Algal Research 8 (2015) 23–29

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Study of bioflocculation induced by *Saccharomyces bayanus* var. *uvarum* and flocculating protein factors in microalgae

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ARTICLE INFO

Article history:
Received 21 July 2014
Received in revised form 21 December 2014
Accepted 29 December 2014
Available online xxxx

Keywords:
Flocculation
Glucanase
Lectins
Microalgae
Yeast

ABSTRACT

Autoaggregation of flocculent microalgae in response to stressing conditions is poorly understood, but it is a promising approach to induce the aggregation of microalgae into flocs and make microalgal harvesting a straightforward and cheap procedure. The effect of the self-flocculating yeast strain *Saccharomyces bayanus* var. *uvarum* on two chlorophytes: the model freshwater microalga *Chlamydomonas reinhardtii* and the novel marine microalga *Picochlorum* sp. HM1, has been investigated. The addition of *Saccharomyces* induces cell aggregation in both microalgal species studied, being the flocculating effect caused by anaerobically grown yeasts almost two-fold the effect of standard aerobically grown yeast. In order to gain more insights into the origin of yeast-induced microalgal flocculation, proteins released into the culture medium by the flocculent yeast *S. bayanus* var. *uvarum* during the fermentative phase of growth were isolated and their ability to induce flocculation was tested. Addition of 0.1 mg mL^{-1} of concentrated flocculating excreted proteins resulted in recovery efficiency values of 95% and 75% for *Chlamydomonas* and *Picochlorum* respectively. The flocculating activity of some plant lectins on the chosen chlorophytes was also evaluated.

1. Introduction

Microalgae constitute a highly heterogeneous group of photosynthetic microorganisms with a capital ecological importance [1] and an enormous biotechnological potential, which has experienced a renewed interest in the last years [2–4]. More than 5000 tons of dry algal biomass is produced and marketed per year with an average value of 1.25 billion of euros [5]. Microalgae and their products are used in aquaculture, as additives for animal feed, in human nutrition, and for production of high-added value compounds used as nutraceutical or dietetic complements [2,4,6,7]. Furthermore, the possibility of using microalgae as feedstock in the production of biofuels, like methane, biohydrogen or biodiesel is being intensively studied [8–11].

Differently from the production of high-added value nutraceutical or dietetic compounds, algal-based biofuels have to compete with the price of fossil fuels. And despite the enthusiasm generated, the cost for microalgal production, harvesting and processing is very far for being competitive for the production of biodiesel. Harvesting of microalgal biomass and its separation from the culture medium is a critical step, which accounts for about 20–30% of the total production cost [12–14]. Classical physical methods of biomass recovery like filtration or centrifugation are energy intensive and only economically feasible for high-value products [15].

Different ways to induce aggregation of individual microalgae into flocs of larger size and to make microalgal harvesting a straightforward and cheap procedure have been proposed. Additions of trivalent cations, cationic polymers or pH adjustment, which reduce the electrostatic repulsion among cells, are the most common chemical procedures to induce flocculation [14,16]. But the chemicals added are an additional cost, can make reusing the growth medium difficult, can disrupt the cells causing release of internal metabolites and can interfere with further downstream processes.

Many microorganisms can spontaneously flocculate. This mechanism is called self- or autoflocculation and is a complex process in which multiple factors are involved [17].

Flocculation of yeasts has been widely studied, due to its relevance to industrial applications such as brewing or wine fermentations or bioethanol production [18]. In yeasts, a family of subtelomeric genes called *FLO*, which encodes specific cell surface lectin-like glycoproteins, known as flocculins, is responsible for flocculation [19]. The flocculins in the cell walls of flocculating cells are capable of binding selectively specific carbohydrate residues of adjacent yeast cells. This mechanism of cell–cell adhesion is mainly mediated by highly specific carbohydrates and Ca^{2+} [20,21]. Recent studies suggest that certain cell wall-remodeling enzymes such as glucanases can also have an important role in yeast flocculation [22].

Naturally bioflocculating microalgal species, such *Scenedesmus obliquus*, or *Tetraselmis suecica* [13] have been described. Furthermore, bioflocculation of non-flocculating microalgal species induced by addition of these flocculating species has been proposed as a promising

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http://dx.doi.org/10.1016/j.algal.2014.12.013
2211-9264/

pre-concentration step in harvesting of microalgae [23]. But unlike yeasts, for which the molecular basis of flocculation is starting to be unraveled, the mechanisms underlying the aggregation of flocculating microalgae are poorly understood. Spontaneous aggregation in flocculating microalgae is usually mediated by extracellular polymer substances excreted into the culture medium. Water-soluble extracts of the marine microalga *Skeletonema marinoi* have been reported to induce flocculation of *Nannochloropsis oculata* [24], and characterization of the flocculating agent isolated from the self-flocculating microalga *S. obliquus* AS6a revealed that self-flocculation of this microalga was mediated by cell wall associated polysaccharides [25]. Agglutination of microalgal cells during sexual reproduction has been studied in certain species. In *Chlamydomonas*, when gametes of opposite mating type are mixed, they immediately undergo cell–cell adhesion via specific hydroxyproline-rich glycoproteins called agglutinins. The sequence and structure of these agglutinin proteins and the sequence of the genes which encode them have been characterized [26].

