), whereas the orange colouration of the colony is more distinct on the MSM. However, most bacterial isolates showed strong orange to red fluorescence on the MSM, which was not comparable to the positive control. In contrast, the extent of colony fluorescence on the complex medium was noticeably weaker. Several groups of researchers have noted that strain pigmentation extinguishes fluorescence, which may have influenced the results in our case, as many of the isolates studied had different colourations [106; 118]. However, after ten days, all cultures on the NB medium showed strong orange-red staining under UV light, too. Therefore, it was not possible to determine which of the isolates accumulated PHA based on this method, and it can be assumed that the fluorescence was not caused by the PHA present.

Nile Red, as a lipophilic dye, is not aimed directly and specifically at proving PHA accumulation, but it can also detect other lipid storage compounds [117]. Therefore, other non-polar components may be the cause of the strong fluorescence in almost all bacterial samples; however, a lipophilic profile has not yet been performed among bacteria. One possible component to which the Nile Red dye may have bound may be triacylglycerol, which belongs to the neutral lipids. The ability to form TAG has been previously demonstrated in the *Rhodococcus sp.*, which accumulates TAG as the main storage compound [119]. Other possible lipophilic components may be rhamnolipids, which belong to glycolipids and are the best characterized among the biosurfactants. Rhamnolipids are naturally produced by *Pseudomonas sp.*, but studies have also found producers among cold-loving bacteria isolated from Antarctica – in addition to *Pseudomonas sp.*, for example, *Psychrobacter sp* [120; 121]. Furthermore, the fluorescence from Nile Red staining of rhamnolipid has been previously successfully captured [122].

4.2.2 Sudan Black B

Since Nile Red staining did not yield sufficient results, Sudan Black B (SBB) was chosen as the second dye for pre-screening. The procedure of the whole staining process is given in Chapter 3.6.1.2. Along Nile Red, SBB is the most used dye for selective staining of PHA granules [123]. SBB is a highly lipophilic agent that exhibits high affinity to the neutral lipophilic compartments. This makes it remarkably applicable in diagnostic medicine [124; 125]. After the staining, colonies that are unable to incorporate SBB should remain unstained, while PHA producers should appear bluish-black [126].

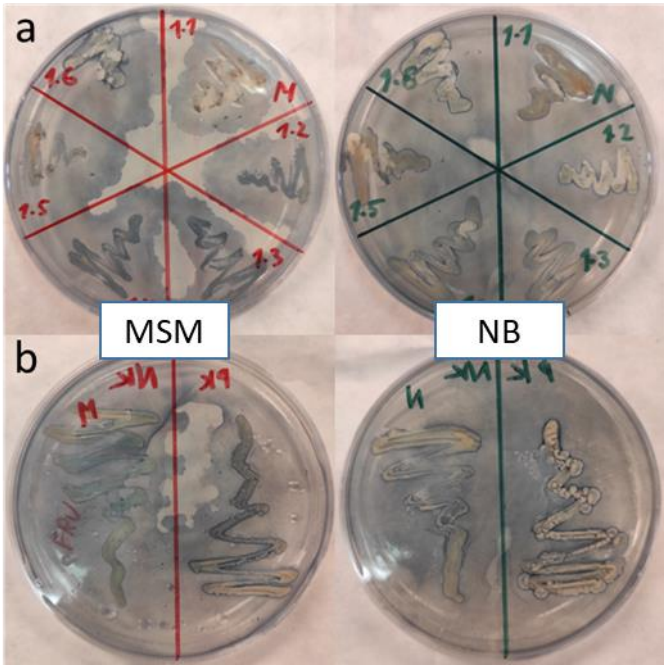

Figure 18 Washed cultures after staining; (a) demonstration of randomly selected bacterial strains from the first set; (b) negative control (C. necator PHB^{-4}) on the left side of the divided plate, positive control (C. necator H16) on the right side; MSM – Mineral salt medium; NB – Nutrient Broth

As can be seen in Figure 18b, negative controls are very difficult to distinguish from positive ones, they seem to be equal. For this reason, it was not possible to objectively evaluate which of the bacterial isolates could be considered as the ones accumulating PHA (the same for MSM and NB medium). Some colonies appeared to be stained only around the edges, some were washed off the agar medium during washing despite very gentle and careful work.

