Polysaccharide quantification using microbial enzyme cocktails

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Abstract:

Polysaccharide quantification is crucial for studying ecological and nutritional processes in microbes, plants, and animals, as well as their role in carbon cycling and sequestration in aquatic environments. Traditional methods hydrolyze polysaccharides into monomers for quantification but are limited to chemically hydrolysable polysaccharides or require characterized recombinant enzymes. Here we use undefined mixtures of enzymes that are secreted by marine microbes or seawater communities to hydrolyze polysaccharides, focusing on colloidal chitin and laminarin as model marine polysaccharides. Colloidal chitin was digested using an enzyme cocktail from a chitin-degrading *Psychromonas sp.* bacterial isolate. By quantifying mono- and oligomers with a 3,5-dinitrosalicylic acid reducing sugar assay and/or liquid chromatography-mass spectrometry, chitin could be quantified down to 62 mg/L and 15 mg/L, respectively, enabling us to monitor microbial chitin degradation. Laminarin was digested using enzymes from an undefined marine microbial community with a lower detection limit of 30 mg/L using a reducing sugar assay. Our findings demonstrate that individual microbes or undefined communities can be readily harnessed as enzyme sources, offering a flexible method for polysaccharide quantification that bypasses limitations in chemical hydrolysis and availability of characterized recombinant enzymes.

Introduction

Biological polysaccharides carry out vital roles, serving as energy storage molecules(Suzuki and Suzuki, 2013), structuring cell walls(Lerouxel et al., 2006) and biofilms(Raszka et al., 2006), and fueling carbon to microbial communities(Arnosti et al., 2021; Cerqueira et al., 2020; Enke et al., 2019). Their abundance in organic matter places them at the center of biogeochemical carbon cycling and carbon sequestration in terrestrial(Horwath, 2007) and aquatic environments(Arnosti et al., 2021). Quantifying polysaccharides is essential to study these biological processes, yet their diverse chemical properties make it difficult to develop universally applicable quantification methods.

The most common approaches to quantify polysaccharides involve hydrolysis into monosaccharides, followed by quantification using colorimetric or chromatographic techniques(Arnosti et al., 2021; Decamp et al., 2021; Schiavone et al., 2014). Typically, hydrolysis is performed with chemical or enzymatic methods. Acid hydrolysis presents an obstacle as different polysaccharides are hydrolyzed with varying efficiencies or not at all(Arnosti et al., 2021), and the harsh conditions can destroy acid-sensitive components(Cao et al., 2017; Lin and Guo, 2015). An important example for resistance to acid hydrolysis is chitin, a linear homopolymer of β-(1,4)-linked N-acetylglucosamine (GlcNAc) monomers(Peter, 1995). As the main constituent of mollusk and arthropod exoskeletons in marine environments(Keyhani and Roseman, 1999), it is one of the most abundant polysaccharides in the ocean. While chitin can be deacetylated into chitosan to enable acid hydrolysis to glucosamine(Yan and Evenocheck, 2012), this method is time-consuming and cannot be done in microtiter plates.

Enzymatic degradation enables the quantification of polysaccharides that cannot be hydrolyzed chemically(Becker et al., 2017; Schiavone et al., 2014; Steinke et al., 2022; Sun et al., 2024). However, a drawback is that identifying enzymes with desired activity may be

challenging, and enzyme expression and purification are time-consuming and may require optimization. As an alternative, commercially produced enzymes may be used. However as in the case of chitinases, they may be prohibitively expensive, restricting their accessibility.

A largely unexplored alternative is to use microbial enzyme cocktails. This involves growing a polysaccharide-degrading bacterium or community on the target polysaccharide to induce the expression of enzymes with desired hydrolytic activity. This mix of undefined secreted enzymes, or cocktail, can be concentrated from the supernatant and used to digest a polysaccharide followed by mono- or oligomer quantification. Enzyme cocktails have previously been used to identify the monomeric composition of polysaccharide substrates(Buck-Wiese et al., 2023; Sichert et al., 2020), and to identify the catalytic capabilities(Pontrelli et al., 2022; Sichert et al., 2020; Unfried et al., 2018) or relative activities(Pontrelli et al., 2022; Sichert et al., 2020) of secreted enzymes.

