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Carotenoid pool of extremophilic bacterium *Deinococcus radiodurans*

Bachelor thesis

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Annotation

The thesis focuses on the carotenoid composition of the extremophilic bacterium *D. radiodurans*. The practical part of the thesis was focused on the composition of the carotenoid pool and the identification of the respective peaks of the chromatogram. Lastly, the influence of the age of the culture and exposure of the UV light was studied and evaluated.

Keywords: *Deinococcus radiodurans*, bacteria, carotenoids, HPLC.

Affirmation

I hereby declare that I have worked on my bachelor thesis independently and used only the sources listed in the bibliography. I hereby declare that in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in the publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abstract

The thesis focuses on the carotenoid composition of the extremophilic bacterium *Deinococcus radiodurans*. In the theoretical part of the thesis *Deinococcus radiodurans* and its survival strategies, especially the carotenoid composition and their arrangement in the cell are studied from available publications. In the practical part, the thesis focuses on the study of the carotenoid composition, influenced by different factors. The carotenoid composition was mainly studied using HPLC using the tertiary gradient designed by Jeffrey et al. (2005) method mainly due to its good suitability for the separation of carotenoids and its use in previous publications. Development of the carotenoid pool over the ageing of cultures, the effect of UV irradiation and comparison of liquid versus solid medium was performed. An attempt was also made at identification of deinoxanthin derivatives using mass spectrometry.

Table of content

Introduction

Deinococcus radiodurans

Deinococcus (D.) *radiodurans* is classified as a member of the Deinococcae family within the Deinococcus- Thermus group. [1] *Deinococcus* lineage includes mesophilic, cryophilic, and thermophilic bacteria. *Deinococcus* bacteria are heterotrophic bacteria living in a rich organic material. All *Deinococcus* species are specific by their ability to survive lethal doses of DNA damaging agents such as gamma-radiation, UV light or desiccation. [2]

General characteristics of *D. radiodurans*

The first isolation of *Deinococcus radiodurans* was achieved from gamma-irradiated canned meat by Anderson et al. in 1956. [3] The genome of *D. radiodurans* has 3.28315 Mbp according to GenBank and codes for 3012 proteins. [4] The genome of *D. radiodurans* consists of two chromosomes, one megaplasmid, and one small plasmid. Genes coded on Chromosome II and the megaplasmid are responsible for the ability to survive long periods of desiccation and starvation. Megaplasmid gives *D. radiodurans* the ability to repair DNA damage. [5] Genes in megaplasmid are predicted to be obtained from horizontal gene transfer from archaea and eukarya. [6] *D. radiodurans* also contains more than one copy of its genome per cell, but no correlation between the number of genome copies and radioresistance has been found. [7]

D. radiodurans is classified as a Gram-positive bacterium based on the Gram staining reaction but resembles Gram-negative bacteria due to its multilayered structure of the cell and lipid composition. [8] *D. radiodurans* is a non-photosynthetic, polyextremophilic, coccal bacterium, which is not motile and does not produce spores. It can exist as one cell or dyads or tetrads, depending on the age of culture. [9,10] It is an easily cultured, chemoorganothrophic proteolytic and aerobic organism, which forms smooth, convex colonies of pink, red and orange colour. It can survive extreme conditions, due to repairing mechanisms of DNA. If laboratory cultured, TGY (casein, yeast extract, glucose, pH~7.00) medium is used and cultured at around 36°C. [11,12] Populations of *D. radiodurans* are considered to form only a minority of the microbiome of the place, compared to other bacteria occupying the same ecosystem. *D. radiodurans* small populations live in the most extreme environments, but the natural habitat of *D. radiodurans* is not known. The *D. radiodurans* is a small part of the microbiome probably because most bacteria are growing faster and invest more into faster and efficient growth and cellular division, but on the other hand, *D. radiodurans* invests more into survival of the cell

and robustness of the cell. So, *D. radiodurans* dominates the microbiome population only in extreme environments like desert, where other bacteria are growing at high rates, but also dying at high rates. As *D. radiodurans* is growing slowly but maintaining viability. [5,10,12]

Abilities of *D. radiodurans* for survival extreme conditions

The name of the species derives from the ability to survive doses of ionizing radiations. For example, high doses of radiation up to 5000 Gy are withstood without loss of viability and can survive up to 20 kGy. [11,12] Compared to *E. Coli*, which couldn't survive an acute dose of 2000 Gy and loses viability at 1000 Gy, *D. radiodurans* needs 10 times higher dose radiation for sterilization of the plates and able to survive 20 times more UV light exposure to *E. coli*. Similarly, the spores of *Bacillus* are not able to survive 10000 Gy and loses the viability of the cell with radiation of 2000 Gy. Most bacteria cannot survive an acute dose of 200 Gy and humans can survive only an acute dose under 10 Gy. [6,13] This resistance to ionizing radiation is predicted to be primarily an evolutionary adaptation to desiccation and UV-light exposure as there is no habitat on the earth, which has as high radiation as this. This prediction is sensible, as other unrelated organisms have resistance to desiccation and radiation, but the resistance is not as extreme. [12]

D. radiodurans is also resistant to damage caused by desiccation and UV light exposure. But the combination of low temperatures (~26°C) and high humidity (~80%) leads to cell inactivation of cells and the slow death of cultures, also a frequent significant change in humidity leads to the slow death of culture. [14] Radiation, UV light, oxidative agents or desiccation leads to similar effects on the DNA. The agents are breaking the double-strands and cause oxidative stress damage. [12] The DNA damage can be direct or indirect. As the DNA can directly absorb the UV light, the UV light can trigger changes in DNA structure. The indirect DNA damage is caused by reactive oxygen species (ROS). ROS can be generated after UV light or radiation exposure, then the ROS trigger the changes in the DNA sequence. The UV light has three subtypes, UVA, UVB and UVC. The UVA light causes only indirect damage, but the UVB and the UVC can cause both direct and indirect DNA damage. [15]

Mechanisms of protection of *D. radiodurans*

Reactive oxygen species and their markers

ROS are capable of damaging DNA, proteins or other cell components. As ROS can be classified as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and superoxide anion (O_2^-). These can act from outside of the cell but can be also the product of cell metabolism. [16,17]

Protection from ROS is mostly focused on proteins, as if they are protected from oxidative damage, they maintain their activity and cell is maintaining their viability. Protein carbonylation is a good marker for irreversible protein damage, which can be then used for comparison of the effectiveness of protein protection. [18]

Protection against ROS

One of the reasons why *D. radiodurans* has high viability is the efficient Reactive Oxygen Species scavenging system. This system could be divided into enzymatic and non-enzymatic parts. The enzymatic part consists of superoxide dismutase (SOD), catalase (CAT), and organic hydroperoxide resistance protein (OHRP), which serve as free radical scavengers. [6,19] Catalases activity is significantly higher in *D. radiodurans* than in non-extremophilic bacteria. For instance, in *E. coli* the catalase activities are 32 times lower than in *D. radiodurans.* There are three types of catalases, four types of superoxide dismutase, which are mostly dependent on metal cations (Mn and Zn/Cu dependent) and two types of peroxidases. This combination of enzymes gives *D. radiodurans an* efficient molecular setup to destroy ROS.

Of the non-enzymatic part, the most important constituents are Pyrroloquinoline–quinone (PQQ), which works as an efficient antioxidant. The PQQ is an important redox co-factor of many enzymes, plays important role in the metabolism of some amino acids and shows high antioxidizing activity. [20] Further manganese cations, which are proposed to combat oxidative stress in bacteria, are found in *D. radiodurans* in the concentration up to 4 mmol/L, which is 15-150 times higher than in most radiosensitive bacteria. [21,22] The small molecules as the complexes of the Mn^{2+} ions bound to peptides, inorganic phosphates, nucleosides, nucleotides, amino acids, which have a higher concentration in the cell envelope have radioprotective and ROS scavenging properties. Those molecules successfully protected *Bam*HI *in vitro* from radiation. [27] *D. radiodurans* has also high manganese and iron ratio, which is 0.24. The relatively high manganese levels are contributing to interchanging iron in Fe-loaded enzymes and thereby protecting the cell from Fenton reaction and oxidative damage. [22] Also, carotenoids are efficient scavengers of ROS. [23]

D. radiodurans is sensitive to different types of antibiotics: antibiotics inhibiting RNA synthesis, antibiotics inhibiting protein synthesis and antibiotics inhibiting cell wall synthesis. [25]

All of the above-mentioned mechanisms are most probably evolved gradually by gene duplication and horizontal gene transfer. Most of the genes responsible for the protection

mechanisms were obtained from archaea or eukaryote and are parts of the DNA repairing mechanism. [6]

Other mechanisms of protection

D. radiodurans has similar metabolic defects, which to some extent acquire radioresistant *Salmonella enterica* serovar Typhimurium, which arises after laboratory exposures of the cultures to multiple cycles of ionizing radiation. [26] As an inability to use TCA cycle products and nucleosides as carbon sources and the inability to synthesize NAD. These inabilities may lead to the aggregation of nucleosides, TCA cycle products, and NAD precursors. Which then help speed up the reparation process of the cell, especially the reparation of the DNA. [19,26]

All of the above-mentioned molecules are expected to create high osmotic pressure, which can be held only by a thick cell envelope as found in *D. radiodurans*. These thick envelopes walls can contribute to better fighting oxidative stress. [27] The thickness of the cell envelope of *D.radiodurans* is 150 nm and consists of several different layers: cytoplasmic membrane, a rigid peptidoglycan layer, compartmentalized layer, interior layer and S-layer. [10,28,29] Compared to *E. coli* which has a thickness of cell envelope of 28.7 nm [30] and the extremophile *Halococcus morrhuae* which has 50 – 60 nm thick cell envelope, [31] *D. radiodurans* has 50 times thicker envelope than *E. coli* and 3 times thicker envelope than *Halococcus morrhuae.* The cell wall of *D. radiodurans* is 70 nm thick, compared to E. Coli, which is 4 nm. [32] The above information may lead to the suggestion that more extremophilic bacteria have thicker cell envelopes than non-extremophilic ones and that a higher cell envelope may have the ability to protect the cell from ROS or radiation.

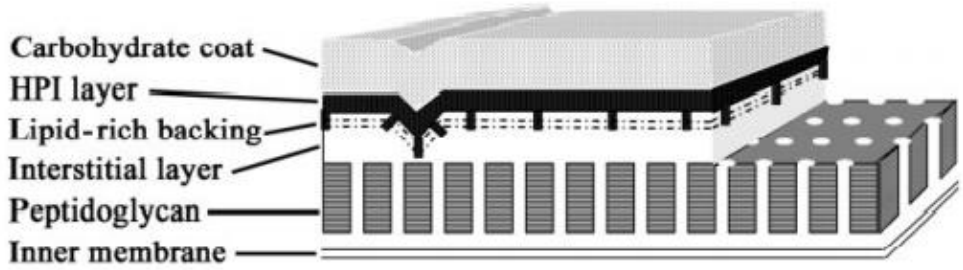

Fig. 1 – Predicted cell wall of *D.radiodurans* [33]

Exponential phase cells of *D. radiodurans* contain electron-dense particles most likely storing polyphosphate. [34] Polyphosphate particles are packed with high molecular weight linear phosphates, which can serve as a source of energy to synthesize ATP, as material for phosphorylation of various molecules as well as for chelation of metals (ex. Ca^{2+}). In

D. radiodurans polyphosphates may operate as orthophosphate which co-act with Manganese cations against ROS. Those polyphosphates most probably do not cooperate to resistance, but most probably help the cell with regeneration after some possible damage. [26] Resistance against ionizing radiation depends on various physiological conditions such as pH, age of culture, growth medium, irradiation conditions. [35] The recovery of cells from high amounts of radiation or oxidative stress is dependent on nutrients, which are needed for generating manganese complexes and manganese dependant enzymes crucial for recovery. [19,27]

Surface layer in *D. radiodurans*

One more possible mechanism of protection against UV, radiation and desiccation in D. *radiodurans* are surface layers. S-layers have many functions from already mentioned protection against environmental influences to cell adhesion to maintaining cell rigidity. S-layers represent about 10-15% of the total protein mass in the bacterial cell. [39] These S-layers consist of proteins associated to the external side of the cell wall and in the case of *D. radiodurans,* the protein binds the carotenoid - deinoxanthin. The proteins which *D. radiodurans* S-layer consists of are HPI – hexagonal packed intermediate, which forms the pores, by connecting its protein monomers to hexameric structure [40] second component is the protein DR_2577 also known as SlpA which is predicted to be important in S-layer organization. [33] The protein SlpA was shown to easily form stable dimers through disulphide bonds. These dimers then form hexamers and with the combination of hexameric DR_2508 also known as HPI and dodecameric DR_0774 channel form a stable S-layer. HPI and DR_0774 form pore structure and the inner pore structure is composed by SplA. [41] An important role in the assembly of the S-layer is played by metal ions, especially in oligomerization and monomerization. The most important role have Cu^{2+} and Fe^{3+}. [42] Protein SlpA is a carotenoid binding protein. It was proposed that the carotenoids of *D. radiodurans* exhibit unusually increased UV absorption and directly serve in UV protection. [43]

Carotenoids

Carotenoids are hydrophobic organic pigments, which play role in different metabolic pathways such as photosynthesis, photoprotection, cell signalling. In photosynthetic organisms carotenoids serve as accessory pigments in light-harvesting complexes, where they increase energy absorption. Carotenoids in photosynthetic organisms also serve as protectors against some intermediate products of photosynthesis and ROS.

