

Extraction and Purification of Exopolysaccharides from Exhausted *Arthrospira platensis* (Spirulina) Culture Systems

Giorgia Sed*, Agnese Cicci, Marco Bravi

Dept. Chemical Engineering Materials Environment, University of Rome La Sapienza, via Eudossiana 18, 00184 Rome.
giorgia.sed@uniroma1.it

Microalgal endo and exopolysaccharides (EPS) are attracting increasing interest for their potential applications in the food, cosmetic and pharmaceutical industries. The standard applications of microbial EPS are as food coatings, emulsifying and gelling agents, flocculants, hydrating agents etc. They present unique biochemical properties that make them interesting from the biotechnological point of view. Their physical-chemical properties are interesting for biomedical applications, since polysaccharides have been demonstrated to possess inhibitory properties against various types of viruses, bacteria and tumors. The purpose of this work is to upgrade the exhausted culture media resulting from the cultivation of the cyanobacterium *Arthrospira platensis* (Spirulina), in order to extract the exopolysaccharides excreted by the cyanobacterium and test their exploitation potential in a cosmetic context (a body cream). The study results include: defining the composition and the productivity of EPS by the Spirulina culture, developing a suitable application method for the DPPH assay in lipophilic matrices, and evaluation of the antioxidant action of these polymers in the cosmetic field.

1. Introduction

The variety of nutrients and bio-active molecules which make up the composition of microalgae, has made them an interesting resource well beyond the original specialty food area. Depending on the microalgal species considered, a different richness in protein, carotenoids, phycobiliproteins, polysaccharides, pigments, vitamins, sterols and essential ω -3 and ω -6 polyunsaturated fatty acids (PUFAs) can be found (Cicci and Bravi 2016). The current most profitable commercial applications of microalgae still mainly reside in food integration, but feed functionalization and cosmetics formulation are stemming as important development areas given the enormous amount of bio-active components, with high added value, deriving from their primary and secondary metabolism (Raposo et al. 2013a). An increasing number of microalgae is then being investigated for their potential in the pharmaceutical domain (Olaizola, 2003; Raposo et al, 2013b), which makes microalgae real "bio-green factories" (Chisti, 2006)

An increasing commitment to microalgal metabolites is observed from the cosmetic industry part which is seeking to find innovative natural additives derived from plants or from microorganisms, to be applied in bio-cosmetic products for the care and well-being of the person that can replace synthetic ones normally in use and under some concern for possible negative effects on consumer health (Borowitzka, 2013).

In this study we outlined an upgrade path for the exhausted culture media resulting from the cultivation of the cyanobacterium *Arthrospira platensis* (Spirulina), in order to extract the exopolysaccharides (EPSs) excreted by the cyanobacterium into the culture medium and test the potential of the recovered EPSs in a body cream formulated therefrom. Biopolymers are secondary metabolites released from cyanobacteria in the surrounding environment, predominantly during the stationary growth phase of the microorganism, having the purpose of protection against tensions of extreme habitats and harmful conditions. The sugar chains configuration depends on many factors related to: culturing conditions, availability and type of nutrients. EPS provide hydroxyl and ketone groups easily attacked by reactive oxygen species (ROS), promoting the stability of the cream and an antioxidant effect to the skin. After defining the composition and the productivity of EPS by the Spirulina culture the potential antioxidant action of these polymers in the cosmetic field was assessed. EPS were separated from the exhausted culture media and used as ingredients of a moisturizing body lotion

(parabens- and nickel-free). The first formulation concerned an EPS-free cream (used as blank in the tests), then samples at 0.5% and 2.5% of EPS were produced. Their antioxidant power over time was checked with the DPPH assay, in normal storage conditions (darkness - room temperature) or stressed conditions (darkness - 45 °C). In addition, such parameters as the pH and the zeta potential were monitored, to evaluate the stability of the produced cosmetic emulsions. To make the cost analysis is primarily assumed to industrial plant, using the of PROII process simulation software. Costing was performed with two objectives in mind: define an operating window of the EPS recovery cost and, even more, directing future experimental investigation efforts toward the main source of product recovery cost.

