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Screening for pigment production and characterization of pigment profile and photostability in cold-adapted Antarctic bacteria using FT-Raman spectroscopy

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ABSTRACT

Microbial pigments can replace synthetic organic pigments which often produced in unsustainable way and can be toxic. Therefore, search for new pigment producing microorganisms is of high interest for industry. In this study, a screening and characterization of pigment profile and photostability in seventy-four newly isolated Antarctic bacteria using Fourier-transform (FT) Raman spectroscopy and HPLC-MS was performed. Screening of the bacterial biomass by FT-Raman identified thirty-seven bacterial strains from the genera *Agrococcus, Arthrobacter, Brachybacterium, Cryobacterium, Leifsonia, Micrococcus, Paeniglutamicibacter, Rhodococcus, Salinibacterium* and *Flavobacterium* as having relatively high pigment content. The impact of growth temperature on the pigment production in the studied Antarctic bacteria was species - specific, while blue light exposure triggered pigment production in majority of the studied bacteria. HPLC-MS analysis of a biomass of a set of ten pigmented Antarctic bacteria identified eighteen different carotenoids and precursors. FT-Raman spectroscopy showed to be suitable for both, semi-qualitative library-independent identification of pigment producing bacteria and determination of pigment profile using spectral library of reference pigment standards. This study provides valuable insights into the pigment production capabilities of Antarctic bacteria and highlighting the potential of FT-Raman spectroscopy for characterizing microbial pigments.

1. Introduction

Pigments have a significant industrial importance and find diverse applications in food, feed, cosmetics, and chemical industry. Majority of pigments used in industry are synthetic [17]. Synthetic organic pigments can be obtained with high purity, consistency, and stability and they can be customized for specific properties depending on the final application [34]. Despite these advantages, there is an increasing trend in the industry to switch from synthetic organic to natural pigments that creates a high demand of naturally sourced pigments [17,34]. This is mainly due to the reported toxicity, pollution potential and many environmental and sustainability concerns of their production. Many food, feed and cosmetic producers tend to include more natural ingredients in their products [17,5]. Also, textile and dye industry searches for natural alternatives of pigments. Therefore, exploring new bio-based sources of pigments and establishing their production becomes a crucial step in elevating sustainability of the modern industry [28].

Naturally sourced pigments usually have plant, insect, mineral ores or microbial origin (bacteria, cyanobacteria, algae, fungi, yeast, archaea) [31,34]. Industrial production of plant-based pigments is often restricted by high extraction cost and availability of plant biomass which often comes as a by-product, rest material or waste of other productions [20]. Establishing agricultural production of plants dedicated to pigment production is challenging and hindered by the European Green Deal which aims to make land use more sustainable and environmentally friendly. Therefore, in recent years, considerable attention has been given to microbe-based pigments, production of which is independent on land use or climate and can be performed in accordance with sustainability standards fulfilling all main EU Bioeconomy strategies.

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Among pigment-producing microorganisms the most promising are bacteria, cyanobacteria, yeast and microalgae which are unicellular and characterized by a rapid uniform growth and ability to utilize various substrates often considered as wastes, side-streams, or by-products [25,31]. Microbes produce a wide range of pigments with different structures and biological properties, such as actinorhodin, carotenoids, flexirubin, melanin, phycocyanin, phycoerythrin etc. [4]. Today, among all carotenoids, six are regarded as industrially significant: astaxanthin, β-carotene, canthaxanthin, lutein, lycopene, and zeaxanthin [22]. But similarly to plants, industrial production of microbe-based pigments is represented by very few examples: astaxanthin (Alga Technologies, Israel; BlueBiotech, Germany; Cyanotech, USA), astaxanthin and mixture of β-carotene, zeaxanthin, cryptoxanthin and lutein (Parry Nutraceuticals, India; Plankton Australia Pty Limited, Australia) [12]. Bacterial pigments with current or potential use as natural food colourants include astaxanthin (a pink-red pigment) from *Agrobacterium aurantiacum* and *Paracoccus carotinifaciens*, rubrolone (a red pigment) from *Streptomyces echinoruber*, zeaxanthin (a yellow pigment) from *Flavobacterium* sp. and *Paracoccus zeaxanthinifaciens* [1]. Limited availability of industrially produced natural pigments of microbial origin replacing synthetic alternatives is mainly due to the lack of knowledge on the availability of microorganisms capable to synthesize certain pigments. According to the Carotenoid Database Japan, there are 702 microorganisms registered as capable of producing natural carotenoids, but many of them produce the same type of carotenoids or their precursors which have no industrial applications [44]. Thus, there is an increasing need in searching, identifying and characterizing new pigment-producing microbes.

Among pigment-producing microorganisms those isolated from polar regions are of special interest since they often possess an ability to synthesize a wide variety of pigments which function as cell photoprotectors and antioxidants allowing them to survive and adapt to extreme conditions such as low temperature and high UV radiation [6,9,30,32,33,36,40]. In addition, it has been reported that pigments from polar microorganisms have unique properties such as high photostability and light-absorbing capability, and higher resistance to UV radiation [32].

Recently, it has been suggested that microbial pigments or even microbial pigmented biomass can be used in solar cells dyeing or dye-synthesized solar cells [23]. Photostability is a critical factor in assessing the feasibility of microbial pigments as photosensitizers for implementation in solar cells [10]. The average illuminance for direct sunlight exposure ranges from 30.000 to 100.000 lx. In the Antarctic, light intensity ranges from almost nothing during winter (max 500 lx during this period) up to 100 000 lx in summer [24].

In this study we performed screening of 74 fast-growing cold-adapted Antarctic bacteria to uncover their capability to produce various pigments. For the screening, we utilized Fourier-transform (FT) Raman spectroscopy which is well-known for sensing pigments and performing qualitative pigment analysis of the biomass [7]. HPLC-MS was utilized for characterizing pigments profile of the extracted pigments for the most promising pigment-producing Antarctic bacteria. Furthermore, we evaluated biotechnological potential of the Antarctic bacteria identified as the most promising pigments producers and studied their pigment photostability in an intact biomass.

2. Materials and methods

2.1. Bacterial strains

Seventy-four fast-growing cold-adapted Antarctic bacteria obtained from the Belarussian Collection of Non-pathogenic Microorganisms, Institute of Microbiology of the National Academy of Science of Belarus (Minsk, Belarus) were used in the study for screening experiment, from them ten strains were selected for detail pigment analysis, evaluation of blue light effect on pigments production and photostability testing (Table S1 in SM). The bacteria were isolated from green snow and meltwater ponds in the Vecherniy District of the Tala Hills oasis, located in the Western part of Enderby Land (East Antarctica) during the 5th (2013) and 7th (2014–2015) Belarusian Antarctic Expedition. All bacteria were identified by 16S rRNA gene sequencing and deposited in Belarussian Collection of Non-pathogenic Microorganisms. Detailed biochemical and physiological characterization was performed and reported previously [45–48].

2.2. Cultivation and sample preparation for screening by FT-Raman analysis

Bacteria were recovered from cryo-preserved cultures by culturing on brain heart infusion (BHI) agar (Sigma Aldrich, USA) for 7 days at 18 °C. A single colony of each strain was transferred into 7 mL of BHI broth (Sigma-Aldrich, USA) in a Duetz microtiter plate system (Duetz-MTPS, Enzyscreen, Netherlands) consisting of 24-square extra high polypropylene deep well microtiter plates (MTPs) with low-evaporation sandwich covers and extra high cover clamps. To obtain enough biomass for FT-Raman analysis, each strain was inoculated into four wells of a microtiter plate. Inoculated MTPs were mounted on the shaking platform of MAXQ 4000 incubator (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 5, 15 and 25 °C, with 400 rpm agitation speed (1.9 cm circular orbit) for 7 days. One well in each plate was filled with a sterile medium for cross-contamination control. All cultivations were done in two independently performed biological replicates.

Bacterial biomass was separated from the growth medium by centrifugation (Heraeus Multifuge X1R, Thermo Scientific, Waltham, MA, USA) at 2330 g, 4 °C for 10 min and washed with distilled water three times. After the washing step, bacterial biomass was freeze-dried (Labconco, USA) until constant weight and stored at −80 °C for further FT-Raman measurements.

2.3. Cultivation and sample preparation for HPLC-MS analysis

Cultivation of the selected strains for analysis by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and evaluation of pigment stability in bacterial biomass was done in shake flasks using BHI broth. First, overnight inoculum culture was prepared in 10 mL BHI broth at 15 °C. Then, shake flasks with 100 mL of BHI broth medium were inoculated with 10 mL of the overnight inoculum. All cultivations were carried out in the shaker incubator MAXQ 4000 (Thermo Fisher Scientific, Waltham, MA, USA) at 15 °C and 400 rpm agitation (1.9 cm circular orbit). All cultivations were done for 7 days, in two biological replicates which were independent cultivation runs performed on different days.

Bacterial biomass was separated from the growth medium by centrifugation (Heraeus Multifuge X1R, Thermo Scientific) at 11,510 g, 4 °C for 10 min and washed with distilled water three times. After the washing step bacterial biomass was freeze-dried (Labconco, USA) until constant weight and stored at −80 °C. Freeze-dried biomass was then used directly for extraction of pigments for HPLC-MS, FT-Raman, and TLC analysis and evaluation of pigments stability. All these steps were carried out under low light conditions to avoid pigment degradation.

2.4. Cultivation under blue light exposure

To determine the effect of light on growth and carotenoid accumulation, cultivation of bacteria in 500 mL baffled shake flasks was conducted using blue (455 nm) LED light as it was reported previously [38]. The level of illuminance for the experiment was in a range of 470–680 lx. Blue light spectra for each experimental setup are depicted on Fig. S1 in SM. The cultivation in blue light was done in ISFX1 Climo-Shaker (Kuhner, Germany) equipped with LED lights using single output LED Driver mix mode (Mean Well, Taiwan). To ensure light-free conditions for control samples, the flasks were also shaded with aluminum foil, and

the glass door of the shaker was covered with aluminum foil. Control samples were collected from cultures incubated in the dark.