From the chemical perspective carotenoids are polyisoprenoids, consisting of C_5 isoprene units joined in a head to tail pattern. Carotenoids are classified into two groups, carotenes, and xanthophylls, according to their chemical composition. Carotenes are chemically classified as hydrocarbon carotenoids and xanthophylls are classified as carotenes possessing one or more oxygen group (ex. hydroxyl group, keto group, ...). [44] Terminal isoprenoid groups often create on the sides of the molecule's six-carbon ring. Three types of these rings exist, and they are labelled with letters from the Greek alphabet. The last linear isoprene unit is labelled as ψ. Carotenoids are characterized by a conjugated system of double bonds with delocalized π-electrons. [45] This system is responsible for pigment properties. The strongly allowed S_0-S_1 electronic transition is responsible for the absorption of light in 300–600 nm and therefore carotenoids are intensely coloured as yellow, orange, or red. [44] The main part of bacterial pigments makes pigment-protein complexes, where pigment is non-covalently bound to the protein. In non-photosynthetic bacteria like *D. radiodurans*, they function as reactive oxygen species scavenging system for cell protection. Carotenoids can degrade under certain conditions i.e. temperature, heavy metals, and other elements, light or oxidants. If carotenoids degrade, their functional group is oxidized or cleaved off, eventually, aliphatic residua are cleaved off with the functional group. The next option is that if carotenoids degrade their structure disintegrate into original C_5 isoprene units.

Carotenoids are able to quench singlet oxygen similarly to tocopherols. Carotenoids and tocopherols are efficient antioxidants and capable of scavenging reactive oxygen species during oxidative stress. [47] As ROS scavengers carotenoids protect cells from DNA damage, protein carbonylation and oxidation of lipids in membranes. [9,49] Carotenoids are also efficient in removing reactive nitrogen species (RNS) such as 2,2-diphenyl-1-picrylhydrazyl. [48]

Carotenoids are able to scavenge radicals in three main ways:

Examples of the reactions with lipid peroxyl radical ($ROO^{\cdot}$)

$$CAR + ROO^{\cdot} \rightarrow ROO - CAR^{\cdot}$$

Forming the resonance stabilized carbon-centred radicals - addition reaction

$$CAR + ROO^{\cdot} \rightarrow CAR^{\cdot +} + ROO^{-}$$

Forming stable radical cations - electron transfer reaction

$$CAR + ROO^{\cdot} \rightarrow CAR^{\cdot} + ROOH$$

Allylic hydrogen abstraction and electron shift - hydrogen atom transfer. [50]

Deinoxanthin

Deinoxanthin is a keto-carotenoid that plays role in *D. radiodurans*, because of strong ROS - scavenging activity, antioxidant activity. It was proven that deinoxanthin has higher scavenging activity against hydrogen peroxide and singlet oxygen than lycopene, β-carotene, zeaxanthin, and lutein. Deinoxanthin has also protective effects on DNA, exhibited approximately 4-fold higher protection of DNA in vitro from hydroxyl radicals than β-carotene. [35] Protective effects on proteins were also recorded. Purified deinoxanthin has a 1-fold higher inhibition of oxidative protein damage than lutein. This means that the inhibition is two times higher. [48]

It was demonstrated that the surface layer protein DR_2577, also known as SlpA binds deinoxanthin in *D. radiodurans*. [43] This protein is ordered in stable hexamers by binding Cu^{2+} or Fe^{3+} ions. Protein hexamer is responsible for S-layer integrity and gives a possible binding site for the deinoxanthin carotenoid, combination of deinoxanthin and the hexamer of the protein DR_2577 is called S-layer deinoxanthin binding complex - SDBC. This protein complex is responsible for UV light resistance, thermostability and resistance to desiccation.

Recently, it was shown that the deinoxanthin pool bound to SDBC consists of several derivatives of the carotenoid. These modifications are most probably deinoxanthin glucoside and 2-deoxydeinoxanthin, and as possible to see in fig. 2, 3 and 4 they have the same carotenoid backbone, it could be predicted that they have similar spectroscopic properties. This also led to the suggestion that SDBC can bind three analogues of deinoxanthin and that SDBC has non-specific binding sides. [50]

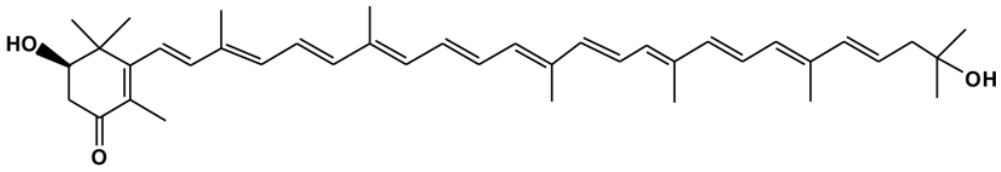

Fig. 2 – Structural formula of Deinoxanthin

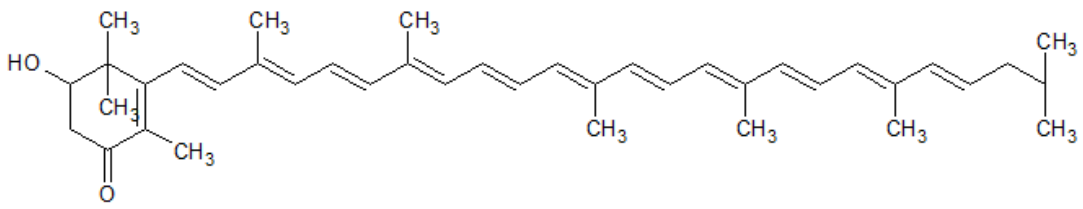

Fig 3 – Structural formula of 2-deoxydeinoxanthin

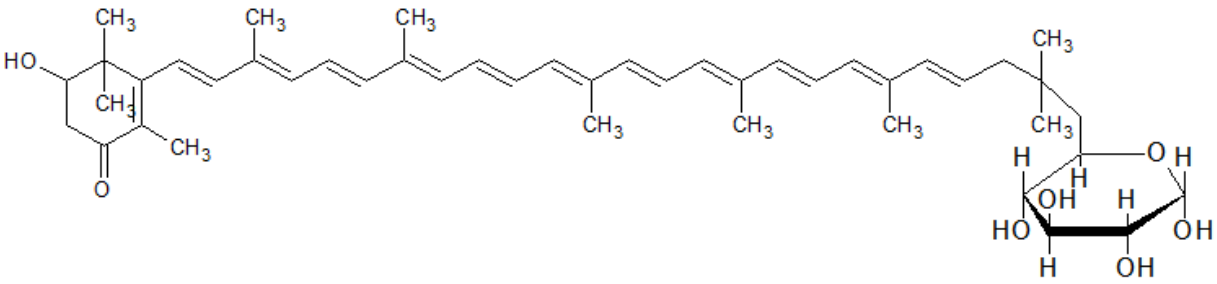

Fig 4– Structural formula of deinoxanthin glucoside

Sensitivity of colourless mutant of *D. radiodurans*

A colourless mutant of *D. radiodurans* is more sensitive to oxidation stress, ionizing radiation, and hydrogen peroxide than the wild type, which concludes that carotenoids from *D. radiodurans* act against oxidative stress. [35] The wild type of *D. radiodurans* is 100-fold less sensitive to hydrogen peroxide than its colourless mutant and 5 orders of magnitude less sensitive than *E. coli.* [36,37]

Aims of the work

Although the unique carotenoid of *D. radiodurans* has been identified decades ago and its role in protection has been intensely studied, the understanding of the mechanistic relation between the pigment and protein part of the protective surface layers of the bacterium has been emerging only recently. The very recent realization that the carotenoid-binding surface protein can harbour several derivatives of deinoxanthin, including ones binding bulky sugar moieties leads to questions such as whether the pigment composition of the surface layer protein is directly related to the function, is there any functional reason for it; does the surface layer protein pigment complement simply reflects the pigment content pf the cell or is the binding selective and what are the mechanisms. To address these issues, it is important to be able to thoroughly characterize the pigment content of the cells depending on the growth conditions and physiological state, as a prerequisite for further biochemical work, such as surface layer protein(s) preparations.

Thus, this thesis tried to address several topics.

i) characterization of the pigment content of *Deinococcus radiodurans* grown using powerful chromatographic methods developed for the analysis of complex mixtures of photosynthetic pigments

ii) testing of different approaches to *D. radiodurans* cultivation and comparison of their effects of pigment composition

iii) observation of the relation between physiological state of the culture and pigment content and effect of certain stress factors

iv) attempt at confirmation of the published pigment assignment of the surface-layer complex using mass spectrometry.

Materials and methods

Basic of applied experimental approaches

HPLC

High performance liquid chromatography is an essential analytical method developed in the 1960s. Separation of particles occurs between the stationary phase and mobile phase. The stationary phase is represented by the column and the mobile phase is represented by a solvent. The ability of the sample to distribute between the stationary and mobile phase affect the separation process.

HPLC could be divided into categories differing in the stationary phase.

Adsorption chromatography - separation is based on the cycle of absorption and desorption processes.
Partition chromatography - separation is based on distribution between mobile and stationary phase.
Ion exchange chromatography - stationary phase has an opposite charge than the sample.

Size exclusion chromatography - separation is based on molecular size. Mostly used HPLC type is adsorption chromatography, which has two modes of action depending on the polarity of phases.

Normal phase - stationary phase is polar and the mobile phase is non-polar. The non-polar phase is eluting first.

Reversed-phase - stationary phase in the non-polar and mobile phase is polar, which means that is the exact opposite of normal phase chromatography.

For changing the polarity of the mobile phase mixture of solvents is used. If the composition of the mobile phase is the same during the whole procedure, then it is referred to as isocratic elution. When the composition of the mobile phase varies through the elution time then it is referred to as the gradient. There are two most important properties for the diffusion of the molecule are the viscosity of solvents and the size of the molecule. For easier analysis solvent with lower viscosity should be chosen over solvent with higher viscosity.

Mobile phase

Solvents are always chosen by their ability to dissolve the sample. Purity, solvents need to maintain high purity HPLC grade samples are used. Gradient mixtures are usually suggested for changing polarity, to dissolve most of the sample. The solubility of phases of the gradients is usually tried starting with a single solvent and increasing the concentration of the second solvent by increasing by 2 % per minute. Up to 3 solvents can be used. Solvents are chosen by polarity, elution strength, low viscosity, compatibility with the detector, and removability = evaporation.

In an automatized machine, another solvent is used to flush the column after analysis. Before the analysis is crucial to degas, it could be done by applying heat, vacuum, ultrasound, or purging with inert gas, nowadays it is done as part of the procedure automatically. The air in the pump can lead to the loss of flow depreciation of the chromatogram.

HPLC system consists of reservoirs of mobile phases, pumps, solvent gradients, solvent programmer, filter, column, detector, recorder, and integrator. Commonly used are also automatic injectors, sample carousels, and collectors.

Methods for inducing samples into the system are automatic samples or injection with a microsyringe. The sample is injected into the holding loop. Loops are designed for exact volume, usually 10 - 20 μL.

Column-stationary phase

For accurate analysis, the correct column with appropriate packing must be chosen. Columns are most of the time encountered by internal diameter (i.d.) from 4.5 to 5 mm and length from 10 cm to 25 cm. Packing of the columns or stationary phases with diameters from 5 to 10 μm in diameter. The chemical composition of stationary varies most common types are silica, styrene-divinylbenzene, polysaccharides, or other polymers.

Pump

Pumps are regulating the flow rate and pressure. In the analysis, the goal is to maintain the same during the analysis. Also, the actual velocity of the sample needs to be high enough to prevent peak broadening, but also too high velocity would not end up in efficient enough results.

