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Endpoint Fragmentation Index: A Method for Monitoring the Evolution of Microbial Degradation of Polysaccharide Feedstocks

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Abstract We describe a simple method for tracking the course of microbial degradation of polysaccharide-rich feedstocks. The method involves determining total polysaccharides present in the feedstock, measured in glucose equivalents, relative to the fractional component of polysaccharides exhibiting 2,3-dinitrosalycylic acid aldehyde activity. The ratio of total polysaccharide to aldehyde activity, defined as the end-point fragmentation (EPF) index, is then calculated and tracked as it shifts as microbial degradation of polysaccharide-rich feedstock progresses. While degradation occurs, the EPF index falls. It bottoms out at an asymptotic limit marking the point in time where further degradation of the polysaccharide-rich feedstock has ceased. The EPF index can be used to follow the progressive breakdown of composting polysaccharide-rich waste. It may also have applicability as a means of tracking the turnover of polysaccharides in other complex environments including soil, sediments, wetlands, and peat bogs.

Keywords Cellulose · Polysaccharides · EPF index · Compost

Introduction

Microbes produce cellulases and other hydrolases which actively degrade polysaccharides [1–4]. Different methods of composting cellulose-rich materials now exist, each adapted for different purposes and feedstocks [5–8]. Each method has advantages and drawbacks. Ideally, composting should short circuit the overall carbon cycle by reducing the flow of carbon into the gCO_2 reservoir. Organic-carbon-enriched soils are better CO_2 sinks because they stimulate plant growth and increase microbial diversity [9–11]. Furthermore, glucose, the basic building block making up the polymeric backbone of cellulose (the most abundant

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carbon source on earth), released into soil near roots, is a rich source of energy promoting plant growth and an up-regulator of nitrogen uptake [3, 12–15]. In addition, polysaccharide-rich waste has potential as a biofuel feedstock [16–19].

Conventional composting involving frequent aeration of waste is labor intensive, costly, and aims to accelerate aerobic respiration and thus results in a significant loss of CO_2 into the atmosphere. Some compost technologies also aggravate the emission of NH_3, and also N_2O and CH_4, two powerful greenhouse gases [20–23]. At the other extreme, some processes, including fermentation under low oxygen tension, low temperature and acidic conditions, and less vigorous aerobic oxidation of cellulose-rich waste, may suppress the emission of CO_2, CH_4, and N_2O [24, 25], but reach early endpoints.

Finding simple, reliable methods of monitoring the turnover of recyclable waste rich in polysaccharides that allow one to track the composting process from beginning to its endpoint, a point of where further composting yields no net benefit, must be done if efficient sequestration of carbon is to be realized. Tracking and interpreting the processing stages between start and endpoint is however difficult and fraught with uncertainty [26–29] even though general requirements and priorities regarding composting principles have emerged [30–32]. Of particular interest are practical methods that would allow analyzing the state of fragmentation of polysaccharides undergoing biodegradation.

In general, polysaccharides can be viewed as a good proxy for biodegradable organic carbon. Current methods used to evaluate the status of biodegradation during composting such as changes in BOD, evolution of total carbon, CO_2 evolution, O_2 uptake, and changes in pH have limitations [30–32]; with respect to monitoring progressive changes in polysaccharides, isotopic analyses, nuclear magnetic resonance (NMR), chromogenic technologies, HPLC, and gas chromatography (GC) analysis are expensive or impractical to conduct on a daily basis. Our goal is to develop a simple, rapid, and robust assay for tracking fragmentation of polysaccharides during composting. We use this as a method to evaluate the status and monitor the progression of biodegradation of feedstock.

Materials and Methods

Cellulose (medium fiber), carboxymethyl cellulose, and 3,5-dinitrosalicyclic (DNS) acid were obtained from Sigma, and 89% phenol was obtained from Mallinckrodt. All other chemicals were reagent grade or better.

To assess the changes in the EPF index in fermentors, we monitored the status of water-insoluble acid-soluble polysaccharides by harvesting leaf residues incubated in serum bottles at varying intervals over a 30-day period. Each serum bottle received 10 g of oak leaves blended with 30 ml of 1 mg/ml glucose solution and 1 ml wheat bran slurry filtrate. The wheat bran slurry was prepared by suspending 5 g of wheat bran enriched in *Clostridia* sp. spores in 50 ml water and passing the slurry through a stainless steel sieve (0.5 mm). The leaf slurries were incubated in 70-ml glass serum bottles sufficient in number to allow for triplicate harvests at different time intervals during the experiment. Bottles were capped with 1-cm thick butyl rubber stoppers and sealed with aluminum caps. Incubation occurred at 30 °C. Gasses were sampled over the course of the experiment up to the point where each incubation bottle was eventually stopped and harvested for analysis of the leaf residues.

