Microalgae as New Sources of Starch: Isolation and Characterization of Microalgal Starch Granules

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Starch is a very important biopolymer used in the modern society. It is a basic element of our diet (35% of daily calories in UE and USA). Moreover, it is widely used in the non-food industries. Indeed, the market of the non-food applications is nowadays growing and it includes chemical additives, bulking agents, and bioplastics productions (e.g. Mater-Bi by Novamont).

The key starch features for industrial applications are: size and shape distributions of starch granules, crystallinity, amylose-amylopectin ratio, thermal properties. These features are critical to address the starch processing in the industrial production. Nowadays the main sources of small starch granules that fulfil the industrial requirements are maize, wheat, rice, oats, and amaranth. However, the reduced availability of arable lands and the increase of food demand ask for alternative sources for starch not in competition with food cultures.

Microalgae are considered a novel highly efficient starch producers. Their starch content can reach the 40%$_W$. They do not require arable land and fresh water for their cultivation. Nevertheless, limited information is available in literature about physico-chemical characterization of microalgal starch. Therefore, additional analysis about molecular weight, crystallinity, and amylose fraction are required to validate the potential industrial applications of this starch type.

The present contribution reports a study on starch granules present in the microalga *Chlorella sorokiniana*. The granules were isolated and a preliminary physico-chemical characterization was carried out. The microalgal starch was characterized by small granules of about 1 µm with a narrow size distribution (key feature for some applications). The molecular weight of microalgal starch is comparable with that of plant-starch sources. The amylose content and crystallinity pattern were similar to cereal starch. Moreover, the high gelatinization temperature of 110 °C makes these granules suitable for system requiring high processing temperature such as for biodegradable materials.

1. Introduction

Starch is the principal storage polysaccharides produced by green plants. Starch consists of two D-glucose polymers: amylose and amylopectin. Amylose has a linear structure with α-1,4-linked glucan, while amylopectin is a highly branched molecule characterized by α-1,4-linked and α-1,6 branching links (Perez and Bertoft, 2010).

Starch is a very important biopolymer with a wide use in human life. It is a basic element of our diet (35% of daily calories in UE and USA) and of the commercially produced starch (50 million ton/year) two third are used in food industries and a third in non-food industries (Morton Satin – FAO Report, 2007). In food industries it performs various functions as thickener, binder, disrupting agent, stabilizer, texture modifier, gelling and bulking agent, useful in the preservation of canned and frozen foods, in the formulation of syrups, essences

and beverages, in confectionery and bakery, snacks and marshmallows (Copeland et al., 2008; Burrell et al., 2003).

Starch use is extended also in non-food industries: it is used for adhesives and rubbers production (already by Romans and Egyptians), as a cement additive to reduce the stabilization time; in paper and textile industry as filling agent and to improve the ink adhesion; it is used also as a bulking agent, humectant and thickening agent in the formulation of cosmetic and pharmaceutical products. Not least is its use in the production of plastic products and for food packaging, food containers and cutlery and agriculture (Perez-Pacheco et al., 2014). Non-food applications have increasing importance also for consumers that become more aware of "green" issues, moreover this kind of application requires specific properties as well as increasing production yield.

Plants starch properties and yield mostly depend on environmental conditions -hardly controllable- so many studies are aimed at chemical starch modification or functionalization (Filippov et al., 2015; Chauhan et al., 2015). The most interesting starch properties are: size and shape distributions, crystallinity, amylose/amylopectin ratio, which influence physico-chemical behaviour during product productions.

In particular, there is a recent interest in small starch granules applications because of their high specific surface area. This property increases viscosity, gives more sites for crosslinking reaction finalized at starch modification, better surface adhesion, high gelatinization temperature and resistance.

Recently, there is interest in small starch granules for peculiar applications: fat replacers in free-fat food formulation; carrier material in pharmaceutics and cosmetics and flavour essences; thick coatings for fabrics, paper, in high quality biodegradable film production (Lindeboom et al., 2014).