Here, we utilized enzyme cocktails for polysaccharide quantification, overcoming pitfalls of chemical hydrolysis and limited recombinant enzyme availability. We collected an enzyme cocktail from the chitin-degrading isolate, *Psychromonas sp*. *Psy*6C06, as a proof of concept to quantify colloidal chitin. Additionally, we used seawater microbial communities to induce a laminarin-degrading enzyme cocktail, which we further purified and used to quantify laminarin. This method simplifies polysaccharide quantification and can be quickly adapted for other polysaccharides and biological systems.

Methods

Chemicals

All chemicals were obtained from Sigma Aldritch unless otherwise specified.

Preparation of colloidal chitin

10 grams of powdered chitin from shrimp shells (Sigma) was dissolved in 100 mL of concentrated phosphoric acid and placed at 4°C for 48 hrs. 500 ml of millQ water was added to precipitate the chitin, which was then vacuum filtered and dialyzed for 72 hours in water with a cellulose dialysis tubing membrane (Sigma). The colloidal chitin was then homogenized using a Bosch SilentMixx Pro blender. The colloidal chitin was autoclaved before use.

LC-MS quantification

Measurements were performed using Liquid Chromatography (Agilent Infinity II UHPLC stack) coupled with an Agilent 6520 Time of Fight Quadrupole Time of Flight Mass Spectrometer in negative mode, 4GHz, high resolution mode.

For quantification of chitin oligomers, an Agilent EC-CN Poroshell column (50x2.1 mm, 2.7 µM) was used isocratically to reduce interference of salts on metabolite ionization(Pontrelli and Sauer, 2021). The buffer contained 90% water, 10% (v/v) acetonitrile (CHROMOSOLVE) and 0.1% (v/v) formic acid. The flow rate was 0.35 mL/min. Prior to measurement, the sample was diluted 20-fold in millQ water. 3 µL was injected every 2 minutes. Data analysis and quantification was performed using Agilent Quantitative Masshunter software.

For time course laminarin pentose degradation, an Agilent EC-CN Poroshell column (50x2.1 mm, 2.7 µM) was used isocratically as described above. Here, the assay was initiated in the Agilent Infinity autosampler by adding 50 µL of 10 mM laminarin pentose to 50 µL of enzyme cocktail, and 3 µL of the enzyme reaction was injected every 3 hours. Data analysis was performed through manual peak integration using Agilent Qualitative Masshunter software.

For quantification of laminarin oligomers of different chain lengths, an Agilent HILIC-Z Poroshell column (100x2.1 mm, 1.8 µM) was used. Buffer A contains 10% (v/v) acetonitrile and 0.3% (v/v) ammonium hydroxide. Buffer B contains 90% acetonitrile (v/v) and 0.3% ammonium hydroxide (v/v). The flow was 0.9 mL/min and the column was kept at 35°C. The gradient is as follows. 0% A for 1 minute, followed by a gradient to 40% A until 5 minutes. This is held for 1 minute before dropping to 0% A and re-equilibration for 3 minutes. 3 µL of sample was injected. Data analysis and quantification was performed using manual peak integration with Agilent Qualitative Masshunter software.

Enzyme cocktail purification

*Psy*6C06 was streaked from -80°C glycerol stocks onto 1.5% agar plates of MB 2216 (Fisher) medium. A single colony was used for an overnight preculture in MB 2216, and the following day the preculture was inoculated 1% into 400 mL of simplified MBL medium with 2 g/l colloidal chitin and shaken at 200 rpm until early stationary phase. Simplified MBL medium is created from several stocks: 4-fold concentrated seawater salts (NaCl, 80 g/L; $MgCl_2$*$6H_2O$, 12 g/L; $CaCl_2$*$2H_2O$, 0.6 g/L; KCl, 2 g/L), 1000-fold diluted sodium sulfate (1 M), 500-fold diluted phosphate dibasic (0.5mM), and 20-fold diluted HEPES buffer (1 M pH 8.2).