2. Materials and Methods

2.1 Growth

The cyanobacteria *Arthrospira platensis* (courtesy of CNR-ISE Sesto Fiorentino) on Zarrouk agar plate, was inoculated in the same liquid culture medium, in 6 cm diameter cylindrical glass tube and grown until it reached the mass concentration of 3.5 g/L. Then it was transferred in 5000 mL cylindrical PCA photobioreactor, with a diameter of 12 cm and grown in axenic conditions, feeding it with filtered and humidified air (flow rate $130 \times 10^3 \text{ Nm}^3/\text{h}$). 16 hours photoperiod of light, provided by cold white fluorescent lamps (400-700 nm, 865 K, 32 W, $80 \text{ mmol photons m}^2 \text{ s}^{-1}$), was followed by a period of darkness equal to 8 hours. The temperature was maintained constant at 28 ± 1 °C. According to Lambert-Beer law, microalgae concentration is directly proportional at the absorbance. The cellular density was correlated with the absorbance measured at a wavelength of 690 nm, corresponding to the absorption peak of chlorophyll by means of a spectrophotometer (UV1800PC by Shanghai Mapada Instruments Co., Ltd).

At the stationary phase of growth, 200 mL of biomass was collected, centrifuged at 15000 rpm for 30 minutes at 18 °C, the exhausted culture medium was stored and the cellular pellet was washed twice with distilled water. Afterwards, the collected algae were transferred in a humidity free glass flask and dried in the vacuum oven at the temperature of 110 °C overnight, the dry weight was calculate subtracting the tare from the gross weight.

2.2 EPS extraction method

The exhausted supernatant was filtered under vacuum with Whatman filter paper, grade 1. In a first set of experiment the same volume of ethanol was added at the solution (Planas Gisbert, 2013). In the second set, a double volumetric quantity of ethanol 95 % v/v, was added to the permeate, and the solution was transferred in a stirred jacketed vessel, refrigerated at the temperature of 4 °C overnight. In this way the solubility of EPS is reduced, forming a white precipitate on the vessel bottom. The water - ethanol solution was removed and the solid precipitate was suspended in 200 mL of H₂O. The next step was a dead-end ultrafiltration with a filter cut-off of 100 kDa, it lasted for 10 hours at a transmembrane pressure of 4 bar. The obtained concentrate EPS solution was put in a oven at 70 °C for six hours until the complete evaporation of water. The white EPS powder was ready for further steps of treatment.

2.3 Metabolites measurement

The total carbohydrates concentration was assayed with the colorimetric test of Dubois (Dubois et al. 1956) and the reducing sugars with the Miller assay (Miller et al. 1959). Both assays were needed to quantify the productivity of microalgal exopolysaccharides. Productivity was recorded twice a week, assaying the exhausted media in the three weeks of biomass cultivation, with the following results: 0.356, 0.373, 0.399, 0.404, 0.444 and 0.456 gEPS/gDryWeight days⁻¹. The ability of EPS to work as radical scavengers was confirmed with the DPPH test on the creams, following the Brand-William's protocol with some corrections (Kedare and Singh, 2011). Results of our cream antioxidant activity in mg/L of Trolox equivalents are reported in the section 3.1.

2.4 Cream formulation and characterization

The base cream cosmetic formulation was developed following European Community guidelines, with natural ingredients: almond oil, shea butter, beeswax, distilled water, starch powder and lavender essential oil. In two different formulations were added respectively the 0.5 % and the 2.5 % of EPS w/w, removing the same quantity of starch from the ingredients.

The shelf-life of the creams was tested, monitoring parameters as colour, odour, pH (pH-meter of Hanna Instruments HI 8418), zeta potential and particle size (Nanosizer-Zeta Sizer Malvern). The measures were effectuated with the zeta potential analyzer at 26 °C, after 30 seconds of ultrasonication, samples were suspended in bidistilled water in a 1 cm cuvette and an electric field with an average intensity of 15 V/cm was applied. Results of these investigation were reported in the Table 1. and Table 2. in section 3.2.

2.5 Stress test

To evaluate the stability of the lotions and to verify the antioxidant effects of EPS, an Accelerated Shelf-Life Test (ASLT) was performed, comparing peculiar parameters already mentioned, for a regularly conserved cream at room temperature in complete darkness and a stressed cream, stored for 10 days at 45 °C in a humidified oven (corresponding at two months period at room temperature, according to European Cosmetics, Toiletry and Perfumery Association).