The current major sources of small starch granules are rice, oats, amaranth, which contain about 30% of small granules (0.5-3 μm) (Wilhelm et al., 1999), but expensive separation process limits their commercial applications. Moreover, the European reduction of agricultural land urges new and sustainable production that avoid the competition with food production.

Microalgae are considered a novel highly efficient starch producers (Brányiková et al., 2010) and they produce only small starch granules with a narrow size distribution range (0.5-2.1 μm) (Tanadul et al., 2014). The recovery of microalgal starch allows to have only enriched small size granules avoiding a wasteful granules separation. As higher plant, microalgae are able to accumulate sugars- synthesized during photosynthesis, as starch granules in the plastids (Ho et al., 2011). Starch concentration of microalgae, especially in chlorophycae strains, may be as large as 40% of the dry biomass when nitrogen depletion conditions establish (Gifuni et al., 2016). Moreover, microalgae as feedstock source are characterized by a wide spectrum of advantages compared to higher plants: i) high growth rate; ii) high photosynthetic efficiency and high level of CO_2 fixed per unit of biomass synthesized (reduction of greenhouse effect); iii) they do not require arable land and fresh water; iii) their exploitation do not compete with food market.

Microalgae cultures have been extensively studied in the last decades, particularly for biodiesel production (Perin et al., 2014; Sforza et al., 2014; Gargano et al., 2016). However, the analysis of the large-scale productions points out that the current technologies to produce liquid fuels are not self-sustainable from an economic point of view. Therefore, the biorefinery approaches to exploit as much as possible all the macro-components of the microalgae – lipids, carbohydrates and proteins – as well as the high-value products become the key issue for the industrial development of microalgal processes. Including starch production in this microalgal biorefinery approach also makes possible to develop an economical feasible process for the small high quality starch granules recovery. Furthermore, to the author knowledge, limited information are available in literature about physico-chemical characteristics of microalgal starch, so additional analysis about molecular weight, crystallinity and amylose content and thermoplastic properties are needed to validate the potential applications of this starch type.

The aim of the present paper is to report on the investigatation regarding the mentioned physical and chemical properties of *Chlorella sorokinana* starch granules. The selected strain belongs to *chlorophyiceae* and it is already used for large scale production. It is suitable for biorefinery application and it is known to accumulate large amount of starch during nitrogen depletion.

2. Materials and methods

2.1 Starch source

Microalgal strain *Chlorella sorokiniana* Shihiraet Krauss ACUF 318, was provided by ACUF collection (http://www.acuf.net). Photoautotrophic batch growth of this strain was carried out in inclined square bubble column photobioreactors in order to obtain the required amount of biomass for starch extraction. Further details can be found elsewhere (Gargano et al. 2016). The photobioreactors were housed in a climatic chamber at the temperature of 25°C, under continue illumination of 300 μE m^{-2}s^{-1}. The flow rate of the gas

sparged at the bottom of the photobioreactors was set to 0.02 vvm. The gas was air additioned with 2% of CO_2. The inorganic medium used was Bold basal medium (BBM) described by Olivieri et al. (2012). The biomass was harvested when nitrogen source in the medium became depleted, to verify if this condition enhances starch accumulation in microalgae.

2.2 Purification of starch granules

Starch granules extraction was carried out on fresh microalgal biomass according to the protocol by Delrue et al. (1992) with some modifications. Briefly, microalgal biomass was suspended in ethanol and mechanically disrupted by beat beating (equipped with glass beads of 0.5 mm). The entire lysate was recovered and heated at 75°C in order to complete cells disruption, fragmentation of the debris and pigments extraction, preserving starch granules structure. The resulting samples were centrifuged at 10,000 g for 20 min. The pellet, containing starch granules and cells fragments, were resuspended in cold distilled water and centrifuged twice through Percoll gradient at 10,000 g for 20 min in order to separate high density starch granules and low density cell debris. The purified starch pellets were washed two times with distilled water and acetone. Then, 10 mg of the obtained starch was assayed by total starch kit (Megazyme - Ireland) to establish the purity of the sample. The purity was calculated as follows:

$$\%\text{Purity} = \frac{m_{\text{Starch measured}}}{m_{\text{Starch assayed}}} \cdot 100 \tag{1}$$

2.3 Starch characterization

Purified starch granules were characterized in terms of granules morphology, average size and size distribution, amylose and amylopectine content, molecular weight, crystallinity and gelatinization temperature.

