Elsevier Editorial System(tm) for Food Research International
Manuscript Draft

Manuscript Number: FOODRES-D-16-01726R2

Title: S-layer proteins as a source of carotenoids: isolation of the carotenoid cofactor deinoxanthin from its S-layer protein DR_2577

Article Type: SI: Carotenoids

Keywords: Deinococcus radiodurans; S-layer; DR_2577; carotenoids; xanthophyll; deinoxanthin; French Pressure Cell; cold extrusion.

Corresponding Author: Dr. Piano Dario, PhD

Corresponding Author's Institution: University of Cagliari

First Author: Domenica Farci

Order of Authors: Domenica Farci; Francesca Esposito, PhD; Piano Dario, PhD; Sabah El Alaoui, PhD

Manuscript Region of Origin: ITALY

Abstract: S-layers are regular paracrystalline arrays of proteins or glycoproteins that characterize the outer envelope of several bacteria and archaea. The auto-assembling properties of these proteins make them suitable for application in nanotechnologies. However, the bacterial cell wall and its S-layer are also an important binding site for carotenoids and they may represent a potential source of these precious molecules for industrial purposes. The S-layer structure and its components were extensively studied in the radio-resistant bacterium Deinococcus radiodurans which for long time represented one of the model organisms in this respect. The protein DR_2577 has been shown to be one of the naturally over-expressed S-layer components in this bacterium. The present report describes a high scale purification procedure of this protein in solution. The purity of the samples, assayed by native and denaturing electrophoresis, showed how this method leads to a selective and high efficient recovery of the pure DR_2577. Recently, we have found that the deinoxanthin, a carotenoid typical of D. radiodurans, is a cofactor non covalently bound to the protein DR_2577. The pure DR_2577 samples may be precipitated or lyophilized and used as a source of the carotenoid cofactor deinoxanthin by an efficient extraction using organic solvents. The procedure described in this work may represent a general approach for the isolation of S-layer proteins and their carotenoids with potentials for industrial applications.

*Manuscript
Click here to view linked References

S-layer proteins as a source of carotenoids: isolation of the carotenoid cofactor deinoxanthin from its S-layer protein DR_2577

Domenica Farci[a,b], Francesca Esposito[c], Sabah El Alaoui[a], and Dario Piano[a]*

[a]Department of Life and Environmental Sciences, Laboratory of Photobiology and Plant Physiology, University of Cagliari, V.le S. Ingnazio da Laconi 13, 09123 Cagliari, Italy;
[b]Department of Molecular Sensory Systems, Center of Advanced European Studies and Research (caesar), Ludwig-Erhard-Allee 2, 52175 Bonn, Germany;
[c]Department of Life and Environmental Sciences, Laboratory of Molecular Virology, University of Cagliari, Cittadella Universitaria di Monserrato, SS554, 09042 Monserrato, Cagliari, Italy;

*Corresponding author, E-mail: dario.piano@unica.it; phone: +390706753505 fax: +390706753535

Abstract

S-layers are regular paracrystalline arrays of proteins or glycoproteins that characterize the outer envelope of several bacteria and archaea. The auto-assembling properties of these proteins make them suitable for application in nanotechnologies. However, the bacterial cell wall and its S-layer are also an important binding sites for carotenoids and they may represent a potential source of these precious molecules for industrial purposes. The S-layer structure and its components were extensively studied in the radio-resistant bacterium *Deinococcus radiodurans*, which for long time represented one of the model organisms in this respect. The protein DR_2577 has been shown to be one of the naturally over-expressed S-layer components in this bacterium. The present report describes a high scale purification procedure of this protein in solution. The purity of the samples, assayed by native and denaturing electrophoresis, showed how this method leads to a selective and high efficient recovery of the pure DR_2577. Recently, we have found that the deinoxanthin, a carotenoid typical of *D. radiodurans,* is a cofactor non covalently bound to the protein DR_2577. The pure DR_2577 samples may be precipitated or lyophilized and used as a source of the carotenoid cofactor deinoxanthin by an efficient extraction using organic solvents. The procedure

34 described in this work may represent a general approach for the isolation of S-layer proteins and
35 their carotenoids with potentials for industrial applications.
36
37
38 **Keywords:** *Deinococcus radiodurans*, S-layer, DR_2577, carotenoids, xanthophyll, deinoxanthin,
39 French Pressure Cell, cold extrusion**.**
40
41
42 **1. Introduction**
43
44 Many species of eubacteria and archaea are characterized by the presence of a regular
45 paracrystalline array of proteins or glycoproteins constituting the outer envelope of the cell wall
46 (Sleytr 1978; Sleytr *et al.* 1993; Bahl *et al.* 1997; Sára and Sleytr 2000). This structural
47 organization, termed Surface layer or S-layer, is a functionalized structure that contributes to
48 provide different properties, from cell shape to cell protection (Beveridge *et al.* 1997; Sleytr and
49 Sára 1997; Fagan and Fairweather 2014; Farci *et al.* 2016). The properties of S-layer proteins, such
50 as stability and self-assembly (Pum *et al.* 2013), make these structures useful targets for developing
51 new materials for nanotechnology (Ilk *et al.* 2011) and for their potential applications in medicine
52 and biotechnology (Xia *et* al. 2011; Sleytr *et al.* 2014). In this respect, several studies have already
53 showed how the S-layers may find important applications (Sleytr and Sára 1997; Sára *et al.* 2005;
54 Sleytr *et al.* 2007). However, the S-layers are complex structures constituted by one or more
55 proteins that are frequently characterized by secondary post-translational modifications (Sára and
56 Sleytr 2000) and cofactor binding (Farci *et al.* 2016). Moreover, because of their strong tendency to
57 auto-assemble (Pum *et al.* 2013), S-layer proteins are soluble only under harsh conditions making
58 very difficult their industrial handling. Considering these features, the heterologous expression of
59 the S-layer components, either for studying their properties or for exploiting them technologically,
60 is not always the obvious option.
61 The bacterial cell wall and its S-layer are also important binding sites for carotenoids (Smarda *et al.,*
62 2002; Farci *et al*. 2016) and they may represent an industrial unexploited source of these group of
63 molecules. The extended research on the S-layer of *Deinococcus radiodurans* (Baumeister *et al.*
64 1992; Baumeister *et al.* 1996), and the detailed procedures developed for its isolation (Farci *et al.*
65 2015) make this system the perfect model for developing procedures aimed to allow a high scale
66 production of homologous S-layer proteins in solution under mild conditions. This external coat is
67 considered to be the result of the assembly of several proteins in which the protein DR_2577, also

68 named Surface Layer Protein A (SlpA), is one of the main components (Farci *et al.* 2015; Farci *et*
69 *al.* 2014). Thanks to its cofactor, the carotenoid deinoxanthin (Farci *et al.* 2016), this protein is also
70 conferring the typical pink color to its cell wall. The deinoxanthin is known for its strong
71 capabilities in scavenging the Reactive Oxygen Species (ROS) (Tian *et al.* 2007) and, in general,
72 for its strong antioxidant properties (Ji 2010). This carotenoid was found to be involved in
73 providing the radio-resistance (Xu *et al.* 2007), but also in protecting the bacterium from UVC light
74 under desiccation (Farci *et al*. 2016). Deinoxanthin is a monocyclic carotenoid containing oxygen
75 binding functions, such as hydroxyl and ketonic groups, organized into a 2-hydroxy-4-oxo-β-end.
76 The biosynthetic pathway of this enzyme has been fully described (Tian *et al.* 2007). Because of its
77 structural features, deinoxanthin falls into the reactive group of xanthophylls, which due to their
78 oxygen content, are more polar than normal carotenoids and also more reactive, explaining its
79 biological properties. Moreover, as typical for carotenoids, a significative contribution to these
80 properties seems to be provided by the long polyene chain and, even if specific studies are missing,
81 its associated hydroxyl group in the C-1' position may play a pivotal role in shaping these properties
82 (Miller 1934; Palace *et al.*1999) (Fig. 1). Being naturally over expressed, DR_2577 represents a
83 primary component of the S-layer structure, suggesting an essential role in the function of this S-
84 layer (Farci *et al.* 2016) and in its integrity (Rothfuss *et al.* 2006). Electron microscopy studies have
85 shown that this S-layer is organized in a repetition of pores in which the elementary components,
86 characterized by an internal 6-fold symmetry, ultimately build the paracrystalline layer (Baumeister
87 *et al.* 1992; Baumeister *et al.* 1996). In this work we have prepared the cell walls according to Farci
88 *et al.* (2015), digested them with lysozyme, in order to dismantle the carbohydrate network, and
89 finally the cell wall suspension was extruded by using a French Press. This last step allowed to
90 collect the protein in solution up to an extent of 1-2% (w/w) of the starting cell pellet after cultures
91 harvesting. By this procedure, starting from a suspension of *D. radiodurans* cell wall fragments, it
92 is possible to obtain in solution the main S-layer protein, DR_2577. As a further step, we extended
93 this strategy on scaled up cultures as a base for continuous French press disruptors, leading to a
94 continuous flow process suitable for industrial applications.
95 Recently, we found that the deinoxanthin is a cofactor non covalently bound to the protein
96 DR_2577 (Farci *et al*. 2016). Considering this protein as a source of deinoxanthin, we either
97 precipitated or lyophilized DR_2577 and subsequently performed a simple step of carotenoid
98 extraction under different organic solvents.
99 Moreover, we also "modulate" the saturation limit of this experimental system decreasing its
100 proneness to auto-assemble by using mild non ionic detergents in low concentrations. The
101 successful application of this approach suggests a new procedure for the high scale production of