2.6 Process simulation

A simplified process for the precipitation of EPS was devised including as key steps: an EPS preconcentration step by membranes (that can also include a dia-filtration sub-step to carry out decontamination from residues of the culturing process); an ethanol-assisted flocculation step; an ethanol recovery step, by distillation. The process was simulated (Figure 1) for the purpose of identifying the size of required equipment and the utility requirements. To recover ethanol at 90% v/v, is required a plate column of 0.61 m of diameter and an height of 6.2 m with 10 plates (N). To improve the ethanol recovery at 95% v/v, the height of the column rises until 8.2 m and the N is 22. The key processes steps generated the Main Equipment Part list the plant cost was estimated upon according to the Guthrie method (Towler, 2012). Best cases in our simulation, assess the final cost for a plant is respectively 329.000 € for the recovery of ethanol at 90% v/v and 360.000 € for the ethanol at 95% v/v. It is possible thanks to an inlet flow rate of 36.17 kg/h and a membrane surface of 11.65 m² in the first case and an inlet flow rate of 32.15 kg/h in the second case, maintaining the same membrane surface.

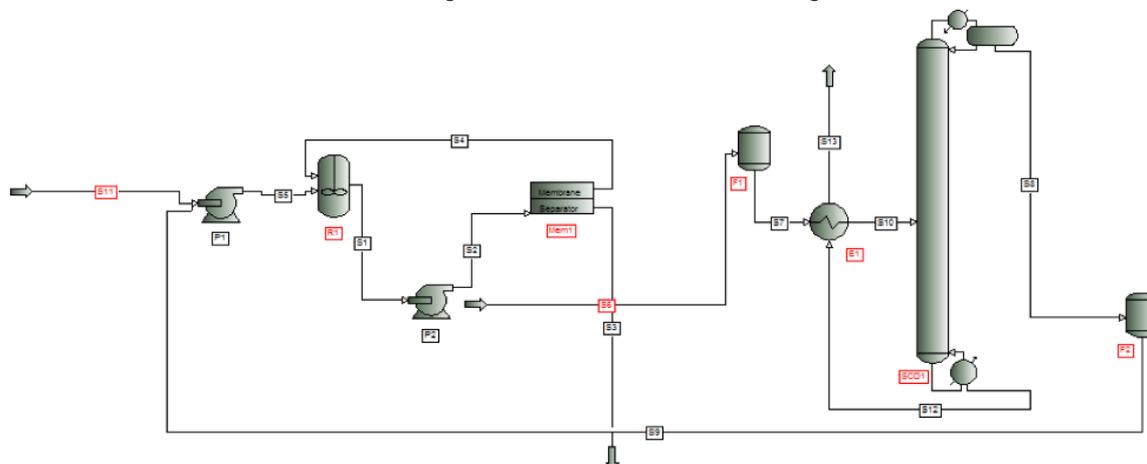


Figure 1. Flow sheet of the process separating the EPS (the exit stream of the flocculated solids is not shown as it is not energetically relevant and not influent from the point of view of the costing).

3. Results and Discussion

3.1 Antioxidant activity

The average productivity of EPS in the mentioned culture condition was 0.405 ± 0.039 gEPS/gDryWeight days⁻¹.

The antioxidant activity is expressed as the percentage of concentration reduction of DPPH stable radical compared with starting solution. In general, it is expressed as EC₅₀, the antioxidant concentration able to reduce the starting amount of DPPH of 50%, and it is expressed in mg of Trolox equivalents. The lavender oil EC₅₀ (even in this ingredient is only in traces), was considered and removed from final values. EC₅₀ for *A. platensis* exopolysaccharides is 60 mg/l of Trolox Equivalents. After the ten days of ASLT there were no alteration in the odour and colour of lotions, even in the cream stored at stressful conditions. Creams antioxidant activity was evaluated measuring the AA% over the time, to define the duration of EPS antioxidant power. Samples were taken from the creams at 0, 48, 168 and 216 hours. Figure 2a. shows the time course of AA% for the creams stored in darkness at room temperature. Starting from an AA% of 100%, it declined over the time in nine days in every sample. The base cream showed the following results: 100% at 0, 94% at 48, 86% at 168 and 80% at 216 hours. The formulations enriched with EPS showed different results, respectively 100%, 95%, 88% and 82% for the cream with 0.5% w/w of EPS and 100%, 95%, 93% and 87% for the cream with the addition of 2.5% w/w of EPS. The AA% behaviour is the same for the first 48 hours, the values seems to settle at 87% for the cream with EPS at 2.5% w/w, 82% for the cream with EPS at 0.5% w/w, the activity for the EPS-free decreases in an approximately linear way. Temperature stressed creams have the same trend with little variations in the final radical scavenging activity, showed in Figure 2b. AA% of lotions stored in the