Granule morphology. The shape of starch was examined by a scanning electron microscopy (SEM). Starch samples were mounted on a metallic slide and the examination was performed with a scanning electron microscopy.

Size distribution analysis. The measure of the average size of microalgal starch granules and size distribution in the sample, was performed using laser granulometer (Master-sizer 2000 Malvern Instruments) after the dispersion of the powders in water under mechanical agitation of the suspension and with the application of ultrasound.

Amylose amylopectine ratio. Amylose content of the *C. sorokiniana* starch was determined using enzymatic assay (Amylose/Amylopectin kit - Megazyme). The amylopectin content is assessed as difference at 100%.

Molecular weight. An estimation of the starch molecular weight was derived from the hydrodynamic volume of the particles, as determined by Dynamic Light Scattering (DLS), considering the volume completely filled by glucose monomer of known molecular weight. DLS measurements were performed with a home-made instrument composed of a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, a photomultiplier (PMT-120-OP/B) and a correlator (Flex02-01D) from Correlator.com. The experiments were carried out at the constant temperature (25.0 ± 0.1) °C, by using a thermostatic bath, at the scattering angle θ of 90°. The scattered intensity correlation function was analysed using a regularization algorithm. The diffusion coefficient of each population of diffusing particles was calculated as the z-average of the diffusion coefficients of the corresponding distributions. Considering that the mixtures are dilute, the Stokes–Einstein equation was used to evaluate the hydrodynamic radius, R_H, of the starch particles, assumed to be spherical, from their translation diffusion coefficient, D.

Crystallinity. X-ray diffraction patterns were obtained with Ni filtered Cu Kα radiation. The powder profiles were obtained with an automatic Philips diffractometer. Starch was placed into the sample holder, and XRD patterns were recorded in reflection mode with 2θ ranging between 5 and 80° operating at 40 kV and 40 mA, at room temperature. The crystallinity was evaluated from the X-ray powder diffraction profiles by the ratio between the crystalline diffraction area and the total area of the diffraction profile.

Thermal properties. Starch gelatinization was measured using a differential scanning calorimeter (DSC 822 Mettler Toledo, Switzerland) with the method reported by Homer et al. (2014). 10 mg of starch was weighed in a small aluminum pan and 20 µL of distilled water were added. The sample was stabilized for 1 hour at room temperature before the measurement. The DSC was calibrated using an empty aluminum pan as a blank. The aluminum pan containing the starch and water was heated at a temperature ranging from 20 to 150 C with a heating rate at 10°C/min. The initial temperature (T_o), peak temperature (T_p), finishing temperature (T_c), enthalpy of gelatinization (ΔH_{gel}) and peak height index were calculated. The thermogravimetric analysis was also carried out using TGA 50 Shimadzu, Japan. 10 mg of starch were heated from room temperature to 400°C at a heating rate 10 C/min.

3. Results

C. sorokiniana was grown under autotrophic conditions at light irradiance of 300 µmol m^{-2} s^{-1}. The biomass was harvested after the first day of nitrogen depletion. This condition was previously found to give the highest starch content and productivity. In particular, the starch fraction reached the 38% of the microalgal dry weight and the starch productivity resulted 0.17 kg m^{-3} day^{-1}.

Starch fraction and biomass productivity of the plants commonly used for starch production are reported in table 1. The values assessed in the present study for *C. sorokinina* strain were also reported. *C. sorokiniana* is characterized by starch fraction lower than the other higher plants but its biomass productivity (as for microalgae in general) is significantly higher than plants species. As a consequence, the starch productivity was higher than that of higher than plant species. Moreover, microalgae can be grown in closed photobioreactors where culture conditions can be controlled, so the amount of biomass and starch produced is not influenced by environmental and climatic conditions.