102 homologous over expressed S-layer proteins in solution that is not only useful for their application
103 in nanotechnologies, but also as industrial source of new carotenoids. This approach has the
104 potential to be applied to insoluble proteins from other characterized and uncharacterised bacterial
105 S-layers.
106
107
108 **2. Material and methods**
109
110 **2.1. Bacterial Strain and Growth Conditions**
111 *D. radiodurans* strain R1 (ATCC 13939) was grown in Tryptone/Glucose/Yeast extract broth (TGY)
112 (Murray 1992) for 24 hours at 30°C, with mixing at 250 rpm. The same growth parameters were
113 used either for growing *D. radiodurans* in flasks into an incubator Aquatron Infors HR or in a
114 bioreactor Minifors Infors. Harvesting of the cells was done by centrifugation at 5000 x g for 10
115 minutes at 4°C. The obtained cells pellet was resuspended in 50 mM Na Phosphate pH 7.8
116 (Phosphate buffer).
117
118 **2.2. Isolation of DR_2577 enriched membranes and cold extrusion**
119 Whole cell membrane fractions were purified at 4°C according to Farci *et al.* (2014). For
120 discontinuous French Press, the pellet obtained after cultures centrifugation, consisting in ~ 4-24 g
121 of cells (1-6 L culture in flasks), was resuspended in Phosphate buffer to a final volume of 15-20
122 mL; for continuous French Press, the pellet, consisting in 80-90 g (8 L culture in bioreactor), was
123 resuspended in Phosphate buffer to a final volume of 100-120 mL. After resuspension, both samples
124 were treated with DNase and disrupted at 4°C, in the first case, by using a discontinuous French
125 Pressure Cell (Thermo) and, in the second case, by using a continuous French Pressure Cell
126 (Constant Systems Ltd). In both cases the disruption was performed at 4°C with three cycles at
127 1600 ψ (psi). Unlysed cells were removed by low speed centrifugation (4°C, 2 x 2000 x g for 10
128 minutes). The final supernatant was centrifuged again (4°C, 48000 x g for 10 minutes) and the pink
129 pellet resuspended in 10-20 mL of Phosphate buffer. To remove surface polysaccharides, the
130 membrane suspension was incubated under agitation (800 rpm) with 100 μg/mL lysozyme for 8
131 hours at 30°C. In order to obtain the protein DR_2577 in solution, a second step of disruption was
132 performed by using a French Pressure Cell (at 4°C with three cycles at 1600 ψ) followed by
133 centrifugation (4°C, 48000 x g for 10 minutes). Pure DR_2577 samples obtained by this step were
134 collected and precipitated by centrifugation (4 °C, 4000 x g for 30 minutes) with PEG4000 10% in
135 50 mM Na Phosphate buffer pH 7.4. After centrifugation the supernatant was discharged and the

136 pellet was dried for 6 hours. Alternatively to precipitation, the protein solution was subjected to a
137 step of lyophilization. In both cases the pure DR_2577 solid samples could be used for
138 deinoxanthin extraction by using organic solvents (Fig. 2).
139
140 **2.3. Size exclusion chromatography**
141 The protein sample obtained after extrusion was either concentrated by PEG4000 precipitation (see
142 paragraph 2.2) and resuspended in Buffer B (50 mM Na Phosphate pH 7.4, 0.06% (w/v) β-DDM) to
143 a final volume of ~200 μL for reaching concentrations up to 3-5 mg/mL protein, or concentrated
144 under flow of argon using an Amicon Stirred cell assembled with a 100 kDa cutoff membrane till a
145 final volume of ~500 μL, reaching concentrations up to 1-2 mg/mL. In both cases the final volume
146 was loaded on a 20 mL size exclusion chromatography column (Superose 6 10/300GL, GE
147 Healthcare) previously equilibrated in Buffer B at a flow rate of 0.3 mL/min. The quantification of
148 the extruded proteins was calculated using the ImageJ software (https://imagej.nih.gov) measuring
149 the area under the elution profile after normalization.
150
151 **2.4. Polyacrylamide gel electrophoresis (PAGE) and protein quantification**
152 For denaturing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), 10%
153 (w/v) separating polyacrylamide/urea gels with 4% (w/v) stacking gels were used (Schägger and
154 von Jagow 1987). Samples denaturation was reached adding Rotiload (Roth) and boiling for 10
155 minutes before loading. Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE) was carried
156 out using 3–12% (w/v) continuous gradient gels, as described in Schägger and von Jagow
157 (1991). DR_2577 samples were mixed with 0.25 volumes of Coomassie Blue Solution 5%, (v/v)
158 Serva Blue G, 750 mM aminocaproic acid and 35% (w/v) sucrose. Electrophoresis was carried out
159 at 205 V for 5 h at 4 °C. Both gels were stained with a Coomassie Brilliant Blue G250 solution.
160 Protein concentration was estimated using a nanodrop ND-1000 spectrophotometer with 2 μL of
161 protein sample.
162
163 **2.5. Deinoxanthin isolation and absorption spectroscopy**
164 Pure DR_2577 samples collected after size exclusion chromatography step were precipitated by
165 with PEG4000 or lyophilized (see paragraph 2.2). Subsequently the samples were subject to an
166 efficient extraction using different solvents, polar (methanol, ethanol and acetone) for the orange
167 form or apolar (chloroform and hexane) for the pink form. In the present work the extraction
168 procedure was performed on ~100-200 mg of isolated DR_2577.
169 The two deinoxanthin forms were analyzed by absorption spectroscopy. Both samples

170 concentrations were adjusted to ~ 0.8 AU in their Vis maximum. Measurements were recorded on
171 an absorption Ultra Micro quartz cell with 10 mm light path (Hellma Analytics) using a Pharmacia
172 Biotech Ultrospec 4000 spectrophotometer at 4 °C in the range of 350-650 nm with an optical path
173 length of 1 cm and a band-pass of 2 nm. Each absorption spectrum was normalized with respect to
174 its Vis absorption maximum.
175
176
177 **3. Results**
178
179 **3.1. Cell disruption by continuos French press allows to isolate homogenous bacterial cell**
180 **walls from scaled up cultures -** A small scale procedure, aimed at the chemical-physical
181 characterization of DR_2577 and its carotenoid cofactor deinoxanthin, was described previously
182 (Farci *et al.* 2016). Starting from this procedure, we calculated the yield and the protein purity
183 (Table 1). These parameters were used as indicators for valuing further attempt of higher scales
184 purifications. Accordingly, yields at several culture volumes were estimated taking into account the
185 amount of bacterial cell walls isolated with respect to the amounts of bacterial cells produced for a
186 given volume of culture (Table 1). For low scale cultures, *D. radiodurans* was grown in Erlenmeyer
187 flasks, finding that from a typical volume of 0.75 L of cultures it was possible to harvest a bacterial
188 cells pellet equivalent to 3-4 g/L. These yields remained almost unvaried, observing just a slightly
189 less than proportional pattern, when the cultures were performed in volumes between 0.75 and 6 L
190 (Table 1). In all these cases, the harvested cells were disrupted with a discontinuous-flow French
191 Press using the same volume (15-20 mL) of final cell resuspension, independently from the starting
192 amount of cell pellet (see materials and methods). After disruption, by two steps of centrifugation,
193 firstly the fraction of unbroken cells was separated from the cell wall fragments, and subsequently
194 the cell wall fragments were separated from the crude extract.
195 With the aim to increase the amount of harvested cells, 8 L of cultures were grown into a bioreactor
196 scaling the harvested amount of bacterial cells from the expected weight of 24-32 g to 80-90 g (10-
197 11 g/L), allowing an increase in the growth efficiency of the bacterial cultures. The higher amounts
198 of cells harvested required the increase of the final resuspension volume (~ 100-120 mL), imposing
199 the exchange of the French Pressure cell breakage from a discontinuous-flow, from which was
200 possible to obtain ~500 mg of cell walls/L of media (~4 g of cells), to a continuous-flow system,
201 leading to a lower efficiency in cell walls extraction (~700 mg/L for ~10 g of cells), but having an
202 easy and high scalable procedure for the cell wall isolation (Table 1). With this procedure, by using
203 a bioreactor and a continuous French Pressure system, it became possible to isolate the pink