Since the initial step in screening approaches to find PHA-producing bacteria usually involves staining intracellular PHA inclusions with fluorescent dyes [70], this strategy was also chosen in this work. Overall, it can be assessed that the results after both stains did not perform as expected. Based on the results obtained, it was not possible to predict which of the isolates can accumulate PHA. Nile Red staining on Petri dishes showed false positive results using psychrophilic and/or psychrotolerant bacteria. The same conclusion was reached by Pernicova *et al*. In their case, these were extremophilic bacteria from the thermophilic and/or thermotolerant group, which also gave false positive results when stained with Nile Red [127]. The literature reports that the SBB dye has been successfully used in several studies for the initial screening of PHA producers, e.g., for bacteria isolated from wastewater and sludge ecosystems [126], from soil [128] or industrial wastes [129; 130]. For cold-adapted bacteria, however, these assumptions have not been confirmed for the reasons given above and therefore both pre-screening methods can be considered unsuccessful in this case.

4.3 Biomass production of selected bacterial strains

Selected bacteria (12) with positive *phaC* and/or *phaC1* genes were chosen for screening as representative samples according to the results of the PCR analysis (intensity of bands on gels, reproducibility of results) and based on good growth ability. Bacterial isolates of four different genera were screened, namely, 8 isolates belonging to the genus *Pseudomonas* (1.20; 1.25; 1.31; 1.32; 1.34; 2.17; 2.33; 2.34), 2 isolates belonging to the genus *Leifsonia* (2.20; 2.27), and the remaining 2 represented members of the genera *Arthrobacter* (2.8) and *Rhodococcus* (2.45). Cultivation was carried out according to the procedure described in Chapter 3.6.2.

For PHA production screening, five different media listed in Table 12 were chosen. The basic approach is to induce the production of PHA based on the reduction of the nitrogen-to-carbon ratio [106]. For this purpose, the mineral salt medium (MSM) with different carbon substrates in excess was used for the four tested media. It has been reported that all extremophiles can grow on glucose as the basic carbon source [131] and in some studies, glucose was also chosen as the best carbon source to produce PHA [132 – 134]. For this reason, one of the tested media (M3) consisted of the mineral salt medium with the addition of 20 g/L glucose as the sole carbon source. Because of the even more pronounced difference in the C/N ratio, an addition of 60 g/L glucose was chosen for Media 5 (M5). In addition to nitrogen limitation, reduced phosphorus content was also shown to affect the higher production of PHA [133]; therefore, Medium 4 (M4) consisted of a base amount of glucose (20 g/L), but in addition, its phosphorus content was halved. Currently, glycerol is becoming a very promising substrate for the production of PHA due to its easy utilization by bacteria and the economic perspective of using low-cost glycerol from the biodiesel industry [135]. Moreover, the ability to produce PHA on this substrate has already been demonstrated in extremophilic species, e.g. thermophiles [57; 127] or halophiles [136]. Medium 3 (M3) therefore contained 20 g/L glycerol in MSM. Cultivation for the production of PHA was also performed on BHI complex medium supplemented with 10 g/L glucose (M1). This medium was chosen based on the findings from work with bacteria in the BioSpec group at NMBU [72 and not published results].

Figure 19 shows the biomass production of all 12 isolates. Cultivation of the second replicate in media M1 – M4 was not successful, so biomass production is averaged for M5 only. The biomass is reported as g dry cells per liter of culture medium (g/L). For quicker reference of the results, each isolate includes the abbreviation of the genus name in addition to its numerical designation used throughout the work.

