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Identification of novel enzymes to enhance the ruminal digestion of barley straw

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Abstract

Crude enzyme extracts typically contain a broad spectrum of enzyme activities, most of which are redundant to those naturally produced by the rumen microbiome. Identification of enzyme activities that are synergistic to those produced by the rumen microbiome could enable formulation of enzyme cocktails that improve fiber digestion in ruminants. Compared to untreated barley straw, Viscozyme® increased gas production, dry matter digestion ($P<0.01$) and volatile fatty acid production ($P<0.001$) in ruminal batch cultures. Fractionation of Viscozyme® by Blue Native PAGE and analyses using a microassay and mass-spectrometry revealed a GH74 endoglucanase, GH71 α-1,3-glucanase, GH5 mannanase, GH7 cellobiohydrolase, GH28 pectinase, and esterases from Viscozyme® contributed to enhanced saccharification of barley straw by rumen mix enzymes. Grouping of these identified activities with their carbohydrate active enzymes (CAZy) counterparts enabled selection of similar CAZymes for downstream production and screening. Mining of these specific activities from other biological systems could lead to high value enzyme formulations for ruminants.

Key words: Carbohydrate active enzymes (CAZymes), rumen nutrition, fiber utilization, Blue Native PAGE, LC-MS/MS, Glycosyl hydrolase.

1. Introduction

Sustainable agriculture and animal production represent grand challenges for humanity in the coming decades (Alexandratos and Bruinsma, 2012). With an ever growing human population and more affluent societies, the global demand for food and meat and milk in particular, is projected to increase substantially (Elam, 2010). Ruminant livestock are in a unique position to satisfy the growing demand for high quality protein as they can produce it from crop residues and food by-products. However, often less than 50% of the energy in low quality forages is digestible by cattle (Hatfield et al., 1999). Consequently, large amounts of cereal grains are fed to finishing cattle to enhance the efficiency of growth. Increased global appetite for meat and milk will further heighten demand for feed grains and could threaten food security in some regions of the world (Tenenbaum, 2008). Consequently, alternative cost-effective feed ingredients that promote the sustainable intensification of beef and dairy production must be identified to meet future global demands. Forages such as alfalfa hay or cereal straws (e.g. barley, wheat) could fulfill this need if technologies to increase the conversion of these substrates into energy in the rumen could be developed.

Globally, $\approx$ 73.9 Mt of crop residues are produced annually, with most of them left to decay in the field or burned (Kim and Dale, 2004). These residues can be used as feed for cattle (Sokhansanj et al., 2006), but due to their low digestibility they are often abandoned. If the digestibility of this material could be increased it would represent a global feed source for ruminants that could be used to offset the use of grain in ruminant production systems. The digestibility of plant cell wall is highly correlated with the structural complexity of plant cell walls (McCann and Carpita, 2008; Badhan and McAllister, 2016). The composition and layered architecture of the plant cell wall, extent of cross-linkages among polysaccharides, degree of lignification, crystallinity, size of micro-fibrils and protein cross-linkages all can contribute to the recalcitrance of plant cell walls (Himmel et al., 2007; McCann and Carpita, 2008). Furthermore, inhibitors that are natural components of the plant cell wall or are generated during hydrolysis can also adversely affect its enzymatic saccharification (McCann and Carpita, 2008).

The rumen harbors a vast array of cellulolytic microorganisms including bacteria, protozoa and fungi that works synergistically to digest plant fiber (Ribeiro et al., 2016). However, the complex plant cell wall is rarely completely digested by ruminal microflora. Limited penetration of cellulolytic microbes into the interior of the plant cell, insufficient retention time of feed within rumen and rate-limiting enzyme activities, have all been reported to act as constraints to ruminal cellulose digestion (Weimer, 1996; Ribeiro et al., 2016). Characterization of total tract indigestible fiber residues (TTIR) could help identify those undigested plant cell wall moieties that escape ruminal digestion and provide insight into factors that limit plant cell wall digestion (Badhan et al., 2015). Metagenomics and metatranscriptomic studies have indicated an absence or scarcity of GH7 (endoglucanase and cellobiohydrolase), GH44 (endoglucanase and xyloglucanase), GH12 (xyloglucanase and endoglucanase), GH52 (β-xylosidase) and GH62 (arabinofuranosidase) activity within the rumen (Dai et al., 2015). It has also been shown that supplementing mixed rumen enzymes with endoglucanase (GH7), arabinofuranosidase or acetyl xylan esterase activity enhances the cellulosic saccharification of mixed rumen enzymes (Badhan et al., 2014; 2015).