oven started from a 100% value for the three formulations and decreased to the value of 73% for the base cream and to the values of 79% and 85% respectively for the 0.5% w/w EPS and 2.5% w/w EPS enriched lotions. Preservative-free lotions maintain the same ability in neutralizing free radicals throughout two months. The EPS contribute in the reaching of an antioxidant activity steady state even after 48 hours. Useful for our investigation could be a new formulation richer in EPS and a longer time-course.

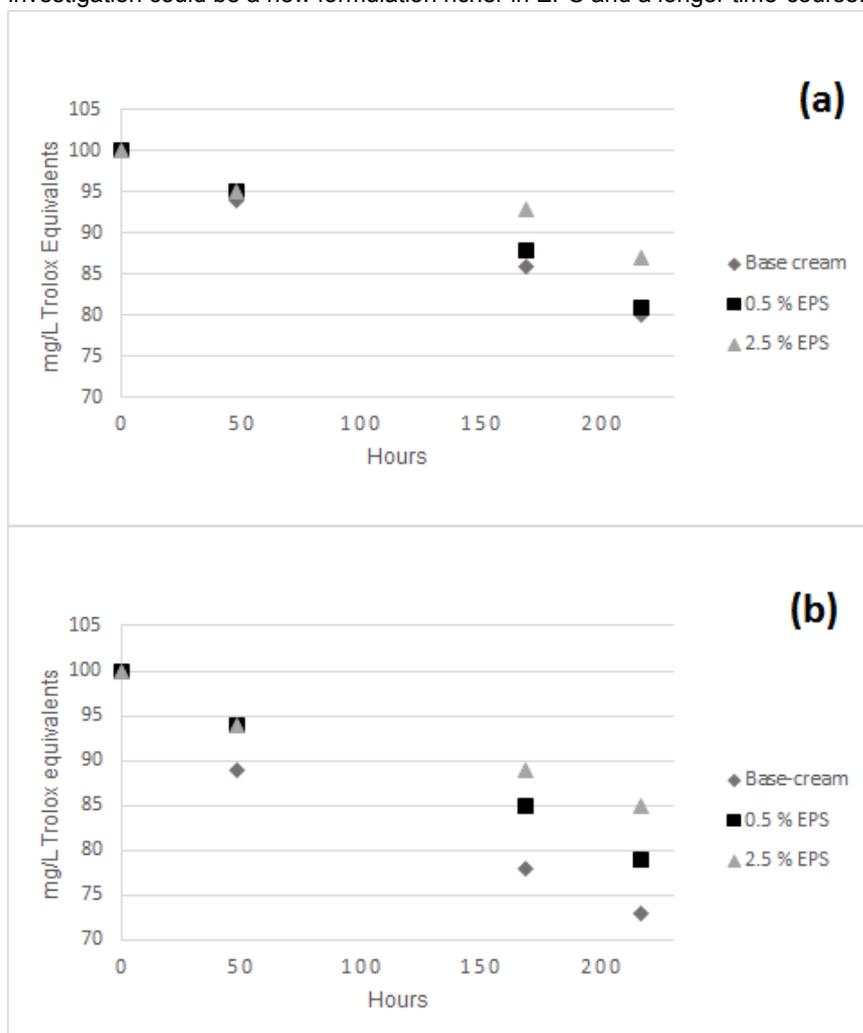


Figure 2. (a) Time course of %AA in the room temperature stored cream; (b) Time course of %AA in the 45 °C stored cream.

3.2 pH and Zeta-potential

The absence of pH stabilizers, preservatives or bactericidal molecules in the formulation could cause a changing in pH values. At time 24 h, the pH value is higher the greater is the quantity of added EPS in the cream. The pH measures are reported in the Table 1.

Table 1. Time-course of pH values of different cream formulations.