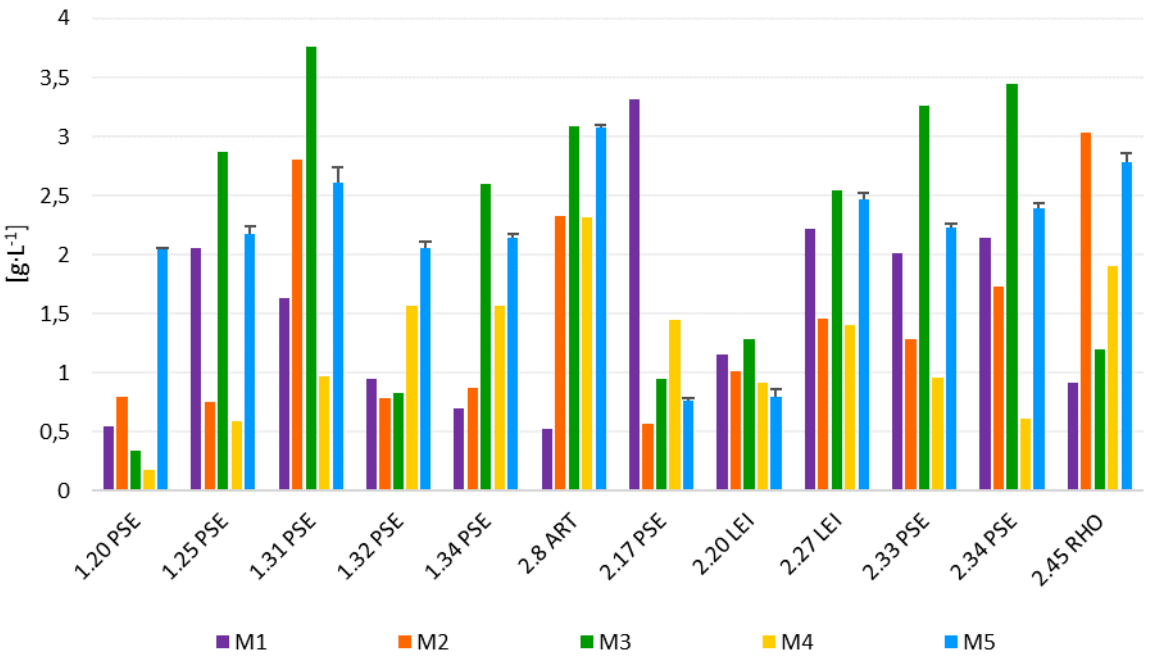

Figure 19 Biomass production of individual isolates on five different media; PSE – genus Pseudomonas; ART – genus Arthrobacter; LEI – genus Leifsonia

From the results, it can be evaluated that MSM with the addition of 20 g/L glycerol (M3) as the sole carbon source recorded the highest biomass production, together with 8 other isolates compared to the other four media. However, an increase in biomass can be seen for MSM with the addition of 60 g/L glucose (M5) for all isolates, which may be slightly surprising as the amount of glucose was quite high, and bacteria isolated from polar regions are generally considered to be oligotrophs [137]. In the overall comparison, the lowest biomass was recorded for the MSM with glucose (20 g/L) and reduced phosphate content – M4, which confirms that an appropriate amount of phosphorus is required for bacterial growth and its reduction became limiting for the growth of the culture [129]. Compared to the literature, psychrotolerant bacteria isolated from Antarctica recorded a maximum of 2.7 g/L (under selected conditions) [138]. For more than half of the tested bacteria (7), this amount was reached/exceeded on at least one of the selected cultivation media, even though culture conditions were not optimized for individual isolates.

4.4 Biochemical profiling of bacterial biomass by FT-infrared spectroscopy

High-throughput screening (HTS) measurement mode of FTIR spectroscopy is a next-generation non-destructive phenotyping technique that enables investigation of the whole biochemical profile of microbial cells [72]. This technique can be used in a broad range of study fields, including molecular biology, metabolic engineering, and others [139]. To this date, little attention has been paid to coupling this method with screening and optimization of PHA production; however, a recent study confirms that HTS can detect even small quantities of PHA in biomass. This finding brings many advantages since the extraction and analysis of microbial compounds are usually time-consuming and laborious [70; 72]. In this work, we decided to adopt the approach of combining FTIR spectroscopy with a high-throughput microculture system (Duetz-MTPS), which has been already successfully applied in the screening of Antarctic bacteria by Smirnova *et al* [72]. The FT-infrared spectroscopy analysis procedure is presented in Chapter 3.6.3 (following the Duetz-MTPS cultivation in Chapter 3.6.2).