2.5. Extractions of bacterial pigments for HPLC-MS and TLC analysis

Extraction of pigments for HPLC-MS was done as follows: each sample of 30 mg of bacterial biomass was treated with 1 mL MeOH (Sigma-Aldrich, USA) at 60 °C and followed by 15 min homogenization in an ultrasonic bath (Crest Powersonic P1100 Ultrasonic Cleaner, USA). After that insoluble material was separated by centrifugation (Centrifuge ELMI CM-50) at 18,000 g for 10 min and supernatant was filtered using PTFE filter with pore size 0.45 μm into 1.8 mL glass vial. Extraction of pigments for thin layer chromatography (TLC) analysis and FT-Raman measurements was done as described by [3].

2.6. HPLC-MS analysis

The extracted pigments were separated using the HPLC-DAD-MS system Agilent Q-TOF 6550 (Agilent Technologies, Santa Clara, CA, USA). The samples in a volume of 5 μL were injected into Thermo Fisher Scientific Hypersil GOLD 1.9 μm HPLC analytical column. The stepped linear gradient of buffers A (0.1 % formic acid (VWR chemicals, USA) in water) and B (0.1 % formic acid and 99.9 % acetonitrile (Sigma-Aldrich, Germany) were distributed as follows: 00–05 min – 50 % buffer A and 50 % buffer B, 05–40 min – gradient from 50 to 100 % buffer B, 40–45 min – 100 % buffer B. The separation of components was monitored using a photodiode array detector at the 190–600 nm range and a mass spectrometer tandem quadrupole-time-of-flight at the 50–1700 *m/z* range. A positive electrospray ionization mode (ESI +) with a time-of-flight TOF detector was used. The peak area (450 nm) representing carotenoids was used for semi-quantitative calculations.

2.7. Thin-layer chromatography

TLC was used to separate pigments extracted from the bacteria for the subsequent analysis by FT-Raman spectroscopy. For TLC the following solvent mixtures were used: (1) Acetone: n-heptane (1:1) [37], 2) Chloroform: methanol (93:7) [35]. Silica Gel 60 F254 (0.25 mm, Merck, Darmstadt, Germany) was activated in the solvent used for the separation before each analysis. All samples were placed on TLC plates, dried, and measured directly with FT-Raman. The following pigments were used as standards: canthaxantin (No. 0380), phytoene (No. 0044), lycopene (No. 0031), zeaxanthin (No. 0119), echinenone (No. 0283), neurosporene (No. 0034), and beta-carotene (CaroteNature GmbH, Münsingen, Switzerland), astaxanthin (Sigma Aldrich, Country) and bacterioruberin extract (32719–43-0, HALOTEK Biotechnologie GmbH, Leipzig, Germany) [21].

2.8. FT-Raman measurements

Raman spectra were acquired in a backscattering configuration using a MultiRAM FT-Raman spectrometer (Bruker Optik GmbH in Ettlingen, Germany). The instrument was equipped with a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser operating at 1064 nm (9394 cm^{-1}) and a germanium detector cooled with liquid nitrogen.

FT-Raman spectroscopy analysis of the pigments in intact biomass was performed using freeze-dried bacterial biomass. Approximately 5–10 mg of the biomass was transferred to flat-bottom 400 μL glass inserts (Agilent, USA). The glass inserts were then placed in a 96-well multi-well holder, and measurements were conducted using a high-throughput setting stage measurement accessory (Fig. S2A in SM).

To measure pure pigment standards using FT-Raman, the pigments were dissolved in chloroform to achieve a final concentration of 1 mg/mL. The pigment solution was then deposited onto a TLC silica plate (Merck, Germany), and the solvent was evaporated. Finally, the plate with the deposited pigments was placed on the Z-motorized stage measurement accessory for subsequent measurements (Fig. S2B in SM).

For the analysis of extracted pigments using FT-Raman, the pigments were deposited onto a TLC silica plate (Fig. S2A in SM). After solvent was evaporated, the plate was placed on a Z-motorized stage measurement accessory for further measurements. Additionally, thin-layer chromatography (TLC) was performed to separate different pigments in the extract. Each pigment fraction on the TLC plate was then measured by FT-Raman by placing it on the Z-motorized stage measurement accessory (Fig. S2C in SM).

For screening experiment, evaluation of blue light effect and photostability testing the acquisition of spectra involved the following parameters: 2048 scans, Blackman–Harris 4-term apodization, a spectral resolution of 8 cm^{-1}, and a digital resolution of 1.928 cm^{-1}, spanning the spectral range from 3785 to 45 cm^{-1}. The laser power used was set at 500 mW. For analysis of pigment extracts and standards on TLC plate the acquisition of spectra involved the following parameters: 128 scans, Blackman–Harris 4-term apodization, a spectral resolution of 4 cm^{-1}, and a digital resolution of 1.928 cm^{-1}, spanning the spectral range from 3785 to 45 cm^{-1}. The laser power used was set at 900 mW. All measurements were performed in two technical replicates for all type of samples (biomass, standards and extracts). The acquisition and control of data were performed using the OPUS software (Bruker Optik GmbH in Ettlingen, Germany).

2.9. Evaluation of pigments stability in bacterial biomass with FT-Raman spectroscopy

To measure pigment stability, 5 mg of the freeze-dried biomass was placed onto a weighing boat (VWR, USA) in a monolayer. Solar simulator Sun 2000 (Abet Technologies, USA) was used for light explosion. Light emission in the full spectral range (280–2500 nm) was used with an AM 1.5G and UVC blocking filters implemented: Atmospheric Edge (AE) filter for terrestrial cells with response below 360 nm and for life sciences, UVC blocking filters for material and life sciences, resulting with the emission in 300–2500 nm spectral range. Light intensity was set at 550 W. Calibration of the instrument was performed before each measurement with digital multimeter (Fluke 175, USA) attached to silicon reference cell (Rera solutions, Netherlands) to ensure same energy of light for each exposure equal to 93.7 mV. Illuminance meter T-10A (Konica Minolta, Japan) was used to measure illuminance before each measurement. The level of illuminance for the experiment was 900 lx. Measurements were performed every 5 h and every 10 h after the first 10 h of measurements. Control samples were placed at the same temperature but covered with aluminum foil to prevent exposure to light. All measurements were done in duplicates.

2.10. Data analysis

2.10.1. HPLC-MS data

The obtained HPLC-MS data were analyzed by Feature (Table S2 in SM) algorithms in Mass Hunter Qualitative Analysis software (Agilent, USA) and by Find by Formula (Table S3 in SM).

2.10.2. PCA analysis of FT-Raman data

For principal component analysis (PCA), FT-Raman spectra of the bacterial biomass were preprocessed in the different way for different set of data. For screening experiment, 2011 raw spectra were preprocessed in the following way: (1) Truncation of data to spectral range 3200–600 cm^{-1}; (2) Baseline correction with rubber band algorithm; (3) Normalization by applying area normalization (integral from 0); (4) Quality test: The peak maximum values within the biomass region (1430–1470 cm^{-1}) and the non-informative region (1800–2000 cm^{-1}) were identified, and the ratio between these maximum values was calculated. Spectra with a ratio lower than 5 were excluded due to the low signal-to-noise ratio. 1885 spectra have passed the quality check and were used in the PCA. For library-dependent experiment of the samples measured on

TLC plate, 118 raw spectra were preprocessed in the following way: (1) Truncation of the region of interest 1570–1460 cm; (2) Baseline correction with rubber band algorithm; (3) Normalization by applying area normalization (integral from 0).

Orange data mining toolbox version 3.31.1 (University of Ljubljana, Ljubljana, Slovenia) was used for the preprocessing and spectral analysis [49,50].

2.10.3. Ratiometric analysis of FT-Raman data

Ratiometric analysis using FT-Raman spectra was used to determine the relative pigment content in bacterial biomass obtained under both optimal and stress growth conditions, while also assessing pigment degradation under exposure to light and temperature [39,16]. For screening experiment, 1885 spectra (preprocessed in aforementioned way) were analyzed. For blue-light exposition experiment, 318 raw spectra were preprocessed in the following way: (1) Truncation of data to spectral range 3200–600 cm^{-1}; (2) Baseline correction with rubber band algorithm; (3) Quality test: The peak maximum values within the biomass region (1430–1470 cm^{-1}) and the non-informative region (1800–2000 cm^{-1}) were identified, and the ratio between these maximum values was calculated. Spectra with a ratio lower than 2.5 were excluded due to the low signal-to-noise ratio. 290 spectra have passed the quality check. For light stability testing, 243 raw spectra were preprocessed in the following way (1) Truncation of data to spectral range 3200–600 cm^{-1};(2) Spectral smoothing by applying the Savitzky − Golay algorithm using a polynomial order of degree 2, derivative order 2 and window size 11 [29]; (3) Quality test: The peak maximum values within the biomass region (1430–1470 cm^{-1}) and the non-informative region (1800–2000 cm^{-1}) were identified, and the ratio between these maximum values was calculated. Spectra with a ratio lower than 2 were excluded due to the low signal-to-noise ratio. 239 spectra have passed the quality check.

After preprocessing, for estimating the relative content of pigments, carotenoid-to-biomass ratio (C/B) was calculated. Specifically, the ratio between peak maxima in the range of 1500–1540 cm^{-1} (related to C = C stretching in polyene chain of carotenoids) and peak maxima in the range of 1430–1470 cm^{-1} (related to CH_2 and CH_3 deformations of lipids, proteins and carbohydrates, thus serving as proxy signal for total biomass) was calculated [7]. Orange data mining toolbox version 3.31.1 (University of Ljubljana, Ljubljana, Slovenia) was used for the preprocessing and spectral analysis [49,50].

3. Results

3.1. Semi-qualitative library-independent screening by FT-Raman spectroscopy

The screening of seventy-four fast-growing Antarctic bacteria using FT-Raman spectroscopy was conducted to investigate their pigment production capabilities at different temperatures. The bacteria were cultivated at three different temperatures (5 °C, 15 °C, and 25 °C) the results of the growth ability present in Table S1. The resulting biomass was analyzed using FT-Raman spectroscopy. This screening is semi-qualitative and is based on the library-independent analysis of FT-Raman data.