Previous crude commercial enzymes assessed in ruminants were formulated primarily for industrial applications and not to confer synergism to the natural enzyme profile of the rumen microbiome (Bhat and Hazlewood, 2001). In fact, commercial enzyme mixtures often contain redundant enzyme activities that are already produced in abundance by the rumen microflora (Meale et al., 2014). Hence an informed and specialized approach towards development of enzyme formulations targeted at enhancing ruminal fiber digestion is needed. The aim of this study was to fractionate a candidate commercial enzyme preparation and through proteomic analysis identify those CAZymes that interact with mixed rumen enzymes in a manner that enhances plant cell wall digestibility.

2. Materials and Methods

Graphical representation as shown in Figure 1 describe the workflow, sequence and interaction of experiments adopted in this study to identify novel fungal enzyme activities with potential to enhance ruminal digestion of barley straw.

2.1. Enzymes

Rumen mixed enzymes (RME) were prepared from rumen fluid collected from six rumen cannulated cows fed 70% barley straw and 30% concentrate (DM basis), comprised of corn dried distiller's grains and canola meal (Ribeiro et al., 2017). Rumen contents were collected 2 h after feeding and strained through four layers of cheesecloth. The collected fluid from each cow was pooled, lyophilized and aliquoted into vials (15ml flacon tubes) for storage. Lyophilized aliquots of rumen fluid were reconstituted in 50 mM sodium citrate (pH 5.0, containing 5 μg/mL tetracycline, 5 μg/mL cycloheximide and 0.02% sodium azide), centrifuged at 38,300 × *g* for 15 min and the supernatant was used as source of rumen mixed enzymes. The commercial enzymes Viscozyme® L was purchased from Sigma Aldrich (Oakville, ON, Canada) while

Dyadic® xylanase plus was obtained from Dyadic International, Florida, USA. Crude extracellular proteins from *Aspergillus niger* strain N402 and *Mycothermus theromphilus* strain CBS 625.91 were obtained from fungal spores cultured for 30 h at 30°C and 45°C, respectively in a liquid minimal medium containing a 1% suspension of barley straw and alfalfa hay as carbon sources (Natvig et al., 2015).

2.2. Micro assay screening

Crude commercial enzymes and fungal enzymes were screened for their ability to enhance the saccharification yield from barley straw incubated with RME using a microassay as described previously (Badhan et al., 2014, 2015). Ground barley straw was mixed in buffered suspension (composition as shown above) at a concentration of 0.5% w/v at pH 6; and 200 µl of this suspension was incubated with additive enzymes (5 mg/g substrate) and RME (5 mg/g substrate) at 39°C for 48 h. The reactions were then centrifuged at 1,500×*g* for 3 min; the supernatants transferred into a clean microplate and heated at 90°C for 10 min to inactivate enzymes. The amount of glucose & xylose released by the enzyme mixture was quantified using the D-Glucose Assay kit (Megazyme, Wicklow, Ireland) as described previously (Badhan et al., 2014). The enzyme preparation that supported the greatest improvement in glucose yield of RME was selected for *in-vitro* batch culture validation.