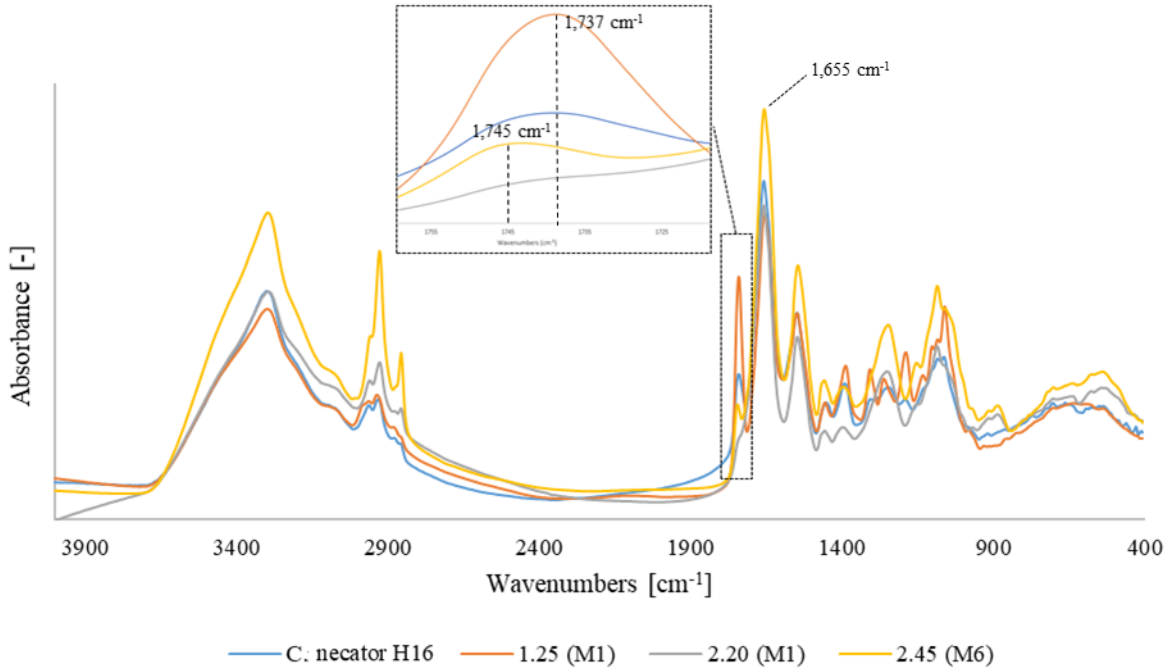

Figure 20 Infrared spectra of biomass – C. necator H16; Pseudomonas peli (1.25) and Leifsonia antarctica (2.20) on M1; Rhodococcus erythropolis (2.45) on M6

Figure 20 shows representative FTIR spectra of *C. necator* H16 and isolates 1.25 (*Pseudomonas peli*), 2.20 (*Leifsonia antarctica*) and 2.45 (*Rhodococcus erythropolis*). Based on literature and measured standards, wavelengths in the region from 1,724 to 1,745 cm^{-1}, which belongs to ester-carbonyl bonds vibrations, were selected for the detection of PHA presence in biomass [70]. *C. necator* H16 is known as a producer of PHA, therefore, it represents a positive control (PC) [98]. The spectrum of isolate 1.25 shows a high signal at 1,437 cm^{-1} (C=O stretching), the same as *C. necator* H16, which indicates PHA accumulation. Although bacteria isolate 2.45 also showed the presence of the peak in the lipid region, its maximum is shifted to 1,745 cm^{-1} which can correspond to lipid production rather than PHA. Although the ability to synthesize and accumulate PHA has already been confirmed in some *Rhodococcus sp.* [140], this genus is also known to produce mycolic acids (Chapter 2.5.2.3; Figure 6), whose structure corresponds exactly to the findings from the obtained spectra – the pronounced peaks attributed to asymmetric and symmetric stretching vibrations of $–CH_2$ and $–CH_3$ (the region of 2,800 – 3,050 cm^{-1}) [69] and also the peak at 3,010 cm^{-1}, which indicates the presence of unsaturated bonds [72].

The ratio of infrared intensities was estimated from signals belonging to carbonyl-ester and amide band I-ester for comparison of PHA synthesis in individual isolates. Based on the literature [70], a wavelength specifically at 1,737 cm^{-1} was used for the potential presence of PHA, as this also corresponded with the highest absorbance values in the lipid region for most isolates. The results for all 12 isolates together with *C. necator* H16 as PC are shown in Figure 21.