204 fragments of cell wall using virtually every starting volume of cells. In this way it would be feasible
205 not only a production in small and middle scale, but also a high scale production with industrial
206 purposes.
207
208 **3.2. Isolation of the main S-layer protein DR_2577 by a cold extrusion of the lysozyme**
209 **digested cell wall fragments -** In small scale experiments the suspension of small cell wall
210 fragments was treated with lysozyme. This essential treatment resulted in the digestion of the
211 peptidoglycan net, leading to the exposure of the underlying DR_2577 layer. Lysozyme digestions
212 longer than 12 hours led to a partial release of DR_2577 in solution. This observation suggested that
213 a mechanic stress could cause the selective release of the DR_2577 in solution, as confirmed by a
214 French Press treatment after lysozyme digestion. This treatment dismantled the main S-layer bulk
215 by making a cold extrusion of the cell wall fragments, rather than causing a normal "explosion"
216 associated to an expansion, as happens in the case of intact cells. After digestion, the extrusion of
217 the cell walls brings to an high efficiency release into solution of the protein DR_2577. Typically,
218 cell walls obtained from 3-4 g of cells (from a culture volume of 1 L), allowed the isolation of about
219 68 mg of protein (yield 1.75 % - Table 1), while the high scale preparation, performed using 8 L
220 culture in bioreactor and disrupted using a continuous french press system, leads to the isolation of
221 about 106 mg of protein for each liter of culture consisting in about ~10 g of cells (yield 0.99% -
222 Table 1). Because of the disruption system used in the scaled up cultures, it was evidenced a lower
223 efficiency in quantity (but not in quality) with respect to the small scale (Table 1).However, this
224 decrease is compensated by the massive production allowed with the continuous flow especially in
225 the case of highly valuable products such as S-layer proteins and carotenoids.
226
227 **3.3. The minimal impurities of DR_2577 extruded samples may be removed by size exclusion**
228 **chromatography -** In previous studies it was reported that this protein has a characteristic
229 oligomeric form, most likely consisting in stable hexamers (Farci *et al.* 2015).
230 DR_2577 samples, isolated by a French Press extrusion of the cell wall fragments, were analyzed
231 by SDS-PAGE (Fig. 3a) showing minor impurities and proving their suitability for deinoxanthin
232 extraction (see paragraph 3.4). The purity of the same samples was further assessed by SEC
233 showing a profile with four main peaks. The first peak, dominant with respect to the others (Fig. 4),
234 was exclusively composed of DR_2577 (Fig. 3a) in its hexameric form (Fig. 3b) (Farci *et al.* 2015).
235 The samples used for the SEC analysis were concentrated prior to injection finding that the
236 saturation limit for this protein pool, due to its auto-assembling properties, is very low. This
237 conclusion rises from the observation of diffused protein sheets formed into the samples while

238 concentrating (data not shown). The critical concentration into which protein sheets start to appear
239 was estimated to be in a range between 1-2 mg/mL, while providing the samples with 0.06% of the
240 mild detergent β-dodecylmaltoside (DDM) helped to reach concentrations of 3-5 mg/mL. This
241 allowed to use an analytical SEC, avoiding protein aggregation and precipitation, with the aim to
242 confirm the DR_2577 samples purity and the dominance of its hexameric form (Fig. 3 and 4). This
243 SEC-mediated approach leads not only to an estimation of the samples purity, but also to a further
244 improvement in their purity. While the improvement of purity by SEC is not essential for the quality
245 of the extracted deinoxanthin, it is fundamental for nanotechnological applications of DR_2577.
246 The graphical analysis of the SEC profile allowed to quantify the purity of the analyzed samples.
247 From this analysis it appeared that the samples are represented by DR_2577 hexamers, as
248 confirmed by SDS-PAGE and BN-PAGE (Fig. 3), for the 74,93% and by residual impurities, mainly
249 DR_2577 monomers, small polypeptides and sugars, for the remaining 25,07% (Fig. 4 inset). For
250 fields of application in which the monodispersity is essential, such as the exploit of S-layers
251 proteins auto-assembling and paracrystallinity properties in nanotechnology, a preparative SEC
252 leads to DR_2577 samples in their dominant oligomeric state.
253
254 **3.4. Efficient deinoxanthin extraction from DR_2577 samples -** As described in Farci *et al.*
255 (2016), the protein DR_2577 binds the carotenoid deinoxanthin. After isolation, the protein can be
256 precipitated by using PEG4000 or lyophilized so that the obtained solid samples can be subjected to
257 a final step of efficient solvent-mediated extraction of the carotenoid deinoxanthin. After solvent
258 extraction, it was possible to remove the apoprotein by centrifugation bringing the pure carotenoid
259 in solution. In order to concentrate and protect the carotenoid from oxidation, the obtained solution
260 was treated with a stream of argon, allowing the deinoxanthin isolation in its dry form and making it
261 suitable for subsequent exchanges of solvent for further uses. Deinoxanthin extraction was
262 performed under five different solvents. Depending on the type of solvent, deinoxanthin can be
263 isolated in one of its two forms (Farci *et al.* 2016). Efficient extractions using polar solvents was
264 achieved either using protic solvents, such as methanol and ethanol, or aprotic solvents, such as
265 acetone. After extraction, in both cases the carotenoid turned from its typical pink color to orange.
266 On the contrary, the use of apolar solvents, such as chloroform or hexane, leads to an efficient
267 extraction retaining the typical pink color observed on fresh cell cultures, isolated cell walls or pure
268 DR_2577 samples. In Table 1 is reported a quantification of the deinoxanthin extraction and the
269 relative yield estimation starting from the dry protein. The extraction efficiency was found to be
270 equivalent in the different solvents tested. A summary is reported in Table 2.
271 After extraction the pink form, extracted with chloroform, and the orange form extracted with

272 methanol, were further characterized by absorption spectroscopy. This analysis showed that the two
273 deinoxanthin forms absorbed differently in the signature region between 400-600 nm, which
274 accounts for the absorption of the polyene chain. In this region the two forms of deinoxanthin
275 differed for a typical shift of ~10 nm, with respect to the main absorption band at 480-490 nm, and a
276 shift of ~15 nm for both shoulders, at 445-460 nm and 510-525 nm (Fig. 5 and Tab. 2). While the
277 orange form is well characterized and its structure is known (Lemee *et al.* 1997), the structure of the
278 pink form still remain unrevealed. As an extension of this work, further studies by using NMR, on
279 the pure deinoxanthin samples, and X-ray crystallography, on the DR_2577 crystals, are being
280 performed in order to identify the structure associated to deinoxanthin in its pink form.
281
282
283 **4. Discussion**
284 S-layer proteins are typically characterized by their ability to auto-assemble (Pum *et al.* 2013; Sleytr
285 *et al.* 2005). This property represents an important feature that makes S-layer proteins suitable for
286 nanotechnological applications, a field in which a nanoscale regular repetition is desirable
287 (Rajagopal *et al.* 2004). However, the bacterial cell walls from different organisms are frequently
288 pigmented by the presence of carotenoids (Smarda *et al.* 2002) and, as in the case of the protein
289 DR_2577 (Farci *et al.* 2016), their S-layer proteins may be the cell wall binding site for these
290 molecules. By way of example, it can be mentioned the carotenoid thermozeaxanthin, a
291 xanthophyll typical of *Thermus thermophylus* (Stark *et al.* 2013) and responsible for the yellow
292 color of this bacterium and its S-layer, or the carotenoids bacterioruberin and salinixanthin typical
293 of extremophiles with S-layer such as *Haloferax* (Fang *et al.* 2010), *Halobacterium* and
294 *Salinibacter* (Jehlička *et al.* 2013). However, in all of these cases there are no studies aimed at
295 proving or disproving a specific binding as the one described between DR_2577 and deinoxanthin.
296 The approach described in the present work may represent a way through which the great
297 carotenoids variability associated to the S-layers can be uncovered and exploited. Accordingly, the
298 S-layer proteins of these organisms may represent not only a traditional candidate for their
299 applications in nanotechnologies, but also an exploitable and important source of carotenoids for
300 industrial purposes.
301 In spite of the S-layers robustness and the presence of precious cofactors, their industrial use in
302 biotechnology and nutraceutics is strongly limited by the harsh conditions frequently required for
303 their isolation and for the solubilization of their subunits (Nuβer and König 1987; Lin and Tan
304 2003). These facts lead to heterogeneity and partial destabilization which may imply the loss of
305 monodispersity, an essential requirement to get a pure sample that may be subjected to a not