Microbial activity was monitored by following changes in O_2 and CO_2 content in the headspace and by tracking formation of volatile organic acids (butyric, isovaleric, and valeric) formed as fermentation progressed. Headspace atmospheric gas analysis was carried out using gas tight syringes; the gas was drawn through the stopper by means of a

needle guidance sampler, and subsequent injection and separation of gases on 3′-silica and a 6′-13X molecular sieve gas chromatography columns on an SRI 310C GC analyzer.

The protocol for the EPF index is summarized in Fig. 1. Amounts of 10–20 g wet slurry residue were transferred onto a stainless steel sieve (0.5 mm mesh size) and washed with ≥1 L tap water. About 1–2 g wet weight (WW) of each washed residue was transferred to a 25 ml screw cap glass tube, then vortexed and incubated for 5 min under ambient room temperature conditions (~21 °C) in 5 ml of 77% H_2SO_4 to dissolve acid-soluble polysaccharides. One milliliter of this acid digest was diluted 16-fold in deionized H_2O (dH_2O); distilled water is also acceptable as a diluent. Samples of the diluted digest were then analyzed for total carbohydrate using the phenol-sulfuric acid (PSA) carbohydrate assay [27, 33], and for DNS reactive aldehyde-reducing activity, an assay commonly used in screening for glucose reactive aldehyde groups [34]. From these measurements the EPF index was calculated as the ratio of PSA activity relative to DNS activity of the acid-dissolved digest.

Results

Xiang et al. [29] attribute solvation and recovery of cellulose in 67% H_2SO_4 to the disruption of hydrogen bonding and conversion of crystalline cellulose into an amorphous state amenable to hydration and solvation. Safarik and Santruckova [27], and Martens and Loeffelmann [40], earlier described good recoveries of cellulose from plant residues in soil treated similarly with H_2SO_4. We found that 77% H_2SO_4 was particularly efficient in solvating cellulose. Using the PSA assay to track recovery of weighed cellulose taken up

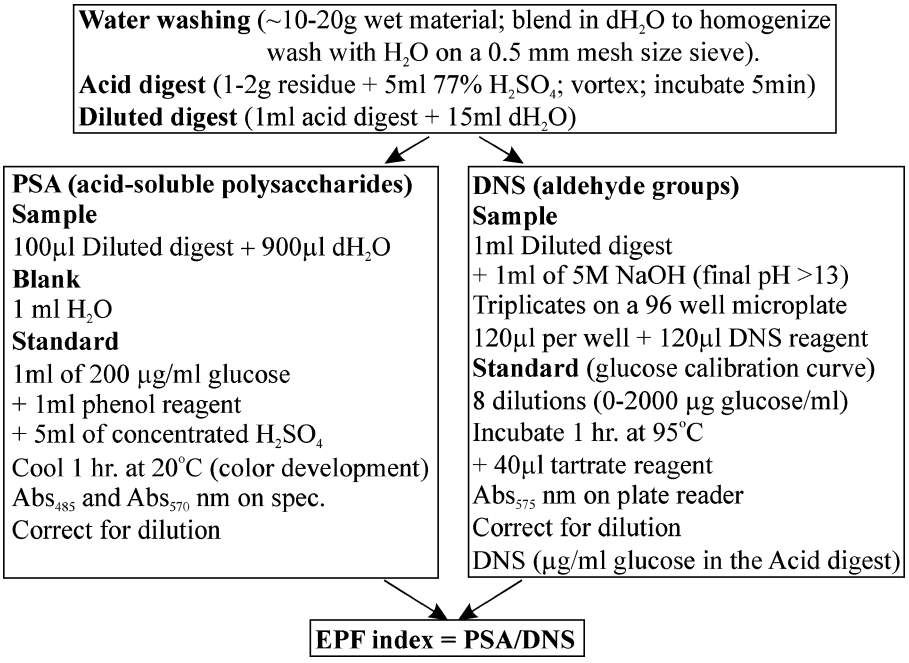

Fig. 1 Summary of the method used to determine the EPF index of water-insoluble acid-soluble polysaccharides

and solvated at room temperature (~21 °C) in 77% H_2SO_4, and glucose as a standard calibrator, we obtained 99±6% (±1 SD, n=5) recovery of cellulose. Solvation of pure cellulose occurred within seconds of mixing the cellulose with H_2SO_4. In 77% H_2SO_4 the cellulose appeared stable on tracking recovery over a period of several hours while at room temperature. The day-to-day coefficient of variation at 200 μg/ml glucose equivalents for the PSA and DNS assays was 4.62% (n=26) and 5.66% (n=26), respectively.