After growth, the medium was centrifuged at 2800 relative centrifugal force for 20 minutes to remove any remaining chitin and cells, then sterile filtered using a 0.2 µM membrane. This supernatant was then concentrated 10 fold using an Amicon stirred cell (Millipore) with a 3 kDa cutoff filter. A protease inhibitor was added to the final concentrate (Roche cOmplete EDTA free protease inhibitor cocktail). 500 µL aliquots were added to 1.5 mL microcentrifuge tubes, which were then snap frozen by placing them in liquid nitrogen before storing at -80°C until use. The concentration of protein in the chitinase enzyme cocktail is 0.07 mg/mL as quantified using Bradford reagent.

For generation of laminarinases from seawater microbes, the same growth and purification protocol was followed, except the medium contained 1 g/l Laminarin from *Laminaria digitata* (Sigma) as the carbon source and 10 mM ammonium chloride as the nitrogen source. Seawater was collected from the North Sea on the shore of Cuxhaven (53°53'04.3"N 8°37'57.1"E), passed through a 5 µM filter, mixed 1:1 with 80% (v/v) glycerol, and stored at -80 °C until inoculation. The concentration of protein in the laminarinase enzyme cocktail is 0.012 mg/mL as quantified using Bradford reagent.

Chitin quantification assay

Frozen enzyme aliquots were thawed on ice. 100 µL of enzyme was added to 100 µL of sample and placed at room temperature until sampling. If the sample is suspected to contain more than 500 mg/L chitin, it was diluted to fall within this quantifiable range. To sample, the assay was centrifuged at 2800 rcf for 1 minute and the supernatant was collected and stored at -20°C until enzyme products are measured using DNS reagent or LC-MS.

Laminarin quantification assay

Frozen enzyme aliquots were thawed on ice. For laminarin oligomer formation, 50 µL of enzyme was added to 50 µL of laminarin pentamer (Megazyme, dissolved in 40 mM ammonium bicarbonate buffer pH 7.8) and placed at room temperature for 24 hours. The reaction mixture was directly quantified using LC-MS as described above. For laminarin quantification, 50 µL laminarin from *Laminaria digitata* was added at various concentrations to 50 µL of enzyme cocktail. Samples were taken at 24 hours and directly quantified using DNS reagent.

DNS reagent preparation

100 mL of water was heated to 70-75°C, then poured over 2 g of NaOH pellets with constant stirring. 2.18 g of 3,5-dinitrosalicylic acid was then dissolved in this mixture, and 30 g of

sodium potassium tartrate (Rochelle salts) were added. The solution was cooled to room temperature and stored in the dark.

DNS reducing sugar assay

25 µL of sample was added to 75 µL of DNS reagent and the mixture was heated to 95°C for 15 minutes in a thermocycler. 80 µL was added to a 384 well plate and a colorimetric readout was measured at OD_{540}.

Assessment

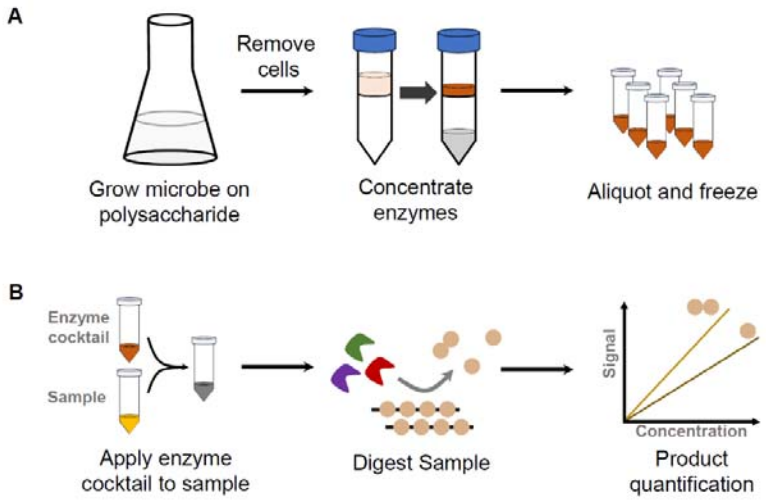

Figure 1

Assay design

A polysaccharide-degrading microbe or complex microbial community is cultured on the polysaccharide until early stationary phase to select for degraders and induce the expression of hydrolytic enzymes. The cells are then filtered to obtain a cell-free supernatant containing secreted enzymes. Enzymes are concentrated tenfold using a 3kDa molecular weight cutoff