Agglutinins and flocculins, as well as many other proteins with agglutinating ability, are lectins. These proteins reversibly and non-enzymatically bind specific carbohydrates and play an important role in a high diversity of mechanisms in which cell–cell or cell–molecule interactions are involved [27,28]. They have been isolated from a diversity of organisms like plants, animals, algae and fungi. Plant lectins are one of the most extensively studied lectins from natural resources due to their high diversity in structure and function. They are involved in physiological plant mechanisms such as plant defense against predators and pathogens or symbiotic interactions between host plants and symbiotic microbes and also have been used in biomedical studies as histochemical reagents to label cells or used to agglutinate erythrocytes in identifying blood serotypes. Some well-known examples are Concanavalin A, from the legume *Canavalia ensiformis*, UEAI lectin from *Ulex europaeus*, or WGA, wheat germ agglutinin, a cereal lectin [27,28].

The aim of this work is to gain more insights into the origin of microorganism-induced microalgal flocculation by identifying protein factors able to induce aggregation of microalgae. We have investigated the effect of the flocculating yeast *Saccharomyces bayanus* var. *uvarum* CECT 1969 and the proteins released into the culture medium by this flocculating yeast, on two chlorophytes: the model and freshwater microalga *Chlamydomonas reinhardtii* and, the novel and marine microalga *Picochlorum* sp. HM1. The study has completed evaluating the flocculating activity of some plant lectins on the chosen chlorophytes.

2. Materials and methods

2.1. Materials

Concanavalin A (CON A), *Dolichos biflorus* agglutinin (DBA), peanut agglutinin (PNA), soybean agglutinin (SBA), *U. europaeus* agglutinin I (UEA I), wheat germ agglutinin (WGA) and *Ricinus communis* agglutinin I (RCA I), were supplied by Vector Laboratories Inc., U.S.A. The lectins listed were provided as 1 mg of salt-free lyophilized powders and were reconstituted in 0.5 mL of 10 mM HEPES saline buffer, pH 8.4, and 0.1 mM Ca^{2+}, except RCA I which was supplied as a 2 mg mL^{-1} solution.

2.2. Microorganisms and culture conditions

2.2.1. Microalgal strains

Freshwater *C. reinhardtii* cell-wall deficient strain 704 (Cw15, Arg7, mt+) was kindly provided by Dr. E. Fernández from the University of Córdoba and cultured photomixotrophically in liquid TAP (Tris–acetate–phosphate) medium [29] at 25 °C under continuous white light irradiation of 100 μE m^{-2} s^{-1}. Saline *Picochlorum* sp. HM1, isolated from the marshlands of Huelva [30], was cultured in F/2 medium with filtered seawater at pH 8 as reported by Guillard and Ryther [31].

2.2.2. Yeast strain

S. bayanus var. *uvarum* CECT 1969 was kindly supplied by the Department of Genetic (University of Seville) and cultured at 28 °C, at initial pH of 4.5, in YPD (yeast extract peptone dextrose) medium containing the following components: peptone (20 g L^{-1}), yeast extract (10 g L^{-1}) and glucose (20 g L^{-1}) dissolved in 1 L of demineralized water. For aerobic growth, cotton capped Erlenmeyer flasks were aerated by shaking at 150 rpm. For anaerobic cultures, yeasts were grown in well-sealed Erlenmeyer flasks at low shaking speed. The pH of the culture medium and the optical density at 620 nm were monitored along fermentation. At the stationary phase of growth, the pH of the medium increased from 4.5 to around 6. Cultures at this stage were used for flocculation assays or for the isolation of the proteins released to the culture medium.

2.3. Protein extraction from S. bayanus fermentation supernatant

Supernatant was separated from a *Saccharomyces* fermentative culture, grown as indicated in Section 2.2.2, by centrifugation at 9500 rpm in a refrigerated centrifuge. Ethanol was added to a final concentration of 10% (v/v) to limit microbial contamination and the pH of the obtained supernatant was adjusted to 7 with 1 M NaOH. The supernatant was stored at −20 °C until further purification. Proteins were precipitated by addition of three volumes of methanol and incubated overnight at 4 °C [32]. Precipitated proteins were collected by centrifugation at 9500 rpm and dissolved in one volume of demineralized water at 1/10 of the initial volume. The same procedure was performed for a YPD culture medium without yeast inoculum, used as a negative control.

2.4. Protein determination

The protein content of the yeast culture supernatant was measured with the Bio-Rad Bradford assay according to the manufacture protocol, using bovine serum albumin (BSA) as standard.

2.5. SDS-PAGE

Denaturing polyacrylamide gel electrophoresis was carried out on a 10% acrylamide gel, in SDS-electrophoresis buffer of pH 8.3, containing: SDS 1 g L^{-1}, Trizma base 3 g L^{-1} and glycine 14.4 g L^{-1}. After migration, the gel was stained with colloidal CBB G-250.

2.6. Flocculation assays

Flocculation experiments were run in small cylindrical glass tubes (20 mL) with a total final volume of 10 mL, or in polystyrene cuvettes (4 mL) with a total volume of 3 mL, when lectins were used as flocculating agents. The initial optical density of the microalgal cultures was measured at 660 nm and it was adjusted to a value of 1 with the suitable volume of the culture medium. At the beginning and at the end of each flocculation assay, temperature and pH were measured to ensure that they were 25 °C and 7.5, respectively.

After addition of the flocculating agent each tube was vortexed vigorously for 8 s and subsequently left without agitation during the settling period. To follow the kinetic of flocculation and to evaluate the recovery efficiency for each flocculating agent in both microalgal strains, small culture aliquots were withdrawn from the top part of the tube and the OD_{660} was measured in a spectrophotometer (Ultrospec 3100 pro) at 0, 15, 45, 90, 180 and 360 min after addition of the flocculating agents, according to Papazi et al. [33] with minor modifications. For lectins, the absorbance was directly followed in the polystyrene cuvettes. Each flocculation assay was run in triplicate (n = 3) to test the reproducibility of the experiment.