306 expensive process of cofactor extraction or to a direct use for nanotechnologies. Also the main
307 protein components of the *D. radiodurans* S-layer are known for having auto-assembling properties
308 (Pum *et al.* 2013; Farci *et al.* 2015), and in this case the handling of this S-layer has traditionally
309 passed through harsh chemical-physical conditions for its isolation and solubilization (Thompson *et*
310 *al.* 1982). Here we describe a mild procedure for obtaining the main S-layer protein DR_2577 pure
311 and in solution providing not only a new tool for the isolation of this S-layer protein, but also for
312 the isolation of its cofactor, the carotenoid deinoxanthin. The same approach described here can be
313 extended to similar S-layer proteins opening enormous potentials in the carotenoids industry with
314 particular relevance to the fields of food chemistry and health.
315
316 **4.1. Cell walls extrusion and selective isolation of the Deinoxanthin binding protein DR_2577 -**
317 As mentioned above, the industrial exploit of S-layer proteins is strongly limited by the harsh
318 conditions required for their isolation and solubilization (Nuβer and König 1987; Lin and Tan
319 2003). In this work, we have modified the procedure to isolate one of the main S-layer proteins of
320 *D. radiodurans* avoiding the use of strong detergents such as Sodium Dodecyl Sulphate and high
321 temperatures (Baumeister *et al.* 1982). In contrast to that, we have treated the homogeneous cell
322 wall fragments with lysozyme, leading to the S-layer exposure due to the peptidoglycan digestion,
323 and subsequently we have subjected these fragments to a sequence of French Press cycles. Through
324 these cycles of compression-expansion at low temperatures (4-10 °C), it became possible the
325 release in solution of the protein DR_2577 maintaining a chemical-physical environment
326 characterized by mild parameters such as physiological buffers and low temperatures in absence of
327 harsh detergents. During the expansion in the French Press cycles, the digested cell wall fragments
328 are subjected to forces with different vectoriality, when compared to intact cells. In particular, intact
329 cells are subjected to radial forces which lead to the cell "explosion", while the cell wall fragments
330 are most likely subjected to tangential forces which lead to dismantle the S-layer in its units. Under
331 these conditions the protein can be isolated, concentrated up to 1-2 mg/mL, precipitated or
332 lyophilized and finally subjected to carotenoid extraction. However, not only the harsh procedures
333 may represent a limiting factor for the correct exploit of the S-layers in technology, but also their
334 unique quality of auto-assembling can itself become a limiting factor. In fact, if on the one hand the
335 auto-assembling ability may be considered as a quality suitable of technological applications
336 (Rajagopal *et al.* 2004), on the other hand this implies a strong limitation in protein handling due to
337 the tendency of having an auto-assembling proneness (Pum *et al.* 2013; Farci *et al.* 2015). This
338 property was shown to be concentration dependent and enhanced by the presence of bivalent cations
339 (Faraldo *et al.* 1988). Accordingly, the use of S-layer proteins in solution is limited by the need of

340 operating with low protein concentrations, compromising significantly the economical convenience
341 to handle these proteins industrially. For other applications, in which may be required
342 concentrations higher than 1-2 mg/mL, the protein starts to auto-assemble forming sheets, which
343 can be easily observed by eye. To get to higher concentrations (up to 5 mg/mL) avoiding these
344 protein formations, the samples can be provided with 0.06% of β-DDM, a mild detergent normally
345 used for membrane proteins purification (Gutmann *et al.* 2007). Under this conditions, higher
346 amounts of samples can be subjected to size exclusion chromatography leading to a further increase
347 in purity and homogeneity separating the dominant hexameric form of DR_2577 (Farci *et al.* 2015)
348 from residual impurities (Fig. 2). The yield of DR_2577 reached by this procedure was calculated
349 with respect to the amount of initial bacterial cells and showed a good persistence of efficiency
350 while passing from the low scale to the middle-high scale procedure, allowing to produce S-layer
351 proteins for industrial purposes by using a continuous flow approach (Table 1).
352
353 **4.2. DR_2577 as a source of deinoxanthin -** The deinoxanthin is known as one of the most, if not
354 the most, powerful carotenoid in terms of antioxidant activity (Ji 2010; Tian *et al.* 2007; Tian *et al.*
355 2009). Moreover, it has several other properties which also justify possible fields of application
356 (Table 3). In general, carotenoids represent an important class of molecules involved in the
357 mechanisms of protection against oxidative stress, playing an essential role in the cell homeostasis
358 (Yamaguchi 2012). Because of their function, carotenoids are molecules that can be employed for
359 preventing oxidative stress (Krinsky 1989a) as food-integrators (Woodall *et al.* 1996) and as drugs
360 in the therapy of several degenerative diseases (Krinsky 1989b). It implies that new sources of
361 carotenoids are essential for both providing new molecules with specific profiles of activity, and
362 also for obtaining new useful and cheap sources that may be used as starting carbon back-bounds
363 for functionalization and semi-synthesis. The present procedure, thanks to the fast and gentle
364 isolation of the native DR_2577 in high purity, provides an easily reachable and cheap source of
365 deinoxanthin which can be extracted in purity by using common solvents (Fig. 2). Even if are not
366 unusual for S-layers, carotenoids that bind these structures are poorly characterized (Burczyk *et al.*
367 1981; Resch and Gibson 1983; Burczyk 1986; Trautner and Vermaas 2013). Previously, we have
368 shown that deinoxanthin can be extracted in two different forms, pink and orange (Farci *et al.*
369 2016), and that, the pink form is found to be folded in the protein DR_2577, suggesting this as the
370 active form *in vivo* (Farci *et al.* 2016). The form extracted depends on the organic solvent used
371 (Table 2, Fig. 2). This suggests that polar solvents are able to convert the deinoxanthin from its
372 dominant pink form to the orange one, that actually represents the commonly studied form of this
373 carotenoid (Lemee *et al.* 1997; Tian *et al* 2007; Tian *et al.* 2009; Ji 2010). While functional and

374 structural studies on the two forms of this cofactor and on the cofactor folded in its protein are in
375 progress, the present procedures of extraction provide two variants of this carotenoid which may
376 well represent two different states of oxidation. This study not only represents a general procedure
377 for the isolation of S-layer proteins starting from different bacterial species, but it also represents a
378 general way for the isolation of their specific carotenoids and cofactors.
379
380
381 **5. Conclusions**
382
383 **5.1. Efficient isolation of S-layer proteins in solution and their use as a source of carotenoids**
384 **or as biotechnological tool -** In the present study, using the protein DR_2577 as a model, we have
385 developed a protocol for its purification under mild conditions that is suitable also for isolating
386 other S-layer proteins. This procedure, allowing the harvest of large amounts of a specific S-layer
387 protein, represents a new strategy for the high scale isolation of their cofactors, *via* microbiological
388 factories. Here we apply this procedure for the isolation of the xanthophyll deinoxanthin from pure
389 samples of the protein DR_2577.
390 Dietary supplements providing pure carotenoids became a common practice as a resulting effect of
391 the gaining in importance of antioxidants in dietetics and in food science. The aim of this
392 supplementation span from food quality improvement, by enhancing its organoleptic properties or
393 preventing degradations, to the nutraceutic by promoting and keeping the good health status of the
394 body. As shown in the case of deinoxanthin and *D. radiodurans*, the production of carotenoids from
395 a specific S-layer may provide a “green” new source of unique carotenoids suitable for a low-cost
396 industrial isolation.
397 The described procedure allows to make available big amounts of pure S-layers proteins in solution.
398 S-layer proteins represent an election tool for several applications in technology, from templates
399 aimed to reproduce replicas with nanoscale regularity for several molecules or elements such as
400 palladium (Fahmy *et al.* 2006) and uranium (Henning *et al.* 2001), to their direct use as surfaces
401 with calibrated porosity (Sára and Sleytr 1987; Ulbricht 2006) or with regular functionalization
402 suitable for the construction of nanoscale filters or sensors (Ilk *et al.* 2011).
403 Considering all these properties and potential applications, the extension of this procedure to other
404 microorganisms will increase the market availability of carotenoids and S-layer proteins with a
405 large diversity of properties.
406
407

