Comparison of methods for glycogen analysis of *in vitro* fermentation pellets produced with strained ruminal inoculum

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Abstract

Microbial glycogen measurement is used to account for fates of carbohydrate substrates. It is commonly applied to washed cells or pure cultures which can be accurately subsampled, allowing the use of smaller sample sizes. However, the nonhomogeneous fermentation pellets produced with strained rumen inoculum cannot be accurately subsampled, requiring analysis of the entire pellet. In this study, two microbial glycogen methods were compared for analysis of such fermentation pellets: boiling samples for 3 h in 30% KOH (KOH) or for 15 min in 0.2 M NaOH (NaOH), followed by enzymatic hydrolysis with α -amylase and amyloglucosidase, and detection of released glucose. Total α -glucan was calculated as glucose $\times$ 0.9. KOH and NaOH did not differ in the α -glucan detected in fermentation pellets (29.9 and 29.6 mg, respectively; $P = 0.61$). Recovery of different control α -glucans was also tested using KOH, NaOH, and a method employing 45 min of bead beating (BB). For purified beef liver glycogen (water-soluble) recovery, BB (95.0%) > KOH (91.4%) > NaOH (87.4%; $P < 0.05$), and for wheat starch (water-insoluble granules) recovery, NaOH (96.9%) > BB (93.8%) > KOH (91.0%; $P < 0.05$). Recovery of isolated protozoal glycogen (water-insoluble granules) did not differ among KOH (87.0%),

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Abbreviations: PTFE, polytetrafluoroethylene; BB, bead beating treatment; KOH, potassium hydroxide treatment; NaOH, sodium hydroxide treatment

NaOH (87.6%), and BB (86.0%; $P = 0.81$), but recoveries for all were below 90%. Differences among substrates in the need for gelatinization and susceptibility to destruction by alkali likely affected the results. In conclusion, KOH and NaOH glycogen methods provided comparable determinations of fermentation pellet α -glucan. The tests on purified α -glucans indicated that assessment of recovery in glycogen methods can differ by the control α -glucan selected.

Keywords: Glycogen, reserve carbohydrate, rumen, amyloglucosidase, method

1. Introduction

Alkaline or physical disruption is used to lyse microbial cells to allow analysis of the glycogen stored therein. A long standing assay for microbial glycogen requires boiling of samples in 30% KOH for 3 h with subsequent purification by centrifugation and ethanol precipitation (Herbert et al., 1971; Daniels et al., 2007). The isolated glycogen is measured with anthrone or phenol-sulfuric acid assays, or through acidic hydrolysis and measurement of released glucose. The 30% KOH method was reported by Herbert et al. (1971) to have originally been published in 1933 (Good et al., 1933) for analysis of glycogen in liver and muscle, and is little changed from the method of Pflüger (1904) which was based on the work of Claude Bernard (1877, p. 302). Although heating with alkali can destroy carbohydrate (Whistler & Bemiller, 1958), the high concentration of KOH used is actually less destructive than lower concentrations, ostensibly due to exclusion of oxygen (Stetten et al., 1958). Prior to wide availability of purified enzymes, such rigorous treatment was required to completely release the glycogen so that it could be isolated for analysis. However, if present, microbial capsular polysaccharide will contaminate the glycogen isolated by this method (Daniels et al., 2007). Consequently, enzymatic release and specific measurement of glucose from glycogen will give a

more accurate value for microbial glycogen than does use of nonspecific condensation reaction methods to detect carbohydrate.