filter, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C. This enzyme cocktail is then thawed and mixed with a polysaccharide sample to initiate hydrolysis. After hydrolysis, the supernatant is collected and quantification of monomers and oligomers is performed using a reducing sugar assay or liquid chromatography mass spectrometry (LC-MS). Akin other enzyme-based quantification methods(Becker et al., 2017; Deng et al., 2022), this assay relies on identical enzyme activity across all samples, producing consistent sets of oligomers and monomers. This provides a quantitative relationship between the amount of polysaccharide digested and the number of reducing sugar ends and degradation products. A calibration curve

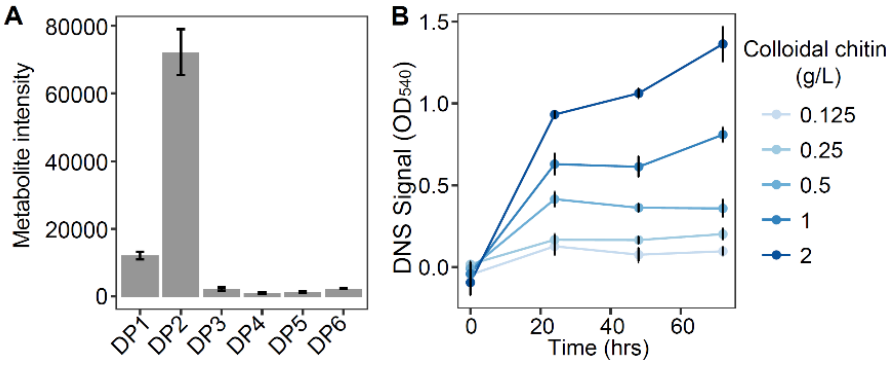

Figure 2

is thus calculated based on digestion of standards with known polysaccharide concentrations. These standards are always digested alongside unknown samples to account for variations in enzyme activity of different cocktail batches, their degradation during storage, or any mono- or oligomers that may be residually present in the cocktails following their concentration.

Quantification of colloidal chitin using enzyme cocktails from a bacterial isolate

We tested the assay design to quantify colloidal chitin with an enzyme cocktail obtained from the chitin-degrading *Psychromonas sp.* bacterium *Psy*6C06 that encodes 13 chitinases(Pontrelli et al., 2022). To verify chitinase activity, the cocktail was incubated for 72 h at equal volumes with a sample containing 2 g/L of colloidal chitin. Produced mono- and

oligomers were determined by LC-MS. Monomeric GlcNAc and oligomers with various chain lengths were detected. The major products are monomeric GlcNAc and dimeric chitobiose, mirroring previous characterization of *Psy*6C06 secreted chitinases(Pontrelli et al., 2022) **(Fig. 2A)**. We tested the time required for the cocktail to completely hydrolyze samples containing up to 2 g/L of colloidal chitin by measuring the formation of reducing sugar ends over time using the 3,5-dinitrosalicylic acid (DNS) reagent **(Fig. 2B)**. Within 72 hours, only samples with 500 mg/L colloidal chitin or less were completely hydrolyzed, as judged from a stable signal. All subsequent experiments therefore used 72 hours for hydrolysis and samples known, or suspected, to contain more than 500 mg/L colloidal chitin were diluted prior to digestion.