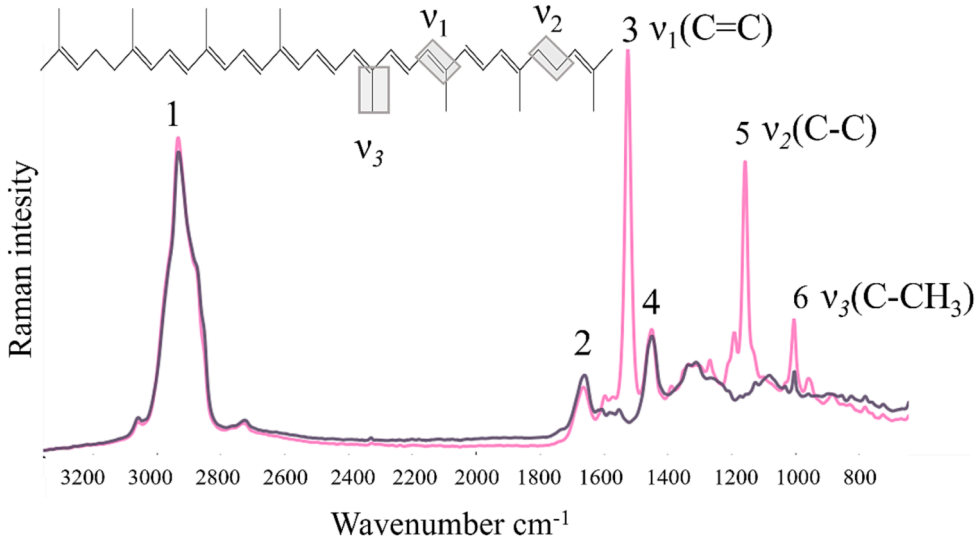
Carotenoid pigments exhibit strong resonance Raman effect. Specifically, the conjugated nature of π-electrons from the polyene backbone causes electronic states of lower energy, leading to strong resonant enhancement of certain vibrational frequencies by excitation lasers emitting in red and near-infrared part of the spectrum. The Raman spectra of carotenoids are dominated by three characteristic vibrational bands: C = C stretching vibration (ν_1), typically observed as peaks in the Raman spectrum around 1500–1520 cm^{-1}, indicating the presence of carbon–carbon double bonds within the carotenoid molecule. Additionally, the conjugated carbon backbone gives rise to C–C stretching vibration (ν_2), visible as peaks in the range of 1100–1120 cm^{-1}. Carotenoids often exhibit a peak around 1000 cm^{-1} related to the C–CH_3 deformation mode (ν_3), allowing the identification of methyl (CH_3) groups in the carotenoid structure [13] (Fig. 1). Based on the visual inspection of FT-Raman spectra it can be concluded that majority of pigments present in the studied Antarctic bacteria are carotenoids.

As a first step of the screening, we performed qualitative analysis to evaluate capability of the studied bacteria to produce pigments. For this, the obtained FT-Raman data were analysed by performing principal component analysis (PCA) for the whole spectral region (3200 – 600 cm^{-1}) where a distinct distribution of samples along the first principal component (PC1) was observed on the score plot (Fig. 2A). The loading plots in Fig. 2B illustrate the weight of each original variable (wavenumbers) on the PCs and the contribution of each spectral feature. The separation along both PC axes was due to changes in the C = C at 1525 cm^{-1}, C-C at 1156 cm^{-1} and C-CH_3 at 1005 cm^{-1} related to carotenoids (Fig. 2B). PCA analysis of FT-Raman data revealed that all bacteria belonging exclusively to Actinobacteria and Bacteroidetes phyla showed presence of pigments in a considerable amount in their biomass based on appearance of carotenoid-specific peaks at high intensity. Among them, strains related to the genera *Leifsonia*, *Cryobacterium*, *Flavobacterium*, and *Rhodococcus* showed the highest absorption values for all pigment specific peaks. Additionally, the PC1 axis exhibited temperature-induced differences (Fig. 2A), particularly noticeable for *Cryobacterium*, where bacteria cultivated at 5 °C were clearly separated from those grown at 15 °C and 25 °C (Fig. 2A).

The PCA score plot for the first and second components of FT-Raman spectra of freeze-dried bacterial biomass is presented in Figs. S3A, S3B and S3C in SM, while the corresponding loadings can be found in Figs. S3D–F in SM. The PCA score plot demonstrates distinct clustering based on genera. Optimal clustering occurred at 15 °C and 25 °C. Further 15 °C was used for the cultivation of samples for reference analysis and for assessing stability and photostability, as not all bacteria could thrive at 25 °C. The loadings depicted in Figs. S3D, S3E and S3F reveal that genera-specific variations in biomass composition are primarily influenced by the ratio of main cellular components, specifically proteins in PC1 (2925 cm^{-1}), lipids in PC2 (2891 and 2854 cm^{-1}) and carotenoids in both PC1 and PC2 (1525, 1156, and 1005 cm^{-1}) (Fig. 2). For instance, at 5 °C *Arthrobacter* and *Leifsonia* displayed the highest pigment production, while at 15 °C, the highest production was observed in *Rhodococcus, Leifsonia*, and at 25 °C, in *Cryobacterium* and *Arthrobacter*.

To assess the impact of temperature on the bacterial pigment profiles, we compared spectra of bacterial biomass obtained after cultivation at different temperatures and examined the presence of shift(s) for pigment related peaks. Overall, the obtained results showed that bacteria share a common spectral fingerprint with similar pigment-specific peaks registered with different intensities (Fig. 3A). However, some genera and species exhibited varying pigment profiles detected as a shift of the peak related to the C = C stretching observed as peaks in the Raman spectrum around 1500–1550 cm^{-1} (Fig. 3B). Thus, it could be seen that based on the peak maxima of the C = C stretching vibrations in carotenoids all bacteria could be split into groups on genus level: (1) *Agrococcus, Brachybacterium, Micrococcus* and *Paeniglutamicibacter* with peak maxima at 1530 cm^{-1}; (2) *Arthrobacter, Leifsonia, Salinibacterium* with maxima of the peak around 1528 cm^{-1}; (3) *Flavobacterium, Cryobacterium, Salinibacterium* with maxima of the peak around 1525 cm^{-1}; (4) *Rhodococcus* with peak maxima at 1520 cm^{-1} and (5) unique spectra with peak maxima at 1506 cm^{-1} for *Arthrobacter agilis* BIM B-1543. Peak shifts were observed among different species within a single genus for *Arthrobacter*, *Rhodococcus, Leifsonia*, except of *Cryobacterium* (Fig. 3B). Additionally, it was noticed that temperature had a discernible effect on the peak shift for *Rhodococcus* strains (Fig. 3B).

As the next screening step, we estimated relative total content of pigments in the bacterial biomass to identify the most promising pigment-producing strains. The estimation of relative total pigment content was performed by calculating carotenoids/biomass (C/B) ratio from the FT-Raman data, which previously was proven to be an effective



Peak №	Wavenumber cm $^{-1}$	Molecular vibration
1	2800-3100	C-H str. (CH, CH_2 and CH_3) carb., lipids, proteins
2	1665	C=O str. (Amide I) proteins, C=C str. lipids
3 ν_1	1500-1550	C=C str. carotenoids
4	1452	CH_2 and CH_3 de def. lipids, proteins, carb.
5 ν_2	1100-1120	C-C str. carotenoids
6 ν_3	1000-1010	C-CH_3 str carotenoids

Fig. 1. Normalized FT-Raman representative spectra of pigmented bacteria *Cryobacterium soli* BIM B-1658 (pink) and non-pigmented bacteria *Shewanella baltica* BIM B-1557 (grey) with main peak assignments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measure [7]. Among the seventy-four studied bacterial strains, thirty-seven strains from 9 genera of Actinobacteria phylum (*Agrococcus, Arthrobacter, Brachybacterium, Cryobacterium, Leifsonia, Micrococcus, Paeniglutamicibacter, Rhodococcus, Salinibacterium*) and one genus of Bacteroidetes phylum (*Flavobacterium*) showed C/B ratio in the range of 0.5 – 10 (Fig. 4, Fig. S4 in SM). Other bacteria had C/B ratio in the range of 0 – 0.5 (Fig. 4, Fig. S4 in SM).

The highest C/B ratio was detected for all strains from genus *Cryobacterium*, for strain *Arthrobacter* sp. BIM B-1549, for all strains from *Leifsonia rubra* and *Rhodococcus yunnanensis* species demonstrated on Fig. 4. Low C/B ratio was detected for *Arthrobacter* sp., *Leifsonia antarctica* and *Rhodococcus erythropolis* species and for strains *Micrococcus luteus* BIM B-1545, and *Brachybacterium paraconglomeratum* BIM B-1571. No pigments were detected for strain *Arthrobacter oryzae* BIM B-1663, *Leifsonia kafniensis* BIM B-1633 and *Salinibacterium* sp. BIM B-1636. Bacteria belonging to Actinobacteria and Bacteroidetes phyla exhibited extraordinarily high C/B ratio indicating high pigment content in the biomass, as depicted in Fig. S4 in SM. Interestingly, while certain genera exhibited relatively similar pigment production across all species and strains (e.g., *Cryobacterium*), other genera showed species-specific variations, resulting in significant differences in the relative pigment content within a single genus, as it was observed for *Arthrobacter*, *Leifsonia* and *Rhodococcus* (Fig. 4).

Analysis of FT-Raman data of bacteria grown at different temperatures showed three main trends of the temperature influencing pigment content in bacterial biomass: (1) increase of relative pigment content with temperature increase, as it was observed for all strains from *Flavobacterium, Cryobacterium, Paeniglutamicibacter* and *Rhodococcus* genera and for majority of *Arthrobacter* sp. *and Leifsonia rubra* strains; (2) increase of relative pigment content with temperature decrease, as it was detected for *Agrococcus citreus* BIM B-1547*, Arthrobacter agilis* BIM B-1543*, Brachybacterium paraconglomeratum* BIM B-1571 and *Micrococcus luteus* BIM B-1545 strains; (3) higher relative pigment content at temperature close to optimal, as it was detected for *Arthrobacter cryoconiti* BIM B-1627, *Arthrobacter* sp. BIM B-1666 and *Leifsonia antarctica* strains. Overall, it can be noted that relative pigment content and its alteration triggered by temperature fluctuations were mainly species-specific and varied considerably (Fig. 4).