408 **Competing interests**

409 The procedure of cold extrusion applied to bacterial S-layer proteins is invented by Dario Piano and
410 protected under the patent number IT-102016000006106 "A selective method for the isolation of
411 bacterial S-layer proteins and its industrial application" (Procedimento per l'estrazione delle
412 proteine degli S-layers batterici e sua applicazione su scala industriale).

413

414

415 **Acknowledgements**

416 This work was carried out with support from the program "FSE SARDEGNA 2007-2013,
417 Legge Regionale 7 agosto 2007, n. 7, Promozione della ricerca scientifica e
418 dell'innovazione tecnologica in Sardegna". DP is grateful to the European Synchrotron
419 Research Facility and the Partnership for Structural Biology (Grenoble, France) for
420 preliminary studies.

421

422

423 **References**

424 Bahl, H., Scholz, H., Bayan, N., Chami, M., Leblon, G., Gulik-Krzywicki, T., *et al*. (1997).
425 Molecular biology of S-layers. *FEMS Microbiol Rev, 20*, 47-98.

426

427 Baumeister, W., Karrenberg, F., Rachel, R., Engel A, Ten Heggeler, B., Saxton, W. O.
428 (1982). The major cell envelope protein of *Micrococcus radiodurans* (R1). Structural and
429 Chemical Characterization. *Eur J Biochem, 125,* 535-44.

430

431 Baumeister, W., Karrenberg, F., Rachel, R., Engel, A., Heggeler, B., Saxton, W. O. (1992). The
432 major cell envelope protein of *Micrococcus radiodurans* (R1). Structural and chemical
433 characterization. *Eur J Biochem, 125,* 535-544.

434

435 Baumeister, W., Barth, M., Hegerl, R., Guckenberger, R., Hahn, M., Saxton, W. O. (1996).
436 Three-dimensional structure of the regular surface layer (HPI layer) of *Deinococcus*
437 *radiodurans*. *J Mol Biol, 187,* 241-50.

438

439 Beveridge, T. J., Pouwels, P. H., Sára, M., Kotiranta, A., Lounatmaa, K., Kari, K., *et al*. (1997).
440 Functions of S-layers. *FEMS Microbiol Rev, 20,* 99-149.

441

442 Burczyk, J., Szkawran, H., Zontek, I., Czygan, F.C. (1981). Carotenoids in the outer cell-wall layer
443 of Scenedesmus (Chlorophyceae). *Planta, 151,* 247-50.
444

445 Burczyk, J. (1986). Cell wall carotenoids in green algae which form sporopollenins.
446 *Phytochemistry, 26,* 121-128.
447

448 Fahmy, K., Merroun, M., Pollmann, K., Raff, J., Savchuk, O., Henning, C., Selenska-Pobell, S.
449 (2006). Secondary structure and $Pd^{(II)}$ coordination in S-layer proteind from *Bacillus sphaericus*
450 studied by infrared and X-ray absorption spectroscopy. *Biophys J, 91,* 996-1007.
451

452 Fagan, R. P., & Fairweather, N. F. (2014). Biogenesis and functions of bacterial S-layers. *Nature*
453 *Reviews Microbiology, 12,* 211-222.
454

455 Fang, C. J., Ku, K. L., Lee, M. H., Su, N. W. (2010) Influence if nutritive factors on C50
456 carotenoids production by *Haloferax mediterranei ATCC 33500* with two-stage cultivation.
457 *Bioresour Technol, 101,* 6487-93.
458

459 Faraldo, M. M., de Pedro, M. A., Berenguer, J. (1988). Purification, composition and Ca2+-binding
460 properties of the monomeric protein of the Slayer of *Thermus thermophilus. FEBS Lett, 235,* 117-
461 21.
462

463 Farci, D., Bowler, M. W., Kirkpatrick, J., McSweeney, S., Tramontano, E., Piano, D. (2014).
464 New features of the cell wall of the radio-resistant bacterium *Deinococcus radiodurans*.
465 *Biochim Biophys Acta, 1838,* 1978-84.
466

467 Farci, D., Bowler, M. W., Francesca, E., McSweeney, S., Tramontano, E., Piano, D. (2015).
468 Purification and characterization of DR_2577 (SlpA) a major S-layer protein from
469 *Deinococcus radiodurans*. *Front Microbiol, 6,* 414. doi:10.3389/fmicb.2015.00414.
470

471 Farci, D., Slavov, C., Tramontano, E., Piano, D. (2016). The S-layer protein DR_2577
472 binds the carotenoid deinoxanthin and under desiccation conditions protect against UV-
473 radiation in *Deinococcus radiodurans. Front Microbiol. 7,* 155. doi:
474 10.3389/fmicb.206.00155.
475

476 Gutmann, D. A. P., Mizohata, E., Newstead, S., Ferrandon, S., Henderson, P. J. F., van
477 Veen, H. W., Byrne, B. (2007). A high-throughput method for membrane protein solubility
478 screening: the ultracentrifugation dispersity sedimentation assay. *Protein Sci, 16,* 1422-28.
479
480 Hennig, C., Panak, P. J., Reich, T., Rossberg, A., Raff, J., Selenska-Pobell, S. S., Matz,
481 W., Bucher, J. J., Bernhard, G., Nitsche, H. (2001). EXAFS investigation of uranium(VI)
482 complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochim Acta,*
483 *89,* 625-31.
484
485 Ilk, N., Egelseer, E. M., Sleytr, U. B. (2011). S-layer fusion proteins-construction principles and
486 applications. *Curr Opin Biotechnol, 22,* 824-31.
487
488 Jehlička, J., Edwards, H. G., Oren, A. (2013). Bacterioruberin and salinixanthin carotenoids of
489 extremely halophilic Archaea and Bacteria: a Raman spectroscopic study. *Spectrochim Acta A Mol*
490 *Biomol Spectrosc, 106,* 99-103.
491
492 Ji, H. F. (2010). Insight into the strong antioxidant activity of deinoxanthin, a unique carotenoid in
493 *Deinococcus radiodurans*. *Int J Mol Sc, 11,* 4506-10. doi: 10.3390/ijms11114506.
494
495 Krinsky, N. I. (1989a). Antioxidant functions of carotenoids. *Free Radic Biol Med, 7,* 617-35.
496
497 Krinsky, N. I. (1989b). Carotenoids and cancer in animal models. *J Nutr, 119,* 123-6.
498
499 Lemee, L., Peuchant, E., Clerc, M., Brunner, M., Pfander, H. (1997). Deinoxanthin: A new
500 carotenoid isolated from *Deinococcus radiodurans*. *Tetrahedron*. 53, 919-926.
501
502 Lin, J., & Tan, T. S. T. (2003). Self-assemply of *Deinococcus radiodurans* S-layer proteins. *Techn.*
503 *Proc. 2003 Nanotech. Conf, Trade Show*. *3,* 39-42.
504
505 Miller, E. S. (1934). Quantitative absorption spectra of the common carotenoids. *Plant Physiol.* 9,
506 693–694. doi: 10.1104/pp.9.3.681
507
508 Murray, R. G. E. (1992). The family *Deinococcaceae*. *The Prokaryotes.* New York: Springer. pp.

