

Saccharification of Brown Macroalgae Using an Arsenal of Recombinant Alginate Lyases: Potential Application in the Biorefinery Process ^S

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Alginate lyases (endo and exo-lyases) are required for the degradation of alginate into its constituting monomers. Efficient bioethanol production and extraction of bioactives from brown algae requires intensive use of these enzymes. Nonetheless, there are few commercial alginate lyase preparations, and their costs make them unsuitable for large scale experiments. A recombinant expression protocol has been developed in this study for producing seven endo-lyases and three exo-lyases as soluble and highly active preparations. Saccharification of alginate using 21 different endo/exo-lyase combinations shows that there is complementary enzymatic activity between some of the endo/exo pairs. This is probably due to favorable matching of their substrate biases for the different glycosidic bonds in the alginate molecule. Therefore, selection of enzymes for the best saccharification results for a given biomass should be based on screens comprising both types of lyases. Additionally, different incubation temperatures, enzyme load ratios, and enzyme loading strategies were assessed using the best four enzyme combinations for treating *Macrocystis pyrifera* biomass. It was shown that 30°C with a 1:3 endo/exo loading ratio was suitable for all four combinations. Moreover, simultaneous loading of endo- and exo-lyases at the beginning of the reaction allowed maximum alginate saccharification in half the time than when the exo-lyases were added sequentially.

Keywords: Endo-alginate lyase, exo-alginate lyase, brown macroalgae biorefinery, brown macroalgae saccharification, brown macroalgae extraction

Introduction

Alginate is an anionic polysaccharide that is widely used as a gelling or thickener agent in food and beverages, while also having applications in the paper, biomaterials, and pharmaceutical industries [1]. Brown algae (Phaeophyceae) are composed of ~40% DW alginate, which acts as their main structural cell wall component [2]. *Pseudomonas* and *Azotobacter* bacteria can also synthesize alginate for cell adhesion and protection, respectively [3]. All sources of alginate are composed of uronic acid monomers: α -L-guluronate (G) and its C5 epimer β -D-mannuronate (M), arranged as homopolymeric or heteropolymeric blocks.

The proportion of these blocks varies between alginates from different organisms, while also depending on several environmental factors [4].

Alginate lyases (E.C. 4.2.2.3 and E.C. 4.2.2.11) can cleave the glycosidic bond between alginate monomers by beta-elimination. There are two types of alginate lyases, endo-cleaving alginate lyases, which produce oligoalginates, and exo-cleaving alginate lyases (“oligoalginate lyases”), which release uronic acid monomers. Both types are present in a variety of organisms including algae, mollusks, fungi, bacteria, and viruses. Their functions are related to the synthesis of alginate cell walls or the degradation of alginate as a carbon and energy source [5]. Alginate lyases

have been assigned by the Carbohydrate-Active Enzymes Database (CAZy) to families 5, 6, 7, 14, 15, 17, and 187 of polysaccharide lyases (PL) based on hydrophobic cluster analysis (<http://www.cazy.org/Polysaccharide-Lyases.html>), or they can also be classified by their substrate preference for G-G, M-M, or G-M bonds [2].

Alginate lyases have various applications. Oligoalginates produced with these enzymes can act as growth promoting and/or activating factors for probiotic bacteria [6], plants [7–10], endothelial cells [11], and immune cells [12–14]. Oligoalginates can also be used to disrupt pathogenic biofilms [15–17], and inhibit colonization of enteropathogenic bacteria [18]. Alginate lyases are also part of medical treatments for disrupting *Pseudomonas aeruginosa* alginate-based biofilms and increasing antibiotic efficacy [19–21]. Additionally, alginate lyases are important tools in laboratories for producing brown algae protoplasts for biochemical and genetic transformation studies. In the past years, uronic acids from brown algae produced by endo and exo alginate lyases have become an attractive and sustainable source of fermentable sugars which could be turned into bioethanol, biogas, or some other commodities provided the appropriate metabolic pathways are present [22–30]. On the other hand, the degradation of alginate has been shown to be favorable for the extraction of mannitol and cellulose from algal biomass for using them as alternative sugar sources [31–36]. Alginate lyases have also been proposed for “enzyme-assisted extractions” of high-value bioactives from brown macroalgae such as carotenoids, fluorescent pigments, fucoidan, and phlorotannins [37–40].

Despite the multiple applications of alginate lyases there are few reports on the production, optimization and utilization of recombinant alginate lyases. Previous publications have dealt with three or fewer recombinant targets, and usually testing either exo or endo lyases, but not both. One of the more complete publications has tested a single exo and a single endo lyase, concluding that it was better to use these enzymes sequentially [24]. Recombinant endo-type enzymes can only be obtained from three commercial sources that report little about enzyme activity in their datasheets (Sigma, Megazyme, and Nippon Gene), while several other companies offer products with no technical specifications. NZYtech offers two recombinant exo-alginate lyases but their use has not been reported in the scientific literature yet. Furthermore, the NZYtech *PaAlg7A* exo-lyase has been previously characterized as an endo-lyase [41]. Given the high cost and low quantities offered for the commercial enzymes, it is unviable to include them in the design of pilot or industrial scale processes

comprising alginate degradation. Nonetheless, few studies have been dedicated to develop the recombinant production of alginate lyases, and most work has focused on the analytical-scale purification of a single enzyme and its characterization [42–47].