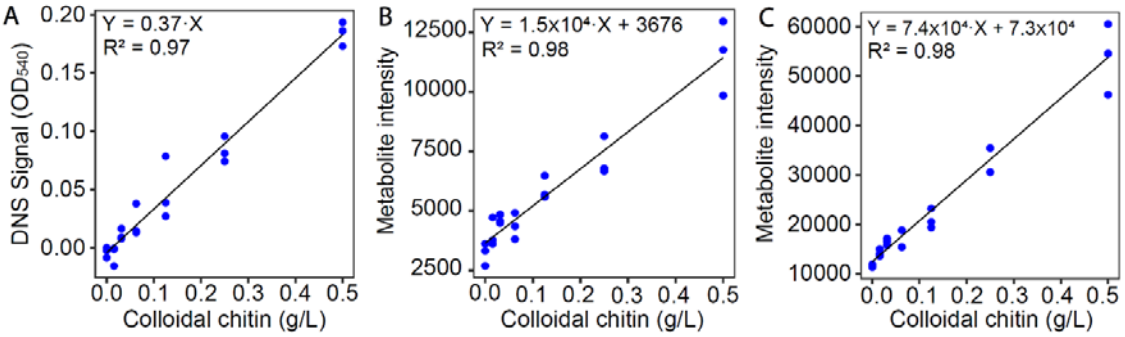

Figure 3

Enzyme cocktails may contain multiple hydrolytic enzymes that degrade polysaccharides other than the target polysaccharide. Thus, if an alternative polysaccharide is non-specifically degraded into mono- and oligomers, colorimetric assays like DNS, which react with many different reducing sugar ends, may overestimate concentrations of the target polysaccharide. Since LC-MS or HPLC can distinguish mono- and oligomers after a sample is digested, these

degradation products can be used as specific readouts for the degradation of a target polysaccharide. As previously demonstrated(Becker et al., 2017; Deng et al., 2022), this is especially useful if the enzyme digest produces known, specific oligomers from a target polysaccharide, which can help distinguish a specific polysaccharide's oligomers even in samples with multiple polysaccharides present. Here, enzyme cocktails from *Psy*6C06 produce GlcNAc and chitobiose as major degradation products. Both can be used as readouts for chitin quantification. However, in cases where it is unknown if other GlcNAc-containing polysaccharides are present in a sample that may be non-specifically hydrolyzed, quantifying chitin based on chitobiose provides a viable option. This is because chitobiose is verified to be specifically produced by the enzyme digest, and direct GlcNAc-GlcNAc linkages are generally rare in natural polysaccharides. We compared the use of chitobiose measured with LC-MS as a readout against the other two readouts: GlcNAc measured with LC-MS and total reducing sugar ends using DNS reagent. We determined the limit of detection (LOD), defined as the lowest concentration with a signal above the background mean plus five standard deviations. Using LC-MS, we obtained LODs of 15 mg/L colloidal chitin with chitobiose as a readout or 500 mg/L with GlcNAc **(Fig. 3ABC)**. Using DNS, we determined an LOD of 62.5 mg/L. These results demonstrate that using chitobiose as a readout not only provides a more specific quantification for chitin, but also allows for the most sensitive readouts.

Measuring colloidal chitin dynamics in bacterial cultures

Quantifying chitin degradation dynamics is important for the investigation of bacterial cultures and marine communities involved in biogeochemical carbon cycling, allowing to link changes in population dynamics with chitin degradation. Using the above enzyme cocktail, we monitored the time-course consumption of colloidal chitin by the chitin-degrading *Vibrio splendidus*, *Vib*1A01(Amarnath et al., 2023; Iffland-Stettner et al., 2023). Aliquots were periodically sampled during growth, centrifuged, and the pellet was briefly boiled to denature

any *Vib*1A01-derived chitinases. The denatured pellet was then incubated for 72 h with a 1:1 ratio of the enzyme cocktail. To quantify degradation products, we first ensured that other components in *Vib*1A01 biomass are not nonspecifically hydrolyzed by the enzyme digest. To this end, we used the enzyme cocktail to digest biomass from a *Vib*1A01 culture grown on

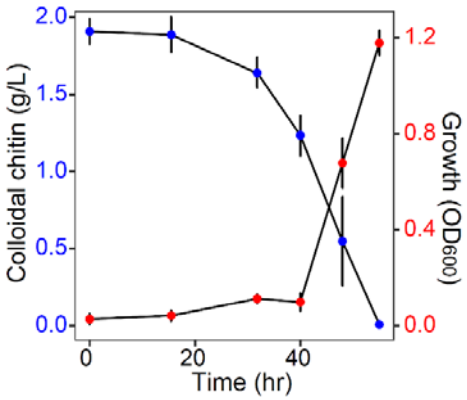

Figure 4

20mM GlcNAc instead of chitin and observed no detectable background signal using DNS reagent (data not shown). We therefore determined reducing sugars in the chitin culture using DNS reagent. Results show a gradual reduction in colloidal chitin corresponding to increased *Vib*1A01 growth **(Fig. 4)**. This highlights the method's utility in quantifying chitin degradation dynamics in bacterial cultures, which has broader implications for researchers studying chitin dynamics in other biological systems.

