Algal Research 27 (2017) 335–344

Contents lists available at ScienceDirect

Algal Research

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

The enzymatic ulvan depolymerisation system from the alga-associated marine flavobacterium *Formosa agariphila*

A. Salinas*, C.E. French

School of Biological Sciences, University of Edinburgh, Roger Land Building, King's Buildings, Edinburgh EH9 3FF, UK

ARTICLE INFO

Keywords:
Green algae
Seaweed
Depolymerisation
Ulvan
Ulva lactuca
Formosa agariphila

ABSTRACT

Green macroalgae represent an attractive source of renewable carbon. Conversion of green algal biomass to useful products requires depolymerisation of the cell wall polysaccharide ulvan. The enzymatic depolymerisation of ulvan is not fully understood and only few enzymes involved in this process have been reported. In this study the first model for ulvan depolymerisation and utilization was built based on a polysaccharide utilization locus from the alga-associated flavobacterium *Formosa agariphila*. It was also shown that *F. agariphila*, is able to grow using biomass from the green macroalga *Ulva lactuca* as its sole carbon source, and enzymes with ulvanase activity are induced by the presence of algal biomass in the culture medium. Enzymes for ulvan depolymerisation from *F. agariphila*, including an ulvan lyase, an unsaturated glucuronyl hydrolase, a xylosidase and two rhamnosidases, were cloned using the PaperClip DNA assembly method and expressed in active form in *Escherichia coli*. These results represent a first step for the design of a microorganism capable of utilizing green macroalgal biomass for the production of biofuels and other valuable bio-products.

1. Introduction

Finding alternative energy sources that are renewable and less damaging to the environment is essential. The possibility of producing biofuels such as ethanol and n-butanol from green macroalgal biomass seems to be an attractive alternative. This is due to a number of desirable characteristics such as no competition for arable land, fast growth rates, absence of structural biopolymers such as lignin, and the capability to grow in saline water [1]. The use of green macroalgal biomass for the production of ethanol [2–6] and n-butanol [7,8], along with acetone and ethanol in a process known as 'ABE' fermentation, have been recently reported. Additionally, this kind of biomass can be used to produce other useful co-products. Ulvan, one of its main cell-wall polysaccharides, is a source of the rare sugar rhamnose, which can be used as a precursor for the synthesis of fine chemicals such as fragrances [9] or the commodity chemical 1,2-propanediol [10]. The production of 1-2-propanediol by clostridial fermentation of rhamnose from green macroalgal biomass was recently demonstrated [7,8].

Species of the genus *Ulva* are green macroalgae found worldwide [9]. *Ulva* are grown or collected for food consumption [11] and responsible for green tides [12], an increasing economic and environmental problem. Currently the generated biomass is of little value; however, up to 54% of algae's dry weight corresponds to cell wall polysaccharides [9], representing an interesting source of renewable biomass to be explored.

The main carbohydrate of *Ulva* is ulvan, a water-soluble cell wall polysaccharide made up mainly of rhamnose (Rha), glucuronic acid (GlcA), iduronic acid (IduA), xylose (Xyl) and sulfate [9]. The predominant repeating disaccharides in ulvan are made up of rhamnose-3-sulfate (Rha3S) linked with either GlcA (Rha3S-GlcA, ulvanobiouronic acid A), IduA (Rha3S-IduA, ulvanobiouronic acid B) or Xyl (Rha3S-Xyl, ulvanobiose 3-sulfate) [9].