2.3. *In-vitro* batch culture of rumen mix microbes with Viscozyme® pre-treated barley straw

Viscozyme® was applied to barley straw at 100 µg (0.01%) and 500 µg (0.05%) protein per gram of substrate DM (barley straw) to determine its ability to improve the *in-vitro* ruminal fermentation of barley straw using ruminal batch cultures as described by Mauricio et al., (1999). Barley straw (0.7 g DM; ground through a 1mm screen) was weighed into acetone-washed, pre-weighed filter bags (model F57; Ankom Technology Corp., Macedon, NY,USA). Enzymes were diluted with buffer (50mM sodium citrate buffer pH 6.0) and sprayed (at two dose rates in triplicate) directly onto barley straw within the filter bags at the designated application rate. Bags were heat-sealed and placed into empty 125-ml amber serum vials and allowed to stand at 39°C for 10 h prior to incubation. Rumen fluid (a strained pool sample collected before morning feeding from four ruminally cannulated Angus × Hereford cows fed 50% grass hay, 30% barley straw, 15% corn DDGS, and 5% mineral/vitamin supplement (DM basis)) and mineral buffer (Goering and Van Soest, 1970) were mixed in 1: 4 ratios under a stream of O_2-free CO_2. Mixed inoculum (65 ml) was transferred to each vial under a stream of O_2-free CO_2. Vials containing inoculum, but no substrate were included as blank controls. Vials were sealed with rubber stoppers and placed on an orbital shaker at 90 oscillations/min and incubator at 39˚C for 48 h. Gas production was measured after 3, 6, 12, 18, 24, and 48 h of incubation by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada). Triplicates vials for each treatment were withdrawn after 12, 24 and 48 h incubation to measure dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), acid detergent fiber disappearance (ADFD), production of volatile fatty acid (VFA) ammonia (NH_3) and pH. After removal, bags were thoroughly rinsed with cold water until the water ran clear, dried at 55°C for 48 h, weighed and subsequently analyzed for NDF, and ADF content to estimate NDFD and ADFD, respectively. Samples (1.5 mL) of the fermentation fluid were collected and transferred to micro-centrifuge tubes (2 mL) containing 300 µL of H_2SO_4 (1%; v/v) for NH_3-N analysis, and another sample (1.5 mL) was collected and acidified with 300 µL of meta-phosphoric acid (25%; w/v) for VFA analysis. Batch culture experiments were repeated in three separate runs.

2.4. Blue native PAGE fractionation of Viscozyme® and characterization of fractionated protein bands.

Viscozyme® samples were desalted and concentrated using Vivaspin 500 centrifugal concentrators (5000 MWCO; Z614009-25EA Sigma-Aldrich, Oakville, ON) and loaded on NativePAGE™ Novex® 4-16% Bis-Tris Gels (1.0 mm, 10 Well; BN1002 Box, Life technologies Inc., Burlington, ON, Canada). Gels were initially run at 100 V for 30 mins, followed by a run at 180 V at 4°C until the dye reached the end of the gel in a Bolt electrophoresis system (Thermo-Fisher Scientific, Ottawa, ON, Canada). A native running buffer kit (BN 2007, Life technologies Inc., Burlington, ON, Canada) was used to generate cathode and anode buffers. All gels were stained with CBB G-250.

The Blue Native (BN) fractionated protein bands were excised and proteins were extracted by incubating gel slices in 1 mL of 50 mM citrate buffer (pH 5.0, containing 0.02% sodium azide) at 4°C for 24 h. Extracted proteins (30 µL) were boiled in (4X) sample loading buffer (#1610747, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) containing freshly added mercaptoethanol (100µL per 900 µL of buffer) and loaded onto SDS PAGE Mini-PROTEAN® Tris-Tricine Precast Gel (10%, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Electrophoresis was then performed according to standard protocols (Laemmli, 1970).

Cellulase and xylanase activity within extracted proteins were assayed against 1 % carboxyl methyl cellulose (CMC) and oat spelt xylan (OSX). Reaction mixtures containing 500 µL of substrate (1% CMC or 1% OSX), 30 µL of individual extracted protein and 470 µL of 50 mM citrate buffer (pH 5.0) were incubated for 1 h at 50°C. The reaction was stopped by adding 2 mL of 3, 5-dinitrosalicylic acid (DNS) and boiling the reaction mixtures at 100 ^{o}C for 10 min. The resulting chromophore was measured at 540 nm.