509 3732–3744.
510
511 Nuβer, E., & König, H. (1987). D-layer studies on three species of *Methanococcus* living at
512 different temperatures. *Can J Microbiol, 33,* 256.
513
514 Palace, V. P., Khaper, N., Qin, Q., Singal, P. K. (1999). Antioxidant potentials of vitamin A and
515 carotenoids and their relevance to heart disease. *Free Radic Biol Med*, 26:746-61.
516
517 Pum, D., Toca-Herrera, J. L., Sleytr, U. B. (2013). S-layer protein self-assembly. *Int J Mol Sci, 14*,
518 2484-501.
519
520 Rajagopal, K., & Schneider, J. P. (2004). Self-assembling peptides and proteins for
521 nanotechnological applications. *Curr Opin Struct Biol, 14,* 480-6.
522
523 Resch, C.M., & Gibson, J. (1983). Isolation of the carotenoid-containing cell wall of three
524 unicellular cyanobacteria. *J Bacteriol, 155,* 345-350.
525
526 Rothfuss, H., Lara, J. C., Schmid, A. K., Lidstrom, M. E. (2006). Involvement of the S-layer
527 proteins HPI and SlpA in the maintenance of cell envelope integrity in *Deinococcus radiodurans*
528 R1. *Microbiol, 152,* 2779-87.
529
530 Sára, M., & Sleytr, U. B. (1987). Production and characteristics of ultrafiltration membranes with
531 uniform pores from two-dimensional arrays of proteins. *J Mem Sci, 33,* 27-49.
532
533 Sára, M., & Sleytr, U. B. (2000). S-layer proteins. *J Bacteriol, 182,* 859-868.
534
535 Sára, M., Pum, D., Schuster, B., Sleytr, U. B. (2005). S-layer as pattering elements for application
536 in nanobiotechnology. *J Nanosci Nanotechnol, 5,* 1939-53.
537
538 Schägger, H., & von Jagow, G. (1987). Tricine Sodium Dodecyl-Sulfate Polyacrylamide-Gel
539 Electrophoresis for the Separation of Proteins in the Range from 1-Kda to 100-Kda. *Anal Biochem,*
540 *166,* 368-379.
541
542 Schägger, H., & von Jagow, G. (1991). Blue Native Electrophoresis for Isolation of

543 Membrane-Protein Complexes in Enzymatically Active Form. *Anal Biochem, 199,* 223-231.
544
545 Sleytr, U. B. (1978). Regular arrays of macromolecules on bacterial cell walls: structure, chemistry,
546 assembly, and function. *Int Rev Cytol, 53,* 1-62.
547
548 Sleytr, U. B., Messner, P., Pum, D., Sára, M. (1993). Crystalline bacterial cell surface layers. *Mol*
549 *Microbiol, 10*, 911-916.
550
551 Sleytr, U. B., & Sára, M. (1997). Bacterial and archaeal S-layer proteins: structure-function
552 relationship and their biotechnological applications. *Trends Biotech, 15,* 20-26.
553
554 Sleytr, U. B., Sára, M., Pum, D., Schuster, B., Messner, P., Schäffer, C. (2005). Self-assembling
555 protein systems: microbial S-layers. *Biopolymers Online.* doi: 10.1002/3527600035.bpol7011
556
557 Sleytr, U. B., Egelseer, E. M., Ilk, N., Pum, D., Schuster, B. (2007). S-layer as a basic building
558 block in a molecular construction kit. *FEBS J, 274,* 323-34.
559
560 Sleytr, U. B., Schuster, B., Egelseer, E. M., Pum, D. (2014). S-layer: principles and applications.
561 *FEMS Microbiol Rev, 38,* 823-64.
562
563 Smarda, J., Smajs, D., Komrska, J., Kryzánek, V. (2002). S-layers on cell wall of cyanobacteria.
564 *Micron, 33,* 257-277.
565
566 Stark, T. D., Angelov, A., Hofmann, M., Liebl, W., Hofmann, T. (2013). Comparative direct infusion
567 ion mobility mass spectrometry profiling of *Thermus thermophilus* wild-type and mutant ΔcruC
568 carotenoid extracts. *Anal Bioanal Chem, 405,* 9843-8.
569
570 Tian, B., Xu, Z., Sun, Z., Lin, J., Hua, Y. (2007). Evaluation of the antioxidant effects of carotenoids
571 from *Deinococcus radiodurans* through targeted mutagenesis, chemiluminescence, and DNA
572 damage analyses. *Biochim Biophys Acta, 1770,* 902-11.
573
574 Tian, B., Sun, Z., Shen, S., Wang, H., Jiao, J., Wang, L., Hu, L., Hua, Y. (2009). Effects of
575 carotenoids from *Deinococcus radiodurans* on protein oxidation. *Lett Appl Microbiol, 49,* 689-94.
576 doi: 10.1111/j.1472-765X.2009.02727.x.

577

578 Thompson, B. G., Murray, R. G. E., Boyce, J. F. (1982). The association of the surface array and the
579 outer membrane of *Deinococcus radiodurans. Can J Microbiol, 28,* 1081-88.
580
581 Trautner, C., & Vermaas, W.F.J. (2013). The *sll1951* gene encodes the surface layer protein of
582 *Synechocystis* sp. Strain PCC 6803. *J Bacteriol, 195,* 5370-5380.
583
584 Ulbricht, M. (2006). Advanced functional polymer membranes. *Polymer, 47,* 2217-62.
585
586 Woodall, A. A., Britton, G., Jackson, M. J. (1996). Dietary supplementation with carotenoids:
587 effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. *Br J Nutr, 76,* 307-17.
588
589 Xia, Y., Nguyen, T. D., Lee, B., Santos, A., Podsiadlo, P., Tang, Z., Glotzer, S. C., Kotov, N. A.
590 (2011). Self-assembly of self-limiting monodisperse supraparticles from polydisperse nanoparticles.
591 *Nat Nanotechnol, 6,* 580-7.
592
593 Xu, Z., Tian, B., Sun, Z., Lin, J., Hua, Y. (2007). Identification and functional analysis of a
594 phytoene desaturase gene from the extremely radioresistant bacterium *Deinococcus radiodurans.*
595 *Microbiology, 153,* 1642-52.
596
597 Yamaguchi, M. (2012). Role of carotenoid β-cryptoxanthin in bone homeostasis. *J Biomed Sci, 19,*
598 36.
599
600

601 **Figures**

602

603 **Figure 1:** Deinoxanthin structure (orange form). In red are indicated the hydroxyl (C-2 and
604 C-1' positions) and ketonic (C-4 position) groups that characterize this xanthophyll. This
605 carotenoid is monocyclic with a typical 2-hydroxy-4-oxo-β-end.

606

607 Figure 2: Schematic flowchart of protein isolation and deinoxanthin extraction.

608

609 **Figure 3:** In a) it is shown a SDS-PAGE of DR_2577 samples isolated by extrusion (left)
610 and on the main peak of the same sample after SEC (right). M indicates the molecular

marker. For both samples the apparent weight of the DR_2577 band is consistent with its theoretical mass (123.7 kDa) when compared with the third band of the molecular marker (120 kDa). In b) it is shown a BN-PAGE of a pure DR_2577 sample obtained after SEC and compared to a molecular marker (M). The apparent weight of the DR_2577 native complex band is consistent with its hexameric form (~ 740 kDa).

Figure 4: SEC profile of a extruded DR_2577 sample. The main peak (red dot) represents the dominant hexameric form of DR_2577 (see also Figure 2). The inset indicates the contribution between the DR_2577 peak (1) and the other peaks (2, 3, 4) represented by impurities. The measurement was recorded at a wavelength of 280 nm.

Figure 5: Absorption spectra of the two deinoxanthin forms: the orange form is extracted with methanol (orange curve), while the pink form is extracted using chloroform (pink curve). Both forms present the typical polyene signal, but shifted each other.

Tables

Table 1: Parameters for the DR_2577 isolation under different growth conditions and their related DR_2577 yields.

	Volume (mL)	Culture (mL)	French Press / Cell Suspension volume (mL)	Cell Pellet (g/L)	Cell Wall Fragments (mg/L)	Cell Wall Fragments Extrusion Volume	DR_2577		Deinoxanthin**	
							(mg/L)	Yield (%)	(mg/L)	Yield (‰)
Flasks*	2000	750	Discontinuous 15/20 mL	3,88	504,80	7-10 mL	68,03	1,75	0.27	0.07
Bioreactor	8000	8000	Continuous 100/120 mL	10,91	704,51	7-10 mL	106,30	0,97	0.44	0.04

* The amounts of harvested cells, isolated cell walls and isolated protein, growth proportionally from 1 (750 mL media) to 8 Erlenmeyer flasks (6000 ml media). For this reason all values are referred to 1L of cultures;

640 ** extractions using different solvents. Yields for different deinoxanthin forms were equivalent.