Laminarin quantification using enzyme cocktails from environmental communities

For polysaccharides where degrading microbes are unavailable or cannot be isolated from the environment, collecting enzyme cocktails from complex environmental microbial communities is an alternative. By growing environmental communities on the polysaccharide of interest as a sole carbon source, degraders will be selected, and their secreted enzymes can be concentrated into a cocktail. We tested this approach for the quantification of laminarin, an algal storage molecule with a β-(1-3)-glucose backbone and single β-(1-6)-glucose side

branches. Seawater collected from the North Sea on the shore of Cuxhaven (53°53'04.3"N 8°37'57.1"E) was used to inoculate a medium with laminarin as the sole carbon source to induce and concentrate laminarinases in a cocktail. To verify the activity of the enzyme cocktail, we digested 5 mM of a β-(1-3)-glucose pentamer, representing the laminarin backbone, for four hours and measured the formation of smaller oligomers using LC-MS **(Fig. 5A)**. The formation of a mix of monomers and smaller oligomers indicates the presence of multiple microbial laminarinases with different activities. We determined the time required to completely digest 2 mM laminarin pentamer (1.6 g/L) using LC-MS and observed a stable signal before 21 hours **(Fig. 5B)**, identifying 24 hours as a suitable digestion time for quantities of laminarin of 1 g/L or less. To demonstrate that DNS reagent can also be used to quantify laminarin, and hence provide a widely accessible measurement method beyond LC-MS, we used it to detect laminarin concentrations between 1 g/L and 0.03 g/L after a 24 hr digestion period and observed an LOD of 30 mg/L **(Fig. 5C)**. These results highlight the utility of environmental microbial communities as a source of enzyme cocktails to quantify polysaccharides.

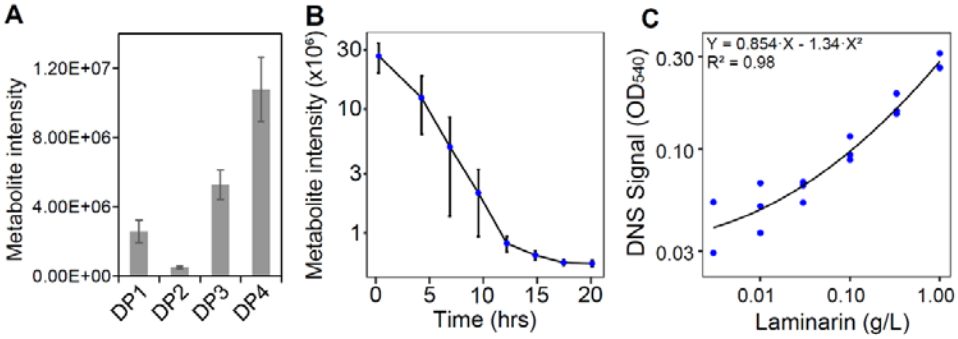

Figure 5

Discussion

In this study, we demonstrate how microbial enzyme cocktails can be used to quantify polysaccharides, exemplified with colloidal chitin and laminarin. Unlike other enzyme-based

polysaccharide quantification techniques, our approach eliminates the need to identify and recombinantly express specific hydrolytic enzymes, by collecting them from cultures of bacteria that are actively degrading a polysaccharide. This is particularly advantageous for polymers whose degradation requires multiple enzymes such as fucoidan(Sichert et al., 2020). Concentrating all secreted enzymes into single cocktails has the additional benefit that potential non-catalytic proteins that enhance activities of hydrolytic enzymes are maintained(Vaaje-Kolstad et al., 2005). Furthermore, we demonstrate that enzyme cocktails can be derived from complex microbial communities, bypassing the need to obtain specific polysaccharide degraders.