Based on the screening experiment a set of ten bacterial isolates showing relatively high pigment content was selected for detail pigment analysis, evaluation of blue light effect on pigments production and photostability testing. In addition, the selected isolates were chosen based on variations in pigments spectral profile (Fig. 3B) and different responses to temperature fluctuations (Fig. 4). The selected bacterial isolates belong to genera *Flavobacterium*, *Arthrobacter, Leifsonia*, *Rhodococcus*, *Agrococcus*, *Cryobacterium,* and *Paeniglutamicibacter* (Figs. 4 and 5) and most of them produce yellow pigments, except *Arthrobacter agilis* BIM B-1543 and *Rhodococcus yunnanensis* BIM B-1621 producing red and orange pigments respectively (Fig. 5).

3.2. *Pigments profile by HPLC-MS*

To perform detailed study of pigment profile of the selected set of pigmented Antarctic bacteria, HPLC-MS analysis was performed, and the results are presented in Table S5. HPLC-MS analysis of the extracted pigments revealed the presence of eighteen different carotenoids and their precursors in the biomass of the selected bacteria (Table S5). Mixtures of different carotenoids were found in all studied bacteria, thus from five to seven carotenoids were identified in each strain depending on the strain (Table S5). Also, precursors of carotenoids such as farnesyl diphosphate and geranylgeranyl diphosphate were detected in 5 out of 10 studied bacteria (Table S5). The following pigments were identified by HPLC-MS in the studied Antarctic bacteria: C40 (Lycopene, beta-carotene, neurosporene, zeta-carotene, phytoene, echinenone, cantha-xanthin, zeaxanthin), C45 (nonaflavuxanthin, dihy-droisopentenyldehydrorhodopin) and C50 carotenoids (flavuxanthin,

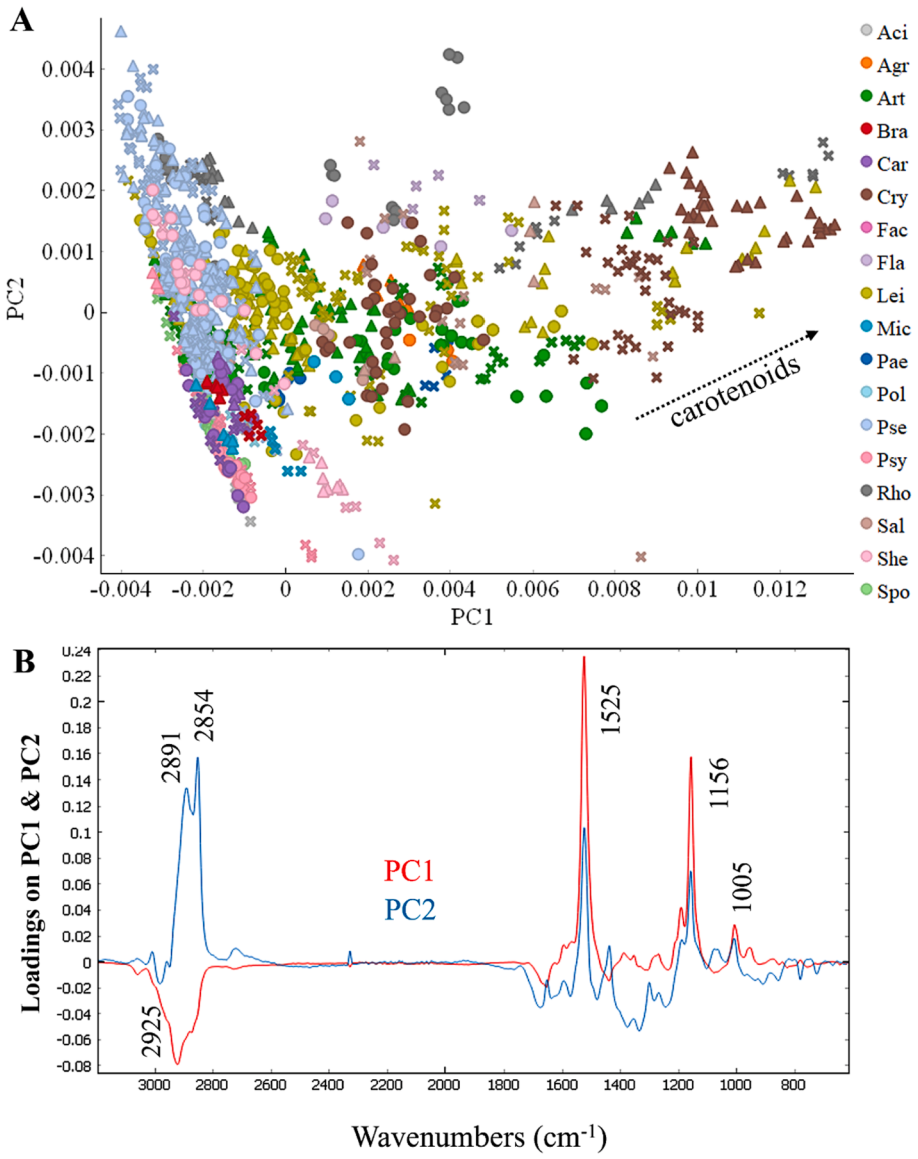


Fig. 2. Principal component analysis (PCA) of the preprocessed FT-Raman spectra of Antarctic bacteria grown at different temperatures ('●' – 5 °C, '×' – 15 °C and '▲' – 25 °C). A – Score plot of PC1 and PC2 components, different colors represent genera (Aci-*Acinetobacter*, Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Car-*Carnobacterium*, Cry-*Cryobacterium*, Fac-*Facklamia*, Fla-*Flavobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Pol-*Polaromonas*, Pse-*Pseudomonas*, Psy-*Psychrobacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*, She-*Shewanella*, Spo-*Sporosarcina*), vector indicates the direction of increasing carotenoid content within the biomass. B – Loading plot of FT-Raman data with the main contributing peaks, PC1 (red) and PC2 (blue). PC1 provided 67 % of explained variance and PC2 provided 12 % of explained variance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decaprenoxanthin, and dihydrobisanhydrobacterioruberin, monoanhydrobacterioruberin and bacterioruberin, (5Z)-bacterioruberin, (9Z)-bacterioruberin, (13Z)-bacterioruberin: 5Z.9′Z-bacterioruberin 9Z.9′Z-bacterioruberin) which were also major group of carotenoids found in these bacteria (Fig. 6A and Table S5).

Further, HPLC-MS data showed that the studied bacteria have pigments with the following amounts of conjugated double bonds (CDBs) 3, 7, 9, 11, 12, 13 and 16 (Fig. 6B, Table S5). The peak area (450 nm) representing carotenoids was used for semi-quantitative calculations based on HPLC-MS data. Thus, phytoene with 3 CDBs was detected only in one strain *Leifsonia rubra* BIM B-1567. Carotenoids with 11 CDBs were the most abundant in bacteria from genera *Arthrobacter, Leifsonia* and *Rhodococcus*. Genera *Arthrobacter* and *Paeniglutamicibacter* were characterized by the presence of carotenoids with 12 CDBs Pigments with 13 and 12 CDBs were detected in *Leifsonia antarctica* BIM B-1638 in small quantities (Fig. 6B, Table S5). Overall, pigments with 11 CDBs were the most abundant in the studied bacteria (Fig. 6B, Table S5). The unknown pigments were summed and shown (Fig. 6B, Table S5).

HPLC-MS data mapped a diversity of pigment profiles for the studied bacteria. For example, *Flavobacterium degerlachei* BIM B-1562 from Bacteroidetes phylum, showed a distinctive pigment profile characterized by the presence of C40 ehinenone, canthaxanthin, and zeaxanthin as the main carotenoids (Table S5). Controversially, bacteial strains from genus *Arthrobacter* showed C50 flavuxanthin, decaprenoxanthin, and dihydrobisanhydrobacterioruberin as the main pigments. The strain *Rhodococcus erythropolis* BIM B-1661 had C40 carotenoids canthaxanthin and zeaxanthin, accompanied by C50 carotenoids decaprenoxanthin as major carotenoids. For the strain *Cryobacterium soli* BIM B-1659, decaprenoxanthin emerged as a main carotenoid. Interestingly, carotenoid profiles of bacterial strains from genus *Leifsonia* varied significantly. For instance, strain *Leifsonia antarctica* BIM B-1638 displayed bisanhydrobacterioruberin as its primary carotenoid, while strain *Leifsonia rubra* BIM B-1567 exhibited C40 lycopene and phytoene as the main carotenoids. The strain *Agrococcus citreus* BIM B-1547