Changes observed as the leaves degraded are summarized in Table 1. Consistent with the progressive drop in pH of the outflow (compost tea), from an initial pH of 5.6 to a final pH of ~3.5, was the accumulation of volatile organic acids, principally isovaleric and valeric (data not shown). Oxygen in the headspace fell sharply, leveling off by around the third day to ≤2%. The CO_2 in the headspace rose steadily through the tenth day, after which it reached a plateau. The EPF index fell sharply during the first 10-day interval from an initial value slightly above 1.4, and bottomed out as it approached 0.5 (Fig. 2).

In a separate set of experiments, pure cellulose (medium fiber) was suspended in dH_2O at a final concentration of 50 mg/ml, centrifuged at 14 K for 5 min, suspended again in water, and re-centrifuged two additional times into a pellet. The washed pellet was suspended in 77% H_2SO_4 at room temperature (approximately 21 °C) and processed in the same manner as the leaf samples to characterize its acid-solubilized cellulose EPF index in the presence of varying concentrations of carboxymethyl cellulose (CMC). This was done by spiking cellulose solubilized samples with CMC before running the PSA and DNS assays. In CMC/cellulose mixtures a strong negative correlation (r=0.84) was found between the abundance of cellulose and the EPF index (Fig. 3).

Discussion

The degree of maturation of compost (i.e., the abundance of small metabolites, the level of cellulose hydrolysis, and the stability of compost during storage and after dispersal in soil) must be considered when processing recyclable wastes [8]. At the same time, to optimize the composting of recyclable wastes rich in useable carbon, and to lower the carbon footprint imparted in processing such wastes, robust methods for monitoring the progress of decomposition are needed. Some of the most common methods for characterizing the quality of compost during processing include an analysis of caloric content, carbon content,

Table 1 Summary of metabolic activity and changes in EPF index of leaves subjected to fermentation[a]

Interval (days)	Headspace CO_2 (μmoles)	Headspace O_2 (μmoles)	pH	DNS glucose equiv (μg/mg WW)	PSA glucose equiv (μg/mg WW)	EPF index
0	0±0	369±5	5.55[b]	nd[c]	nd[c]	nd[c]
3	597±26	35±9	4.00±0.02	48±7	68±10	1.414±0.13
10	745±16	40±1	3.69±0.08	60±5	38±8	0.636±0.09
17	742±42	40±1	3.81±0.12	58±8	31±2	0.534±0.04
27	711±58	39±1	3.53±0.05	54±5	27±4	0.497±0.03

[a] Average ±1 SD of readings obtained on samples run in triplicate at each interval as indicated in table

[b] pH of blended leaf sample slurry measured immediately before samples were transferred into fermentation bottles and capped with butyl rubber stoppers

[c] No measurement was made in the beginning of this experiment because this value was known from prior measurements on the leaf stock (EPF ~1.4–1.5)

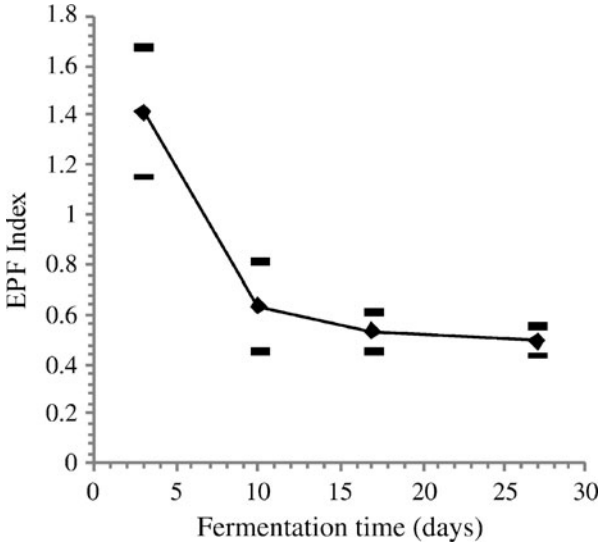

Fig. 2 The evolution of the EPF index (i.e., PSA/DNS ratio) during the wet acid fermentation of leaves. The plotted index data is the average of triplicate samples analyzed at each interval shown. *Upper* and *lower bars* represent ±2 SD range