Detector

There are two sorts of widely used detectors: selective and universal. Universal detectors give a similar response for most compounds. Selective detectors give different responses depending on molecular structures. Special types of selective detectors are absorbance, fluorescence, and

UV detectors, which are the most selective and sensitive of these. Detectors usually absorb in three regions UV light, visible light, and infrared light. [51]

Absorption spectroscopy

Absorption spectroscopy is one of the most widely used analytical methods for determining concentrations of light-absorbing species. This method can be used as a qualitative and quantitative method, which determines molecules in the relation to their specific spectra. Absorption spectroscopy is a wide term that determines all types of spectroscopic techniques as UV-visible, fluorescence, circular dichroism, and infrared spectroscopy.

The principle of absorption spectroscopy is based on Lambert-Beer law, which describes properties of materials in which light passes light. Mathematically Lambert-Beer law can be expressed as $A = -\log\frac{I_0}{I} = \varepsilon cl$, where *Io* is the incoming intensity, *I* is the intensity of radiation leaving the sample; c and l denote (molar) concentration and pathlength (sample thickness), respectively, and ε is the wavelength-dependent constant, extinction coefficient, specific for the chemical species.

Instrument for absorption spectrometry is called a spectrophotometer which measures absorbed intensity in the sample. In UV-visible spectrophotometers a beam of light from UV or visible regions is passed through a monochromator, then the light passes through the sample, and then the light is detected via a detector. UV-visible spectrophotometers consist of a light source, sample holder, monochromator, detector, and a device to display the data. The standard light source is the deuterium arc and tungsten filament lamp which generates light in the range 190 - 800 nm. [52]

Mass spectrometry

Mass spectrometry is an analytical method for determining the m/z ratio, which states for mass to charge ratio of ions. These ions are then determined qualitatively and quantitatively by their m/z and abundance.

The mass spectrometer consists of 3 main parts: ionizer, the mass analyser, and detector, all of which are operated under a high vacuum. The ionizer does not have to be operated under vacuum if atmospheric pressure ionization methods are used. The instrument can have also separate parts for introduction, evaporation, and ionization of the sample. The analysed sample has to be ionized prior to the analysis, ionization may be thermal, by use of the electric field, or

by the impact of electrons, ions, or photons with high energy. The ionization process is mostly performed at a high vacuum to prevent the collision of molecular ions with other molecules. After ions are generated, they are accelerated into the mass analyser, where they are separated according to their mass to charge ratio using a magnetic or electric field or their combination. Then ions are passed onto an ion detector, where ions produce an electrical current, which is then amplified and detected. [53]

The downside of mass spectrometry is the consumption of the sample. Other analytical methods like chromatography, NMR, or IR spectroscopy don't modify or consume the sample, mass spectrometry consumes the sample, but it can be considered as a non-destructive method as it consumes only small amounts of the sample in the range of micrograms or less. This leads to the conclusion, that mass spectrometry is a great analytical method for analysis small amounts of samples. [54]

Growth media

A growth medium or culture medium can be liquid, semi-solid, or solid and are designed to support the growth of cell cultures or microorganisms. Agar-agar, gelatine, or silica gels are used for full or partial solidifying of the media. Growth of culture is easier in liquid media, because of a constant flow of nutrients, water, and oxygen. Disadvantages of liquid cultures are easy contamination and poor recognition if the culture is pure.

In point of view of the composition of media, media are divided into two sections: Synthetic media, which have well-defined structures and compositions, usually with the source of carbon, source of nitrogen, vitamins, growth factors or solidifying agents as agar-agar. And natural media, which are complex and not well defined, are usually made by hydrolysis of gelatine or other protein or by soil extract.

Selective media are used for favouring of growth of selected organisms, this media can contain antibiotics, different specific growth factors, compounds that favour the growth of specific types of microorganisms (ex. Eosin methylene blue, which favours the growth of coliforms) or absence of elemental nutrients (ex. nitrogen absence for favouring the growth of nitrogen-fixing bacteria). [55] The Czech collection of microorganisms has its own collection of different types of media**.**

Inoculation methods

The most widely media used for inoculations are agar plates and liquid media. For agar plates are many options of inoculation. The streak technique is the most widely used inoculation technique. It can be used for purification cultures, keeping pure cultures, or growing higher quantities of culture, when still ensuring the culture is not contaminated by other organisms. Streak technique has different patterns for spreading the cultures. Different patterns are used for various situations and different organisms. For streak techniques inoculation loops or inoculation, needles can be used, these can be plastic, single used, and sterilized or metal that needs sterilization in flame or ethanol prior to the inoculation. The whole procedure is done in sterile conditions in a flow box, which is sterilized with ultraviolet light type C before and after the inoculation procedures. The air in the flow box is filtrated through a nano-filter, to prevent contamination from the air in the flow box.

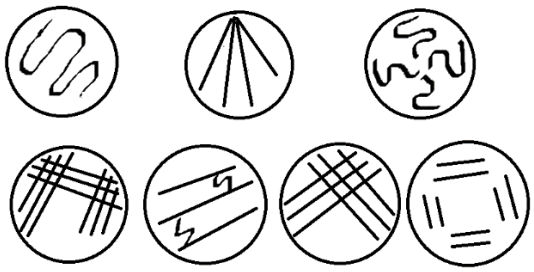

Fig 5 - Different spreading patterns for streak plate methods [56]- modified

Another possibility is inoculation with an inoculation spreader ("hockey stick"), using liquid media inoculum. Inoculation spreaders can be plastic, readily sterilized, and single-use or made out of glass which needs to be sterilized prior to inoculation using ethanol and flame. The exact volume is withdrawn from the liquid culture with a sterile pipette and poured on the agar plate. Inoculum is then spread with a sterile inoculation spreader. For inoculating from liquid media to liquid media sterile tip for an automatic pipette is used and the exact volume is withdrawn from a grown culture which is then added to the media. If no liquid culture is present part of the culture from agar culture is scraped with sterile inoculation loops and then mixed into liquid media. [56]

Growth of bacterial culture

The growth of the bacterial culture has few distinct phases which depend on the number of bacterial cells, type of microorganisms, and the length of the cultivation time.

Lag phase

In this phase, cells are adapting to the new environment or media. Cells are producing enzymes, growing, maturing, and prepare for cell division.

Phase of faster growth

Part of the growth curve where is the culture showing fast growth.

Log phase

Part of cell growth that is characterized by doubling the number of cells, growth is intensive, cells are growing and reproducing until the nutrient supply in the media is limiting. All cells are reproducing at the maximal possible rate.

Phase of slower growth

Bacteria are slowing their intensity of metabolism and storing their metabolites.

Stationary phase

Cell reproducing rate slows down. The number of dead cells is equalizing with the number of newly created cells, the concentration of nutrients is decreasing, the lifespan of bacteria could decrease depending on the concentration of nutrients, endospores then can be formed.

Death phase

Nutrients are almost consumed, cells are consuming their nutrients supply and poisoning themselves with their own metabolic waste.

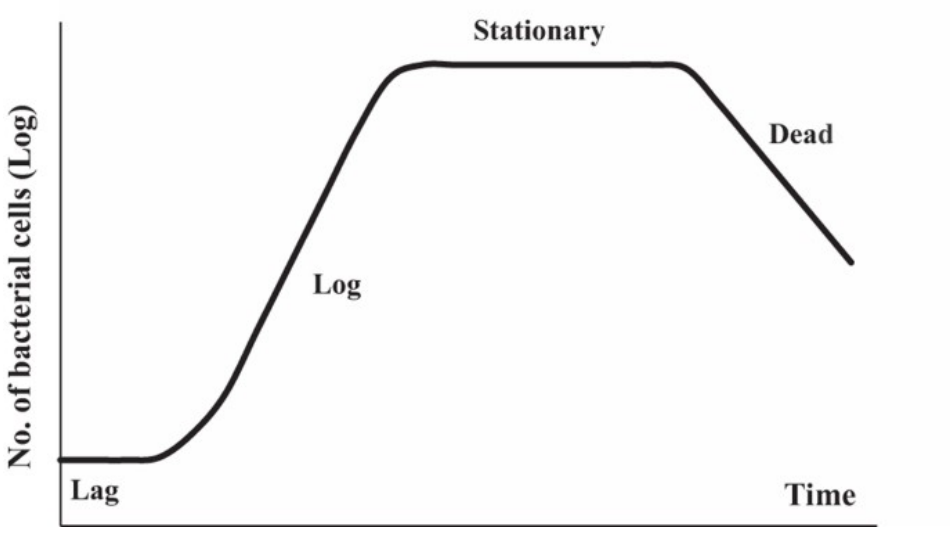

Fig- 6 - Graph showing the dependency of the logarithm of the number of newly formed colonies in time. Part A shows the lag phase, B log phase, C stationary phase, and D death phase of growth. [57]

Methods

Preparation of media and growth of bacterial culture

Culture of *D. radiodurans* was purchased from the Czech Collection of Microorganisms, at MUNI Brno. First CCM 8 media was prepared. This medium consists of 5 g of Yeast extract, 5 g of peptone, 10 g of glucose, 20 g of agar (Optional, only for agar plate media), and 1 L of distilled water, pH of media is then adjusted to 7,2.

Then media was sterilized through autoclave and poured into Petri dishes. Petri dishes were then sealed with parafilm and stored in the fridge until use. For liquid media, media was first poured into flasks, sealed and sterilized through an autoclave, then stored in the fridge until use.

Once a week or once two weeks the culture was inoculated to a Petri dish with the use of an inoculation loop. The technique of streaks was used. The Petri dish was labelled and stored in the incubator with a regulated temperature of 30°C.

After a certain amount of time culture was scraped into Eppendorf tubes, labelled, and stored in the freezer until pigment extraction. [56]

Pigment extraction

All samples were treated in this fashion: a sample of bacteria was resuspended in 1500 μL of methanol. The sample was vortexed and cells were broken by sonification. Then it was

centrifuged, resulting supernatant was withdrawn and the remaining pellet was dissolved again in 1500 μL of methanol, and the procedure was repeated. For the third time procedure was repeated with the use of acetone instead of methanol. The resulting supernatants were mixed and the pellet was discarded, dried, and stored in a freezer until analysis. [58]

Pigment analysis

Sample of pigments was dissolved in 200-300 μL of methanol and placed into HPLC flask and analysis was performed. The system used for analysis consisted of Pump controller delta 600, automatic injection system, and PDA detector 2996 (Waters USA), and for analysis reverse C18 column. Linear gradient with flow rate 1 ml/min had the following composition Solution B (80:20 methanol with 0.5 M Ammonium acetate), Solution C (90:10 Acetonitrile with water), and Solution D (100% ethylacetate). [59] For other analysis pigments were dried and stored in a freezer.

Growth rate measurement

Sample of pigments was dissolved in 200-300 μL of methanol and placed into HPLC flask and analysis was performed. The system used for analysis consisted of Pump controller delta 600, automatic injection system, and PDA detector 2996 (Waters USA), and for analysis reverse C18 column. Linear gradient with flow rate 1 ml/min had the following composition Solution B (80:20 methanol with 0.5 M Ammonium acetate), Solution C (90:10 Acetonitrile with water), and Solution D (100% ethylacetate). [59] For other analysis pigments were dried and stored in a freezer.

Illumination of cultures with UV light

1 mL of inoculum was pipetted on premade media on a Petri dish and spread with a sterile microbiology spreader (Hockey stick). Petri dishes were then left under UV light for the exact amount of time. Every time control, which was covered from UV light, was made. All Petri dishes were sealed with parafilm, labelled, and incubated until colonies were visible. Then colonies were collected and stored in Eppendorf tubes in the freezer (- 80°C) until pigment extraction. [56]

Results

The carotenoid composition of the *D. radiodurans* was mostly studied using HPLC using the gradient Jeffrey method (2005) and chromatograms were extracted at wavelength 480 nm, which is most suitable for this type of carotenoids. For additional identification of the peaks, the absorption spectra were used.