Unfortunately, alginate lyases are regarded as “specialty” enzymes; therefore the mainstream biotechnology research community has neglected them as targets for production, instead favoring plant-biomass-degrading enzymes. It remains then a responsibility for the brown macroalgae research community to develop alginate lyases as an everyday low-cost tool for biorefinery and biochemical research. The first aim of this work is to establish such an approximation by using widespread cultivation and genetic tools for improving expression of endo-alginate lyases in *E. coli*. We then expand these results for the production of exo-alginate lyases. Past efforts for the saccharification of alginate into its monomers have relied on the use of a single pair of an endo and exo lyase, without testing combinations of different enzyme targets. Our expression platform has allowed us to produce and test 7 endo and 3 exo- lyases as 21 combinations for the saccharification of alginate in order to confirm if the selection of enzyme couples could be adjusted for enhancing product yield. Four enzyme pairs were then further tested with *Macrocystis pyrifera* biomass to analyze their differential response to temperature, enzyme load, and the order of addition of endo/exo lyases in the saccharification reaction.

Materials and Methods

Strains and Growth Conditions

The following *E. coli* strains were used in this work: DH10B for cloning procedures, BL21 (DE3), BL21 (DE3) pLysS, BL21 (DE3) Rosetta2, and BL21 (DE3) pLysS Rosetta2 were employed as hosts for optimizing recombinant alginate lyase expression (see Supplementary Information). Cloning work was performed using LB medium supplemented with 100 µg/ml ampicillin and/or 34 µg/ml chloramphenicol, 37°C growth temperature, and 200 rpm orbital shaking. All transformations were done with chemically competent cells [48]. See below for expression experiments growth condition. Cloning work was performed using LB medium supplemented with 100 µg/ml ampicillin and/or 34 µg/ml chloramphenicol, 37°C growth temperature, and 200 rpm orbital shaking. All transformations were done with chemically competent cells [48]. See below for expression experiments growth conditions.

DNA Constructs

All 18 alginate lyase coding sequences used in this work are presented in Table 1. The sequences for *alyA-D2*, *alyB*, *alyD*, *aly-SJ02*, *OalA*, *OalB*, and *OalC* were amplified from the *E. coli* strain

Table 1. Alginate lyase proteins expressed in this study.

Name	Species	Family	Expressed amino acids	NCBI Protein	Reference
Edo-lyase					
AlyA-D2	<i>Vibrio splendidus</i> 12B01	PL7	376-580 (205)	EAP94921.1	[42]
AlyB	<i>Vibrio splendidus</i> 12B01	PL7	18-522 (505)	EAP94922.1	[42]
AlyD	<i>Vibrio splendidus</i> 12B01	PL7	22-345 (324)	EAP94925.1	[42]
Aly-SJ02	<i>Pseudoalteromonas</i> sp. SM0524	PL18	175-400 (226)	618885355	[43]
AlyPEEC	<i>Pseudoalteromonas elyakovii</i>	PL18	166-398 (233)	4322372	[44]
AlgmsP	<i>Microbulbifer</i> sp. 6532A	PL7	80-358 (279)	319993261	[46]
AlyL1	<i>Agarivorans</i> sp. L11	PL7	24-349 (326)	AIY68670.1	[43]
A1-II	<i>Sphingomonas</i> sp. A1	PL7	414-641 (228)	BAB03312.1	[47]
A1-II'	<i>Sphingomonas</i> sp. A1	PL7	81-308 (228)	BAD16656.1	[45]
Exo-lyase					
Cly*	<i>Cellulophagalytica</i>	PL7	12-355 (344)	WP_038507284.1	This work
ScbB*	<i>SCB49 unidentifed bacterium</i>	PL7	17-339 (323)	EDM45391.1	This work
Atu3025	<i>Agrobacterium tumefaciens</i> C58	PL15	1-776	NP_357573.1	[62]
OalA	<i>Vibrio splendidus</i> 12B01	PL15	1-692	EAP93067.1	[61]
OalB	<i>Vibrio splendidus</i> 12B01	PL17	1-735	EAP93062.1	[61]
OalC	<i>Vibrio splendidus</i> 12B01	PL17	1-717	EAP93063.1	[61]
Cat*	<i>Croceibacter atlanticus</i>	PL7	17-348 (332)	WP_013188042.1	This work
Lpe*	<i>Lewinellapersica</i>	PL7	20-349 (330)	WP_026231793.1	This work
ScbA*	<i>SCB49 unidentifed bacterium</i>	PL7	24-366 (343)	EDM45394.1	This work

BAL1611. All seven coding sequences contain the codons from the original marine host [30]. The sequence for Atu3025 was amplified from genomic DNA from *Agrobacterium tumefaciens* C58. CDS for *alyPEEC*, *algmsP*, *alyL1*, *a1-II*, *a1-II'*, *Cat*, *Cly*, *Lpe*, *ScbA*, and *ScbB* were codon optimized for *E. coli* bias and synthesized. N-terminal secretion signal peptides and lipoprotein signal peptides were predicted with SignalP 4.1 and LipoP 1.0, respectively, and were not included in the expression constructs [49, 50]. All genes were PCR-amplified to add restriction sites and/or Gateway recombination sequences using primers from Table S1. Ligase-based cloning was performed for expression vectors pET22b+, pETDuet1, pMXB10, pTYB21, pN-HIS10, and pNative (see Table S2). The latter two vectors were constructed for this work using the pET22b+ backbone. pN-HIS10 has ten His codons before the NdeI site in pET22b+. pNative has a stop codon before the XhoI site in pET22b+. All genes and expression vectors were digested with NdeI or AseI (towards start codon), and XhoI or Sall (towards the stop codon). NdeI/AseI and XhoI/Sall have compatible ends and were alternatively used depending on the presence of secondary restriction sites.

Gateway expression vectors pETG-10A, 20A, 30A, 40A, 41A, 50A, 52A and 60A (see Table S2) were obtained from the Protein Expression and Purification Facility, European Molecular Biology Laboratory, Heidelberg. Gateway LR cloning was performed using primers shown in Table S1 as previously described [51].