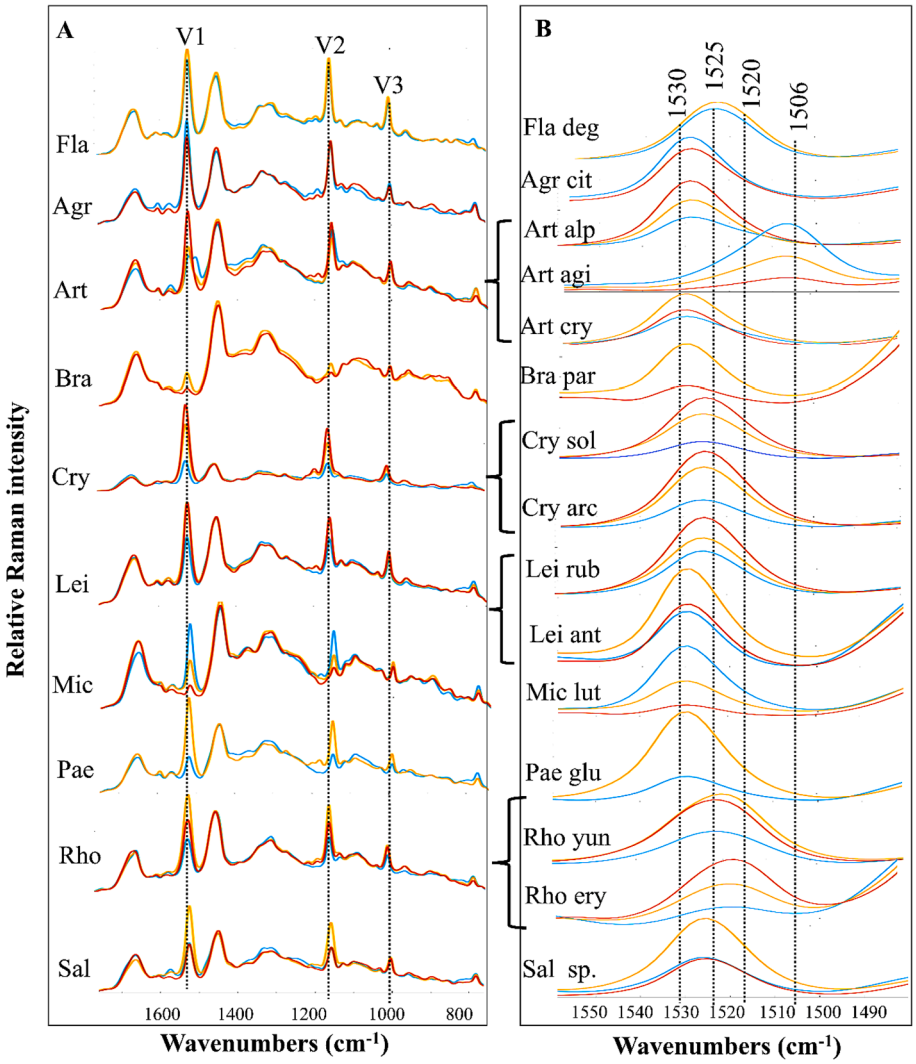


Fig. 3. A- Temperature-averaged FT-Raman spectra of bacterial biomass for different genera grown at different temperatures (blue – 5 °C, yellow – 15 °C and orange – 25 °C). B- Position of the peak related to the C = C stretching vibrations in carotenoids observed for different genera grown at different temperatures (blue – 5 °C, yellow – 15 °C and orange – 25 °C). Strains related to *Flavobacterium, Brachybacterium* and *Paeniglutamicibacter* genera did not grow at all three tested temperatures (see Table S1). Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Fla-*Flavobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium.* Spectra are vertically off-set for better visualization.

exhibited relatively low levels of carotenoids, with only C45 and C50 variants being detected. However, for certain bacterial species such as bacteria related to genera *Agrococcus*, *Paeniglutamicibacter* and strains *Arthrobacter agilis* BIM B-1543 and *Arthrobacter cryoconiti* BIM B-1627 numerous carotenoids remained unidentified, underscoring the complexity of their pigment profiles and the need for further analysis (Table S5).

3.3. Library-dependent analysis of carotenoids by FT-Raman

FT-Raman spectroscopy can be highly specific in identifying pigment-producing microorganisms and is often used for semi-qualitative screenings. We evaluated the potential of this technology to perform detailed pigment profiling of the extracted and purified pigments using reference spectral library of pigment standards. For this, eight commercially available pigment standards selected based on the HPLC-MS data were measured by FT-Raman to establish a reference spectral library. This reference library was used to analyse composition of the bacterial pigment extracts and single pigments purified by TLC and measured by FT-Raman. The recorded spectral data were analysed by PCA, and the results are presented in Fig. 7.

The PCA results revealed clear correlation between pigment standards and some bacterial extracts (Fig. 7A), where all bacterial extracts and purified pigments were grouped into several clusters along the first principal component (PC1) axis, which correlated with Raman shifts of the peak at 1500–1550 cm^{-1} related to variations in pigment structures (Fig. 7B). These peak shifts are dependent on the pigment's structure and the number of conjugated double bonds in carotenoids. The first component effectively separates all samples based on the peak position and shift between 1530 cm^{-1} and 1506 cm^{-1} (Fig. 7B).

The first well-separated cluster comprises of the extracts and purified pigments from the strains *Paeniglutamibacter antarcticus* BIM B-1657, *Agrococcus citreus* BIM B-1547, *Leifsonia antarctica* BIM B-1638, *Arthrobacter cryoconiti* BIM B-1627, and *Arthrobacter* sp. BIM B-1549 (Fig. 7A). These extracts are grouped with neurosporene standard (9 CBDs, no oxygen) and exhibit similar peak maxima around 1530 cm^{-1} (Fig. 7A, C). Interestingly, the grouping of the purified pigments from *Arthrobacter cryoconiti* BIM B-1627 (TLC fraction 3 and 5) appear slightly dislocated than neurosporene standard along PC1, suggesting the presence of other pigments with longer chain lengths of conjugated double bonds (Fig. S5). The second cluster is represented by the pigment extracts and purified pigments from *Flavobacterium degerlachei* BIM B-

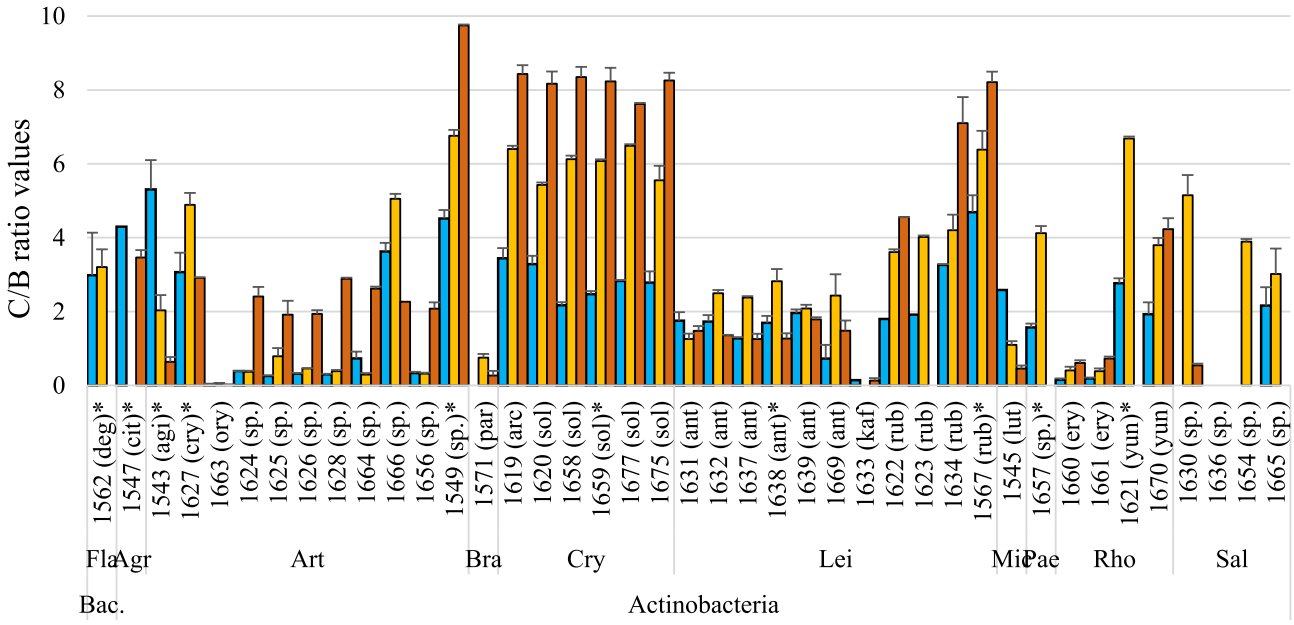


Fig. 4. Ratiometric analysis based on FT-Raman spectra of pigmented bacterial biomass obtained after cultivation at different temperatures (blue – 5 °C, yellow – 15 °C, and orange – 25 °C). The standard deviation was calculated for genera that were represented by two or more strains. Genera: Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Fla-*Flavobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*. * - strains selected for future analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

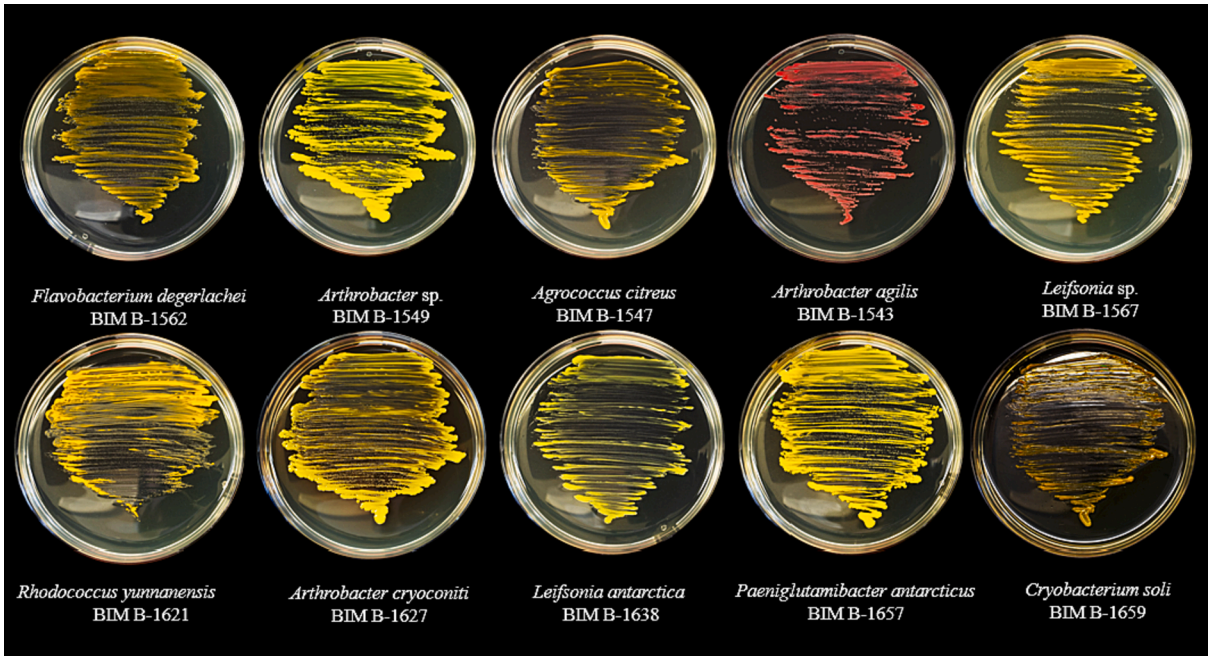


Fig. 5. Petri dish images of the strains selected for further study.