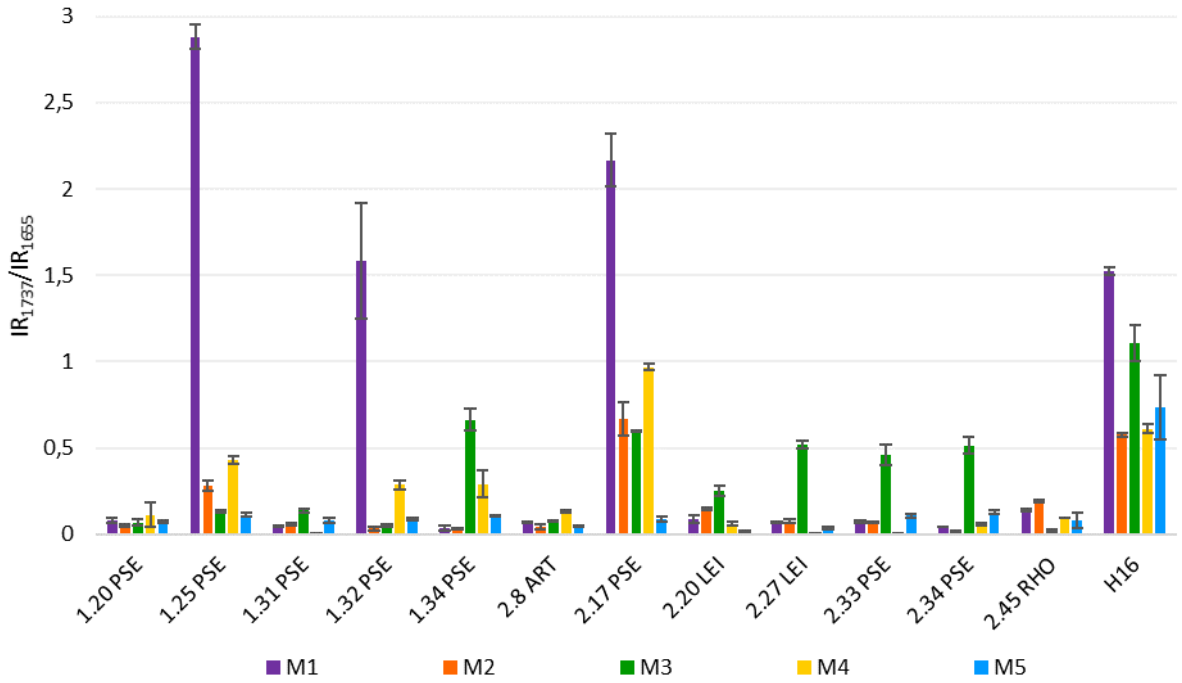

Figure 21 Comparison of PHA accumulation at Antarctic bacteria based on infrared intensities ratios at 1,737 and 1,655 cm $^{-1}$; PSE – genus Pseudomonas; ART – genus Arthrobacter; LEI – genus Leifsonia

From the data recorded in Figure 21, it can be assessed that 5 of the tested isolates (1.20, 1.31, 2.8, 2.20, 2.45) can be evaluated as non-accumulating PHA, as no production was recorded on any of the media. Visibly, the best results were obtained for the three isolates belonging to *Pseudomonas sp.* (1.25, 1.32, 2.17) on BHI medium supplemented with 10 g/L glucose (M1), whereas, for the first two isolates mentioned, this medium can even be described as the only one on which significant production was observed. There is no evidence in the literature of increased production of PHA on a complex substrate. In this case, we hypothesize that the higher production could have been caused by some of the components of the complex medium (amino acids, trace elements, vitamins, etc.), which is stimulating the synthesis of PHA itself. In the literature, production was tested on mineral media with and without nitrogen limitation. The results showed that PHA concentration and productivity increased under nitrogen starvation in *Pseudomonas* genera, but only in the case of *Pseudomonas antarctica*, PHA yield was not induced by nitrogen limitation [141]. Although this was not a nutrient-rich medium, this finding may be related to the increased production of PHA without limitation in bacteria isolated from polar regions. Another medium on which the presence of PHA was potentially detected is M3, which contained 20 g/L glycerol in MSM. On M3, as the only one of the five media, increased accumulation of PHA was observed in four isolates (1.34, 2.27, 2.33, 2.34). Although there are studies reporting that phosphate concentration does not affect the synthesis of PHA [142], M4 was detected as the second highest ratio for isolate 2.17 and smaller ratios were also observed for the other three isolates (1.25, 1.32, 1.34). From the results, isolate 2.17 appears to be the most universal producer, with production detected on four of the media examined (M5, with its high glucose content, meanwhile, did not show production in any of the 12 isolates). Overall, when identifying the most promising potential producer of PHA, the results of biomass yields should also be considered (Figure 19). *C. necator* H16 was only used in this case as PC to compare the results (mainly spectra) after FTIR analysis, therefore it is not appropriate to compare the production of PHA in terms of their quantity since it was not cultivated under its optimal conditions.