Their same nomenclature was maintained. Non-synthetic genes required the addition of 17 bp attL1-T1 and 14 bp attL2-T1 adapters by PCR before cloning. A second round of amplification was necessary to obtain the full 49 bp attL1-T1 and attL2-T1 sites, which allowed for efficient LR recombination. All synthetic genes already included 17 bp attL-T1 adapters, thus requiring a single PCR with one set of primers before gateway cloning (attL1-T1-For and attL2-T1-Rev primers).

All PCR amplifications were performed using the X7 high-fidelity Pfu polymerase [52]. Synthetic genes and primers were purchased from Integrated DNA Technologies (USA). Restriction enzymes were acquired from New England Biolabs (USA). T4 ligase and Gateway LR Clonase II were obtained from Invitrogen (USA). Sequence confirmation was performed by MacroGen Inc. (Korea).

Standard Expression Protocol

The inoculation protocol was based on "Strategy 3" from [53]. After chemical transformation of the expression host, the complete mix was transferred to grow overnight in 7 ml LB liquid medium plus 100 µg/ml ampicillin and 34 µg/ml chloramphenicol (only ampicillin for BL21 (DE3) alone). The OD₆₀₀ of the cultures were then recorded, and the expression cultures were inoculated to a 0.05 final OD₆₀₀. Expression cultures consisted of 20 ml of ZYM-5052 autoinduction medium [54] in 60 ml Erlenmeyer flasks,

grown at 25°C, and 200 rpm orbital shaking. The standard expression protocol also considered: BL21 (DE3) Rosetta2 as the host; harvesting at 42 h, and expression vector pET22b+, unless otherwise stated.

Preparation of Protein Crude Extracts

Briefly, 2 ml of the autoinduction cultures were centrifuged and resuspended in 600 µl TBS pH 7.5. Samples were sonicated twice in Eppendorf tubes for 10 sec each time, with a 1/8" tip at 8 W power using a XL-2000 sonicator (Misonix, USA). Samples were then centrifuged at 20,000 g for 10 min at 4°C. The clear supernatant was then recovered and used for the enzymatic assays.

Enzymatic Assays for Testing Expression of Endo- and Exo-Alginate Lyases

Recombinant protein production is reported as soluble alginate lyase activity in this work. All enzymatic assays were normalized to the same volume of original culture media loaded per reaction. For endo-lyases, 37.5 µl of the soluble protein supernatants were mixed with 1 ml of the reaction buffer: 50 mM Tris-HCl pH 8.0, 0.5% w/v sodium alginate from brown algae (W201502, Sigma), 400 mM NaCl, 1 mM CaCl₂. Reactions were incubated for 4 h at 30°C with 200 rpm orbital shaking. For exo-lyase initial assays were performed using 50 µl of the soluble protein supernatants mixed with 1 ml of the reaction buffer: 50 mM Tris-HCl pH 7.5, and 1% w/v sodium alginate from brown algae (Sigma). Reactions were incubated for 6 h at 30°C with 200 rpm orbital shaking.

The resulting reducing sugar ends were then quantified with a microplate DNS assay using a glucose standard curve for comparing the amount of released oligoalginate reducing ends [55]. Absorbance was measured at 550 nm with a UVM 340 microplate reader (Asys, Eugendorf, Austria). Negative controls were always included containing the equivalent amount of soluble protein from the *E. coli* host without an expression plasmid incubated with the corresponding substrate.

Enzymatic Assays for Testing Different Combinations of Exo/Endo-Lyases

Briefly, 50 µl of exo-lyase and 37.5 µl of endo-lyase crude protein preparations (Section 2.4) were incubated with 1 ml of 0.5% alginate Tris-HCl pH 7.5, for 2 h at 30°C, 200 rpm agitation. For the quantification of uronic acid reducing ends, 25 µl of reaction products with 75 µl of copper reagent were boiled for 10 min. Samples were incubated on ice for 5 min, and 50 µl of Nelson's reagent plus 100 µl of distilled water were then added [56]. Each mixture was centrifuged at 2,600 g for 5 min and the absorbance of each sample was measured at 595 nm in a microplate reader (UVM340). A standard curve was prepared using glucuronic acid (Sigma).

Statistical Analysis

The results were subjected to one-way ANOVA analysis and

Tukey's HSD test ($p < 0.05$) for multiple comparison using Graph Pad Prism software.

Enzymatic Reactions Using Algae Biomass

Crude protein enzyme preparations were quantified using the Bio-Rad Bradford assay (USA). 0.3 mg of total protein from the crude preparations of an exo and an endo-lyase (0.6 mg total protein) were loaded in each reaction containing 220 mg of acid-pretreated dry *Macrocystis pyrifera* biomass [25] in a final volume of 1.5 ml 0.5 M Tris-HCl pH 7.5. The high concentration of Tris buffer was required due to the high acidity of the algal substrate. Duplicate reactions were incubated for 6 h, at 30°C, 200 rpm shaking, unless otherwise stated. Released uronic acid reducing ends were quantified as in Section 2.6.

Thin Layer Chromatography (TLC)

The plates of silica gel 60 F254 (Merck Millipore) were developed in n-butanol-acetic acid-water (3:2:2 (v/v/v)) for 4 h, followed by drying of the plates. Reaction products were visualized with a sulfuric acid-ethanol (1:4 (v/v)).

Results and Discussion

Selection of Alginate Lyase Targets

Endo and exo-alginate lyases selected for this work are shown in Table 1, along with their sequence information and the corresponding primary references. Endo AlyA-D2, AlyB, AlyD, and Aly-SJ02 were selected because these enzymes were found suitable for alginate degradation and conversion into ethanol (*E. coli* strain BAL161) [30]. Only domain 2 of AlyA was cloned because it has been reported that the full protein is less active [42]. After a survey through the literature, additional genes coding for AlyPEEC, AlgmsP, AlyL1, A1-II, and A1-II' were selected for codon optimization and synthesis based primarily on reported V_{max} and the ability to cleave all three M-M, G-G, and M-G bonds. Selected enzymes belong primarily to polysaccharide lyase family 7 (PL7) according to the CAZy database. This family constitutes 39% of the total 1,618 enzymes classified so far among the seven alginate lyase families. Also the PL7 family constitutes 40% of the 91 alginate lyases characterized to date [57]. Enzymes within the PL7 family share the same β -jelly roll folding pattern and the disposition of the amino acid in the reaction center. The more recently classified PL18 family also shares these characteristics [58]. Endo-lyases from these two families are the subject of the present work in order to obtain results that could be useful for a broader range of research groups.