Less extended treatment of bacteria may suffice for measurement of glycogen if the cells are damaged sufficiently to allow access of amylolytic enzymes for hydrolysis of glycogen to glucose. Such an approach has been taken for analysis of glycogen in yeast using different types and lower concentrations of alkali incubated at or near boiling (Gunja-Smith et al., 1977; Becker, 1978; Parrou and Francois, 1997). For bacteria, heating with 1.0 N NaOH for 5 min at 100°C was reported to “effect complete extraction of protein from all bacteria, yeasts and moulds tested” (Herbert et al., 1971). One could infer, inversely, that such conditions could allow access of added enzymes to bacterial cell contents. An alternate method involving the use of 0.2 M NaOH boiled for 15 min has also been used to disrupt bacterial cells for analysis of microbial protein (Chen and Russell, 1988). Physical disruption through bead beating is yet another method used to lyse bacterial cells, particularly for the extraction of DNA (Henderson et al., 2013). A recently described bacterial reserve carbohydrate assay employed 15 min at boiling temperatures followed by a total of 45 min of bead beating for analysis of reserve carbohydrates in ruminal microbes grown *in vitro* from washed cells (Hackmann et al., 2013). If less lengthy treatments adequately disrupt bacterial cell walls to allow analysis of glycogen comparable to the 30% KOH treatment, they could greatly reduce the time required to perform the assay with no loss of accuracy.

Many of the glycogen assays described in the literature are applied to pure cultures or washed cells which can be accurately subsampled for analysis. However, cultures and centrifuged pellets from *in vitro* fermentations inoculated with strained ruminal fluid are visibly nonhomogeneous, showing layers of residual feed, glycogen-laden protozoa, and microbial mass.

Such pellets or cultures are not amenable to accurate subsampling for analysis. Accordingly, to insure accuracy, the entire pellet from a batch culture fermentation vessel must be analyzed. A search of the literature did not find comparisons of glycogen analysis methods as applied to such nonhomogeneous fermentation pellets.

The published microbial glycogen methods also vary in the carbohydrate used to assess α -glucan recovery. Typically, some form of purified, water-soluble glycogen from animal sources is used (e.g., shellfish glycogen, Gunja-Smith, et al., 1977; beef liver glycogen, Hackmann et al., 2013). However, the glycogens present in isotrichid protozoa (Oxford, 1951) and in the ruminal bacteria *Fibrobacter succinogenes* (Gong and Forsberg, 1993) are present in the form of water-insoluble granules. The differing physical characteristics among α -glucans may affect their recovery in different glycogen analyses. The alkali treatment and heating components of glycogen assays are analogous to those used in starch assays to gelatinize insoluble starches to allow their enzymatic hydrolysis (McCleary et al., 2002; Hall, 2015a).

The objective of this study was to evaluate specific aspects of microbial glycogen analysis. Methods using 30% KOH and 0.2 M NaOH were compared for analysis of glycogen in entire fermentation pellets prepared with strained, mixed ruminal inoculum. The two alkali methods and a bead beating method (Hackmann et al., 2013) were evaluated for their impact on recovery of purified α -glucan sources.

2. Materials and Methods

2.1. Substrates

Substrates included purified carbohydrates used to determine carbohydrate recovery, as well as centrifuged pellets from *in vitro* fermentation. Purified substrates included beef liver glycogen (type IX; G0885, Sigma Aldrich, St. Louis, MO), wheat starch (S-5127, Sigma, St.

Louis, MO), corn starch (S-4126, Sigma Aldrich, St. Louis, MO), and isolated protozoal glycogen (Hall, 2015c, submitted)(Table 1). Corn starch was used as the control sample in the fermentation pellet analysis comparisons; it is used as the control standard in our laboratory for α -glucan analyses. Wheat starch was selected for the comparison of control α -glucans because it has a granule size more variable (2 to 36 μm diameter) than that of corn starch (5 to 20 μm diameter; Jane et al., 1994), with the size of some granules approaching that of protozoal glycogen granules (1-2 μm diameter; Oxford, 1951). It was chosen to represent a broad range of granule sizes that would require gelatinization for complete recovery. As to the protozoal glycogen isolation, briefly: protozoal glycogen granules were isolated from cultures of mixed rumen microbes grown on glucose. Isotrichid protozoa laden with glycogen granules were allowed to sediment in separatory funnels and were decanted into tubes. Centrifugation in 0.9% saline with supernatant discarded, and sonication in saline to destroy cell structure was followed by repeated suspensions and centrifugations first in 0.9% saline and then in 18-megohm water. Visible contaminating cell debris was physically removed at each step. The final material was frozen and then freeze dried.