A limitation of this approach is that cocktails may contain other hydrolytic enzymes that break down alternative polysaccharides in complex samples, inflating quantified values. One way to overcome this is by performing controls to rule out background interference. For example, we showed that the *Psy*6C06 enzyme cocktail does not digest polysaccharides in *Vib*1A01 bacterial biomass, meaning there is no interference when measuring bacterial degradation of colloidal chitin. LC-MS can monitor specific mono- and oligomers, aiding in quantifying the target polysaccharide, especially if the enzyme cocktail produces polysaccharide-specific oligomers like chitobiose. However, LC-MS may still fall short for complex samples, as different polysaccharides may have similar monosaccharide compositions, skewing readouts due to nonspecific hydrolysis of alternative polysaccharides. This may occur in the case of laminarin which is solely composed of glucose and would be difficult to quantify when alternative glucans and glucanases are also present. Nevertheless, the presented approach is suitable when non-specific hydrolysis can be ruled out or when measuring polysaccharide-specific oligomers. It is also suitable when the polysaccharide composition is known, such as when specific substrates are provided to microbial isolates as sole carbon sources or after extraction procedures isolate the target polysaccharide from complex environmental samples.

This work showcases the utility of microbial enzyme cocktails as an easily accessible means for quantifying polysaccharides, and provides a specific protocol for colloidal chitin that is currently missing for this abundant marine polysaccharide(Pontrelli et al., 2022; Salas-Ovilla et al., 2019; Shen et al., 2010; Szabo et al., 2022). By requiring only a microbial isolate or community that secretes hydrolytic enzymes, our approach is highly transferable to other polysaccharides and biological systems. Our findings highlight the potential to harness natural microbial communities as enzyme sources, offering a flexible method for measuring a wide range of polysaccharides.

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Figure legends

Figure 1: Assay design and workflow A) Enzyme cocktail preparation involves growing a microbe, or microbial community, on the polysaccharide as a sole carbon source, followed by filtering cells and concentrating enzymes using a 3 kDa molecular weight cutoff membrane. The concentrate is aliquoted and stored until use. B) Polysaccharide quantification entails mixing the enzyme cocktail with a sample to initiate digestion, leading to the formation of monomers or oligomers. Post-digestion, the products can be quantified using LC-MS, HPLC, chemical, or other methods.

Figure 2: Activity of chitin enzyme cocktail. A) GlcNAc oligomers (Degree of polymerization, DP 1-6) formed after 72 hr digestion of 2 g/L colloidal chitin by the enzyme cocktail, measured using LC-MS. B) Samples with varying concentrations of colloidal chitin digested with equal volumes of enzyme cocktail. Liberation of mono- and oligomers is measured every 24 hours for 3 days using a DNS reducing sugar assay (with an OD_{540} signal). Experiments were performed in triplicate. Error bars represent standard deviation from the mean.

Figure 3: Assay sensitivity. Decreasing concentrations of colloidal chitin are applied to enzyme cocktail and digested for 72 hours. A) Detection of total reducing sugar ends with the DNS reagent. B) Detection of GlcNAc by LC-MS. C) Detection of dimeric chitobiose by LC-MS. A linear regression of signal intensity per chitin concentration is shown.

Figure 4: Chitin concentrations during bacterial growth. OD_{600} of chitin degrader *Vib*1A01 when growing on chitin as a sole carbon source (Red). Colloidal chitin concentration

at each of the measured timepoints (Blue). Error bars represent standard deviation of the mean of three biological replicates.

Figure 5: Laminarin quantification with seawater community enzymes: A) Mass spectrometry measurement of glucose oligomers (Degree of polymerization, DP1-4) after 4 hour digestion of a laminarin pentamer by a laminarinase enzyme cocktail expressed by a seawater microbial community. The horizontal bar is fold change of 1 compared to a no-enzyme control. B) Time course digestion of 2 mM laminarin pentamer using the enzyme cocktail, measured using LC-MS. C) Digestion of different laminarin concentrations by an enzyme cocktail, quantified with DNS reagent and a readout of OD_{540}. A polynomial regression is shown and the LOD is 30 mg/L. Error bars represent standard deviation of the mean of three biological replicates.