1562, *Leifsonia* sp. BIM B-1567, and *Cryobacterium soli* BIM B-1659, which are grouped with zeaxanthin (11 CBDs, oxygen) and show the same peak maxima at 1525 cm^{-1}. Notably, for *Flavobacterium degerlachei* BIM B-1562, spectra of the pigments extract and purified pigment (TLC fraction 2) are separated, indicating that the pure pigment separated by TLC differs from the extract and that the extract likely consists of several pigments with different structures (Fig. S5). For *Cryobacterium soli* BIM B-1659, it is obvious that purified pigments (TLC fractions 1 and 2) exhibit closer proximity to the extract, while fraction 3 appears separate from the rest, suggesting the presence of chemically different pigments with longer chain lengths of conjugated double bonds. Fraction 3 of *Cryobacterium soli* BIM B-1659 is located between cluster of beta-carotene/echinenone, and canthaxanthin/astaxanthin. Purified pigments from *Leifsonia* sp. BIM B-1567 are grouped together, suggesting their structural similarity, and closely grouped with beta-carotene and echinenone, which could indicate predominance of this type of carotenoid structure. The extract and TLC fractions from *Rhodococcus yunnanensis* BIM B-1621 shows presence of different pigments. Thus, the extract of all pigments is grouped together with one purified pigment (TLC fraction 3) and have the same peak maxima as zeaxanthin 1525 cm^{-1}, while other purified pigments (TLC fractions 1 and 2) are grouped distinctly and have the same peak maxima as lycopene at 1519 cm^{-1},

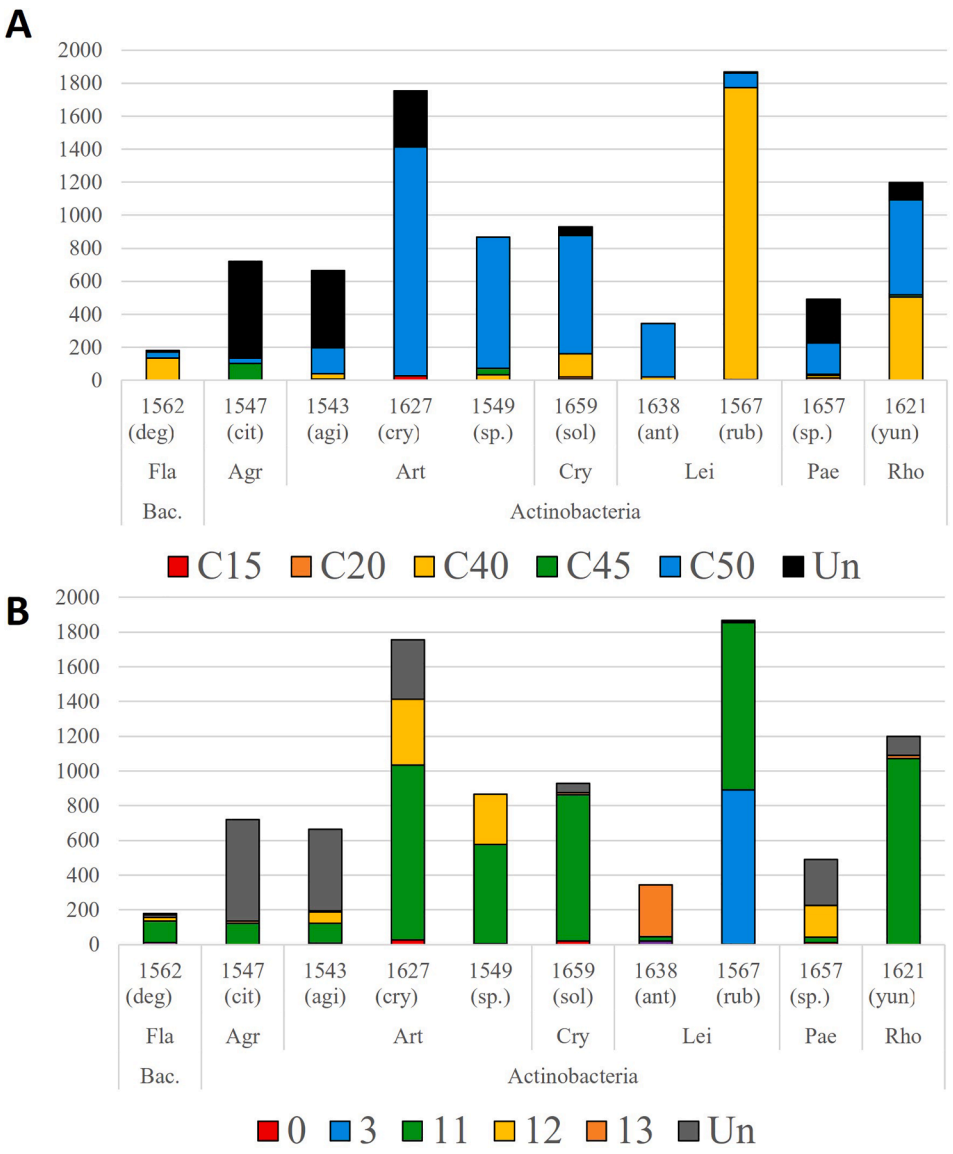


Fig. 6. Summed peak area of extracted carotenoids with different number of conjugated double bounds (A) and different chain lengths (B). Genera: Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Fla-*Flavobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*.The peak area exceeding 20 was displayed.

indicating the presence of pigments with different structures (Fig. S5A, B). The most distinct cluster is represented by the extracts from *Arthrobacter agilis* BIM B-1543, spectra of which are grouped together with spectra of bacterioruberin (16 CDBs) (Fig. S5A, C). A significant increase in the number of conjugated double bonds leads to a pronounced shift of bacterioruberin to lower wavenumbers, with maxima at 1506 cm^{-1} and 1482 cm^{-1}, distinctly separating it from all other pigments (Fig. 7A, B). PCA analysis reviled that bacterial pigment profiles are complex and contain more than one type of carotenoids. Based on the result of separation of the bacterial extract by TLC it was detected that for bacteria such as *Cryobacterium soli* BIM B-1659, *Rhodococcus yunnanensis* BIM B-1621, and *Arthrobacter cryoconiti* BIM B-1627 the extract contains several different pigments with different structure and different conjugation chain length. For bacteria *Leifsonia antarctica* BIM B-1638, *Flavobacterium degerlachei* BIM B-1562, and *Arthrobacter agilis* BIM B-1543 pigments separated by TLC are grouped together, which indicates their structural similarity.

3.4. Evaluating biotechnological potential and testing photostability

To determine biotechnological potential of the selected pigment-producing Antarctic bacteria we evaluated their biomass and pigment production under conditions triggering pigments production (blue light exposure). The experiment was performed at 15 °C since six out of ten selected strains exhibited a poor growth at 25 °C (Fig. 3). The highest biomass production was recorded for the strains *Paeniglutamicibacter antarcticus* BIM B-1657 (~5 g/L), followed by *Arthrobacter* strains (from 2 to 3.5 g/L) and *Rhodococcus yunnanensis* BIM B-1621 (up to 2 g/L) (Fig. 8). When comparing biomass production at 15 °C between control samples (no light exposure) and samples after light exposure, it was observed that exposure to blue light had a genus and species-specific impact on pigment production. Thus, for *Flavobacterium degerlachei* BIM B-1562, *Arthrobacter cryoconiti* BIM B-1627 and *Paeniglutamicibacter antarcticus* BIM B-1657 the biomass production was higher after blue light exposure (Fig. 8), while for *Agrococcus citreus* BIM B-1547, *Arthrobacter agilis* BIM B-1543, *Leifsonia rubra* BIM B-1567 and *Rhodococcus yunnanensis* BIM B-1621 the biomass after exposure was lower compared to the control samples, and for the rest of bacterial isolates no