Table 1. Analyzed α -glucan values for purified carbohydrates, % of DM

Substrate	n ¹	Mean	SD ²
Beef glycogen	2	98.1	0.07
Protozoal glycogen	2	98.3	0.01
Wheat starch	2	99.5	0.57
Corn starch	2	99.6	0.50

¹ Total number of replicates per analysis.

² SD = standard deviation.

2.2. Fermentation pellet preparation

Rumen inoculum was obtained from 2 lactating Holstein cows maintained under protocols approved by the University of Wisconsin College of Agriculture and Life Sciences Animal Care and Use Committee. Donor cows were fed a total mixed diet consisting on a dry matter basis of 312 g/kg corn silage, 224 g/kg alfalfa haylage, 63 g/kg whole linted cottonseed, 4 g/kg dextrose, and 395 g/kg mixed concentrate supplemented with vitamins and minerals to meet National Research Council (2001) recommendations. Ruminal contents obtained from each cow within 2 h post-feeding were strained through 4 layers of cheesecloth and the rumen fluid maintained under CO₂. Equal volumes of rumen fluid from each cow were measured and filtered through an additional 4 layers of cheesecloth into the same Erlenmeyer flask maintained at 39°C in a water bath with CO₂ bubbled continuously through the inoculum. Inoculum was stirred under CO₂ to achieve homogeneity before addition to fermentation vessels.

Fermentation pellets were produced in an anaerobic *in vitro* batch culture fermentation using 3 g glucose/L as the substrate in individual sealed borosilicate glass fermentation tubes (Hall, 2015d) using Goering and Van Soest (1970) medium. Each vessel contained 20 mL of medium, 1 mL of reducing solution, 5 mL of strained ruminal inoculum, and 0.5 mL of autoclaved 159 g glucose/L in aqueous solution. Vessels with medium, reducing solution, and glucose solution were pre-warmed in a water bath at 39°C and the headspace flushed with CO₂ before addition of ruminal inoculum. Vessels were incubated for 2 h in tube racks in an incubating orbital shaker at 39°C and 160 rpm (Innova 40 bench top incubator shaker, 19 mm orbit, New Brunswick Scientific, Edison, NJ). Racks were set on their sides within the incubator so that the long axis of the tubes was positioned parallel to the motion of the shaker to continuously mix substrate, inoculum, and medium. Vessels were harvested at 2 h as previous fermentations with glucose

showed this to be the time of maximal glycogen accumulation under the fermentation conditions used (M. B. Hall, unpublished).

Entire contents of fermentation tubes were prepared for individual α -glucan analyses. The measured carbohydrate is referred to as α -glucan because it may represent microbial glycogen or residual dietary starch introduced with the inoculum. Ruminant ciliate protozoa ingest and slowly digest starch granules (Jouany and Ushida, 1999), and thus have the potential to introduce feed α -glucan, in addition to intracellular glycogen introduced by protozoa or bacteria. Microscopic examination of protozoa in strained ruminal inoculum from cattle fed starch-containing diets shows feed starch granules present within the protozoa (M. B. Hall, unpublished). Vessel contents were transferred quantitatively with 0.9% NaCl rinses to tared 50 mL high speed centrifuge tubes (#361694, Beckman Coulter, Inc., Brea, CA). The samples were twice centrifuged at 13,000 $\times g$ for 45 min at 5°C with the supernatant decanted and discarded each time, and pellets resuspended in 0.9% NaCl before the second centrifugation. Tared centrifuge tubes with pellets were weighed and grouped to allow pellets of similar weight to be analyzed in both alkali methods in a given analytical run. Fermentation pellets used ($n = 8$) averaged 1.20 ± 0.02 g wet weight. For the NaOH treatment, pellets were quantitatively transferred to 50 mL beakers using about 20 mL of 0.2 M NaOH; transferred samples were capped with aluminum foil and frozen at -20°C. For the KOH treatment, pellets were stored frozen in centrifuge tubes at -20°C. Samples were stored frozen for 10 and 15 days before analysis.