641

642

643 **Table 2:** Extraction of deinoxanthin in its two different forms, pink and orange by using

644 different solvent types.

645

type of solvent	Polar solvents		Apolar solvents
	Protic	**Aprotic**	
solvents tested	methanol ethanol	acetone	chloroform, hexane
deinoxanthin color	**Orange**		**Pink**
solvent used	**methanol**		**chloroform**
absorption bands (nm)	445; 480; 510		460; 490; 525

646

647

648

649

650

651

652

653

654

655

656 **Table 3:** Functional activities of deinoxanthin and possible applications as reported in

657 literature.

658

Type of activity	Possible applications	Reference
Apoptosis induction in cancer cells	Chemotherapy	Induction of apoptosis by deinoxanthin in human cancer cells (Choi *et al*, 2014).
Antioxidant properties in vivo and in vitro	Chemotherapy Dietary supplementation Therapeutic supplement (e.g. Neurodegenerative diseases)	Insight into the strong antioxidant activity of deinoxanthin, a unique carotenoid in *Deinococcus* (Ji, 2010). Evaluation of the antioxidant effects of carotenoids from *Deinococcus radiodurans* through target mutagenesis, chemiluminescence, and DNA damage analyses (Tan *et al.*, 2007).
UVB-C filter	Dermatology, Cosmetics	The S-layer protein DR_2577 binds deinoxanthin and under desiccation conditions protect against UV-radiation in *Deinococcus radiodurans* (Farci *et al.*, 2016).

		Solar selective pressure in the photobiology of *Deinococcus radiodurans*. (Farci *and* Piano, 2016).
Algicidal activity	Treatment of algal blooms	The first evidence of deinoxanthin from *Deinococcus sp. Y35* with strong algicidal effect on the toxic dinoflagellate *Alexandrium tamarense* (Li *et al.*, 2015).