4.5 Quantification of PHA by gas chromatography

The GC-FID method was chosen to confirm the presence of PHA and for quantification, as it is the most established method for these purposes. A total of 10 samples were selected based on previous FTIR results, namely those which showed a high ratio of IR intensities at 1,737 and 1,655 cm^{-1} (Figure 21) – 1.25 (M1), 1.32 (M1), 2.17 (M1), 2.17 (M2), 1.34 (M3), 2.17 (M3), 2.27 (M3), 2.33 (M3), 2.17 (M4), 2.34 (M4); together with an additional one sample belonging to 2.45 (*Rhodococcus erythropolis)* discussed in the previous chapter.

Based on the GC results, Figure 22 shows the total content of PHA (in general) in dry biomass. Six of the eleven samples were tested positive for the presence of PHA, namely: *Pseudomonas peli* (1.25), *Pseudomonas peli* (1.32) and *Pseudomonas sp* (2.17). In this genus isolated from Antarctica, the production of PHA has also been confirmed from a variety of carbon substrates [143]. In the remaining five samples (1.34, 2.27, 2.33, 2.34, 2.45), the amount of PHA was negligible (less than 0.2 % in DCW) and therefore these isolates can be considered non-accumulating PHA. However, the data are burdened by the fact that only three basic standards were used in the GC analysis (more were not available).

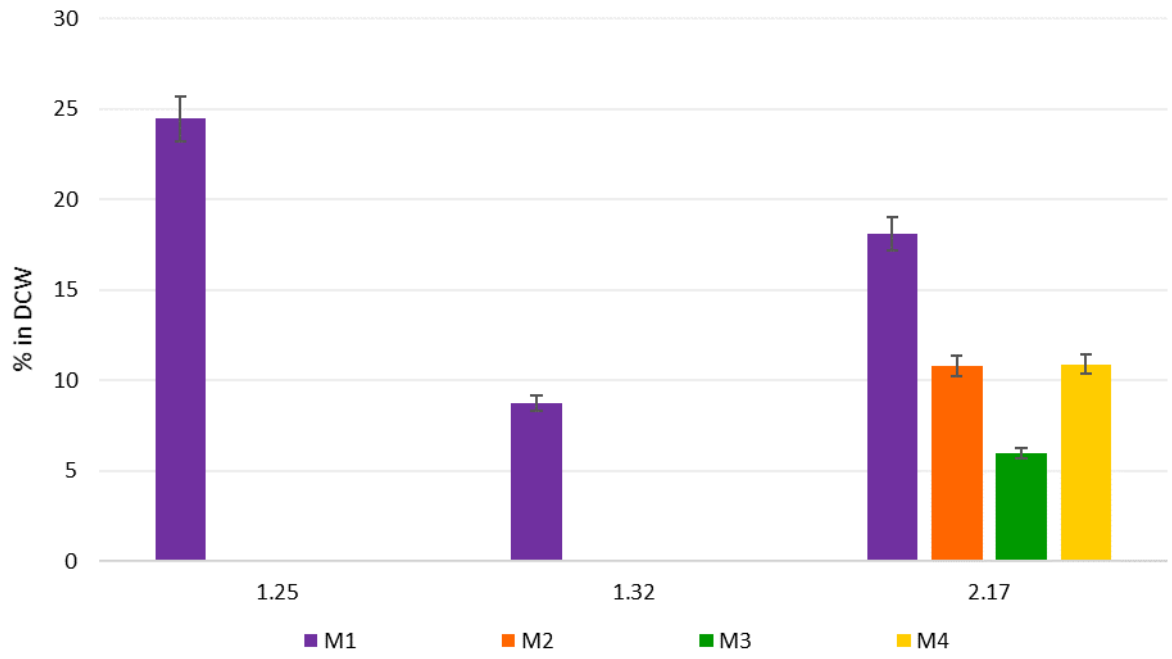

Figure 22 Total content of PHA in dry cell weight

Pseudomonas peli (1.25) isolated from temporary melted water ponds accumulates the highest amount of PHA in biomass (up to 25 %, Figure 22) followed by *Pseudomonas sp.* (2.17) with 18 %. It has been reported that the percentage of PHA can reach up to 76.1 % of DCW in psychrotolerant bacteria under optimal conditions, which specifically refers to the genus *Pseudomonas* [144]. In our pre-screening experiments with non-optimal conditions, the maximum percentage reached almost 25 % in DCW for *Pseudomonas peli* (1.25). In further studies, the culture parameters need to be optimized. Despite the higher percentage of PHA in the biomass of isolate 1.25, isolate 2.17 was capable of higher production of PHA relative to the total biomass production. After calculation, the amount of PHA was 0.5 g/L for isolate 1.25, whereas it was 0.6 g/L for isolate 2.17, which could be more beneficial

for biotechnological purposes. What is more, isolate 2.17, as the only one in this study, produced PHA from other carbon sources (glycerol).