Survey of pigment composition of whole cells

In Fig. 7, we show the HPLC chromatogram of whole cells of *D. radiodurans* detected at 480 nm. As a reference, we also present the analysis of pigment composition of the purified surface-layer carotenoid binding complex (SDBC). This trace corresponds to the chromatogram published in Adamec et. al, 2020. As seen, the SDBC pigment-protein complex contained three major carotenoids, labelled them 1-2-3 in the figure in the agreement of the cited work. Those peaks were tentatively assigned to deinoxanthin-glucoside (1), deinoxanthin (2) and 2-deoxydeinoxanthin (3), based on the data published earlier. [50,60]

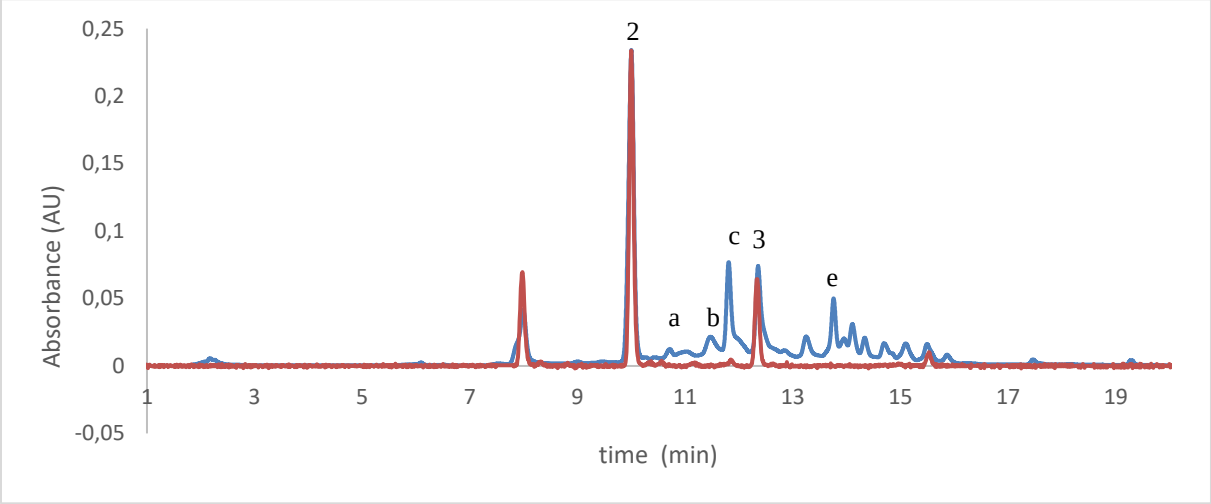

Fig. 7 – Chromatograms obtained from three-week-old cells (blue) and from SDBC protein from Adamec et. al 2020 (orange). Detection wavelength 480 nm.

As seen in Fig. 7, the chromatogram obtained from three-week-old cells is much more complex than the chromatogram obtained from the SDBC. The three same peaks which are proposed to be the deinoxanthin and its analogues present in SDBC can be readily identified. The peaks have a relative shape and the intensity of the peaks is almost the same. For better identification of the peaks in chromatograms, the absorption spectra of each peak were taken. The absorption spectra can help with the identification of the chemical nature of the compounds and similarities

between those compounds. As compounds with similar chemical structure have similar absorption spectra with the similar and maximum absorbance.

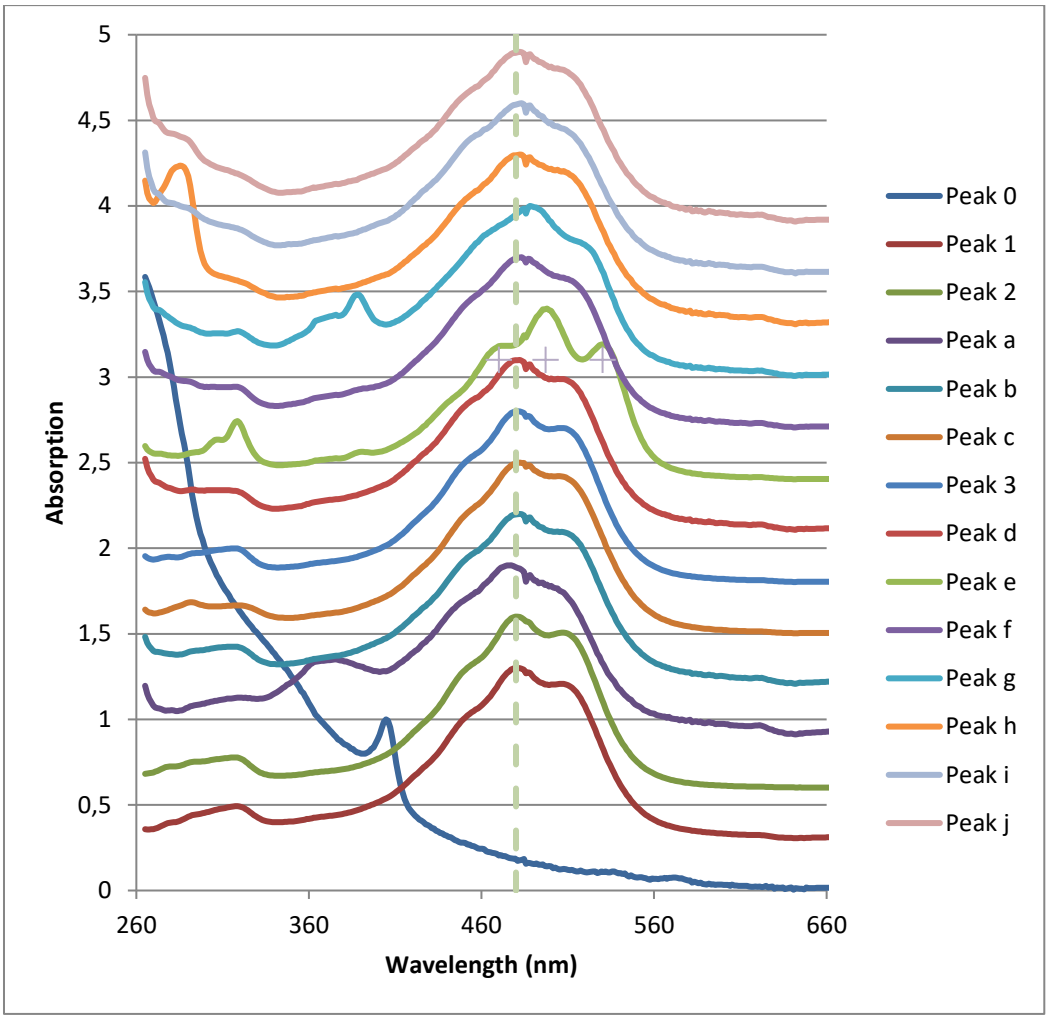

Fig. 8– Absorption spectra of the labelled peaks from Fig. 7

In Fig. 8 we show all the absorption spectra of the labelled peaks from Fig. 7 to facilitate comprehensive comparison. A more detailed analysis follows.

The peak labelled 0 represents the elution solvent front. However, as evidenced by the associated spectrum in Fig. 7, at this stage a compound absorbing at ~405, 535 and 570 nm leaves the column. From the position at the front of the elution, i.e. in the most polar solvent, it can be deduced that this compound is very polar. Its absorption maxima indicate that it is some species of tetrapyrrole, i.e. heme. [62]

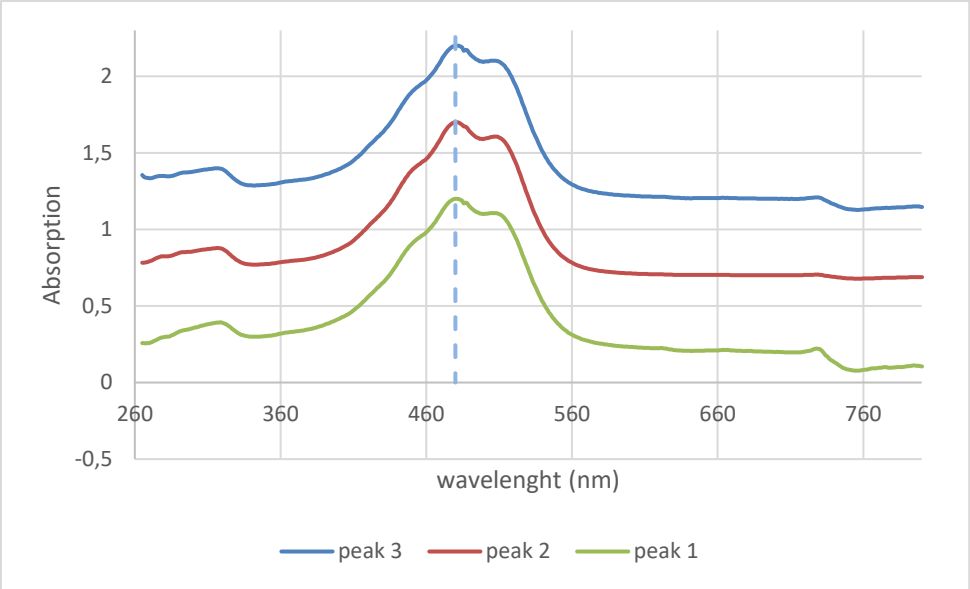

Fig. 9 – Absorption spectra of peaks 1-3

In Fig. 9 we show the spectra of peaks 1-3. In agreement with published reports, the spectra have a maximum at 480 nm and their shapes agree with deinoxanthin, as published earlier [50]. The shape of the spectra, which is similar for all three of them and has a shape typical for the keto-carotenoids, that are typically characterized by poorly-resolved vibronic subbands in moderately polar solvents. This leads to the prediction, that the compounds have the same basic structure and changes are most probably only in their ψ end, which does not influence the absorption but does influence the molecule's polarity. In any case, the fact that the spectra of compound 1-3 are essentially identical, although their elution times are different mean that the chemical modification of the molecule does not affect the conjugated system of π-electrons.

There is a number of peaks between 10 to 16 minutes. As seen in Fig. 8, spectra of most of them are very similar to peaks 1-3 which suggests that these too are derivatives of deinoxanthin. Two compounds, labelled a and g have a marked absorption band in the range of 300-400 nm. Those peaks likely represent cis-conformation of carotenoids that have the important feature of "bending" the carotenoid in a specific position, the carbons are on the same side of the double bond, compared to the trans, where they are on opposite sites. Compared to the trans-structures, the cis forms of the carotenoids can be recognized by absorption peaks between 300 nm and 400 nm and a slight shift of the main absorption band to higher energies. [63] The spectra of the two putative cis-carotenoids are shown in Fig. 10 for better comparison. It can be seen that the maximum of the compound a, which elutes at 10,7 minutes, is indeed blue-shifted compared to deinoxanthin and can thus be a cis form of it. On the other hand, the other cis-carotenoid

previously marked as g eluting at 14,7 is red-shifted and thus should correspond to cis form of a carotenoid absorbing more to the red than deinoxanthin.

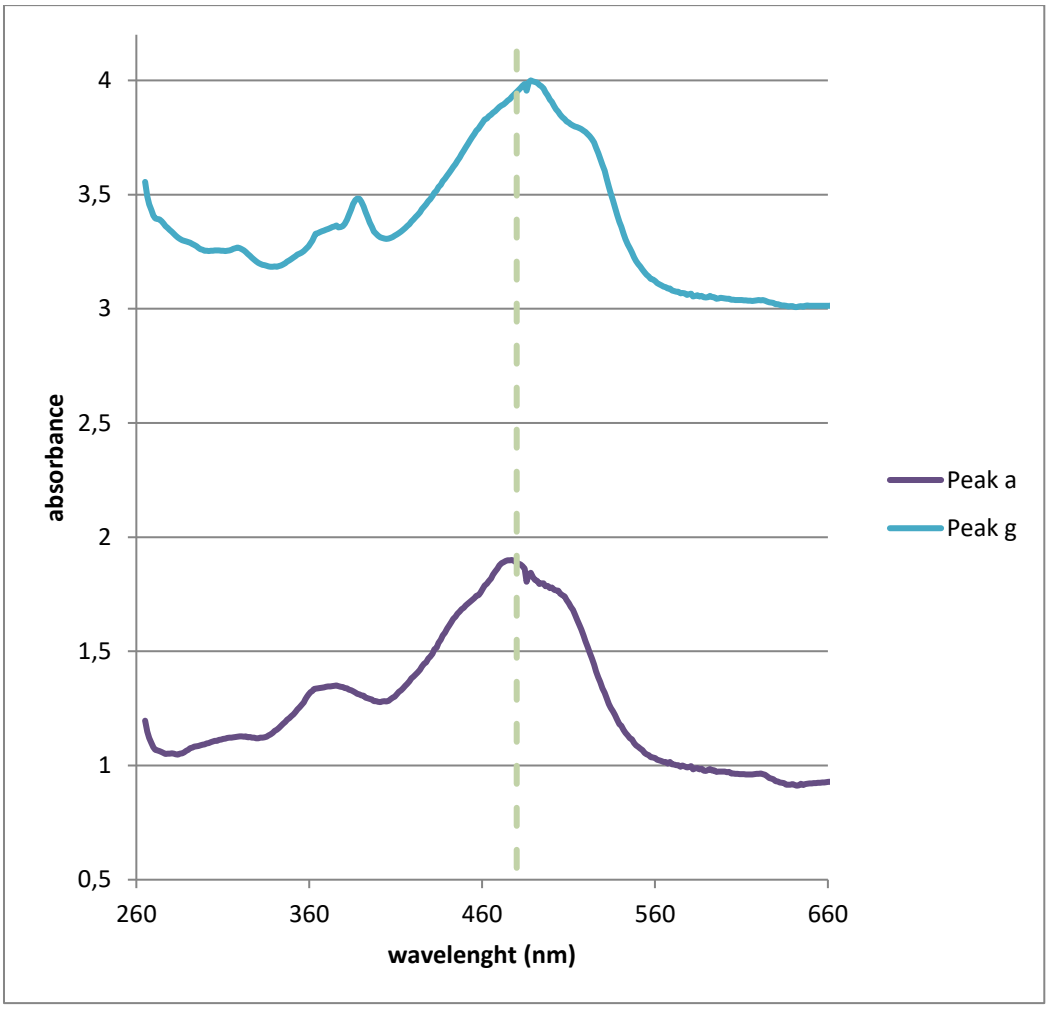

Fig. 10 – cis carotenoids spectra extracted from the peaks a and g

Of particular interest is the compound labelled e (eluting at 13.75 min). This carotenoid has a red-shifted absorption. Compared to deinoxanthin, its maximum is at ~497 nm and the lowest vibronic band is at 530 nm. This is about 20 nm lower energy than deinoxanthin. Also, the structure of the vibronic bands of this carotenoid is more resolved than deinoxanthin. The shape of the spectrum resembles lycopene, a carotenoid that *D. radiodurans* is known to synthesize as an intermediate product during deinoxanthin synthesis. [64] However, lycopene in solvents absorbs at most 507 nm [65] whereas the compound e absorbs at longer wavelengths (see Discussion).