Table 1: Comparing starch fraction and productivity of the most used plant species (Kulp et al., 2000; www.FAO.org) and microalga C. sorokiniana (this study).

Species	Starch fraction (%on DW)	Biomass productivity (ton ha^{-1} y^{-1})	Starch productivity (ton ha^{-1} y^{-1})
Corn	78	8	6
Rice	80	10	8
Potato	85	17	15
Oat	61	6	4
Amaranth	69	8	6
C. sorokiniana	40	150	58

The starch powder produced according to the optimized protocol for microalgal starch purification was characterized by 87% of purity.

The produced starch granules were characterized from the morphological point of view by means of SEM. Pictures of *C. sorokiniana* starch granules are reported in the figure 1. Granules shape resulted ovals and spheres, similar to wheat and potato starch. Even if there are many aggregates, no composite granules are present.
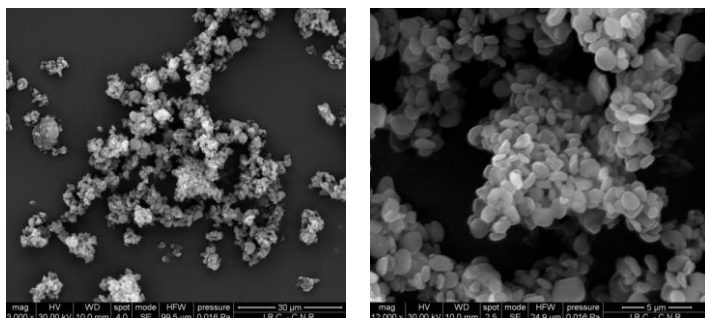

Figure 1: SEM micrograph of granular starch isolated from C. sorokiniana.

Figure 2 shows the results of granulometric analysis in terms of size distribution of the sample particles. The 80% of the analysed particles have a mean diameter of 1.5 µm, the size distribution range was 0.8-5.3 µm, the last 15% of particles exceeding 5.3 µm are probably associated to the impurities of the sample. These data are in agreement with that reported by Tanadul et al. (2014), moreover the microalgal starch is effectively enriched in small starch granules, more than 80%, with respect to the other plant sources for which small granules count the 30% of the total (Wilhelm et al., 1999). This feature is particularly advisable for applications as fat replacers in free-fat food formulation, as carrier material in pharmaceutics, as one-layer-thick honeycomb coatings in the cosmetic, paper, textile industries and for bioplastics film formulation for garbage bags and agricultural mulches (Lindeboom et al., 2004).
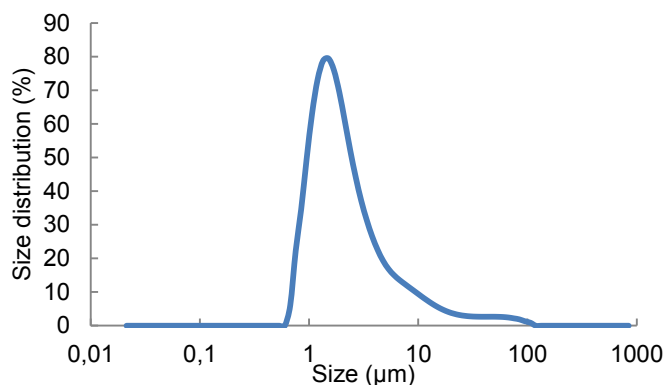
Figure 2: Size distribution of starch powder obtained from C. sorokiniana.

The molecular weight of microalgal starch resulted about $6.35 \cdot 10^8$ kDa, comparable with that plant sources (10^8-10^{12} kDa). Molecular weight strongly influences starch properties, high molecular weight are required for bioplastic production. It is connected to the amylopectin content of starch, since the highly branched polymer has the highest molecular weight.