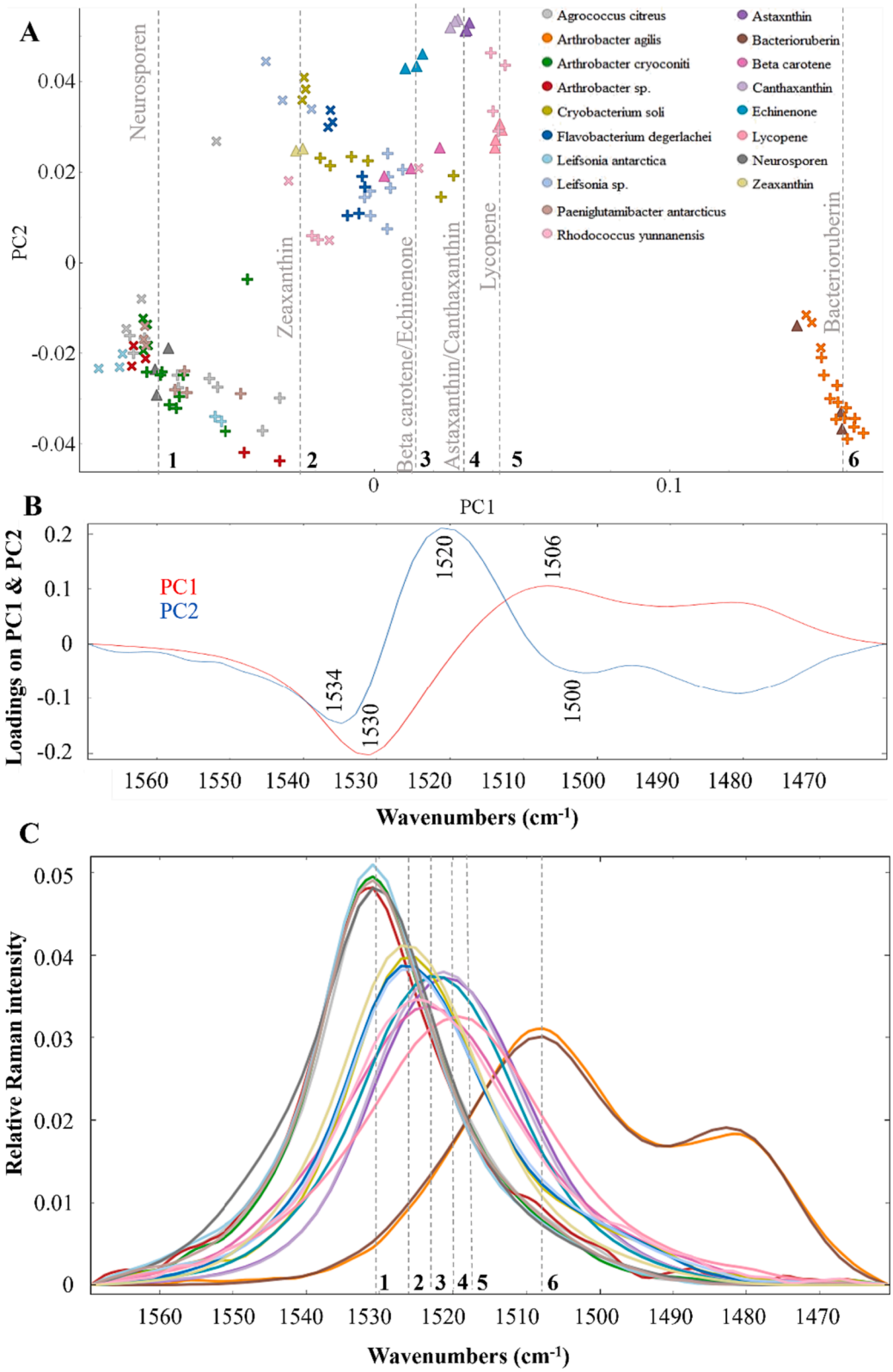


Fig. 7. A – Score plot of PC1 and PC2 components where different colors represent genera and standards from the reference spectral library and shapes represent types of the sample: '×' – bacterial pigment extracts, '▲' – pigment standards; +-purified pigments. B – Loading plot of FT-Raman data with the main contributing peaks, PC1 (red) and PC2 (blue). PC1 and PC2 provided 83 % and 12 % of explained variance, respectively. C – Raman shift between different samples in the peak between 1600 and 1500 cm^{-1} related to C = C stretching vibrations in carotenoids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

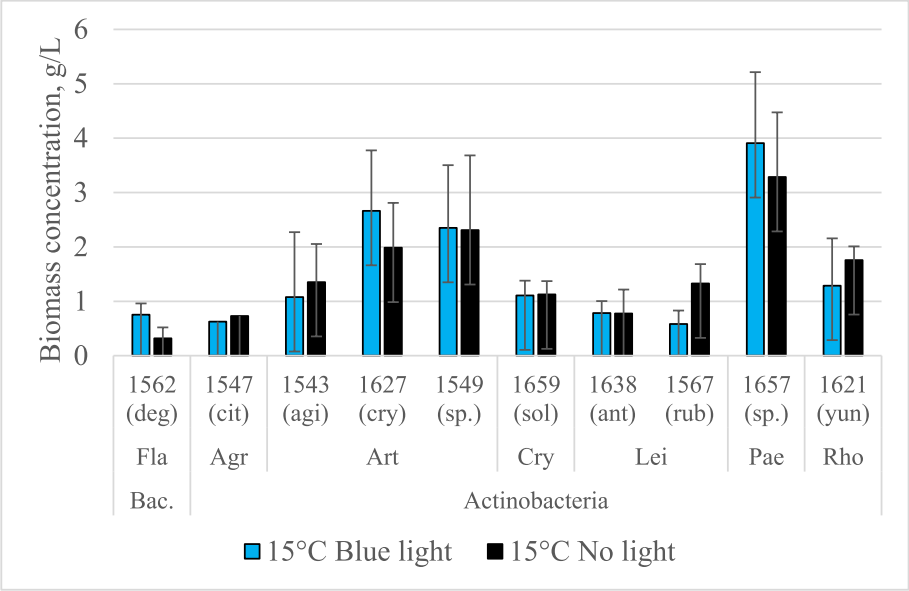


Fig. 8. Biomass production at 15 °C under the blue light exposure and no light exposure (control). Genera: Fla-*Flavobacterium*, Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

light effect was detected (Fig. 8).

In addition to biomass production, the effect of blue light on pigment production at 15 °C was evaluated using FT-Raman spectroscopy, specifically by quantifying the C/B ratio of the biomass spectra (Fig. 9). For *Flavobacterium degerlachei* BIM B-1562, *Agrococcus citreus* BIM B-1547, *Arthrobacter agilis* BIM B-1543, *Cryobacterium soli* BIM B-1659, *Leifsonia antarctica* BIM B-1638, *Paeniglutamicibacter antarcticus* BIM B-1657 and *Rhodococcus yunnanensis* BIM B-1621 the C/B ratio was higher when the strains were exposed to blue light, indicating that these strains exhibited light-induced production of carotenoids (Fig. 9). The biggest effect of blue light exposure was detected for *Cryobacterium soli* BIM B-1659, *Agrococcus citreus* BIM B-1547 and *Flavobacterium degerlachei* BIM B-1562 (Fig. 9).

To assess the photostability of selected bacteria, freeze-dried biomass was exposed to light at an illuminance value of 900 lx for 60 h, covering a spectral range from 300 to 2500 nm. To determine the photostability of the pigments, carotenoids/biomass (C/B) ratio was calculated. The results revealed a pronounced pigments photodegradation effect in the pigmented bacterial biomass after exposure to light leading to a noticeable decrease in the C/B ratio (color bars) compared to the control samples without light exposure (white bars) (Fig. 10A). Thus, *Flavobacterium degerlachei* BIM B-1562, *Arthrobacter* sp. BIM B-1549, *Rhodococcus yunnanensis* BIM B-1621 and *Leifsonia rubra* BIM B-1567, demonstrated a notably higher rate and speed of photodegradation after light explosion compared to other bacterial strains, thus the C/B ratio decreases fast especially during first 20 h of light exposure (Fig. 10A, B). Interestingly, these bacteria have short C40 carotenoids as prevalent. The lowest degradation rate of the pigments was observed for *Arthrobacter agilis* BIM B-1543, *Arthrobacter cryoconiti* BIM B-1627, and *Paeniglutamibacter antarcticus* BIM B-1657 which have C50 carotenoids as predominant. The fluctuations in the control samples were insignificant during the first 5 h of exposure and remained stable thereafter. After 60

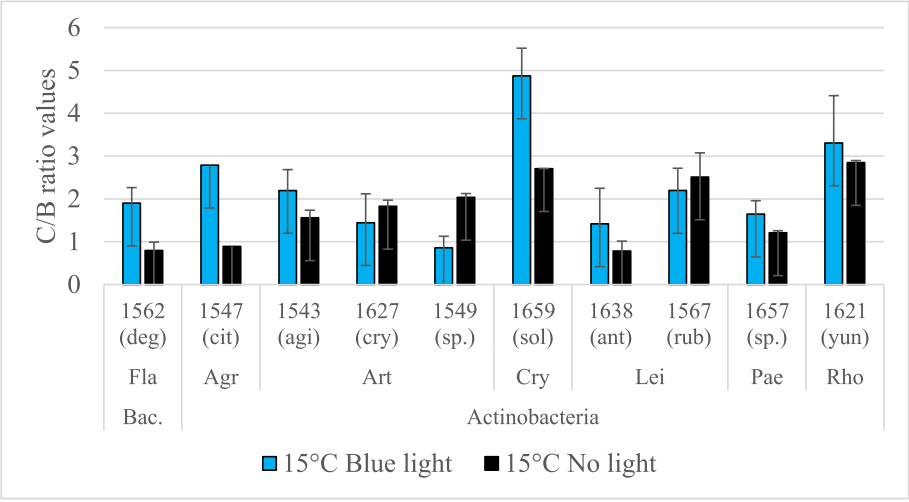


Fig. 9. Relative pigment amount at 15 °C under the blue light exposure and no light exposure (control) measured by quantifying the C/B ratio of the freeze-dried biomass. Genera: Fla-*Flavobacterium*, Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

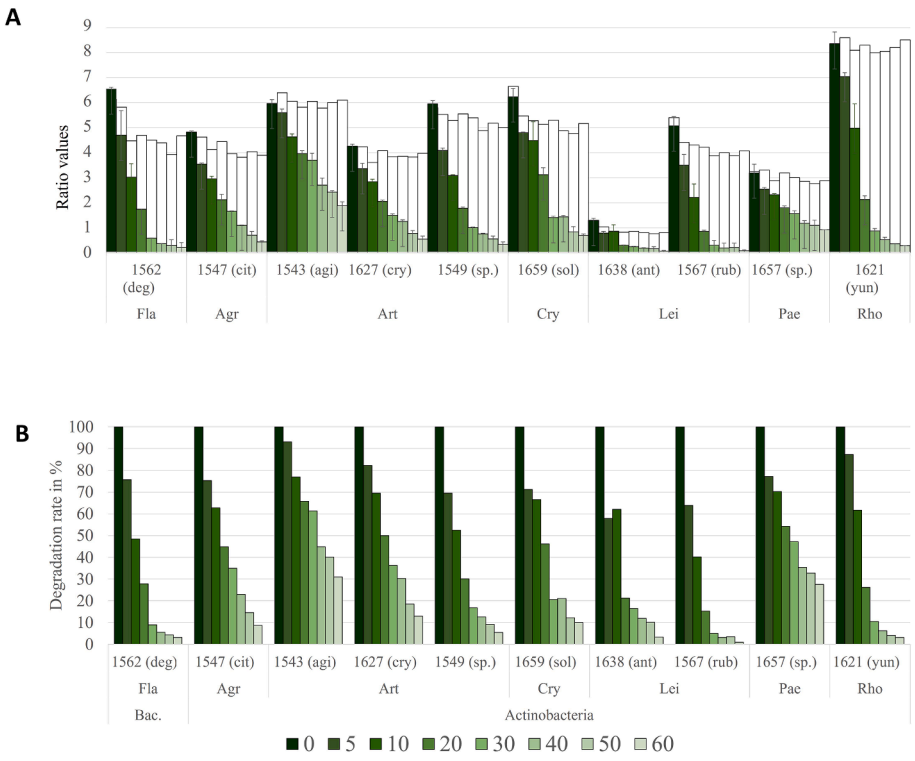


Fig. 10. A-Ratiometric analysis and B- Ratiometric analysis normalized by time 0 of FT-Raman spectra of the freeze-dried bacterial biomass exposed to light for 5, 10, 20, 30, 40, 50, 60 h, Colors represent exposure time, white color represent control samples. Genera: Fla-*Flavobacterium*, Agr-*Agrococcus*, Art-*Arthrobacter*, Bra-*Brachybacterium*, Cry-*Cryobacterium*, Lei-*Leifsonia*, Mic-*Micrococcus*, Pae-*Paeniglutamicibacter*, Rho-*Rhodococcus*, Sal-*Salinibacterium*.

h of light exposure, *Arthrobacter agilis* BIM B-1543 and *Paeniglutamicibacter antarcticus* BIM B-1657 exhibited the most promising results in terms of pigment stability. No peak shift or change in the profile was detected with FT-Raman spectroscopy for the samples exposed by light (Fig. S6).