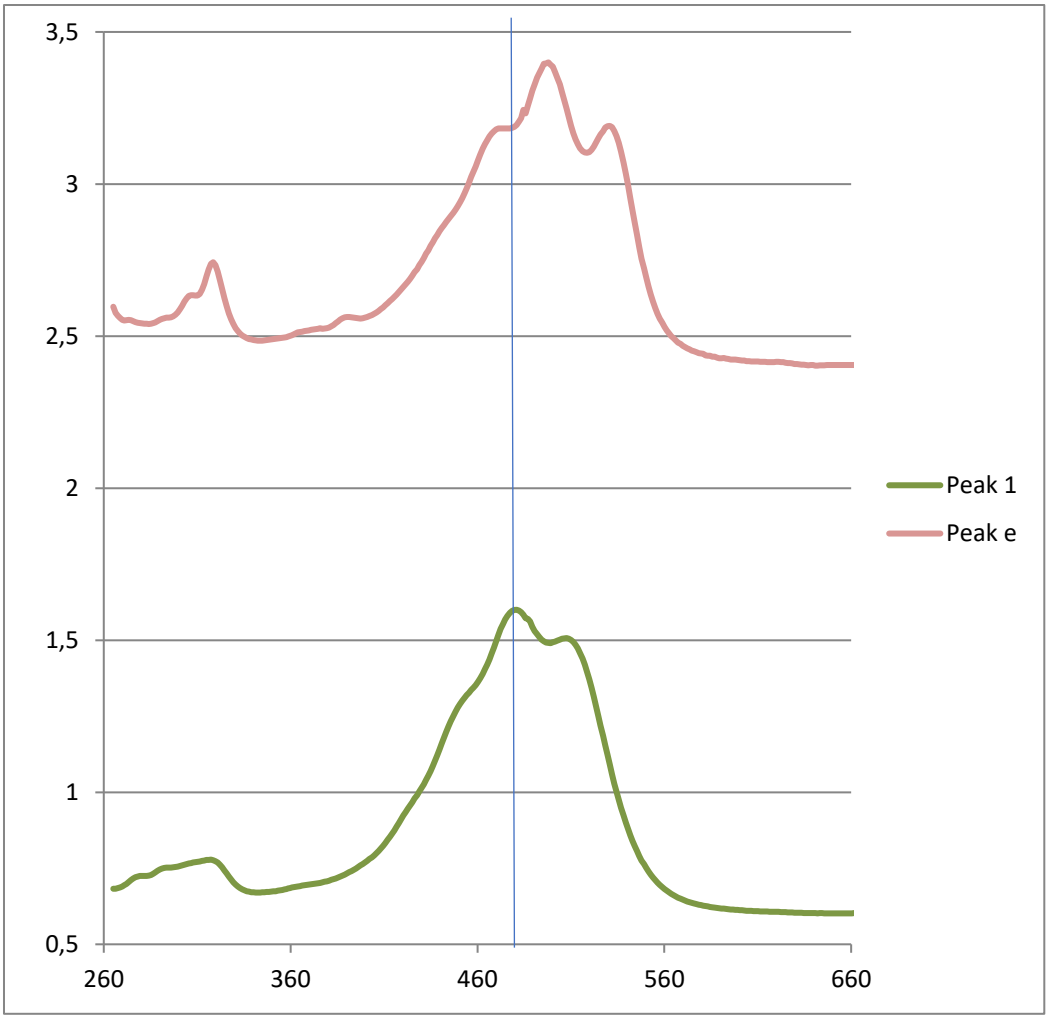

Fig. 11 – Absorption spectra of the peak e and peak 1

Effect on the cellular carotenoids

In the following course of the work, we focused on the possibility of changes in the composition of the carotenoid pool in *D. radiodurans* with the age of the culture. One of the motivations for this research direction was the observation of colour development of differently old cultures of *D. radiodurans*, as shown in Fig. 12.

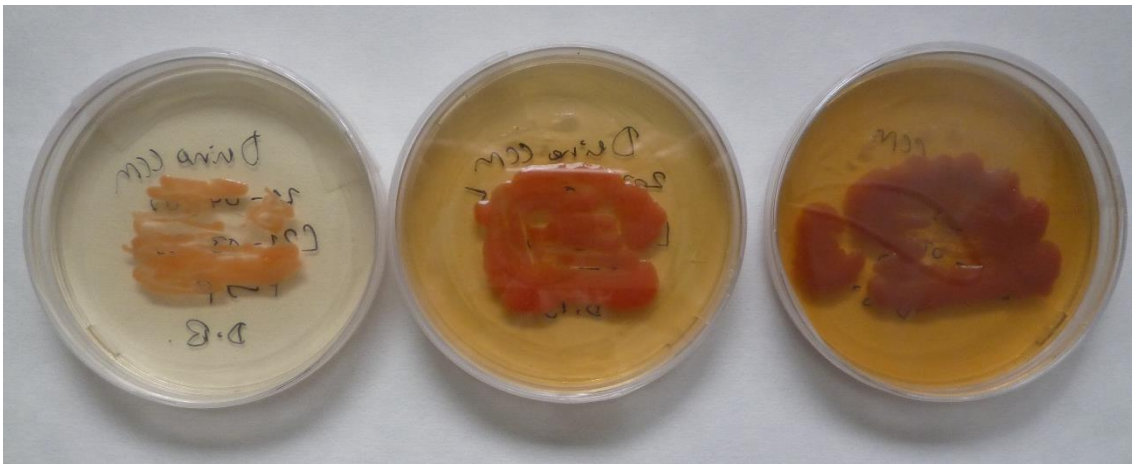

Fig. 12- Differently old cultures of *D. radiodurans*

With the prolonged incubation at 30°C, the agar plates dry out. Since the carotenoids in *D. radiorudans* are associated with the protection against unfavourable conditions, including desiccation, it is interesting to see whether the composition of carotenoid pools changes with the age of culture under our experimental conditions. As all of those cultures were grown in the same media and same environment, the possibility of environmental influence was rejected. The possible factors for changing the colour may be the change of the carotenoid composition, the ageing of the agar and thus changing the background colour and giving the cells darker and more saturated colour, last two reasons may be the growth of more cells and denser culture, which will obviously result in more saturated colour or overall higher amount of carotenoids in the cells.

Graph n. 13 shows the comparison of chromatograms obtained from differently old cultures obtained at 480 nm. The chromatograms were normalized through the deinoxanthin peak, which appears at 10 minutes, for easier comparison of the chromatograms. Overall, the chromatograms do not exhibit conspicuous differences, neither the shape nor retention times of elution peaks differ significantly. The most significant shape change occurs in peaks around 12 minutes, which are identified from Fig. 8 as the derivates of the main carotenoid – deinoxanthin.

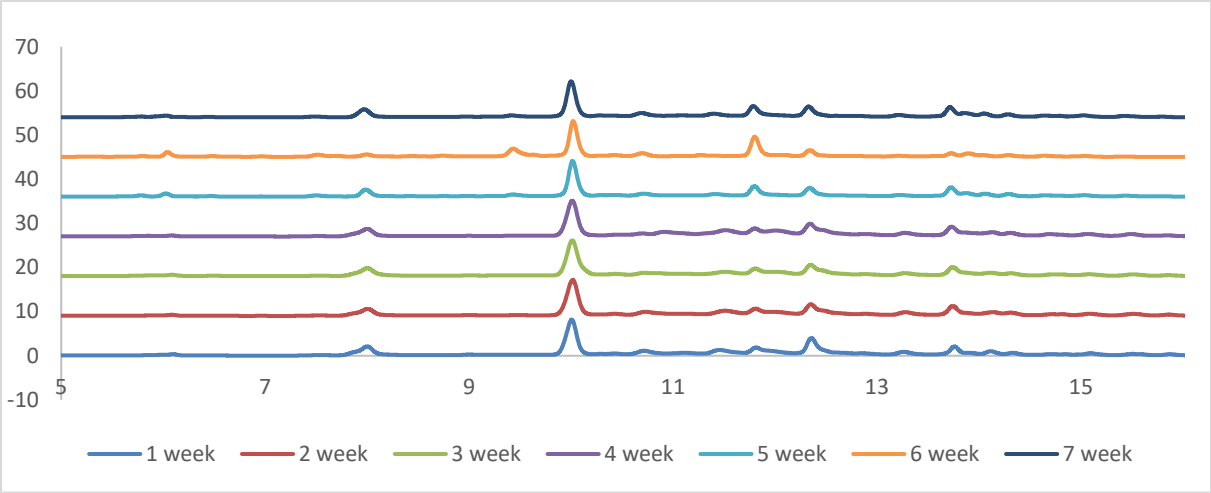

Fig. 13 – HPLC chromatograms obtained from differently old cultures of *D. radiodurans*

Tab. 1 – Areas of the chromatograms from Fig. 13 and their ratios. Values are mean ± standard deviation.

Age of culture	1 week	2 week	3 week	4 week	5 week	6 week	7 week
area under the whole chromatogram	0,456 ± 0,1597	0,386 ± 0,0617	0,416 ± 0,0776	0,433 ± 0,0587	0,427 ± 0,02	0,436	0,411
area under the 3 peaks of SDBC	0,181 ± 0,0309	0,169 ± 0,01532	0,158 ± 0,0033	0,163 ± 0,0052	0,146 ± 0,0061	0,122	0,159
Proportion of SDBC carotenoids to all pigments	0,422 ± 0,0772	0,443 ± 0,0321	0,391 ± 0,0610	0,383 ± 0,0420	0,342 ± 0,002	0,279	0,387
peak 1 / all SDBC carotenoids	0,198 ± 0,0789	0,165 ± 0,0199	0,375 ± 0,209	0,171 ±0,0259	0,138 ± 0,0313	0,0709	0,171
peak 2 / all SDBC carotenoids	0,471 ± 0,168	0,540 ± 0,0491	0,574 ± 0,0117	0,554 ± 0,0181	0,621 ± 0,0260	0,744	0,570
peak 3 / all SDBC carotenoids	0,261± 0,011	0,295 ± 0,0329	0,259 0,0253	0,275 ± 0,104	0,242 ± 0,0053	0,185	0,259

The peak previously labelled as c is increasing in intensity as the cells are older. However, the peak previously labelled as 3 has decreasing intensity trend with the increasing age of the cells. The main difference is in the 7-week old culture when the intensity of both peaks is almost equivalent. Then it is unlikely that the significant change in colour intensity and shade will be caused only by a small change in carotenoid composition. The change is most probably caused by a combination of factors as the darkening of the agar, higher density of the cells. However, the conclusion can be made that the carotenoid composition of *D. radiodurans* changes during its lifetime.