2.3. Glycogen methods

2.3.1. Fermentation pellet analysis

The two alkali methods used for lysis of fermentation pellets were boiling samples in 30% KOH for 3 h (KOH; Daniels et al., 2007) and boiling samples in 0.2 M NaOH for 15 min

(NaOH; Chen and Russell, 1988). Two analytical runs were performed on separate days with all samples in duplicate in each method. For the analysis of fermentation pellets, reagent blanks and purified corn starch control samples were included. Beakers containing fermentation pellets and 0.2 M NaOH were allowed to thaw to ambient temperature before analysis. For KOH, frozen fermentation pellets were quantitatively transferred from the centrifuge tubes into 50 mL beakers using about 30 mL KOH solution. As per the approach in which 50% KOH was added to wet cells to achieve a final KOH concentration of 30% (Daniels et al., 2007), the KOH concentration used with the fermentation pellets was 31.2% to achieve a final KOH concentration of approximately 30%. Frozen pellets were used with KOH because attempts to transfer unfrozen pellets with ~31% KOH resulted in a viscous, sticky material that was difficult to transfer quantitatively. Problems with quantitative transfer could result in decreased α -glucan detection due to failure to transfer the entire sample, and increased variability in values obtained for replicates. All beakers were covered with polytetrafluoroethylene (PTFE) sheets held in place by aluminum foil. The PTFE barrier was used because otherwise the foil degraded during boiling with KOH, though this was not an issue with NaOH. Samples were maintained in a boiling water bath for 3 h for KOH and 15 min for NaOH.

After boiling, all samples were allowed to cool to ambient temperature. Sample pH were adjusted to 5.0 using concentrated HCl and acetic acid for KOH and 10% acetic acid solution for NaOH. pH probes were rinsed into the samples using 0.1 M sodium acetate buffer, pH 5.0.

To each NaOH sample was added 200 U of amyloglucosidase (E-AMGDF, Megazyme International, County Bricklow, Ireland) in 0.1 M sodium acetate buffer, pH 5.0, and 0.1 mL of heat stable α -amylase (thermostable amylase HTL, approximately 8300 bacterial amylase units; BioCat, Inc., Troy, VA) (Hall, 2011). The amounts of both enzymes were proportionally

increased for KOH to provide the same average dosage of enzymes per mL for KOH as in NaOH. Samples were stirred on a magnetic stir plate, capped with aluminum foil, and then incubated at 50°C for 2 h in a circulating water bath, with stirring at 1 h. After incubation, samples were quantitatively transferred and brought to volume with water in 100 mL volumetric flasks. Samples for glucose analysis were centrifuged at 10,000 x g for 10 min at ambient temperature. Enzymatically released glucose in the supernatant was detected with the glucose oxidase – peroxidase method described in AOAC Method 2014.10 (Hall, 2015a). Detected α -glucan was expressed as glucose x 0.9, to correct for weight gain when hydrolyzing 162 g/mol anhydroglucose in α -glucan to 180 g/mol free glucose.