Most bacterial exo-alginate lyases are classified into families PL15 and PL17 [2]. There are only two examples of

characterized PL7 exo-alginate lyases [27, 59]. PL7 exo-lyases are interesting because they are smaller than their PL15/PL17 counterparts, ranging between 300-400 amino acids in length versus the 700-800 residues of the latter. This might be beneficial for facilitating expression and folding in a heterologous host, while it also reduces the cost and complexity of gene synthesis. Given these advantages we decided to target this sub-type of enzymes for expression in *E. coli*. The sequence of *Zobellia galactanivorans* AlyA5 PL7 exo-alginate lyase was blasted against the NCBI database, and results were filtered to obtain a sub-set of enzymes with at least 95% sequence coverage and 55% sequence identity. A distance tree was generated based on pairwise alignments to AlyA5 using BLAST tree view default parameters. Five targets were selected, each one being a representative from different branches of the distance tree [60]. All five targets have the three loop insertions that are characteristic to the exo AlyA5 primary sequence and tertiary structure, which differentiates these enzymes from the PL7 endo-lyases (Fig. S1) [59]. Additionally, Atu3025, OalA, OalB, and OalC endo-lyases (oligoalginate lyases) were also selected for expression because they have been satisfactorily implemented for the saccharification of alginate [30, 61, 62].

Optimization of Recombinant Expression Conditions

All of the characterized enzymes from Table 1 have been previously expressed in *E. coli*, either in a soluble or insoluble form; therefore we maintained this expression platform in our experiments. Current methods for high-throughput protein expression have successfully implemented the T7 promoter and Studier autoinduction method, given the advantages of higher cell density upon induction, reduced sample manipulation and monitoring, and IPTG cost savings [54, 63]. This method can also be coupled with a simpler inoculation strategy, in which the transformed competent *E. coli* cells are directly grown in autoinduction media, without significant effects in protein yields [53]. The latter protocol considers autoinduction at 25°C, which is in agreement with results obtained in an additional high-throughput survey for soluble recombinant expression in *E. coli* [64]. Since our target alginate lyases derive from marine bacteria, which thrive at temperatures below 37°C, we set our standard protocol for autoinduction at 25°C for 42 h, with 0.05 final OD₆₀₀ inoculation density, as in [53]. The pET22b+ expression vector, with a C-terminal His-tag, was used for our standard protocol. The combination of the aforementioned strategies result in a streamlined protocol that was implemented for optimizing three critical

parameters: expression host, protein fusion partner, and harvesting time.

High-throughput surveys have demonstrated that selection of an appropriate expression strain is a key parameter for recombinant protein production in *E. coli* [63, 64]. Based on these results, four *E. coli* strains were selected because they allowed for soluble expression of the highest number of genes: BL21 (DE3); BL21 (DE3) pLysS; BL21 (DE3) Rosetta2, and BL21 (DE3) pLysS Rosetta2, for expressing three alginate lyases (AlyA-D2, AlyB, and Aly-SJ02). Fig. S2 shows that strain selection can cause substantial differences in soluble alginate lyase expression, and that even enzymes from the same PL family, like AlyA and AlyB, may not express to similar levels with the same *E. coli* host. BL21 (DE3) Rosetta2 strain allowed for high soluble expression for all three enzymes, and it gives flexibility for expressing non-codon optimized genes, since it carries a plasmid with 7 rare tRNA [65]. Strains carrying the pLysS plasmid expressed the lowest levels of active enzyme, which could be due to the amidase activity of LysS lysozyme (cell wall degradation) and the T7 polymerase inhibition present in these hosts [66, 67].

Overexpression of proteins in *E. coli* often results in accumulation of inactive and insoluble aggregates, which require labor-intensive and low-efficiency refolding protocols. Addition of protein fusion-tags to the recombinant target can enhance solubility, while some of them also facilitate detection and purification [68]. In this study we used a set of 14 expression vectors (Table S2) for determining which fusion partners might yield the best accumulation of soluble and active enzyme using our standard expression protocol in BL21 (DE3) Rosetta2. We expressed the same three alginate lyases from the above experiments with all 14 tags, and the enzyme activity results are shown in Fig. S3. All three targets expressed well without the addition of a tag, which is indicative of the soluble nature of alginate lyases. The proteins still expressed well with the addition of an N-, or C-terminal Histidine tag, which might constitute a viable alternative for detecting and purifying alginate lyases. The three best vectors were: pETG40A, 41A, and 60A. pETG40A and pETG41A have an N-terminal maltose-binding protein (MBP), without or with a 6xHis-tag, respectively. pETG60A has an N-terminal N utilization substance protein A tag (NusA) along with a 6xHis-tag. These results are in agreement with previous large protein expression surveys [64]. In contrast, our results also show that some tags should probably be avoided for alginate lyase expression, like N-terminal Sce VMA chitin-binding-domain/intein (pTYB21), and N-terminal His-tag/Glutathione

S-transferase (pETG30).