Cream	pH at 24 h	pH at 168 h	pH at 240 h
Base (rt)	6.7	6.6	6.6
0.5 % EPS (rt)	10.7	9.6	9.3
2.5 % EPS (rt)	11.9	11.4	11.3
Base (45 °C)	4.4	8.4	8.6
0.5 % EPS (45 °C)	9.8	9.8	9.7
2.5 % EPS (45 °C)	11.3	10.3	10.5

A stabilizer of pH, e.g. ascorbic acid, is necessary to neutralize the basic effect of *A. platensis* EPS, because it could act as pH buffer, maintaining it constant in every formulation even in the thermal stressed cream. The pH value of EPS-free cream stored at 45 °C redoubles in a week and it is probably due to the effect of high temperature storage.

Table 2. reports zeta-potential values recorded in 10 days, no substantial modifications were detectable, except for the base-cream, even if the value of electrophoretic mobility is still low to suppose an emulsion destabilization. The formulation with the 0.5 % EPS has the best stability. A major quantity of EPS can stabilize and emulsify the cream, lowering the zeta-potential over time.

Table 2. Time course of zeta-potential of different cream formulations stored at 45 °C.

Cream	Time 0 hours		Time 216 hours	
	ζ-pot [mV]	Dim.Part. [nm]	ζ-pot [mV]	Dim. Part. [nm]
Base (45 °C)	-77	595.4	-52	511.4
0.5 % EPS (45 °C)	-75	342.4	-71	297.7
2.5 % EPS (45 °C)	-57	420.1	-100	245.6

3.3 Cost analysis of EPS recovery

The results cost of the EPS recovery process were found to be mostly dependent upon the initial concentration of exopolysaccharides at the time flocculation is carried out. Since the EPS concentration inherited by the upstream process cannot be modified (it is determined by the culturing process of *A. platensis*), the only way to do this is concentrating the EPS themselves by a membrane process before carrying out the flocculation, in order to reduce the volume of ethanol which is required. However, EPS concentration is likely to cause membrane fouling during the operation and the likeliness of irreversible fouling, that requires premature replacement of membrane modules, rather than a slow performance decay that only requires the replacement of membrane modules after months or years, cannot be forecasted without a specific experimentation that enables the determination of threshold flux.

The results of the sensitivity study carried out with the flowsheet model and the costing model shows that EPS recovery cost depends much more on the concentration level of EPS in solution than it depends on the decontamination that is carried out by dia-filtration. Keeping in mind that objectives of this analysis are defining an operating window of the EPS recovery cost and directing future experimental investigation efforts toward the main source of product recovery cost, it was found that the former is bracketed by the 0.299 €/g_{EPS} (best case, recovery of ethanol at 90% v/v) and 0.985 €/g_{EPS} (worst case, ethanol at 95% v/v) limits, while the latter requires to a precise identification of threshold flux of membrane separation of EPS from the exhausted culture medium.

4. Conclusions

The proposed EPS extraction protocol from exhausted media of *A. platensis* results technically simple, which should warrant a good potential for profit; however, the operating cost window (0.3-1.0 €/g_{EPS}) is very sensitive to membrane processing conditions. The productivity of EPS at steady state, in standard conditions of growing (28 °C, synthetic balanced culture medium), is 0.405 and could be improved stressing the biomass with extremes culturing conditions. The EPS extraction was performed with the addition of ethanol in a refrigerate jacketed vessel, this is the most expensive phase of the protocol. The second step of filtration avoid the dead-end ultrafiltration, and improve the recovery of EPS, the time of treatment and polarization of biopolymer. Redoubling the ethanol volume utilized for the precipitation it could be obtained a raise of 20% in the EPS production (from 3.30 g to 3.98g). On an industrial point of view, it will be interesting improve the EPS purity with a tangential ultrafiltration, adapted to avoid fouling problems. The use of microbial EPS in high added value products, e.g. cosmetic creams, justifies the expenses due to the solvents (ethanol and isopropyl alcohol). Exopolysaccharides emulsifying and stabilizing actions find expression at small concentrations and lend improved properties to a w/o emulsion texture (as the proposed one), resulting in a better consistency, more spreadability and cutaneous uptake. At low concentration, EPS show antioxidant abilities in a lipophilic cosmetic emulsion. Enhanced AA% could be reached raising the EPS content in the formulation, paying attention to avoid the alteration of the rheological properties (texture, fluidity and spreadability) of the cream.

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