2.3.2. Purified substrate analysis

Two analytical runs were performed on separate days with samples and reagent blanks analyzed in duplicate for each assay in each run. All samples were suspended or solubilized in water before they were dispensed into reaction vessels. For analysis with alkali, 0.12 g of a purified sample was weighed into a 50 mL beaker, 30 mL of distilled water was added, and the mixture stirred with a magnetic stir bar to fully suspend the sample. A repeating, positive displacement pipette (Repeater M4, Eppendorf North America, Hauppauge, NY) was used to take up the suspended sample and rapidly dispense 5 mL into each of four 25 x 150 mm glass screw cap tubes with PTFE liners. Distilled water (5 mL) was added to tubes used as reagent blanks. To each KOH tube was added 5 mL of 60% KOH, and to each NaOH tube 5 mL of 0.4 M NaOH was added, to give final concentrations of 30% KOH and 0.2 M NaOH for KOH and NaOH, respectively. Samples were maintained in a boiling water bath for 3 h for KOH and 15 min for NaOH. Samples were allowed to cool on the bench for 30 min. Samples were adjusted to pH 5.0 using previously determined volumes of acid. Sodium acetate buffer (10 mL; 0.1 M,

pH 5.0) was added to each tube. Heat stable α -amylase (80 μ L for KOH, 70 μ L for NaOH) and 200 U / mL amyloglucosidase in sodium acetate buffer (800 μ L for KOH, 700 μ L for NaOH) were added to provide the same enzyme units per milliliter to both treatments and in the same proportions per milliliter used for AOAC Method 2014.10 (Hall, 2015a). Samples were then incubated for 2 h at 50°C with vortexing at 1 h of incubation. After incubation, distilled water (16 mL for KOH, 14 mL for NaOH) was added to each tube, and tubes were inverted to mix. Samples were analyzed for released glucose and α -glucan was calculated as described for analysis of fermentation pellets.

Bead beating of purified samples with enzymatic analysis for α -glucan was performed as described by Hackmann et al. (2013; Mini-Beadbeater 16, model 607, Biospec Products, Bartlesville, OK). In lieu of the cell pellets of the original method, 0.25 mL of purified carbohydrate solubilized or suspended in water was used. Purified sample (50 mg) was weighed into an 8 mL screw cap vial, 5 mL of distilled water was added, and the carbohydrate suspended or solubilized through vortexing and inversion of the sealed vial. A repeating, positive displacement pipette (Repeater M4, Eppendorf North America, Hauppauge, NY) was used to take up the suspended sample and rapidly dispense 0.25 mL into each of 4 bead beating tubes. Distilled water (0.25 mL) was added to tubes used as reagent blanks. Sodium acetate buffer (0.75 mL; 0.2 M, pH 5.0) was then added to each tube. For each substrate, 2 tubes were subjected to bead beating and 2 tubes used as controls. Control tubes were processed by the same procedure as the bead beaten (treated) samples, except that they were held at ambient temperature during the time that treated samples were bead beaten. Enzymatic hydrolysis was carried out through addition of 56 μ L of a 100-fold dilution of amyloglucosidase (E-AMGDF, 3,260 U/mL, Megazyme International, County Bricklow, Ireland) in 0.2 M sodium acetate buffer

(pH 5.0) to each sample, and incubation at 57°C (procedure of T. J. Hackmann, Univ. of Florida, personal communication) for 24 h. After incubation, samples were centrifuged at 1000 x g for 10 min at ambient temperature. Enzymatically released glucose in the supernatant was detected with the glucose oxidase – peroxidase method described in AOAC Method 2014.10 (Hall, 2015a). α -glucan was expressed as glucose x 0.9.

2.4. Other analyses

Purified substrates were analyzed for α -glucan using an enzymatic-colorimetric assay for starch (AOAC Method 2014.10; Hall, 2015a). Beef glycogen was also subject to the starch analysis performed with or without gelatinization at 100°C in each of two analytical runs. The ungelatinized samples were treated identically to the gelatinized samples, but were incubated at ambient temperature rather than at 100°C for the gelatinization step.