and biological oxygen demand [8, 35, 36]. A diversity of methods also exist for monitoring the progression of polysaccharide degradation including chemical quantification of different polysaccharides, assays of cellulolytic and amylolytic activities in the waste stream, isotopic tracking of labeled organics, and NMR and NIR spectroscopy [5, 6, 32, 37–39]. Such methods offer a snapshot of the overall abundance of different materials in compost, and the biochemical dynamics of the process, but they provide insufficient information regarding the degree of decomposition relative to progression to an endpoint as decomposition is occurring.

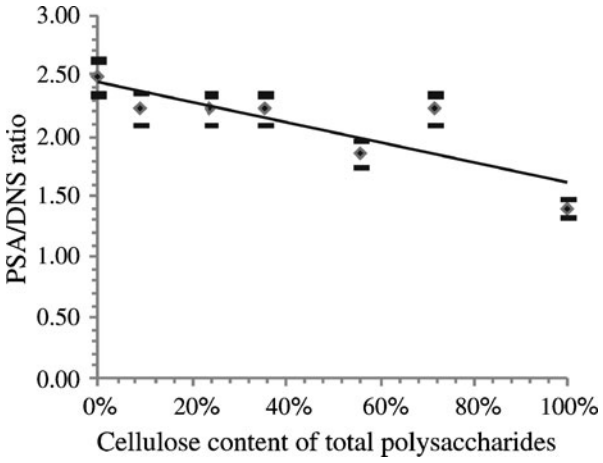

Fig. 3 Changes in the EPF index of acid-soluble cellulose as a function of its abundance in mixtures with CMC. *Upper* and *lower bars* represent the ±2 SD range

Our measurements showed good recovery (i.e. dissolution) of purified commercial fibrous cellulose using 77% H_2SO_4 as a solvating agent. The concentration of H_2SO_4 (77%) used in solvating polysaccharides was established by varying the H_2SO_4 concentration and measuring optimal (e.g., maximal) recovery of pure cellulose brought into the soluble (aqueous) fraction. At the mechanistic level H_2SO_4 disrupts the crystalline structure of cellulose, breaks up hydrogen bonds, and thus facilitates dissolution of the polymer in water.

The EPF index as defined in this study is the ratio of glycosyl units (measured by the PSA carbohydrate assay) to end group aldehyde units (measured by the DNS assay). This ratio reflects the progressive decomposition (e.g., shortening) of polysaccharides as they break down into smaller units. Hence, the relative ratio of total glycosyl units in a polysaccharide to aldehyde reactive end groups can be anticipated on purely structural grounds to shift as the polysaccharide undergoes hydrolysis. In the case of celluloses, most of the glucose units are linked in β-1,4-glycosidic bonds formed between C1 and C4 of the glucose units [41, 42]. Hemicelluloses, which make up roughly 20% of plant biomass, are structurally similar to cellulose except that they are shorter polymers with less crystalline properties, comprised of mixed glycosyl units with xylose, mannose, galactose, and various other hexose and pentose units substituted within the backbone of the their polymers [43, 44]. Starches and amylopectins, on the other hand, while similar in structure to cellulose, and hemicelluloses, exist in nature as α-1,4-glycosyl polymers, and like cellulose and hemicelluloses have in common reactive end terminal reducing activity [45, 46]. All of these polysaccharides, if present and undergoing biodegradation in feedstock, would be expected to show shifts in their respective EPF indexes.

Thorough washing of the residue recovered from feedstocks undergoing microbial degradation (in order to eliminate water solutes) before initiating the acid solvation of the residual polysaccharides (Fig. 1) is very important in this method. It eliminates nonspecific reducing equivalents that otherwise interfere with the DNS assay.

In the case of cellulose, as degradation of the polymer progresses to an asymptotic endpoint, its EPF index would be expected to reach a point where further changes in the ratio of total glycosyl equivalents relative to aldehyde activity bottoms out. The reasons for reaching an endpoint could be that the polymer is completely degraded. It could also mean that some sites within the polymer chain not yet degraded remain inaccessible to cellulases, possibly because molecules such as lignin, caging the cellulose fragment, shield the susceptible site from enzymatic attack.