4. Discussion

In this study we performed a high-throughput screening and comprehensive characterization of the relative pigment content and profile for seventy-four Antarctic bacteria isolated from green snow and temporary meltwater ponds. According to FT-Raman analysis, majority of the studied bacteria identified as pigmented were able to produce carotenoids. Thirty-seven strains from nine genera *Agrococcus, Arthrobacter, Brachybacterium, Cryobacterium, Leifsonia, Micrococcus, Paeniglutamicibacter, Rhodococcus,* and *Salinibacterium* and *Flavobacterium* displayed relatively high total pigment content, as indicated by FT-Raman data analysis. Strains from many of these genera have been previously reported as pigment producing [18,43,28,33]. Semi-quantitative analysis based on the calculation of ratio of carotenoids versus proteins (C/B) using FT-Raman spectra showed that bacteria from genera *Arthrobacter, Cryobacterium, Leifsonia* and *Rhodococcus* have the highest total pigment content. Important to note, while several studies previously reported pigment analysis of Antarctic bacteria biomass, many bacterial species characterized in the study have not been previously analysed for pigment production.

Carotenoids protecting bacterial cells against the harmful effects of solar radiation by preventing DNA damage, countering reactive oxygen species formation, and modulating membrane fluidity in cold environments like Antarctica [36]. A strong influence of temperature and UV radiation on bacterial pigment production was reported previously [27]. We observed that changes of the relative pigment content (C/B ratio) triggered by temperature are mainly species specific, and it can vary considerably. Temperature triggered changes in pigment profile, estimated by analyzing presence of peak shifts, varied between different genera while it was conserved within single species. This indicates that temperature-triggered metabolic cell responses associated with the pigment profile changes can be conserved within a single genus. This was not reported previously, and more research would be needed involving large and balanced set of bacteria. For most of the strains increase in pigment production with temperature increase was detected as it was shown for *Flavobacterium, Arthrobacter* sp., *Cryobacterium, Leifsonia rubra, Paeniglutamicibacter* and *Rhodococcus* and for few strains such as *Arthrobacter agilis* BIM B-1543 and *Micrococcus luteus* BIM B-1545 an opposite effect of decrease in pigment production at elevated temperature was detected that possibly connected to the regulation of cellular membrane fluidity at low temperatures [9].

The pigment profiles obtained by HPLC-MS for many Antarctic bacteria were consistent with the published results in the literature. Among all carotenoids identified in the Antarctic bacteria, six are regarded as industrially significant - astaxanthin, β-carotene, canthaxanthin, lutein, lycopene, and zeaxanthin [22]. Thus, a presence of C40 ehinenone, canthaxanthin, and zeaxanthin, beta-carotene in *Flavobacterium degerlachei* BIM B-1562 recorded in our study was in accordance with the previously reported for *Flavobacterium frigidarium* [11] *Flavobacterium* sp. [43], while production of flexirubin was not detected in our study [41]. *Flavobacterium* has been mentioned in the literature as a promising producer of zeaxanthin with productivity 500 mg/L [26]. For *Arthrobacter* bacteria, similar profile of C50 carotenoids (flavuxanthin, decaprenoxanthin, and dihydrobisanhydrobacterioruberin) was reported earlier [2,43]. Interestingly, bacterioruberin detected in *Arthrobacter agilis* BIM B-1543 was previously reported for other *Arthrobacter agilis* strains [9] was not identified by HPLC-MS but identified by FT-Raman using reference spectral library. For *Cryobacterium soli* BIM B-1659 production of lycopene and decaprenoxanthin were detected similarly as in previous studies [43]. Our study for the first-time reports production of C40 canthaxanthin and zeaxanthin and C50 decaprenoxanthin for *Rhodococcus erythropolis* BIM B-1661. For

some bacterial strains, such as *Paeniglutamicibacter antarcticus* BIM B-1657, *Arthrobacter agilis* BIM B-1543 and *Arthrobacter cryoconiti* BIM B-1627, several pigments were unidentified by HPLC-MS. This can be due to that some carotenoids are highly instable [36].

Evaluation of biomass productivity under the blue light exposure triggering pigment production showed that *Paeniglutamicibacter antarcticus* BIM B-1657 and *Cryobacterium soli* BIM B-1659 could be promising strains to explore further for the production of lycopene and dihydrobisanhydrobacterioruberin. Lycopene is a widely used food industry pigment with growing interest due to its health benefits, including antioxidant, anti-cancer, and cardioprotective properties [22]. The biomass production for pigment producing strains reported in the literature is in a range from 3 to 12 g/L and the carotenoid content ranged from 0.4 mg/g to 7.4 mg/g or from 0.3 mg/L to 500 mg/L [15,26]. The primary objective of this study was to conduct a high-throughput screening and characterization of the pigment producing Antarctic bacteria, while the proper quantification of carotenoids content, aside the rough estimate by C/B ratio, was not performed. Also, it is important to note that cultivation conditions used in the study are not optimized for pigments production, and further studies need to be performed for optimizing cultivation parameters and media composition. While BHI broth medium has been used as a production medium in some previous studies for canthaxanthin production by *Gordonia jacobea* MV-26 [42].

In this study, blue light exposure triggered pigment production in all studied pigment producing strains. This phenomenon had previously been reported for gram-positive Actinobacteria from genera *Arthrobacter*, *Leifsonia*, *Paeniglutamicibacter* [38], *Rhodococcus* [8] and *Flavobacterium* [19]. It was also shown for *P. aeruginosa* NR1 that no light, red, and blue light are optimal conditions for maximizing extracellular pigment production, whereas yellow and green light are favorable for achieving the highest biomass and intracellular pigment production [27]. To the authors knowledge, light-inducible pigment production observed for *Agrococcus* and *Cryobacterium* is reported for the first time.

Carotenoids are chemically unstable molecules due to their high degree of unsaturation, causing oxidation as a primary cause of their degradation. Additionally, external factors like temperature, light, or pH can trigger significant qualitative transformations in carotenoids through isomerization reactions [51]. Photostability is a critical factor in determining the suitability of carotenoids as photosensitizers for use in solar cells. The conventional method for assessing the photostability of bacterial pigments involves pigment extraction and measuring absorbance decay at various time intervals during light exposure [23]. In our research, we, for the first time, used FT-Raman spectroscopy to directly evaluate the photostability of bacterial pigments in intact freeze-dried biomass, eliminating the need of pigment extraction. The obtained results showed that bacterial biomass containing mainly C40 carotenoids was less photostable than biomass with C50 carotenoids. The highest photostability was observed for *Arthrobacter agilis* BIM B-1543 producing red pigment bacterioruberin, *Arthrobacter cryoconiti* BIM B-1627, and *Paeniglutamibacter antarcticus* BIM B-1657. The higher photostability of the red pigments was previously shown [23]. The experimental conditions described in the paper were quite severe, and we anticipated relatively rapid degradation. The Antarctic bacteria were subjected to elevated light stress, which is a common occurrence during the Antarctic summer, marked by intense UVB and UVA radiation.

In this study we demonstrated exceptionally versatile potential of FT-Raman spectroscopy for pigment analysis of microbial biomass. Fourier-transform Raman spectroscopy (FT-Raman) has recently emerged as a promising technology for the characterization of bacterial pigments. FT-Raman spectroscopy offers non-destructive analysis, without extensive sample preparation, making it valuable for rare or limited samples. Additionally, it can be conducted in a high-throughput manner, allowing for the screening of many isolates. Compared to conventional (dispersive) Raman measurements, FT-Raman spectroscopy uses high-wavelength near infrared (NIR) laser excitation, thus diminishing risk of detrimental effects, such as sample heating, photodegradation and strong fluorescence [14]. Even notwithstanding detrimental effects, FT-Raman spectra are often superior to the corresponding spectra obtained by dispersive Raman spectrometers since, in the FT-Raman spectra, carotenoid bands are not completely obscuring signals of proteins, carbohydrates and other compounds [14]. Notably, FT-Raman spectroscopy has been used to build databases of Raman spectra for various bacterial pigments, facilitating rapid and reliable characterization of pigmented microorganisms [13]. Carotenoids stand out as remarkable pigments in Raman spectroscopic analysis of microbial communities since resonance Raman effect enables measurement of very low concentration of these pigments [13]. In this study we showed that FT-Raman allows to (i) perform large high-throughput library-independent screenings to identify pigment producing microorganisms, (ii) estimate relative total pigment content, (iii) determine pigment profile when reference spectral libraries are available, (iv) determine temperature and light effect on pigments production, (v) determine photostability of the pigments in intact biomass.

5. Conclusion

This study showed a high relevance of screening and studying polar bacteria for identifying new potential pigment producers. More than half of the studied Antarctic bacteria were able to produce pigments and many of them of high industrial importance. Some Antarctic bacteria such as *Leifsonia, Cryobacterium, Flavobacterium* and *Rhodococcus* exhibited the high levels of pigment content achieved under non-optimized cultivation conditions. In addition, in this study we demonstrated that FT-Raman spectroscopy is truly powerful analytical tool for both semi-qualitative screenings and descriptive analysis of pigmented microorganisms.

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CRediT authorship contribution statement

Volha Akulava: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Dana Byrtusova:** Investigation, Writing – review & editing. **Boris Zimmermann:** Conceptualization, Methodology, Validation, Visualization, Writing – review & editing. **Margarita Smirnova:** Investigation, Writing – review & editing. **Achim Kohler:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Uladzislau Miamin:** Supervision, Writing – review & editing. **Leonid Valentovich:** Supervision, Writing – review & editing. **Volha Shapaval:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotochem.2024.115461.

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