659

Figure_1
Click here to download high resolution image

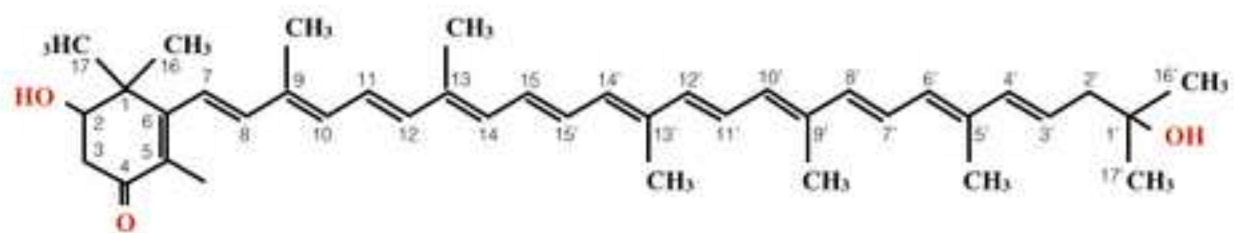


Figure_2
Click here to download high resolution image

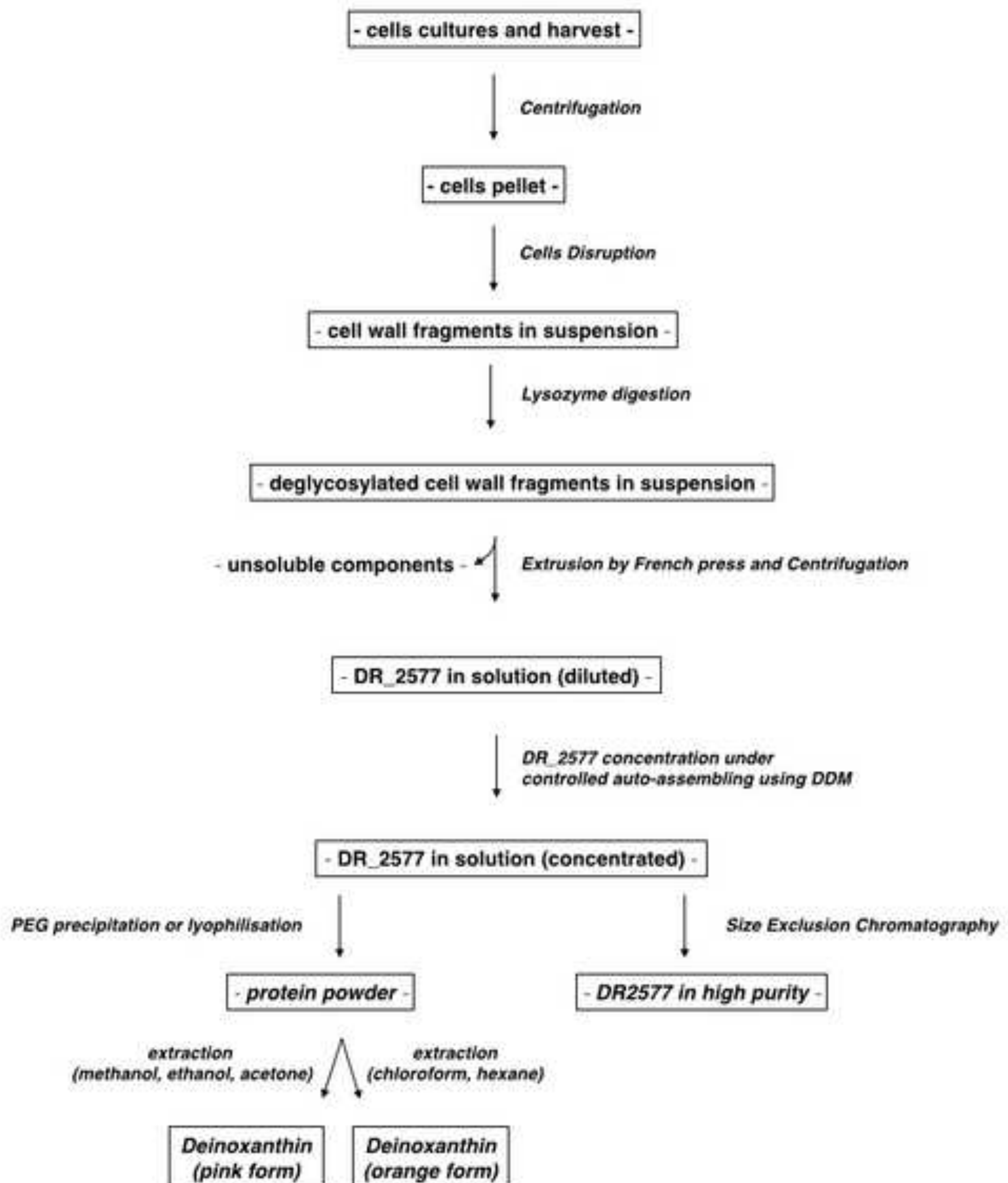


Figure_3
Click here to download high resolution image

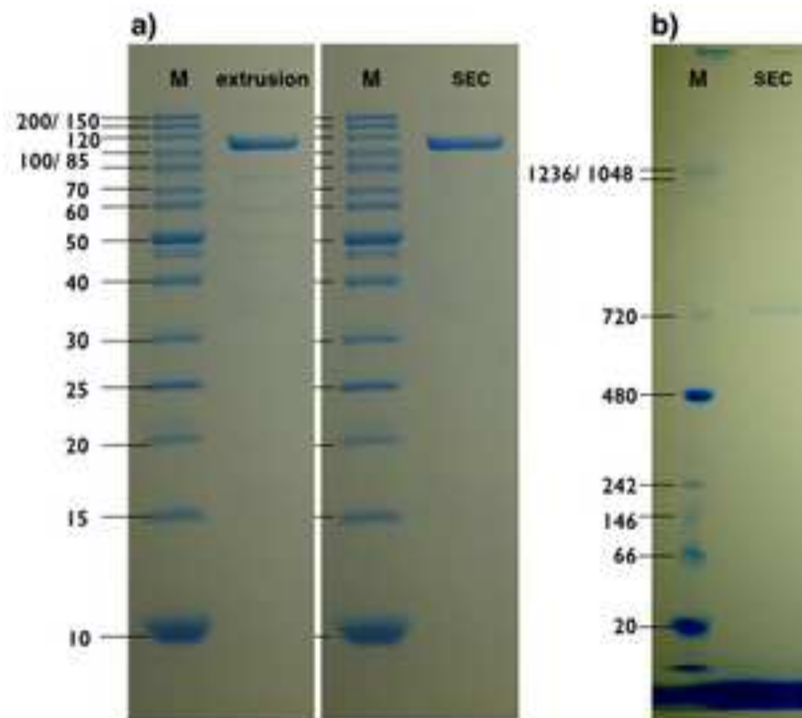
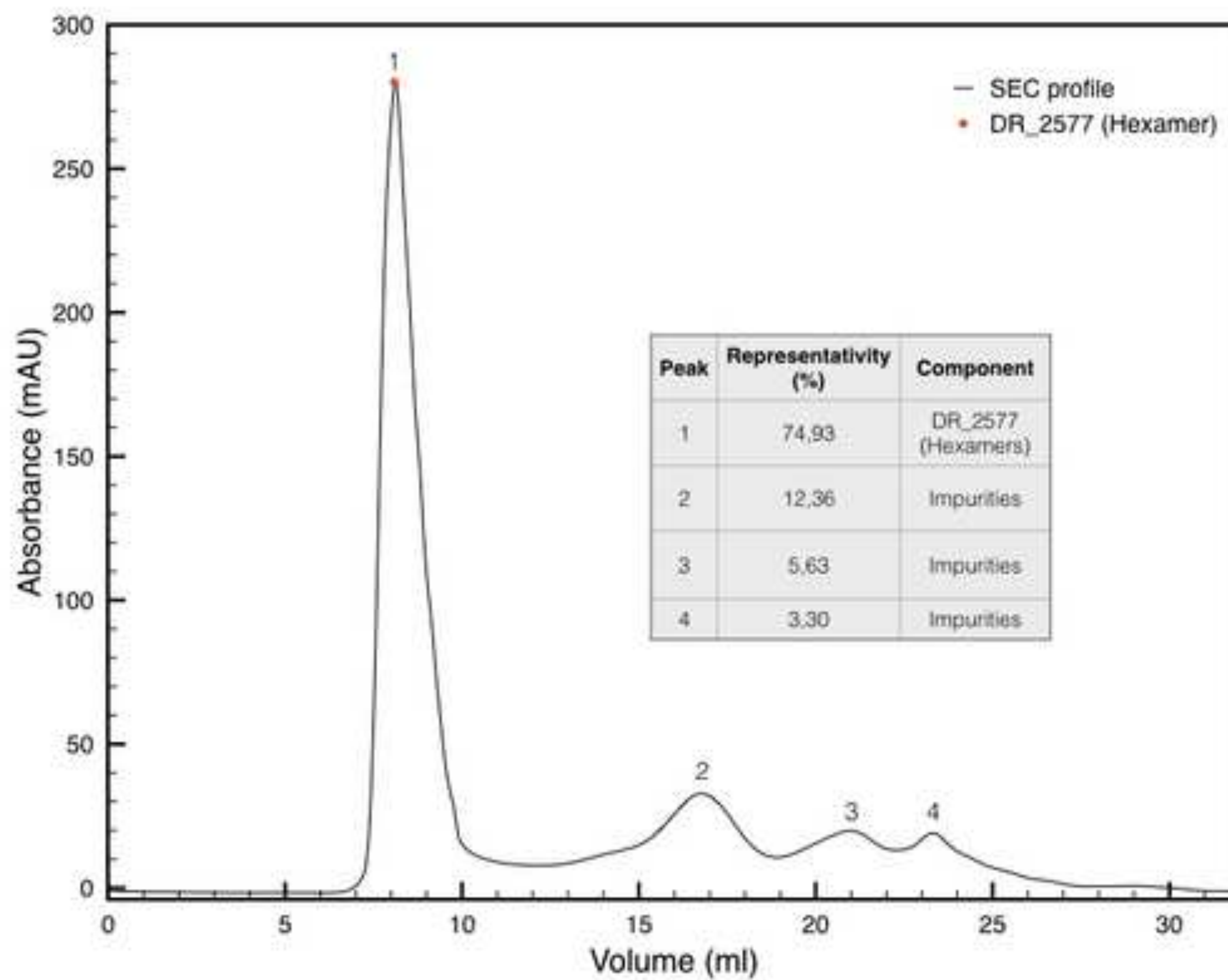


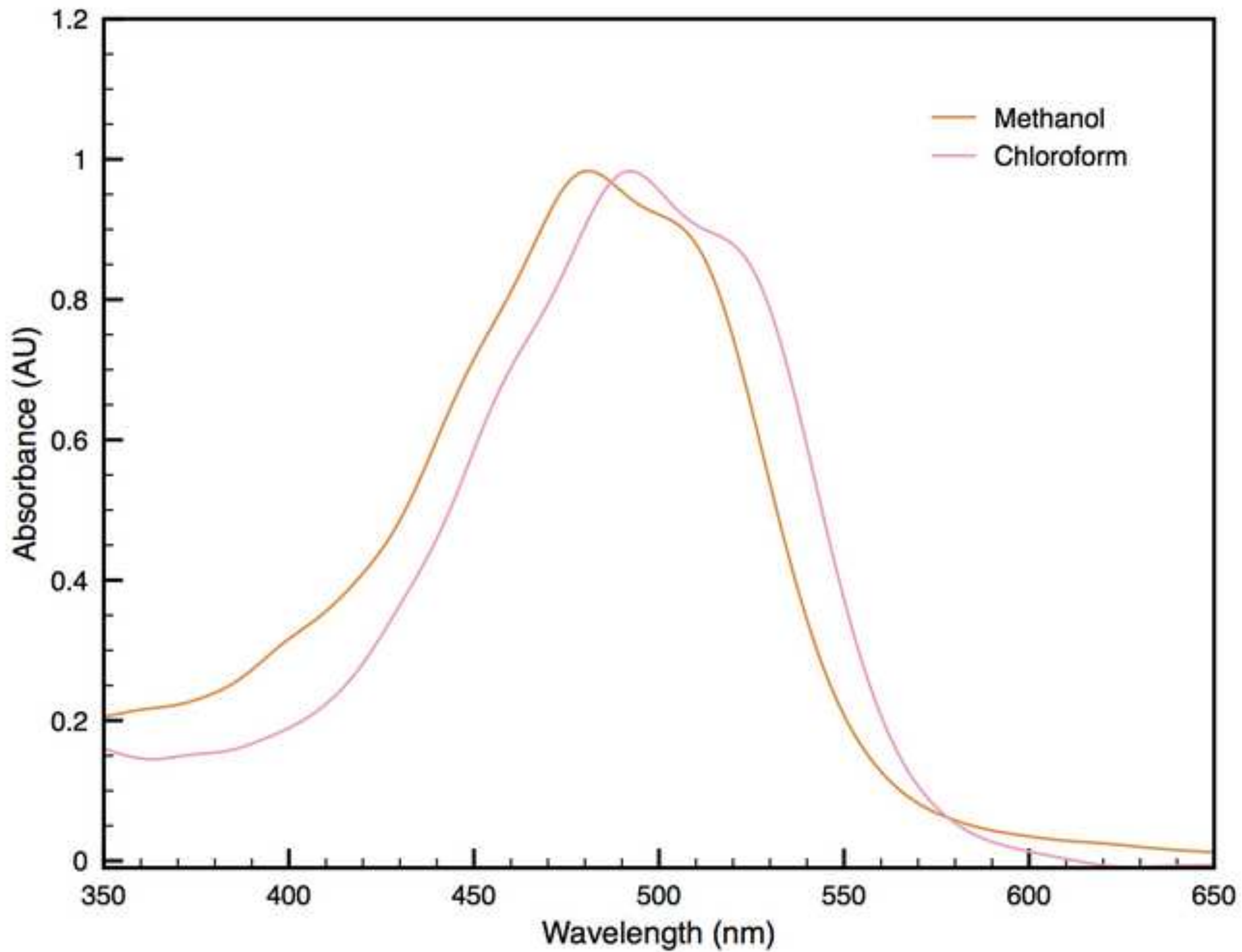
Figure_4

Click here to download high resolution image



Peak	Representativity (%)	Component
1	74,93	DR_2577 (Hexamers)
2	12,36	Impurities
3	5,63	Impurities
4	3,30	Impurities

Figure_5
Click here to download high resolution image



*Graphical Abstract
Click here to download high resolution image