It was noticed that culture density reached saturation before the 42 h autoinduction time proposed by [53], given the high inoculation density employed by these authors (0.05 final OD₆₀₀). Therefore, we analyzed the effect of shorter autoinduction times, while maintaining this inoculation level, which could ultimately result in higher protein yields, shorter experiments, and lower overall production costs. A preliminary screening with autoinduction of AlyA-D2, AlyB, and Aly-SJ02 at 25°C showed that harvesting at 23 h generated higher alginate lyase activity compared to longer culture times (data not shown). Therefore, the same three strains were sampled every three hours between 8 and 20 h of culture time, and the recombinant enzyme activity results are shown in Fig. S4. It is apparent that harvesting at 8 or 11 h results in very low enzyme yields. It is likely that glucose, the expression repressor, is depleted from the autoinduction medium between 8 and 11 h, thus triggering the accumulation of the recombinant proteins past 11 h. All three enzyme activities reached a peak at 14 h after inoculation. AlyA-D2 and Aly-SJ02 activities start diminishing after 17 h, while AlyB activity increases slightly between 14 and 20 h. These different trends are most likely related to the stability of each enzyme under these culture conditions, and their sensitivity towards the host proteases.

Optimized Protocol for the Expression of Additional Alginate Lyases

An optimized protocol has been generated for testing the expression of all alginate lyases targets from Table 1. The following conditions were implemented:

- *E. coli* BL21 (DE3) Rosetta2
- Expression vectors pNative, pETG41A, or pETG60A
- ZYM-5052 autoinduction medium
- 0.05 final OD₆₀₀ inoculation, 14 h harvesting time at 25°C, 200 rpm

Results are shown in Fig. 1. The proposed protocol allowed us to express eight out of nine alginate endo-lyases, and three out of nine exo-lyases. The process only takes 14 h of culture time at 25°C, which could prove favorable when taking into account energy costs of the fermentation. The enzyme extract was normalized to 0.3 mg of total protein for each type of lyase. The overall results suggest the conclusion that N-terminal 6xHis-MBP is the partner of choice for expressing endo-lyases, while no partner seems to be the best option for exo-lyases.

Endo A1-II' was the only enzyme that strictly required

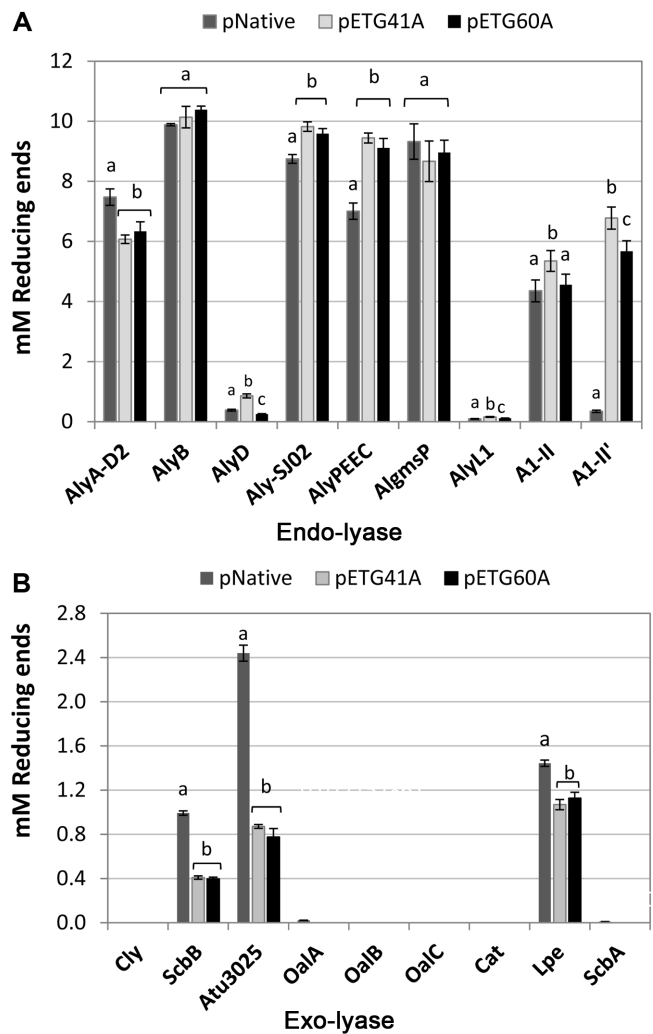


Fig. 1. Optimized protocol for the expression of 18 different alginate lyases in *E. coli*.

Three different vectors were tested using the parameters from Section 2.3. (A) Endo-lyase targets. (B) Exo-lyase targets. Enzymatic assays were performed as described in Materials and Methods. Plasmid pNative: no tag fused to the target protein; pETG41A: N-terminal 6xHis/Maltose-binding protein tag; pETG60A: N-terminal NusA/6xHis tag. Error bars represent the standard deviation of duplicates. For each enzyme mixture, means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

the presence of a fusion partner for soluble expression, probably for enhancing folding and/or solubility. Endo AlyA-D2, and the three exo-lyases (Atu3025, Lpe, and ScbB) had the highest activity when expressed in the native form. This could be related to negative steric effects on enzyme function caused by the exogenous fused polypeptide. This in turn could be alleviated by adding a protease site for cleaving the fusion partner, or by

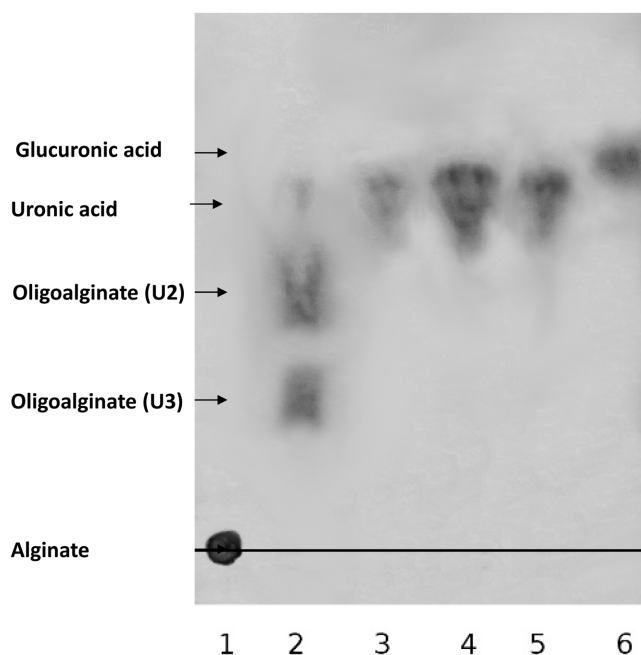


Fig. 2. Thin-layer chromatography of the hydrolysis products of alginate.