Comparing the results from infrared spectroscopy (Figure 21) and gas chromatography (Figure 22), the amount of PHA detected in each medium correlates indicating that the FTIR method provided a suitable method for the selection of potential PHA producers. The higher ratio estimated for the remaining five samples (Figure 21) can be assigned to ester-carbonyl bond vibrations caused by other types of molecules, most likely lipids, or fatty acids. This theory was confirmed by gas chromatography, where peaks with different retention times than PH standards were. GC analysis did not confirm the presence of PHA even for isolate 2.45, therefore it can be genuinely surmised that in this case the production and accumulation of the aforementioned mycolic acids may be involved, which is typical for the genus *Rhodococcus* (2.5.2.3).

Since three standards were used in the CG analysis, it was also possible to recalculate the percentage of each standard in the isolates in which PHA were detected. Based on this, the profile of PHA recorded in Figure 23 was constructed.

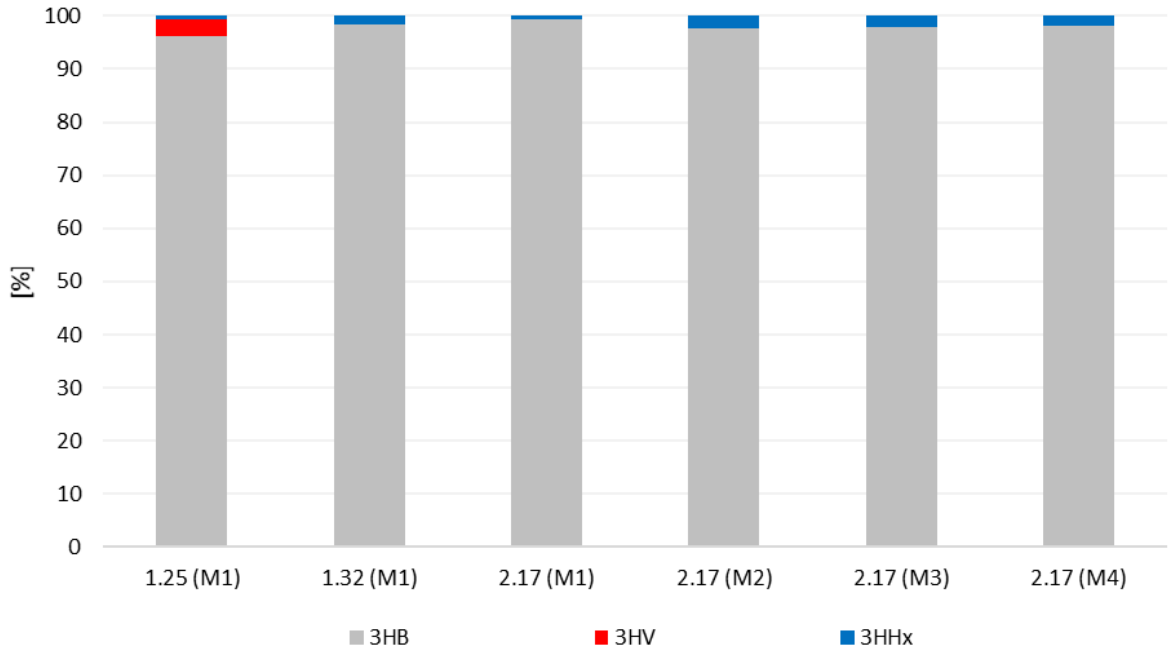

Figure 23 Profile of PHA

The analysis showed that the 3HB monomer was predominantly represented in all isolates (over 95 %). Because the selected three isolates represent only members of *Pseudomonas sp.*, based on the knowledge it was expected that the production of mcl-PHA should also be recorded [29; 31]. In the case of the standards used, the medium chain length PHA monomer was represented by 3HHx. As can be observed from Figure 23, the ability to synthesize this polymer was confirmed in all three isolates and even in each of the media, which mainly confirmed the genotype-level analysis performed at the beginning of the measurements (Table 17 and Table 18). Among all tested samples, isolate 1.25 on BHI medium with 10 g/L glucose (M1) was the only one able to synthesize short chain length copolymer P(3HB-*co*-3HV). The ability to synthesize this copolymer has been observed in *Pseudomonas sp.* in several cases and is reported to depend on the used carbon source [24; 145]. It is noted that by using a precursor carbon source as a co-substrate in addition to the main carbon source, 3HV monomers are most frequently incorporated. In particular, organic acids, alcohols or some amino

acids have been studied for co-substrates [146]. Since the BHI medium is a complex medium, it is most likely that the precursor carbon sources were amino acids, while the main source was, obviously, glucose.