Qualitative evaluation of the number of intact bacteria remaining after α -glucan analysis was performed on KOH and NaOH treated fermentation pellets from the second analytical run to assess whether a difference in efficacy of cell lysis existed between treatments. A bacterial viability assay (LIVE/DEAD BacLight fluorescence-based assay, L7012, Invitrogen Corp., Carlsbad, CA) was used. After enzymatic hydrolysis and being brought to volume, samples were inverted to mix and approximately 1 mL of liquid was taken from each fermentation pellet replicate for each treatment, for a total of two samples per treatment. Samples were stained on individual slides, and 12 separate fields per slide were examined microscopically (FITC filter, 10× ocular, 40× objective, Zeiss Axioskop microscope, Carl Zeiss Microimaging Inc., Thornwood, NY). Dead intact microbial cells stain red and intact microbial cells with uncompromised membranes stained green in this assay.

2.5. Statistics

Data sets were analyzed as randomized complete blocks. “Treatment” was included in the statistical model as a fixed effect and “analytical run” as a random variable. Means separation was performed with the Tukey-Kramer test. Data for the purified α -glucan recoveries were analyzed by substrate. For bead beating, α -glucan recoveries for control and bead beaten (treated) samples were analyzed with a model including the factors: treatment, substrate, and substrate x treatment; “analytical run” was included as a random variable. All statistical analyses were performed with the Mixed Model procedure of SAS (Ver. 9.4, SAS Institute, Inc. Cary, NC). Significance was declared at $P < 0.05$.

3. Results

3.1. Analysis of fermentation pellets

The KOH and NaOH treatments did not differ in the amount of glycogen detected in fermentation pellets produced from *in vitro* fermentations with strained ruminal inoculum ($P = 0.61$; Table 2). The recovery of control corn starch samples was greater with NaOH than with KOH ($P < 0.01$). Sample solution volumes after addition of enzymes were averaged 56 mL for KOH and 36 mL for NaOH. Microscopic examination of KOH samples after enzymatic hydrolysis showed some, but very few intact, red-staining cells including spirochetes, many of them caught in masses of amorphous, gel-like debris. The NaOH samples showed more, but still very few, intact red-staining cells including spirochetes and cocci, and a very rare light or green-staining cell (all large cocci). The amorphous masses noted in KOH were absent in NaOH; relatedly, the KOH sample solutions were cloudy, whereas those for NaOH were clear.

3.2. Recovery of purified α -glucan

Glycogen determination methods differed in recovery of purified α -glucan, with their effect differing by α -glucan source (Table 3). Beef glycogen, a water-soluble α -glucan, showed the

Table 2. Effect of alkali methods on measurement of α -glucan in *in vitro* fermentation pellets¹

Substrate	n ²	30% KOH	0.2 M NaOH	SED ³	P-value
Pellet α -glucan, mg	4	29.9 (0.92)	29.6 (0.60)	0.52	0.61
Control corn starch recovery%	4	91.6 (0.41)	96.7 (0.28)	0.29	<0.01

¹ Values are least squares means. Mean standard deviations for within run replicates are in parentheses.

² Total number of replicates per treatment, 2 replicates per treatment per analytical run.

³ SED = standard error of the difference.

greatest recovery with bead beating, then with KOH, and the lowest with NaOH ($P < 0.01$).

In contrast, wheat starch showed the greatest recovery with NaOH, followed by bead beating, and then KOH ($P < 0.01$). There were no detected differences among methods for protozoal glycogen, with the recoveries also being numerically similar ($P = 0.81$). Numerically, the standard deviations for within-run duplicate samples were noticeably larger for bead beating and NaOH with protozoal glycogen than for other treatments and substrates.

Table 3. Effect of glycogen analysis methods on α -glucan recovery of purified substrates¹

Substrate	n ²	30% KOH	0.2 M NaOH	Bead beating	SED ³	P-values
Beef glycogen	4	91.4 ^b (0.83)	87.4 ^c (0.98)	95.0 ^a (0.47)	0.55	<0.01
Protozoal glycogen	4	87.0 (0.83)	87.6 (1.56)	86.0 (2.78)	2.24	0.81
Wheat starch	4	91.0 ^c (0.36)	96.9 ^a (0.31)	93.8 ^b (0.56)	0.62	<0.01

¹ Values are least squares means. Mean standard deviations for within run replicates are in parentheses.