Compared to the enzymatic depolymerisation of carbohydrates from brown and red algae, the saccharification of ulvan has been investigated less extensively. The first enzyme with ulvan lyase activity was reported by Lahaye et al. [13]. The ulvan lyase was obtained from a Gram-negative bacterium isolated from muds containing decomposing *Ulva* sp., and it was shown to cleave the linkage between Rha3S and GlcA, generating an unsaturated uronic acid at the newly created non-reducing end. However, no amino acid sequence of the enzyme or further characterization of the bacterium that produces it was provided. *Nonlabens ulvanivorans*, a marine member of the phylum Bacteroidetes isolated from the faeces of the sea slug *Aplysia punctata* fed with *Ulva* sp., was shown to be an efficient ulvan degrader [14]. An ulvan lyase

Abbreviations: CAZymes, carbohydrate-active enzymes; CBM, carbohydrate-binding module; GH, glycoside hydrolase; HTCS, hybrid two-component system; PBS, phosphate buffered saline; PL, polysaccharide lyase; PUL, polysaccharide utilization locus; TBDR, TonB-dependent receptor; TR, transcription regulator; UGL, unsaturated glucuronyl hydrolase

* Corresponding author.
E-mail address: a.a.salinas-vaccaro@sms.ed.ac.uk (A. Salinas).

http://dx.doi.org/10.1016/j.algal.2017.09.025
Received 10 December 2016; Received in revised form 27 September 2017; Accepted 27 September 2017

from *N. ulvanivorans* was purified, sequenced, and heterologously expressed in *Escherichia coli* by Collen et al. [15]. Biochemical characterization of the ulvan lyase showed that it was an endolytic enzyme able to cleave ulvan between Rha3S and GlcA or IduA via a β-elimination mechanism. The released oligosaccharides had a degree of polymerization higher than two and contained an unsaturated uronyl residue at the non-reducing end. Considering that no characterized homologues were found in the databases, the enzyme was designated as the first member of a novel polysaccharide lyase family. Recently, a new family of ulvan lyases, showing no homology with ulvan lyase from *N. ulvanivorans*, was identified in three bacteria from the Alteromonadales order by Kopel et al. [16]. The novel ulvan lyases were heterologously expressed in *E. coli* and characterized to determine their mode of action. In contrast with the ulvan lyase from *N. ulvanivorans*, these enzymes only cleave between the residues Rha3S and GlcA.

In addition, an unsaturated glucuronyl hydrolase (UGL) from the glycoside hydrolase family 105 (GH105) was found in the genomic environment of the ulvan lyase from *N. ulvanivorans* [17]. The UGL was shown to specifically cleave the unsaturated uronic residues generated by the ulvan lyase. Considering the proximity within the genome of the ulvan lyase and the UGL from *N. ulvanivorans*, it has been proposed that they might be part of a polysaccharide utilization locus [16]. Considering the complex structure of ulvan, it is clear that several other enzymes such as other lyases, rhamnosidases, xylosidases and sulfatases must be involved and their identification is crucial in order to understand the entire enzymatic system that leads to the saccharification of this biopolymer [15].

Many seaweed-associated bacteria are able to enzymatically decompose algal cell walls [18], thus, are an interesting source of ulvanolytic enzymes. Recently, the complete genome of *Formosa agariphila*, a member of the Bacteroidetes isolated from green alga *Acrosiphonia sonderi*, was reported [19], revealing a broad potential for algal polysaccharide degradation. In particular, a polysaccharide utilization locus (PUL) described in the study seems to have all the enzymes required for the complete saccharification of ulvan.

In order to better understand the enzymatic saccharification of ulvan, *F. agariphila*'s capability to utilize this polysaccharide was investigated. To do that, the potential PUL for ulvan depolymerisation was manually curated and analysed in-silico by comparative genomics. Additionally, the capability of *F. agariphila* to grow using the green alga *U. lactuca* as the sole carbon source was assayed and the induction of enzymes with ulvanase activity when this alga is present in the culture medium studied. Finally, the genes of the PUL identified as carbohydrate-active enzymes (CAZymes) were cloned into *E. coli* and heterologously expressed in order to validate their predicted activities.