5 CONCLUSION

Large-scale industrial production of PHA as bioplastics is still hampered by several factors despite having started decades ago. In this thesis, the first screening and selection of potential PHA producers among an interesting group of extremophilic microorganisms were performed, namely 78 bacterial isolates from East Antarctica. Bacteria from polar regions represent a very promising and prospective source of PHA-producing microorganisms, which made their investigation with a focus on this production the main objective of this work.

The first part of the work was devoted to the analysis at the genotypic level, as the genetic predisposition represents the main prerequisite for the possible production and subsequent accumulation of PHA. During this analysis, all bacterial isolates were subjected to polymerase chain reactions aimed at detecting genes for key enzymes responsible for the synthesis of PHA – PHA synthases class I and class II. By PCR method 36 polar isolates were pre-selected in which one/both genes of interest were detected. However, during this analysis, frequent occurrence of non-specific or two products was observed (despite the measures put in place to prevent them), which could be avoided in the future by using other primer pairs, as the ones used by us were designed based on gram-negative bacteria and the sample of bacteria examined also consisted of gram-positive representatives.

In the next section, we focused on the phenotypic expression of PHA production also along with the optimization of production conditions in the form of five different cultivation media. As a typical pre-screening method, staining of cultures on agar plates was performed using two available fluorescent dyes, Nile Red and Sudan Black B. Unfortunately, psychrophilic and/or psychrotolerant bacteria recorded false positive results, which made it impossible to identify and select potential producers at the phenotypic level. Therefore, 12 isolates were selected based on the results from the PCR analyses and cultivated at five different media. The chosen approach of combining FTIR spectroscopy with a high-throughput microculture system (Duetz-MTPS) yielded positive results, as the cultivation could be carried out in smaller volumes with a larger number of samples at a time, and the subsequent FTIR-HTS analysis correctly predicted the presence and the amount of PHA in the biomass for each sample. The best scores in terms of PHA production (up to 25 % of PHA in DCW) were obtained for representatives from the *Pseudomonas* genus, surprisingly on the complex BHI medium with glucose addition. Since in this case, the setting of stress conditions in terms of nutrient limitation was not involved and despite this, the highest PHA production was recorded, it is assumed that the medium contains some of the components that stimulated the synthesis of PHA (amino acids, vitamins, trace elements, etc.). When comparing PHA production plotted against biomass, the best results (0.6 g/L) were recorded for *Pseudomonas sp.* isolated from green snow in the East Antarctic region.

In the final stages of the work, gas chromatography was performed and, in the predicted samples, the presence of PHA was proved. Among them, poly(3-hydroxybutyrate) was the most abundant, however, the presence of mcl-PHA was also demonstrated in all tested isolates belonging to the *Pseudomonas* genus, confirming the data obtained in the initial work devoted to genetic analysis.

It can be summarized that the main objective of the work was fulfilled and potential promising producers of PHA were found among the tested polar bacteria. Subsequent studies need to be performed to optimize culture conditions for the scale-up of PHA production from psychrophiles and/or psychrotolerants and combine the low-cost substrates and waste materials to achieve economic sustainability.

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7 LIST OF ABBREVIATIONS

PHA	Polyhydroxyalkanoates
TEM	Transmission electron microscopy
FTIR	Fourier transform infrared spectroscopy
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
FAMEs	Fatty acid methylesters
RFLP	Restriction fragment length polymorphism
sp.	Species
TAG	Triacylglycerols
PCR	Polymerase chain reaction
MSM	Mineral salt medium
NB	Nutrient Broth
BHI	Brain Heart Infusion Broth
EDTA	Ethylenediaminetetraacetic acid
PC	Positive control
NC	Negative control
rpm	Revolutions per minute
HTS	High-throughput screening
SBB	Sudan Black B
DCW	Dry cell weight
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HHx)	Poly(3-hydroxyhexanoate)