Values within rows with different superscripts differ, $P < 0.05$.

² Total number of replicates per treatment, 2 replicates per treatment per analytical run.

³ SED = standard error of the difference.

Bead beaten samples and controls not subjected to bead beating did not differ in recovery of isolated α -glucan (treatment x substrate: $P = 0.16$; Table 4). The larger between-duplicate, within-run standard deviations noted for protozoal glycogen in the bead beaten tubes were also seen with the control tubes.

Beef glycogen samples analyzed with or without gelatinization did not differ in α -glucan detected (Table 5; $P = 0.56$).

Table 4. Comparison of bead beating control and treated purified substrates for α -glucan recovery¹

Substrate	n ²	Control	Treated	SED ³	Trt ⁴	<i>P</i> -values	
						Sub ⁴	Trt x Sub
Beef glycogen	4	96.2 (0.75)	95.0 (0.47)	1.14	0.46	<0.01	0.16
Protozoal glycogen	4	88.2 (2.37)	86.0 (2.78)				
Wheat starch	4	92.2 (1.21)	93.8 (0.56)				

¹ Values are least squares means. Mean standard deviations for within run replicates are in parentheses.

² Total number of replicates per treatment, 2 replicates per treatment per analytical run.

³ SED = standard error of the difference.

⁴ Trt = treatment = bead beaten or control, Sub = substrate.

Table 5. Effect gelatinization on α -glucan analysis of beef glycogen¹

Treatment	n ²	Ungelatinized	Gelatinized	SED ³	<i>P</i> -value
α -glucan, % of DM	4	96.7 (0.43)	96.5 (0.35)	0.28	0.56

¹ Values are least squares means. Mean standard deviations for within run replicates are in parentheses.

² Total number of replicates per treatment, 2 replicates per treatment per analytical run.

³ SED = standard error of the difference.

4. Discussion

The similarity in glycogen analysis results between the established sample processing method using 30% KOH with 3 h of boiling treatment, and an alternate method using 0.2 M NaOH with 15 min of boiling indicates that one protocol may be substituted for the other with the type of fermentation pellet tested. In order to estimate the amount of microbial glycogen produced in a fermentation, given that the inoculum can introduce feed starch granules and previously made microbial glycogen, measures of α -glucan from zero hour fermentation blanks should be subtracted from α -glucan measurements at subsequent time points to correct for introduced α -glucan.

The presence of intact dead (red-staining) bacterial cells in KOH was surprising, given the extended treatment of the samples. The cells were found predominantly in the amorphous material of unknown composition, suggesting that that material may have somehow protected the

318 cells or simply entrapped remaining intact cells. It has been reported that even with 30% KOH
319 treatment of bacterial cells, mechanical disruption may be necessary to disrupt Gram-positive
320 cells for analysis (Daniels et al., 2007, p. 469). In NaOH, red-staining cells were not associated
321 with other materials. The presence of green-staining cells in NaOH indicated the presence of
322 cells for which the integrity of membranes was not compromised. The very few such cells noted
323 and similarity of glycogen results to that of KOH suggest that the number of cells not disrupted
324 by NaOH was not sufficient to substantially affect the measurement of microbial glycogen.