The Tab. 1 summarizes the quantitative results of the experiments, showing areas of all peak and the areas under the peaks corresponding to the SDBC-bound carotenoids, peaks 1-3, as well as their ratios. As seen in the table, the proportion of the three SDBC carotenoids to the whole pigment pool shows a slightly decreasing trend, from 2-week old cells to 6-week old cells. The data from week 7 do are in clear contrast to this trend, however, since this age was only studied once, the true error of this data point is not known and it might be just due to an error in sample processing. The relative proportions of the SDBC peaks respective peaks did not show any strong trend apart from a gradual increase of the proportion of the deinoxanthin (peak 2), which increased from about 50 % of the SDBC carotenoids to about 70 %. However, the scatter of these data is very large, precluding any strong conclusions. The cells with the highest deviations are 1-week old cells and 3-week old cells. Again, the 6 week and 7-week cells had only one sample, therefore the standard deviation is not calculated for them.

Effects of the UV irradiation

In the following step, we investigated the possibility of manipulating the carotenoid composition of bacteria using UV light radiation. The hypothesis was driven by consideration of *D. radiodurans* survival strategies and if possibly *D. radiodurans* can produce carotenoids in different ratio for better protection against UV radiation. For the inoculation of the cells, the diluted liquid culture was used as a media and the inoculation spreader was used instead of the inoculation needle and culture on agar media. This type of inoculation was performed for a better dispersion of the cells to ensure that the culture is uniformly exposed to UV. The experiment was performed by exposure of the culture plates to the sterilizing UV light in the flowbox used for culture maintenance.

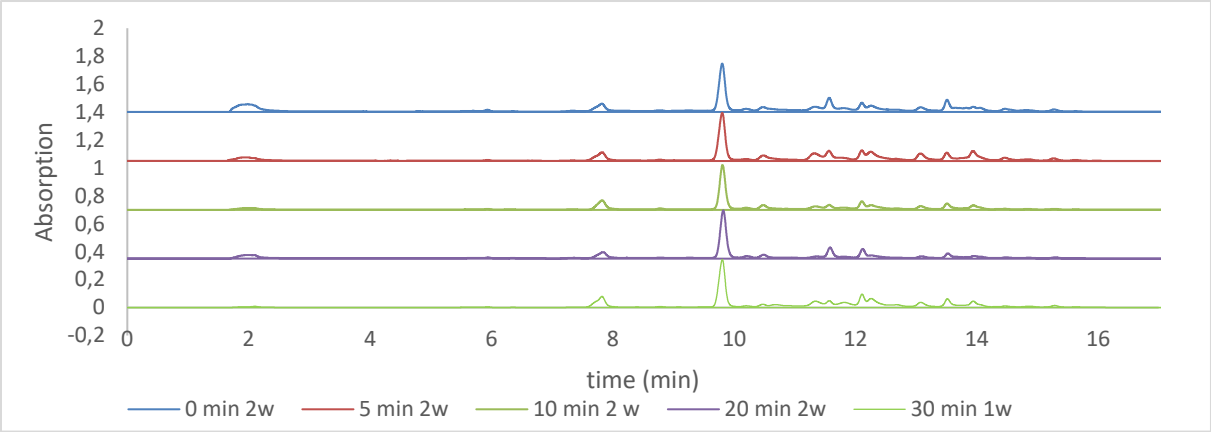

Fig. 14 – Chromatogram obtained from UV enlighted cells

Tab. 2 - Quantitative analysis of carotenoid pools of *D. radiodurans* cells irradiated with UVC light; carotenoid ratios, mean ± SD

Time duration of the UV exposure	0 minutes	5 minutes	10 minutes	20 minutes	30 minutes
Mean area under the whole chromatogram	0,269 ± 0,0448	0,332 ± 0,0783	0,251 ± 0,0217	0,249 ±0,0437	0,266 ± 0,0085
Mean area under the 3 main peaks	0,121 ± 0,00788	0,126 ± 0,0126	0,122 ± 0,0087	0,117 ± 0,0077	0,133 ± 0,0061
Mean ratio between area under 3 main peaks and whole chromatogram	0,461± 0,069	0,394 ± 0,0593	0,490 ± 0,0343	0,478 ± 0,0542	0,5 ± 0,014
Mean ration between area under peak 1 and 3 main peaks	0,019± 0,0379	0,175 ± 0,0133	0,179 ± 0,0162	0,171 ± 0,0350	0,212 ± 0,0226
Mean ration between area under peak 2 and 3 main peaks	0,599 ± 0,226	0,576 ± 0,0571	0,592 ± 0,0399	0,620 ± 0,0395	0,544 ± 0,026
Mean ration between area under peak 3 and 3 main peaks	0,213 ±0,0269	0,249 ± 0,0623	0,229 ± 0,0369	0,210 ± 0,0109	0,244 ± 0,0373

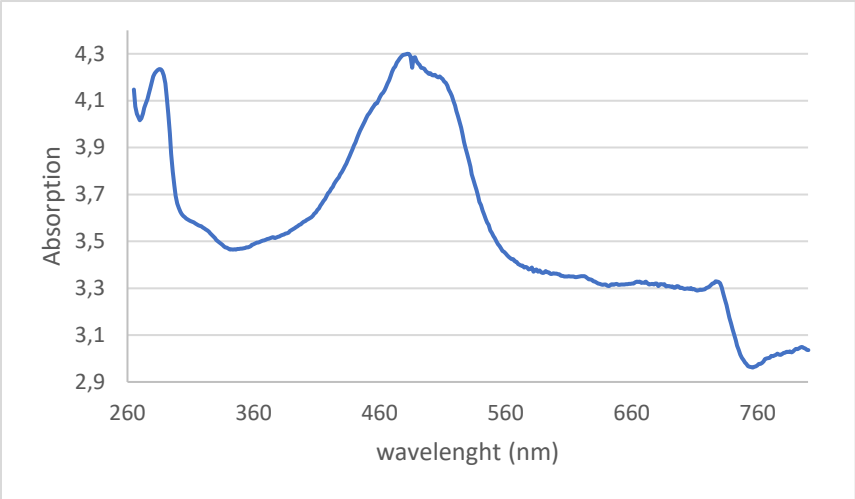

Fig. 15 – Absorption spectra of peak h

It should be noted that exposure to UV light up to 30 minutes did not prevent the growth of the cells of *D. radiodurans*, confirming the known resistance of the organism to ionizing radiation. The results of the pigment analyses of the irradiated cultures are presented in graph Fig. 14 and summarized in the Tab. 2.

As before, the graph shows chromatograms at 480 nm. It can be seen that there are no marked differences in the appearance of the chromatograms. This is also supported by the quantitative data shown in Table 2. The proportion of the SDBC carotenoids to the total pigment pool does not change with the duration of the UV exposure, remaining in the range of 40-50 %. The relative abundances of the three main SDBC carotenoids did not exhibit major trends with UV illumination, either, maintaining the ratio of 1 : 2 : 3 of roughly 0,2 : 0,6 : 0,2.

The ratios of the areas of the peaks were highest in the sample obtained from cells illuminated for 10 minutes, the lowest values were observed for the non-illuminated cells, however, the consistent trend in the ratios was not observed. It may also be due to the high scatter of the area values, as indicated by the large standard deviation. In conclusion, the results are rather inconclusive and for some trustworthy values, the experiments should be repeated in higher number to suppress the effect of outliers.

Liquid culture

In the further step of the work, we analyzed the properties of the *D. radiodurans* cultures grown in a liquid medium. The composition of the medium was the same as for plates and the cultures were incubated at 30°C like the agar plates. The liquid culture is the one most commonly used

in the mass production of the cell material for biochemical studies. In this type of cultivation, the cells maintain better access to the food source that can be absorbed by the whole surface of the cells while in agar plates the nutrient uptake can be spatially limited. Moreover, contrary to the agar plates, the cultures are not subjected to drying (although nutrient depletion ultimately does occur). Hence, the different amount of stress makes the comparison of the carotenoid pool composition in liquid culture and the agar plates interesting. As a first step, we analyzed the growth of the bacterial culture by measurement of the light scattering at 650 nm as a function of cultivation time. Since in the UV experiments the bacteria exhibited the expected resilience against adverse conditions, we decided to test their resistance against common household antibacterial agents, the chlorine bleach (SavoTM) and the tea-tree oil.

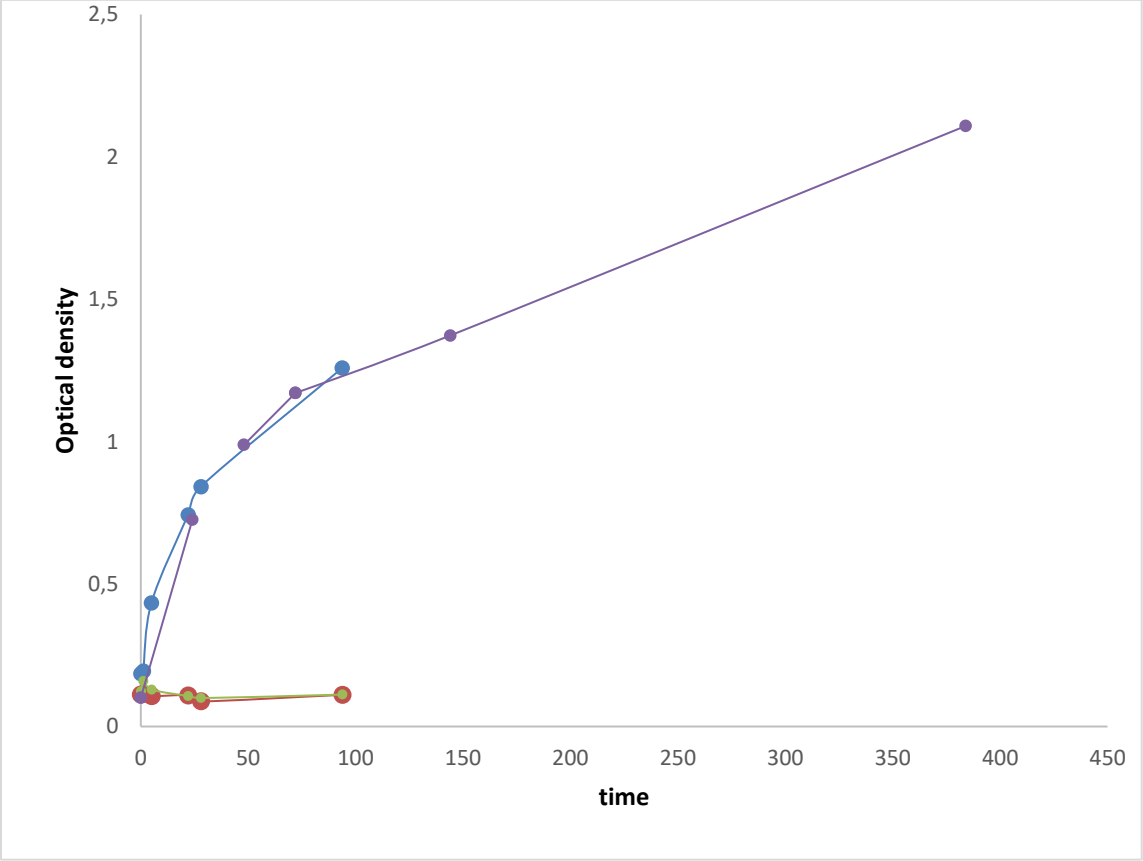

Fig. 16 - Growth curve of *D. radiodurans* in liquid culture as measured by light scattering. Colours denote different replicates of the experiment. The green and brown symbols show the cultures supplied with 5 % chlorine bleach and 5 % tea tree oil, respectively.

In Fig. 16, we show the examples of growth curves of *D. radiodurans* in liquid culture in the TGY medium. The growth curve of culture influenced Chlorine-based bleach labelled orange, and the growth curve of culture influenced by tea tree oil is labelled green. All cultures were

grown in the same media, with the same inoculum and in the same environment to prevent any statistical errors and results can lead to a clear comparison.

Untreated culture growth curve has a logarithmic shape. It is possible to predict that the lag phase is short, less than 2 hours. Also, the shape of the curves suggests that the exponential phase of the growth likely does not extend to more than 10 hours, and from about 15-20 hours the growth starts to slow down. However, the culture continued to grow for more than 15 days.

After thirty hours of bacterial growth, growth slows down and slowly passes into a stationary phase. The stationary phase will come around ninety hours of growth of the culture. It could be predicted that the time for reinoculation into new media is around ninety hours of growth, to maintain the viability of the cells and the quality of the culture. But experiments was proven that even 4-week old cell cultures have high viability and are able to form new colonies. This corresponds with publications [4,9,11] that discussed *D. radiodurans* survival strategies. As its strategy is about prolonging the life of the cells rather than reproducing cells, which die after a short period of time.