In our experiments, comparing results in the early phase of the study with those toward the end of the 27th day (Table 1), it can be seen that aldehyde activity held constant while total glucose equivalents fell as microbial degradation of the leaf residues progressed. Furthermore, the EPF index shows that the fermentation reached an endpoint near the tenth day into the study. The change in the EPF index implies that the polysaccharide polymers from the water-insoluble fraction were undergoing turnover with portions of the polymers freed of the residue during the workup of the samples for analysis; furthermore, beyond about 10 days into the fermentation polysaccharides were no longer broken down.

Data in Fig. 3 show that mixing CMC with cellulose in varying proportions resulted in a linear relationship between the abundance of reactive aldehyde groups measured by the DNS assay relative to reactive glycosyl groups measured by the PSA assay. CMC is a derivative form of cellulose formed by reaction of cellulose with alkali and chloroacetic acid. The conversion of hydroxyl to carboxymethyl groups makes it much more water soluble albeit chemically distinct from cellulose.

The derivatization of CMC however also changes its reactivity in the PSA assay. We obtained a value for the EPF index of ~1.5 for pure cellulose and ~2.5 for CMC. By measuring DNS and PSA activities and calculating the EPF index of known mixtures of these two polymers, it can be seen (Fig. 3) that the EPF index tracks differences in polysaccharide aldehyde/glycosyl reactivity expressed as the composition of the mixture shifted from pure CMC to pure cellulose. These results confirm that the EPF index registers changes in the ratio of polysaccharide reactive PSA activity to aldehyde DNS activity as they occur.

In principle the EPF index ought to be useful in tracking the biodegradation of feedstocks that are difficult to degrade including lignocelluloses complexes. These latter polymers do not easily degrade due to the comingling of lignin and crystalline cellulose polymers in complex structures which are difficult for lytic enzymes to access. On fundamental grounds, as lignocellulose complexes are opened up during biodegradation, unmasking DNS aldehyde reactive end groups and PSA reactive glycosyl units making up the backbone structure of cellulose entrapped within the lignocellulose complex, a shift in the EPF index would however ensue. The EPF index, in short, is a simple functional assay for following fragmentation of feedstock materials having PSA and DNS reactivity irrespective of the polysaccharide feedstock. So long as these two chemical moieties are present, it should in principle be feasible to apply this assay in tracking the biodegradation of a given feedstock to its end point where further fragmentation of the polysaccharides contained in the feedstock is no longer occurring.

Water-soluble polysaccharides freed from feedstock as its decomposition progresses are not measured by this procedure. Obviously, for a more comprehensive examination of the entire degradation process this method could be applied while also tracking water-soluble polysaccharides.

Lastly, selection of a sieve used for washing fermented residues before analysis is somewhat arbitrary and can be viewed as a sizing element in tracking residues broken down into a given size. Depending upon the mesh size of the sieve, different size fragments may pass freely through the sieve, and the assay results are defined to some limited extent by the sieve size used to process samples subjected to EPF analysis. Useful information as to the progress of the turnover of residue feedstock can be obtained without going to the extreme of attempting to capture all of the insoluble matter in a digest. Providing the sieve size is not changed while tracking fermentation by EPF index analysis, a plot of EPF index versus fermentation period should provide reasonable insight as to when the endpoint of hydrolysis is reached.

Conclusions

We describe in this manuscript a method for tracking the degradation of polysaccharides in wet compost. Cellulose, hemicelluloses, and other polysaccharides, when tracked by PSA and DNS assays yield colorimetric reactions indicative of the ratio of total glucose equivalents present in their polymer backbone relative to reactive aldehyde activity, defined as an EPF index. The EPF index can be used to track and mark the point in processing polysaccharide-rich waste where processing conditions are no longer necessary or efficient. This assay is direct and relatively simple to perform. It can be adapted to field conditions as well. Furthermore, progressive changes in the EPF index serve as a marker of the global hydrolysis of all water-insoluble acid-soluble polysaccharides from a sample, and thus are less sensitive to sample heterogeneity. The EPF index may be used in many applications for

studying the evolution of composting activity, but could also prove useful in tracking the overall decay of polysaccharides in soil, leaf litter, sediments, wetlands, and peat bogs.

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