325 The differences in recovery of purified substrates among the assays raises issues regarding
326 selection of control samples and their relevance to assessing efficacy of assays. Beef glycogen is
327 a water-soluble α -glucan that apparently does not require gelatinization. Consequently, it is
328 literally open to the effects of the treatments from the time of their initiation, whereas wheat
329 starch and protozoal glycogen both require gelatinization to allow enzymes or water access to the
330 entirety of the α -glucan molecules (Hall, 2015b). Alkali can both gelatinize α -glucan (McCleary
331 et al., 2002) and destroy polysaccharide (Whistler and BeMiller, 1958), depending on the type,
332 concentration, temperature, and length of reaction time. It is likely that the competing effects of
333 gelatinization to solubilize granules and varying degrees of destruction of solubilized α -glucan
334 are the basis for the observed results for alkali treatments. The lower recoveries obtained for
335 beef glycogen with KOH and NaOH relative to bead beating may reflect the longer period in
336 which beef glycogen was available for reaction with and degradation by the alkalis. In contrast,
337 wheat starch and protozoal glycogen require gelatinization to become solubilized and become
338 available for reaction with alkali or enzymes. Potentially, they would have been protected from
339 alkaline degradation until they were gelatinized and solubilized. The shorter time that wheat

starch was subject to NaOH at boiling temperatures may be the reason for the greater recovery in NaOH.

Alkaline degradation of carbohydrates generally begins at the reducing end of the molecule (Whistler and BeMiller, 1958). Sodium borohydride is known to reduce sugars to the corresponding sugar alcohols (Abdel-Akher et al., 1951), and can thus protect carbohydrates from alkaline degradation by converting the reducing end sugar. Treatment of rabbit liver glycogen with sodium borohydride did not protect the carbohydrate from alkaline degradation (Stetten et al., 1958). Similarly, in the present study, addition of 3 mg sodium borohydride/mL of alkaline solution did not increase the amount of glycogen detected in fermentation pellets in KOH or NaOH (data not shown).

The high recovery of beef glycogen obtained with bead beating may relate to the lack of need to gelatinize that substrate for α -glucan analysis. It is not obvious that the physical disruption of bead beating would affect a soluble substrate, a point borne out by the similar high recoveries of beef glycogen in control or bead beaten treatments, and with or without gelatinization in the α -glucan assay. The 95% recovery obtained with bead beating is equivalent to the recovery found for spiking cell pellets with the carbohydrate (Hackmann et al., 2013). The bead beating protocol did employ a 15 min boiling temperature step which may at least partially gelatinize the granules of protozoal glycogen or wheat starch prior to physical disruption. However, the lower recovery for wheat starch and low recovery for protozoal glycogen with bead beating may reflect an inability of the treatment to fully open the granules to enzymatic attack. The lack of detected differences between control and bead beaten samples suggests that the bead beating itself was not a key factor affecting analysis of purified samples.

The results with protozoal glycogen showed that none of the treatments achieved a >90% recovery. Unlike bacteria, protozoal cells do not require the more extreme treatments for lysis and release of cell contents. The protozoal glycogen granules do need to be gelatinized to allow complete enzymatic hydrolysis (Hall, 2015b). It is possible that the alkaline treatments resulted in partial destruction of the protozoal glycogen, whereas the bead beating treatment did not adequately destroy the granule structure to allow enzymatic attack; with all assays resulting in incomplete recovery.

None of the evaluations of purified substrates address the lysis of cells that is necessary to release bacterial glycogen for analysis. The amount of time required for lysis alters the amount of time that intracellular carbohydrates are subject to the effects of the treatments, including alkaline gelatinization and degradation. Physical disruption of cells through bead beating has been shown to be efficacious in releasing DNA, and thus bacterial cell contents (Henderson et al., 2013), it would not have the potential to degrade carbohydrate as alkali can, but may have little effect on opening granule structure to enzymatic attack. It would seem, then, that evaluation with purified substrates, by themselves or through spiking of samples, serves only to prove the recovery of already released materials in the assay, and the recoveries will differ with the α -glucan chosen. They will not assess time dependent effects related to release of glycogen from lysed microbes and destructive effects of treatments. The purified substrates will not assess efficacy of the procedures in recovering the carbohydrates of interest that are held within cells.

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