Culture treated with the tea tree oil grew slightly in the initial phase, but then the culture had decreasing optical density, which leads to the assumption that culture was slowly dying and the new cell was not rising in high enough number for keeping viability of the cultures. Similarly, culture influenced by the chlorine-based bleach had slight growth in the initial phase, but then the optical density of the culture was decreasing. As those disinfectants are easily vaporized, the cultures may survive and start to grow again after the vaporization and partially the consumption by the bacteria. Hence, despite its resistance against ionizing radiation, the bacterium is clearly susceptible to common disinfecting treatments. And these disinfectants in minimal concentrations are sufficient to clean and disinfect surfaces after work with D. *radiodurans*.

The pigment content of the liquid culture was also studied. In the Fig. 17, we show the usual chromatogram at 480 nm for the 2-week old liquid culture, compared to three examples of bacterial cultures of the same age grown on agar plates. As seen in the plot, the pigment content of the cultures was similar, however, close inspection reveals that the carotenoid composition of the liquid culture was probably simpler than for the agar plates. In particular, in the liquid culture, the three SDBC carotenoids appeared to be more prominent and in comparison, the amount of other carotenoid species was lower. The only exception was the red-shifted carotenoid at about 14.75 min, which was present in similar amounts in the plates and the liquid

culture. Unfortunately, this experiment was performed only once and thus more work is needed to confirm this observation.

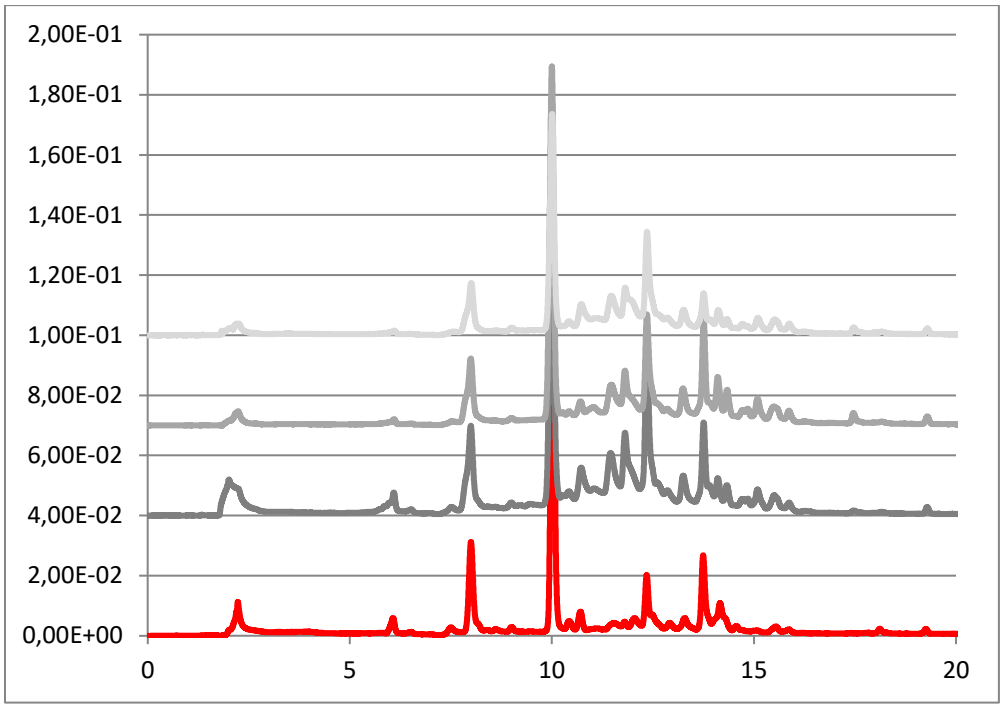

Fig 17. Comparison of pigment content of 2-week old *D. radiodurans* culture grown in liquid medium (red) and on agar plates (grey, data from three different agar plate cultures). HPLC chromatograms detected at 480 nm.

Mass spectrometry

In the previous sections, it was assumed, based on the published data, that the three main carotenoids found in the cells of *D. radiodurans* correspond to deinoxanthin, deinoxanthin glucoside and 2-deoxydeinoxanthin. However, this assignment has not actually been rigorously tested. Hence, during the course of the work, we collected samples of the peaks 1-3 for mass-spectrometry analysis. The pigments were collected, dried under vacuum or nitrogen stream and stored at -20°C. This is the usual approach in carotenoid storage.

In the following plots, we show the results of the mass spectrometry analysis of the peaks 1, 2 and 3.

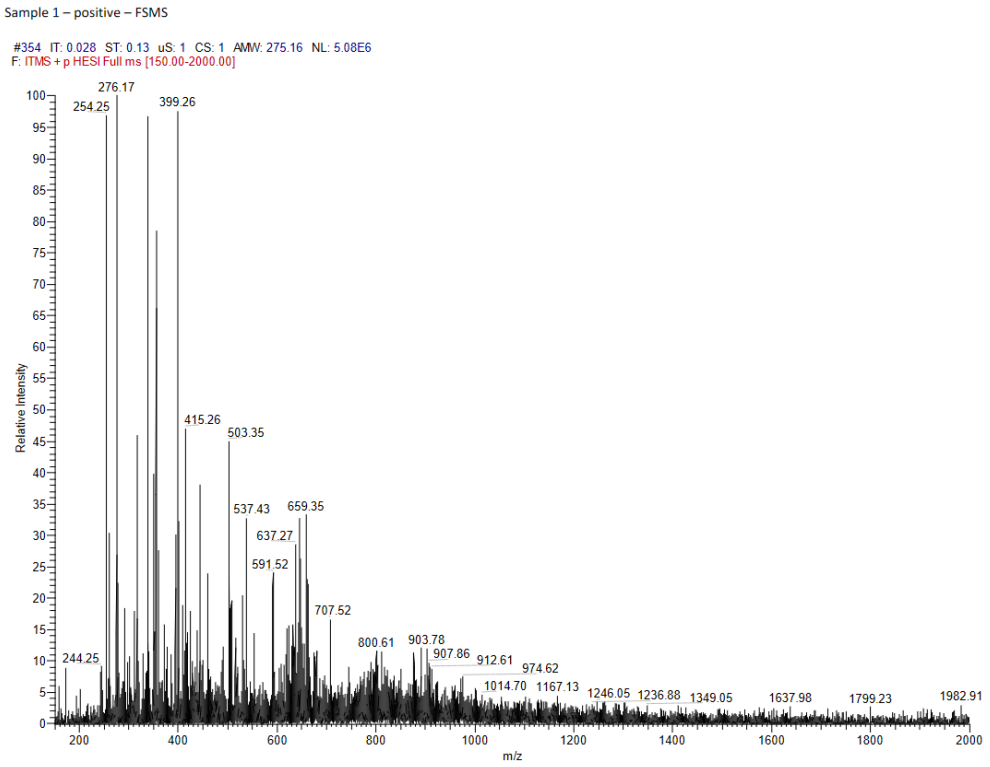

Fig.18 – Mass spectrum of first major peak of HPLC chromatograms

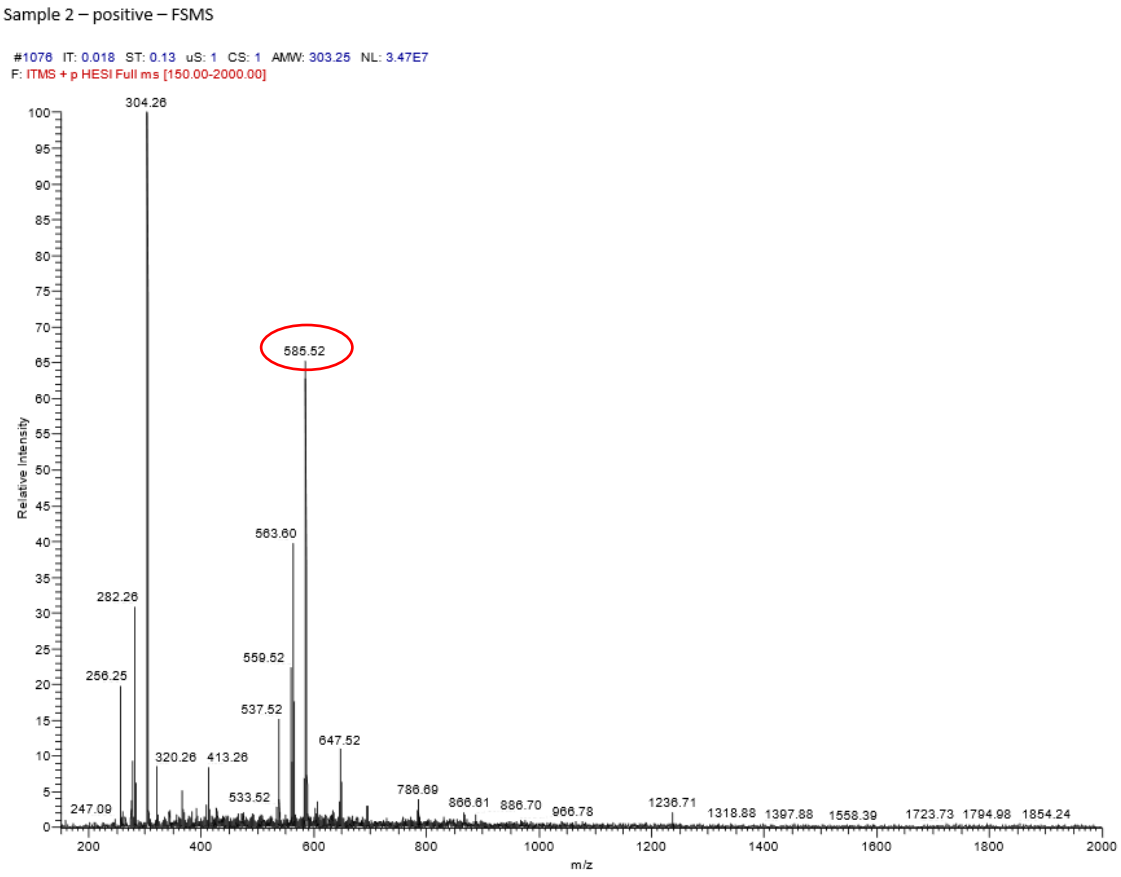

Fig. 19 - Mass spectrum of the second major peak from HPLC chromatograms

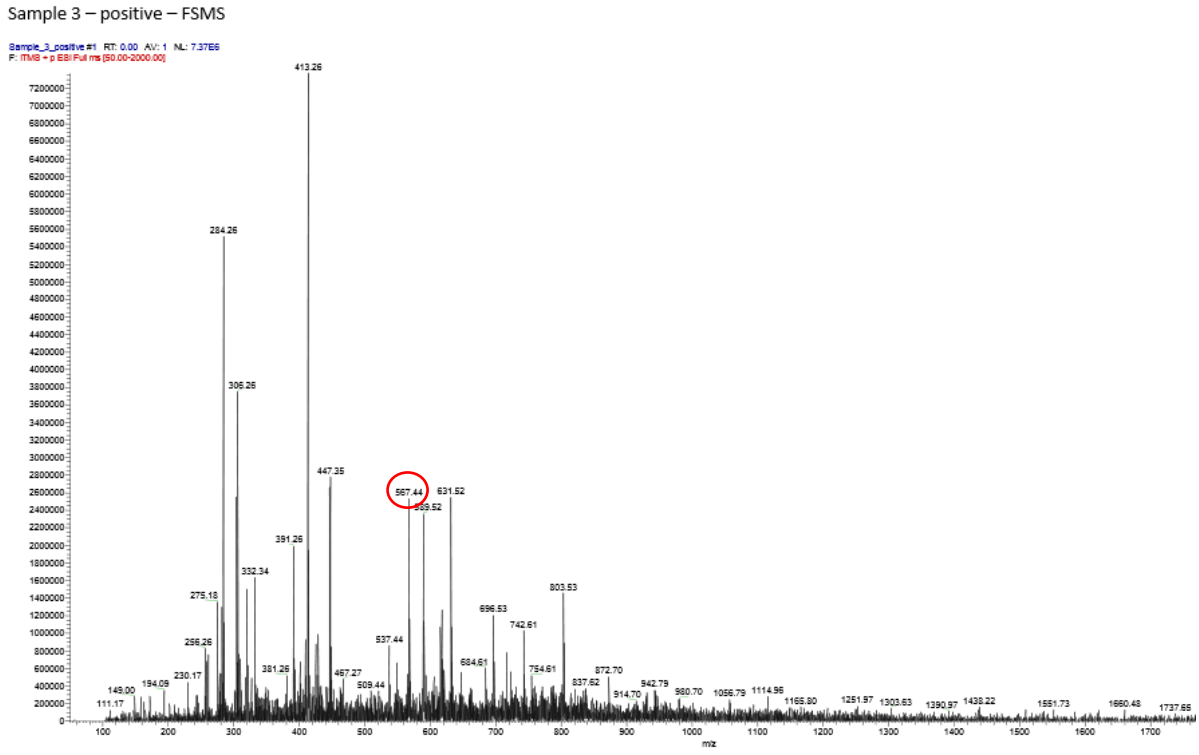

Fig. 20 - Mass spectrum of the third major peak from HPLC chromatograms

The Fig n. 18 shows mass spectra of the peak previously labelled as 1. The peak was predicted to contain the deinoxanthin glucoside. However, the graph does not show any peak containing similar m/z values to the deinoxanthin glucoside molecular weight (m/z 745, [61]) The Fig. 19 shows mass spectra of the content of peak 2 from HPLC analysis, which was predicted to be deinoxanthin. The spectra show a high peak at 585,52 m/z, which corresponds to the molecular weight of the deinoxanthin. The peak at 585,52 m/z, could be taken as proof, that the second peak in HPLC spectra contains indeed deinoxanthin. We see similar patterning as in Lemeee et. al 1997[66], which studied the main pigment of *D. radiodurans* - deinoxanthin. The graph shows not only the patterning shown for deinoxanthin but also various peaks with a large range of molecular weight. This leads to the suggestion that the peak did not contain only one compound, but rather contain a complex mixture of various compounds, most probably proteins or peptides, which were not visible in the original chromatogram.