Lane 1, alginate + no enzymes; Lane 2, endo-Aly-SJ02; Lane 3, endo-Aly-SJ02 + exo-Lpe; Lane 4, endo-Aly-SJ02 + exo-ScbB; Lane 5, endo-Aly-SJ02 + exo-Atu3025; Lane 6, glucuronic acid standard. Reactions were prepared as in Section 2.6. 8 μ l of each reaction (0.5% alginate) and standard (1 mg/ml) were loaded per lane.

including an optimized peptide linker sequence.

Additionally, different pHs for all expressed targets based on the literature were tested (data not shown), and the ones utilized in this paper represent conditions that work well with all enzymes.

Confirmation of Exo-Lyase Activity of ScbB and Lpe

Thin-layer chromatography was performed in order to

confirm the release of urinate monomers due to the exo-lyase activity of the novel enzymes ScbB and Lpe. Fig. 2 shows that alginate is degraded into a mixture of oligoalginates in the presence of the Aly-SJ02 endo-lyase by itself (Lane 2). When combined with Atu3025, a confirmed exo-lyase, the alginate degradation products resolved into a shorter band (Lane 5), with a size similar to the glucuronate monomer (Lane 6). Lanes 3 and 4 show a product of similar size as in Lane 5, without the detectable presence of larger oligoalginates, confirming that Lpe and ScbB have exo-lyase activity. These results support that the unique sequence and structural characteristics of *Z. galactanivorans* ALyA5 PL7 exo-alginate lyase could be valuable for predicting exo-lyase activity in PL7-like enzymes (Fig. S1) [59].

Alginate Saccharification Using a Combinatorial Approach

Seven recombinant endo-lyase preparations were combined with three exo-lyase extracts, resulting in 21 enzyme cocktails for the saccharification of alginate. Table 2 shows the product yields from the saccharification of purified alginate using these 21 combinations along with controls containing the endo-lyases by themselves. When comparing the overall results for the three exo-lyases, it is apparent that ScbB yielded the lowest amounts of reducing sugar ends. On the other hand, Atu3025 and Lpe produced overall higher yields, and behaved similarly with the corresponding endo-lyases. Interestingly the endo-lyase partner that resulted in the highest yields for ScbB (AlgmsP) was the least efficient partner for Atu3025 and Lpe. Conversely, the better suited partners for Atu3025 and Lpe (AlyPEEC and Aly-SJ02, respectively) resulted in low product yields for ScbB. Moreover, the endo-lyase that resulted in the lowest yield for ScbB (AlyB), produced high quantities of reducing ends with Atu3025 and Lpe. Additionally, A1-II' endo-lyase was one of most productive

Table 2. Degradation of purified alginate using 21 combinations of endo- and exo-alginate lyases.

Endo-Lyase	Exo-Lyase			No Exo-Lyase
	ScbB	Atu3025	Lpe	
AlyA-D2	0.072 \pm 0.020	0.538 \pm 0.035	0.545 \pm 0.031	0.011 \pm 0.002
AlyB	0.059 \pm 0.010	0.523 \pm 0.034	0.579 \pm 0.017	0.093 \pm 0.021
Aly-SJ02	0.226 \pm 0.012	0.578 \pm 0.093	0.679 \pm 0.044	0.075 \pm 0.005
AlyPEEC	0.195 \pm 0.002	0.612 \pm 0.056	0.552 \pm 0.077	0.032 \pm 0.001
AlgmsP	0.342 \pm 0.012	0.390 \pm 0.047	0.382 \pm 0.036	0.137 \pm 0.025
A1-II	0.140 \pm 0.042	0.490 \pm 0.032	0.427 \pm 0.051	0.076 \pm 0.035
A1-II'	0.297 \pm 0.002	0.455 \pm 0.032	0.558 \pm 0.004	0.173 \pm 0.044

The mean values correspond to the equivalent of mg/ml of glucuronic acid as the standard represents the standard deviation of duplicates. Samples were prepared as described in Materials and Methods.

partners for ScbB and Lpe, while at the same time was one of the least proficient partners for Atu3025. The relative inconsistency for any single enzyme to produce the highest yields is indicative of interdependency between the endo and exo type of enzymes. This is probably due to the substrate bias that is characteristic for a given alginate lyase. Oligoalginates produced by endo-lyases differ in length and G/M bond composition depending on the specific enzyme. Some oligoalginates would be substrates with higher affinity for some specific endo-lyases, which also have biases for length and glycosidic bond composition [2]. Therefore it is important to find an optimal couple of enzymes for a particular process and biomass, and to do this it is necessary to produce a wide array of targets, such as the ones presented for the first time in this work. Additionally, it is also important to match enzymes according to their optimum temperature, pH and buffer composition. Fortunately most of characterized alginate lyases are produced by bacteria from marine environments, and have similar requirements [5]. Furthermore, an optimal couple of enzymes for one algal biomass might not be necessarily the best for other algae species. It is well documented that each species has a unique G/M composition, which can also vary according to age, morphology of the alga, geographical location, and harvest season [4]. Finally, the possibility of using more than one endo and/or exo lyase per reaction should not be discarded. This notion is supported by the presence of several of these genes in a single organism. For example,