Isolated protein fractions were also assayed for their ability to enhance the saccharification of barley straw by RMEs in the microassay as described above. Ground barley straw (5 mg) was suspended in 150 μL sodium citrate buffer (0.5% w/v; pH 6) and incubated with BN fractionated enzymes (300 μL) from Viscozyme® and RME (10 mg/g DM) at 39°C for 24 h. The amount of glucose released by the enzyme mixture was quantified using D-Glucose Assay kits (Megazyme, Wicklow, Ireland).

2.5. In-gel digestion and MS analysis

BN fractionated bands that enhanced saccharification yield of RME were excised, de-stained, reduced, cysteine-alkylated and in-gel digested with sequencing grade modified trypsin (Promega, Madison, WI, USA) as described by Wasiak et al., (2002). Solutions of 1% formic acid (FA) with increasing concentrations of acetonitrile (ACN) were used to extract peptides from gels and the extracts were dried in a speed vac prior to being re-suspended in 90 µl 5% ACN: 0.1% FA.

2.5.1. LC-MS/MS Analysis

Peptide digests (2 µL) were resolved using a PicoFrit column (15 cm x 75 µm, New Objective, Woburn, MA, USA) packed with Jupiter 5 mm, 300 Å, C18 resin (Phenomemex, Torrance, CA, USA) connected in-line with a Velos LTQ-Orbitrap mass spectrometer (Thermo-Fisher, San Jose, CA, USA). A linear gradient using a mixture of solvent A (3% ACN: 0.1% FA) and solvent B (99.9% ACN: 0.1% FA) was used with the gradient initiated using 1% B, which was linearly increased to 27% B in 7 min, further increased to 52 % B in 2 min followed by an increase to 90% B, where it was held for 3 min.

The capillary voltage on the nanospray source was adjusted from 1.9 to 2.1 kV to achieve the optimal spray plume at 10% B. Mass spectrometry survey scans spanning the 350 to 2000 *m*/*z* range were completed in the Orbitrap analyzer at 60000 resolutions. The best ten double, triple or quadruple charged ions with intensities > 5000 counts were targeted as candidates for CID MS/MS fragmentation in the LTQ-Velos ion trap. Optimal accumulation times were set automatically using adaptive automatic gain control with a maximum accumulation time of 150 msec. Selected ions were placed in a dynamic exclusion list for 7 sec and reacquired again if still detected within a 20 sec window. The MSMS scan range was automatically adjusted based on precursor m/z and charge. Selected ions were fragmented using a normalized collision energy set at 35% and an isolation window of 2 m/z.

Proteome Discoverer 1.3 (Thermo-Fisher) was used to process raw mass spectrometric data. Spectra were searched against the *Aspergillus aculeatus* (i.e., source organism of Viscozyme®) genome sequence available at the Joint Genome Institute (https://genome.jgi.doe.gov/Aspac1/Aspac1.home.html). Sequence searches were undertaken using a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 ppm. An iodoacetamide derivative of cysteine was specified as the fixed modification and methionine oxidation as the variable modification. Peptide scores supporting a lower than 1% false discovery rate was used to identify proteins. Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS were classified together to satisfy the principles of parsimony.

2.6. Phylogenetic analysis

Characterized sequences from CAZyme families were extracted, trimmed, and aligned with LC-MS sequences as previously described using the SACCHARIS pipeline (Jones et al., 2018) . Briefly, SACCHARIS retrieves indicated CAZyme sequences from the CAZy database (http://www.cazy.org) (Lombard et al., 2013). Trimmed sequences of embedded CAZyme modules are extracted using dbCAN (Yin et al., 2012) and aligned with MUSCLE (Edgar et al., 2004). Phylogenetic models are generated by RAxML (Stamatakis, 2014) or FAstTree (Guindon and Gascuel, 2003), using the best-fit model determined by ProtTest (Darriba et al., 2011). Final trees are plotted using FigTree (http://tree.bio.ed.ac.uk). Trees are inspected for sequences of interest and manually highlighted.