2. Materials and methods

2.1. Organisms and growth conditions

F. agariphila KMM 3901^T was obtained from DSMZ (catalog number DSM-15362). Marine broth (MB) or MB agar were used for routine *F. agariphila* culturing. The composition of this culture medium is a modification of the Marine Broth 2216 (Becton Dickinson, US), which contained 35 g/l sea salts (Sigma, US), 1 g/l yeast extract and 5 g/l peptone. Liquid cultures were incubated at 21 °C with shaking at 200 rpm. Solid culture was incubated at room temperature. *E. coli* JM109 was obtained from New England Biolabs and routinely grown in Lysogeny Broth (LB) or LB agar. Culture was at 37 °C with shaking at 200 rpm. For recombinant cells, chloramphenicol (40 mg/l) was used for selection.

2.2. DNA assembly

DNA assembly was carried out using the PaperClip DNA assembly method as recently described in [20]. DNA parts were amplified by PCR from plasmid DNA or *F. agariphila* colonies using their corresponding upstream forward (UF) and downstream reverse (DR) oligonucleotides as primers. PCRs were performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, US) according to the manufacturer's instructions. Samples were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. pSB1C3 (http://parts.igem.org) was used as the backbone and P_{lac}-*lacZ′* (BBa_J33207, http://parts.igem.org) added upstream of the gene to add a *lac* promoter and for blue-white selection on X-gal plates. Specific ribosome-binding sites (RBSs) for each construct were added as short intervening sequences between the half clips according to [20]. RBSs were designed using the RBS calculator [21] with a target translation initiation rate of 10,000 on the RBS calculator proportional scale. The cloning strategy is diagrammatically presented in Fig. S1. Oligonucleotide sequences are listed in Table S1. All constructs were sequence verified by The GenePool (Edinburgh) and are listed in Table S2.

2.3. Ulvan purification

Dry *U. lactuca* biomass was obtained from La Herradura de Guayacán bay, Chile, and was kindly provided by Dr. Cristian Bulboa from Universidad Andrés Bello, Chile.

Alcohol insoluble residue (AIR) of *U. lactuca* was obtained as described by Fry [22]. Ulvan was extracted from the *U. lactuca* AIR using ammonium oxalate as a chelating agent [22]. A suspension containing 500 mg of AIR in 5 ml 0.2 M ammonium oxalate (pH 4 adjusted with formic acid) was incubated for 2.5 h at 85 °C and then stirred overnight at 37 °C. The sample was centrifuged at 4500*g* for 30 min and the supernatant saved. The pellet was washed with 5 ml of deionized water and centrifuged at 4500*g* for 30 min. The supernatants were combined and dialyzed against deionized water using a 3.5 K molecular-weight cut off dialysis cassette (Thermo Fisher Scientific, US) and then freeze-dried.

The *U. lactuca*, *U. lactuca* AIR and purified ulvan samples were analysed by thin layer chromatography (TLC) (see Supplementary material 2.3). In agreement with van der Wal et al. [7], the main sugars observed in all the samples were rhamnose, xylose, glucose and glucuronic acid.

2.4. F. agariphila growth assays

F. agariphila colonies grown on MB plates for 3 days at room temperature were used to inoculate flasks containing 50 ml of a 35 g/l sea salts (Sigma, US) preparation supplemented with 1 g/l yeast extract and different carbon sources. The carbon sources used were 5 g/l peptone and 10 g/l autoclaved *U. lactuca* biomass. The flasks were incubated at 21 °C with shaking at 200 rpm and OD 600 nm recorded to determine growth.

2.5. Heterologous protein expression

E. coli colonies with the desired constructs, were used to inoculate vials containing 5 ml of LB with 40 μg/ml chloramphenicol. Each vial was incubated overnight at 37 °C with shaking at 200 rpm and 1 ml transferred to a flask containing 50 ml LB. The new culture was incubated under the same conditions until OD 600 nm ~0.6 was reached and protein expression was induced with 90 μg/ml isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight.

2.6. Cell fractionation

The cells were harvested by centrifugation (5000*g* for 30 min at 4 °C) and the supernatant saved as the extracellular fraction. The pellets were resuspended in 1 ml of phosphate buffered saline (PBS) and lysed by ultrasonication using 10 cycles of 10 s bursts (12 μm amplitude) and