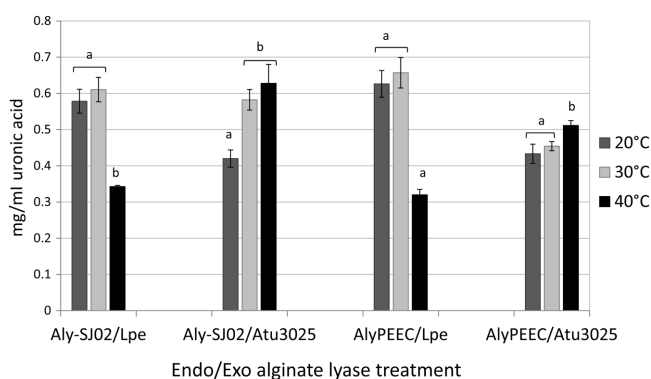


Fig. 3. Endo+Exo alginate lyase treatment of algal biomass at three different temperatures.

Acid pre-treated alga was incubated for 6 h and 200 rpm at the indicated temperatures. Reactions contained: 0.3 mg of total soluble protein crude extract for each enzyme, 220 mg alga, in 1.5 ml final volume of 0.5 M pH 7.5 Tris buffer. Error bars represent the standard deviation of duplicates. For each enzyme mixture, means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

the well-studied *Vibrio splendidus* 12B01 has 4 functional endo-lyases and 3 exo-lyases [42, 61].

Activity of Alginate Lyase Pairs at Different Temperatures

According to the product yield results from Table 2, four alginate lyase pairs were selected for further testing: Aly-SJ02/Lpe, Aly-SJ02/Atu3025, AlyPEEC/Lpe, and AlyPEEC/Atu3025. Fig. 3 shows the results from treating *M. pyrifera* biomass (brown algae) for 6 h at three different temperatures: 20°C, 30°C, and 40°C. This incubation time was selected in order to favor enzymes that are thermally stable over time, so they continue to catalyze the reaction. These temperatures were selected because most alginate lyases are derived from marine bacteria, and are active at 20°C or less, although they usually achieve maximum activity between 30°C and 40°C [2, 5]. None of the enzyme combinations performed best at 20°C. Incubation at 40°C marginally favored AlyPEEC/Atu3025, while not having a relevant effect for Aly-SJ02/Atu3025 when compared to the yield at 30°C. On the other hand, pairs containing Lpeexo-lyase were negatively affected by the highest incubation temperature, indicating that this enzyme might be unstable at 40°C. Overall, incubations at 30°C yielded consistently higher product accumulation for all four enzyme couples, therefore this temperature was used for subsequent experiments. These results are in accordance with the optimal temperature range for many characterized alginate lyases from marine bacteria [5].

Effect of Endo/Exo Lyase Load Ratio on Product Yield

It is expected that the endo and exo lyases will not catalyze their respective reactions at the same rate for a given pair of enzymes. It is also likely that one of the enzymes could be more stable over time than the other. It is then necessary to test if adding different proportions of endo and exo lyase would affect the final product yield. Fig. 4 shows the results from the saccharification of algal biomass using four different endo/exo lyase enzyme loads: 1:1, 1:3, 3:1, and 3:3. A 1X enzyme load corresponds to 0.3 mg of total soluble protein from the crude *E. coli* extract for each enzyme. It is apparent from this Figure that the reactions are close to being saturated by a 1:1 enzyme load for the four enzyme cocktails. There are no clear advantages to adding three times more endo-lyase for any of the enzyme combinations. Furthermore, in some cases the extra endo-lyase load caused a reduction in product yield. This might be due to the inhibition of the reaction by all the additional impurities present in the *E. coli* crude extract. This can be directly observed in Fig. S5, which shows that the addition

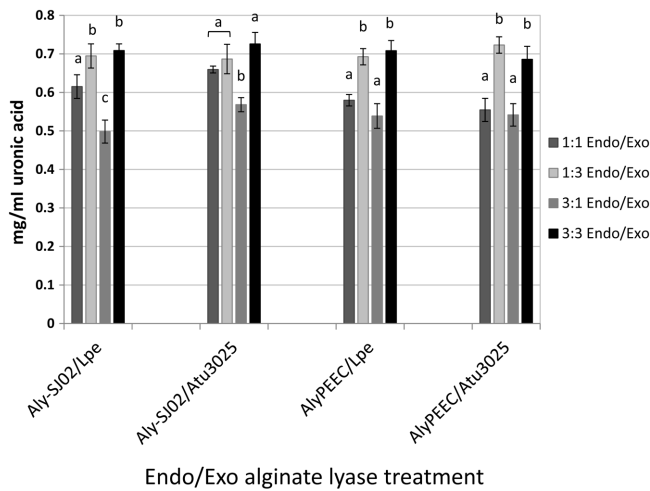


Fig. 4. Effect of endo/exo enzyme load ratio on alginate saccharification.

A 1X enzyme load corresponds to 0.3 mg of total protein from the crude extracts. Each reaction contained 220 mg of alga in 1.5 ml final volume of 0.5 M pH 7.5 Tris buffer. Treatment was performed for 6 h, 30°C, 200 rpm. Error bars represent the standard deviation of duplicates. For each enzyme mixture, means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

of 0.6 mg total protein of an untransformed *E. coli* extract causes the reduction of product yield for a 1:1 enzyme load. For this reason, and since the endo-lyase reactions seem to have excess catalyst, it is plausible to think that a higher product yield might be achieved by loading less crude extract for certain endo-lyases. On the other hand, the reactions were not negatively affected by a 1:3 or 3:3 endo/exo ratios when compared to the 1:1 ratio, leading us to think that the additional exolytic activity countered the inhibitory effects of the crude extracts. Only in the case of reactions containing AlyPEEC was there an advantage from adding three times more exo-lyase than endo-lyase (20% product increase). A 1:3 enzyme ratio was maintained for our final enzyme trials since it did not result in lower product yields for any of the cocktails and was superior for the ones containing AlyPEEC when compared to a 1:1 ratio.