The amylose/amylopectin ratio was also measured. Amylose content of starch from C. sorokiniana is 17%, amylopectin content is 83%. The amylose fraction is nearby that of cereals starch (19-28%). The amylose/ amylopectin ratio is mainly influenced by biological origin and environmental factors, then probably amylase content could be increased changing microalgal culture conditions in order to obtain a good film forming starch. Indeed, high-amylose starch shown to form stronger and stiffer thermoplastic films, but amylopectin fraction contribute to increase swelling properties that lead to gel and film formation.

In the figure 3, XRD pattern of *C. sorokiniana* starch is reported. Peaks at 15° and 23°, and a double peak at 17 and 18° 2θ are clearly recognizable. This pattern indicates a crystalline structure classified as A-type, typical of cereal starches (Pérez and Bertoft, 2010). The crystallinity of the starch was about 30%, straightly linked to the amount of crystalline and amorphous structure (amylopectin fraction)
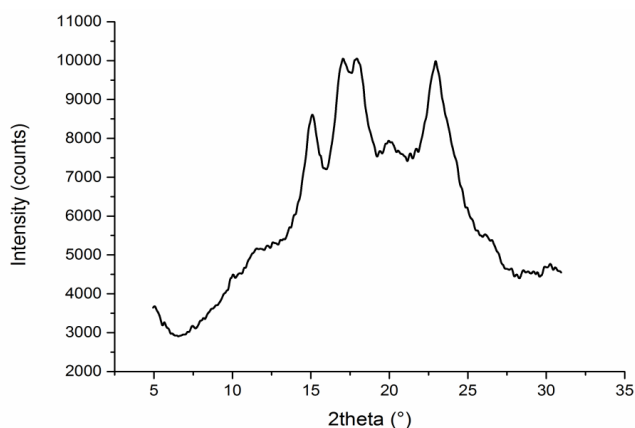

Figure 3: XRD diffractograms of C. sorokiniana starch.

The thermal properties of starch granules were also analyzed in order to assess the thermal processability of the starch in the industrial field (food and plastics industries). For microalgal starch, the gelatinization started at 24°C (T_o) and it was completed at 97°C (T_c), with a peak at 69°C (T_p). The gelatinization enthalpy was 5.49 Jg^{-1} (ΔH_{gel}) with a peak height index of 1.55°C. Gelatinization properties seemed similar to other starch sources, except for the onset temperature; probably the swelling of the starch started at lower temperature because of the higher specific surface (associated to small starch granules) which facilitate the water diffusion into starch granules and the crystallinity loss. The thermal degradation and stability assays were also carried out. The total weight loss, after the complete combustion, was 85.81%. The first step of the thermogravimetric analysis, showed a rapid dehydration of the sample below 100°C for approximately 10% of the weight. The second step, representing the degradation of amylose and the amylopectine breakage, occurred in the range 250-350°C. The third step, of the complete combustion took place at 349°C. The thermal decomposition temperature was 321°C, indicating a good thermal stability, since it do not decompose under 190°C (Elmi Sharlina et al., 2017).

4. Conclusions

Microalgae are a sustainable source of starch for industrial applications and they are characterized by far outweigh productivity than higher plants. *C. sorokiniana* starch granules purification was optimized and the physico-chemical characterization showed interesting features. Small granules with narrow distribution range find application as additives in cosmetics, pharmaceutics, food and plastics industries. Crystallinity, molecular weight and thermal properties are very close to starch from other sources, suggesting microalgal starch as valid alternative to plant starch in several fields.

Reference

Brányiková I., Maršálková B., Doucha J., Brányik T., 2011, Microalgae-novel highly efficient starch producers. Biotechnology and Bioengineering, 108 (4), 766-776.

Burrell M.M., 2003, Starch: the need for improved quality or quantity- an overview, Journal of Experimental Botany, 54, 451-456.

Chauhan K., Kaur J., Singh P., Sharma P., Sharma P., Chauhan G.S., 2015, An Efficient and Regenerable Quaternary Starch for Removal of Nitrate from Aqueous Solutions. Industrial and Ingegnering Chemistry Research, 55 (9), 2507–2519.