The Fig 20 shows mass spectra from the content of peak 3 resulting from HPLC analysis. The mass spectra show several peaks, but the most important one is the peak at 567,44 m/z, which corresponds to the calculated molecular weight of the 2-deoxydeinoxanthin 566 g/mol. This could be taken as one of the proofs that the third major peak is 2-deoxydeinoxanthin, this also corresponds to the polarity of compound and elution time in HPLC.

Altogether, when compared to published mass spectra of *D. radiodurans* carotenoids, e.g. [61], our results suggest a much more complex mixtures of compounds and many impurities. We thus recorded the absorption spectra of the samples used for mass spectrometry measurements. These are shown in Figs 21, 22, 23 and compared to the original spectra of peaks 1-3 as obtained fresh from the HPLC. One can see that the shape of the spectrum around the maximum absorption is slightly different, with the MS samples showing smaller resolution of bands. This is most likely due to the difference in solvents. The MS samples were dissolved in methanol, which is expected to produce less-resolved spectra. However, more important is the much larger absorption in the UV region. This leads to the suggestion that the compound probably undergoes some chemical changes or degraded. These chemical changes can be the reason for the unsuccessful MS analysis.

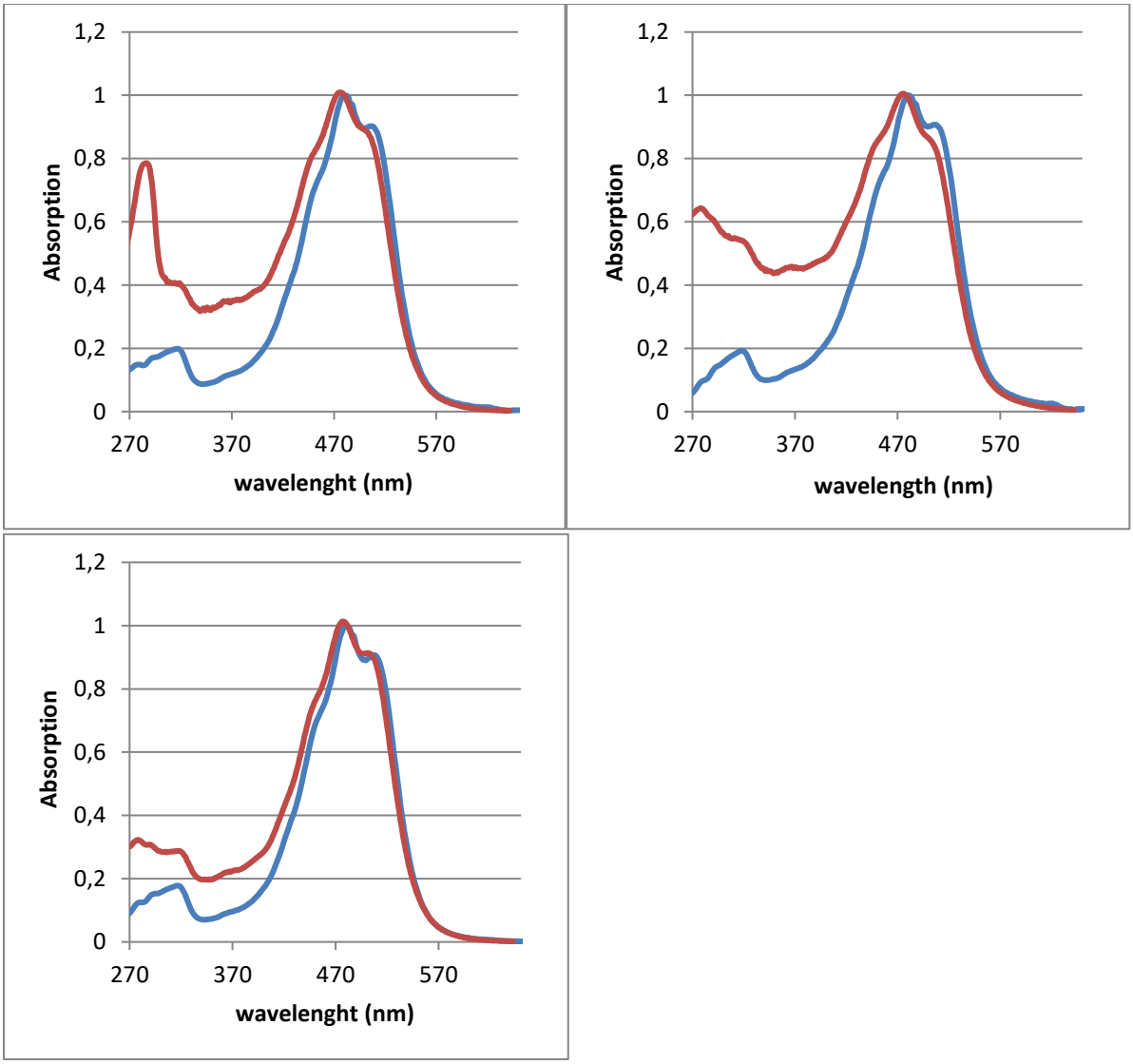

Fig. 21, 22, 23– Absorption spectra (from upper left) peak 1, (upper right) peak 2 and peak 3 (Lower left) Blue spectra showing the absorption before the MS and orange after the MS.

Discussion

The whole-cell carotenoid extract mostly agrees with the previous publications mentioning the whole-cell carotenoid chromatograms, particularly Lemee et al., 1997 [65] and Adamec et al., 2020. [51] The shapes of the whole-cell chromatograms are similar, but it is possible to note the differences. The differences are most pronounced between Adamec and my work compared to Lemee. The differences are most probably caused by the different gradient method. The Jeffrey gradient method [59] which was used for the analysis of the whole-cell carotenoid extract in my work and Adamec et al. 2020, is the method invented for the examination of the pigments from photosynthetic organisms. The photosynthetic organisms have a highly complex pigment composition; therefore, the method is extremely powerful for resolving a wide range of compounds. Indeed, using this this method, the presence of different deinoxanthin analogues in the surface protein was uncovered.

However, while the gradient used is quite an efficient method for separation of the complex carotenoid pool, it is still not perfect. The complexity of the pigment mixture in cells is very high. So, there is a possibility, that the carotenoid pool may be in reality even more complex than shown. Also, the number of compounds binding the surface protein may be higher. Therefore, in future research, the employment or invention of methods with better separation can be used for the analysis of the carotenoid pool.

Based on the spectral information, the carotenoid pool is mostly composed of keto-carotenoids. Most of the compounds contained in the carotenoid pool are the derivates of the main carotenoid deinoxanthin. These conclusions can be based on the fact that the carotenoids have poorly resolved subbands. Moreover, as shown in the plots of the mass-spectrometry samples, the resolution of the shape of the main absorption bands of the carotenoids changes with the solvent. The deinoxanthin is a highly effective ROS scavenger, therefore it is expected that *D. radiodurans* will have a high amount of deinoxanthin and its derivates because it is able to survive the extreme amounts of ROS evolving with UV light, desiccation and radiation. It also corresponds to the carotenoids found in other extremophilic bacteria, as in their carotenoid pool consist mainly of the modifications of the main carotenoid. [68]

Among the carotenoids found in the bacteria, a compound of interest is the one represented by the peak labelled as e, eluting at about 14.75 min. The peak has the absorption spectra shape highly similar to the Lycopene, but as was already mentioned the absorption maxima were

shifted into the longer wavelength. The highest absorption of the Lycopene with similar solvents is recorded to be 470 nm or 490 nm. So, the prediction was made that the compound has to have two more conjugated bonds. An example of such carotenoid, with 13 conjugated bonds is spirilloxanthin, the carotenoid abundant in the purple bacteria. However, so far no reports on spirilloxanthin in *D. radiodurans* have been published, to my knowledge.

The results of the experiment with the illumination with UVC light had a similar result as in Farci et al., 2016. [43] Also, the information given in Slade & Radman, 2011[10] shows the high resistance against the UV light. The wild type of culture of *D. radiodurans* did survive the long UV exposure without a loss in viability. This corresponds with the findings which were observed in these results. The cell life cycle was not affected; however, some small differences were found in chromatograms obtained from cells, which were exposed to UV for different period of times. The most significant change was found in the peak previously labelled as h. Those differences in the carotenoid composition may be triggered by UV-light exposure. Because the UV-light exposure can lead to the creation of the ROS, thus the cell needs more ROS-scavengers for its protection and maintenance of viability.[16] Also, the UV can trigger the DNA mutations in the cells but considering the *D. radiodurans*'s ability to repair its DNA the effect doesn't have to be taken to account.

The differently old cultures of *D. radiodurans* differ in the colour shade and intensity of the cultures. In the Fig 14 was possible to see the changes in peaks c and 3 in the chromatograms, but those changes most probably were not the largest contributors to the colour change. The colour change was most probably a combination of the higher density of cells on the agar and the background colour of the aged agar and the culture drying. But the change in the carotenoid composition may have a different reason. Also when the culture grows older, more protection from the stress is needed, but also protection from its own metabolic waste, which can contain toxic compounds, possibly even ROS. This is most important if the bacteria live in a closed environment, like cell culture, the metabolic waste is cumulating and the waste can endanger the following growth of the culture. Also, the growth of the culture, a drop of nutrient concentration and the starvation of the culture may cause the ROS formation. [69]

The attempts at confirmation of the assignment of the identity of the deinoxanthin derivates using mass spectrometry were not very successful. However, the main pattern of m/z Fig. 19 corresponds to the already published information. [66, 67] The results showed a large variety of the peaks with m/z from 200 to about 2000. This was probably caused by the lack of purity

of the sample. Inspection of absorption spectra of the analyzed compounds showed major differences from the original carotenoids indicating that the procedure of drying and storage of the carotenoids is not appropriate and different approaches of sample handling should be tested. Also, this brings the suggestion of the additional purification of the sample just before the analysis. With respect to this, it is interesting to note that the degradation product of the peak 3 acquired a strong UV absorption, previously not present in the carotenoid. This spectrum strongly resembles the compound labelled h in the whole-cell chromatograms. It is possible that the presence of this compound in the analyses of the whole cells thus reflect not only the state of the pigment pools in cells but can be due to the also sample preparation.

Conclusions

From experiments presented in the results, it can be concluded, that *D. radiodurans* is a bacterium without special needs for cultivation and can be grown easily to obtain high enough amounts of bacteria for performing analysis.

D. radiodurans also can survive quite long exposures of the UVC light without loss of viability of the cells without a need for significant changes in its carotenoid composition.

The pigment content of the cells does not change much during the ageing of the culture. The ratios between the areas of the main three carotenoids known to be bound in the surface protein do not change substantially.

The pigment pool of *D. radiodurans* always contains three main carotenoids, respectively the Deinoxanthin, deinoxanthin glucoside and 2-deoxydeinoxanthin.

The carotenoid composition in *D. radiodurans* changes with increasing UV exposure. The changes are not significant but are visible in normalized chromatograms.

The mass spectrometry results did not give a clear result. It appears that the storage of the pigments might cause degradation of the sample. The stored samples should be purified before future analysis, other ways of sample storage should be investigated.

This brings us to future work ideas. It would be interesting to investigate more the resistance against the oxidative agents, as there were tried only two types of oxidative agents and trying the larger variety of the concentrations of each oxidative agent. Also investigating the survival rates after the exposure of the UV light and comparing the results with common bacteria like *E. coli* and some extremophilic bacteria with higher survival rates.

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