Exo-Alginate Lyase Loading Strategy into the Reaction

It has been shown that loading an alginate exo-lyase after the endo-lyase has generated most of the oligoalginates results in higher product yields when compared to simultaneous addition of both enzymes [24]. We therefore compared these two strategies with our four enzyme couples to test whether this behavior was enzyme-dependent (Fig. 5). Incubations were carried out for 9 h,

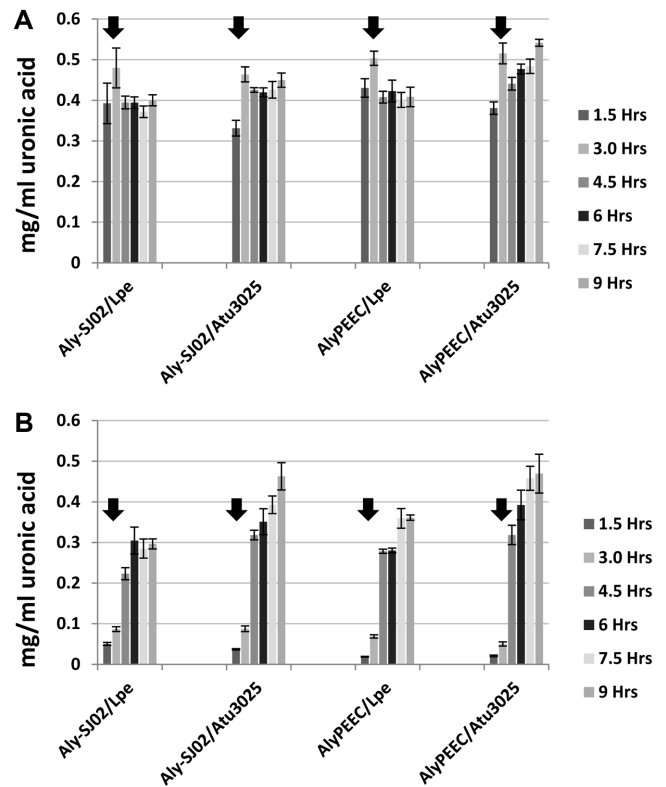


Fig. 5. Simultaneous versus sequential enzyme loading strategies.

(A) 0.3 mg of total protein from exo and endo-lyase preparations were simultaneously loaded at the beginning of the experiments. (B) 0.3 mg total protein from the endo-lyase preparations were loaded at time point 0 h; the same amount of exo-lyase preparations were loaded after time point 3 h. The black arrows indicate at which point the exo-lyases were added. In the case of the simultaneous strategy (A), the arrows indicate that the reactions were diluted with an equivalent volume of buffer instead of the exo-lyase preparations. Reactions contained 220 mg of acid-treated alga in an initial volume of 1.5 ml, 0.5 M Tris pH 7.5 buffer, incubated at 30°C, 200 rpm. 100 μ l samples were withdrawn every 1.5 h for uronic acid quantification. Error bars represent the standard deviation of duplicates.

with samples withdrawn every 1.5 h. Exo-lyases were added after 3 h in sequential loading experiments (Fig. 5B), at that point reactions for simultaneous enzyme load were also diluted by an equivalent volume (approximately 25% volume increase). The latter explains the reduction in product concentration from time point 3 to 4.5 h in Fig. 5A. At the end of the experiments, three of the enzyme pairs generated more product when the lyases were added simultaneously when compared to sequentially (13–35% yield increase). Only Aly-SJ02/Atu3025 responded with a marginal decrease in yield when the enzymes were added

simultaneously. These trends seem to be in contrast with results from [24]. However these authors tested only one enzyme couple in which the exo-lyase was unstable at their reaction conditions (40°C). A sequential strategy is beneficial in this case because the oligoalginates produced by the endo-lyase will be present at high concentration when the unstable exo-lyase is added to the reaction. Given the results from Fig. 3, it is likely that the exo-lyases tested in our work are stable at 30°C, therefore an advantage was observed when they were added along with the endo-lyases at the beginning of the reaction. Fig. 5 also shows that simultaneous enzyme load allowed attainment of maximum product yield at earlier time points for three of the cocktails (4.5 h), while AlyPEEC/Atu3025 reached the highest accumulation after 9 h. Sequential load experiments reached their maximum yield at 7.5–9 h, except for Aly-SJ02/Lpe, which peaked at 4.5 h. Shortened reaction times were also attained by [24] with a simultaneous enzyme load strategy.

Brown algae biorefineries hold great potential for the sustainable production of biofuels and bioactive ingredients. However there are no enzymatic tools for pilot scale work for enhancing the extraction and saccharification processes for phaeophytes. Even at the laboratory scale, there is limited availability of commercial alginate lyases for algae biotechnology. The present study has expanded the available repertoire of alginate lyases, while at the same time demonstrating that they can be easily expressed and implemented for everyday laboratory work. Moreover, this work has shown that testing a wide array of exo/endo alginate lyases can lead to enhanced saccharification results for a given biomass, probably due to the differences in substrate biases of these enzymes. Having several enzyme options would also prove valuable for allowing more flexibility when designing extraction processes from algal biomass from different species. Likewise, lyases could be selected based on the temperature and/or pH conditions from a previous step in the process in order to save time and costs. It was also demonstrated that shorter reaction times could be implemented with at least four different exo/endo lyase combinations when these enzymes were added simultaneously. This was in contrast with previous results showing an advantage when a thermally unstable exo-lyase is added after the oligoalginates have been released by the endo-lyase [24]. The latter disparity pinpoints the importance of working with enzymes that are stable in the reaction conditions, and has direct impact on the design of time-efficient algal degradation processes.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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