Copeland L., Blazek J., Salmen H., Tang M.C., 2008, Form and functionality of starch, Food Hydrocolloids, 23, 1527-1534.

Delrue B., Fontaine T., Routier F., Decq A., Wieruszeski J.M., Van Den Koornhuyse N., Maddelein M.L., Fournet B., Ball S., 1992, Waxy Chlamydomonas reinhardtii: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin, Journal of Bacteriology, 174, 3612-3620.

Elmi Sharlina M.S., Yaacob W.A., Lazim A.M:, Fazry S., Lim S.J., Abdullah S., Noordin A., Kumaran M., 2017, Physicochemical Properties of Starch from Dioscorea pyrifolia tubers, Food Chemistry, 220, 225-232.

Filippov S.K., Sergeeva O.Y., Vlasov P.S., Zavyalova M.S., Belostotskaya G. B., Garamus V.M., Khrustaleva R.S., Stepanek P., Domnina N.S., 2015, Modified hydroxyethyl starch protects cells from oxidative damage, Carbohydrate Polymers. 134, 314-323.

Gargano I., Olivieri G., Andreozzi R., Marotta R., Marzocchella A., Pollio A., 2016, Biodiesel production in outdoor cultures of Scenedesmus vacuolatus, Chemical Engineering Transaction, 49, 397-402.

Gifuni I., Olivieri G., Pollio A., Franco T.T., Marzocchella A., 2016, Autotrophic starch production by Chlamydomonas species, Journal of Applied Phycology, 1-10, DOI: 10.1007/s10811-016-0932-2.

Ho S.-H., Chen C.-Y., Lee D.-J., Chang J.-S., 2011, Perspectives on microalgal CO2-emission mitigation systems – a review. Biotechnology Advances, 29 (2), 189–198.

Lindeboom N., Chang P.R., Tyler R.T., 2004, Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches. A review, Starch Journal, 56, (3-4), 89-99.

Morton Satin, 2012, Functional properties of starches, FAO publication report.

Olivieri G., Gargano I., Andreozzi R., Marotta R., Marzocchella A., Pinto G., Pollio A., 2012, Effects of CO2 and pH on Stichococcus bacillaris in laboratory scale photobioreactors, Chemical Engineering Transactions, 27, 127-132.

Perez S., Bertoft E., 2010, The molecular structures of starch components and their contribution to the architecture of starch granules: a comprehensive review, Starch Journal, 62, 389–420.

Pérez-Pacheco E., Moo-Huchin V.M., Estrada-León R.J., Ortiz-Fernández A., May-Hernández L.H., Ríos-Soberanis C.R., Betancur-Ancona D., 2014, Isolation and characterization of starch obtained from Brosimum alicastrum Swarts Seeds. Carbohydrate Polymers. 101, 920-927.

Perin G., Segalla A., Basso S., Simionato D., Meneghesso A., Sforza E., Bertucco A., Morosinotto T., 2014, Biotechnological optimization of light use efficiency in nannochloropsis cultures for biodiesel production, Chemical Engineering Transaction, 37, 763-768.

Sforza E., Gris B., de Farias Silva C.E., Morosinotto T., Bertucco A., 2014, Effects of light on cultivation of Scenedesmus Obliquus in batch and continuous flat plate photobioreactor, Chemical Engineering Transaction, 38, 211-216.

Tanadul O.-u.-ma., Vandergheynst J.S., Beckles D.M., Powell A.L.T., Labavitch J.M., 2014, The impact of elevated CO2 concentration on the quality of algal starch as a potential biofuel feedstock, Biotechnology and Bioengineering, 111, 1323-1331.

Wilhelm E.P., Mullen R.E., Keeling P.L., Singletary G.W., 1999, Heat Stress during Grain Filling in Maize: Effects on Kernel Growth and Metabolism. American Society of Agronomy, 39 (6), 1733-1741