2.7. Statistical analysis

Batch culture data were analysed by analysis of variance using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA). The model included the fixed effects of treatment, time, treatment × time, and the random effects of run and replicate vials within run, with time included as repeated measure. Degrees of freedom were adjusted using the Kenward-Roger option and the covariance structure for repeated measurements was selected based on the lowest Akaike and Bayesian information criteria values. Differences among treatments were tested using the PDIFF option. The sums of squares were further partitioned by orthogonal contrasts to test for differences between Viscozyme® and the control (no enzyme). Significance was declared at $P \leq 0.05$ and a tendency was described at $0.05 < P < 0.10$.

3. Results and Discussion

Commercial enzyme mixtures have largely failed to result in consistent improvements in fiber digestion in the rumen. A number of factors could be responsible for this outcome, including the presence of enzymes in these mixtures that are redundant to those produced in the rumen (Adesogan et al., 2014; Ribeiro et al., 2016). Optimum ratios of hydrolases in accordance with cell wall composition and their synergistic interaction are critical for efficient biomass deconstruction (Banerjee et al., 2010). Likewise identification of rate limiting enzyme activities within the rumen would seem to be a critical step towards formulating an effective enzyme additive for ruminants (Adesogan et al., 2014; Arriola et al., 2017). Screening exogenous fibrolytic recombinant enzymes with a conventional *in-vitro* batch culture technique involves growing rumen mix microbes on cellulosic substrates in the presence of exogenous enzymes. Various fermentation parameters (e.g., gas production, DM, NDF disappearance) or metabolite profiling (e.g., VFA production) are used to estimate effectiveness of an enzyme additive at improving ruminal fiber digestion. *In-vitro* batch culture methods require milligram quantities of recombinant enzymes, limiting the number of candidate enzymes that can be evaluated (Meale et al., 2014). Accordingly, complex fungal or bacterial crude enzyme preparations or complex commercial enzyme additives can be more readily assessed using *in-vitro* batch culture. However, such batch culture based screening methods are also limited as they only identify if the complex enzyme additive increases ruminal fermentation, but fail to provide information on enzymatic activities within the complex that are responsible for any improvement. To overcome these limitations, a microassay was developed with the sensitivity and ability to screen exogenous recombinant enzymes at low doses (micrograms levels) for their potential to enhance the saccharification of lignocellulose in the presence of mixed rumen enzymes (Badhan et al., 2014; 2015). In addition to screening enzymes, combining the microassay with statistical design enables the formulation of enzyme cocktails for efficient plant cell wall deconstruction (Badhan et al., 2014). The RME used in microassays for this study were collected from cows fed barley straw to ensure that the spectrum of enzymes expressed by rumen microbes was tailored for the effective saccharification of barley straw. An advantage of this assay is that it is adaptable to any forage by enriching the rumen microbial populations through feeding the targeted forage prior to the collection of RME.

3.1. Micro assay screening of enzyme additive

Figure 2 show that the addition of enzymes from various sources to RMEs enhanced the saccharification of barley straw. The crude extracellular proteins from *Mycothermus theromphilus* released more glucose as compare to the extracellular proteins from *Aspergillus niger*. Likewise Viscozyme® released more glucose from barley straw than Dyadic® xylanase (Figure 2A) and the other enzymes examined. Consequently, Viscozyme® was selected for further analysis. Viscozyme® displayed a dose-dependent relationship for glucose release from barley straw when added to RME (Figure 2B). Compared to RME control, Viscozyme® increased glucose yield by 20% and 37%, at a protein load of 10 mg and 20 mg per g of barley DM, respectively. Microassay screening of four different crude enzyme preparations resulted in the selection of Viscozyme® for further study as it exhibited the highest positive interaction with RME amongst all tested enzymes (Figure 2).

3.2. *In-vitro* batch cultures.

Compared to control incubations, addition of Viscozyme® increased the DMD ($P \leq 0.02$) and tended to increase NDFD ($P \leq 0.08$) with no improvement in ADFD (Table 1). Increasing the concentration of Viscozyme® from 0.01 to 0.05% did not result in additional improvement in digestibility of DM, NDF or ADF. Addition of Viscozyme® increased total VFA ($P<0.001$), and acetate concentrations ($P<0.001$) as well as the acetate to propionate ratio ($P<0.05$), but had no effect on ammonia, propionate or butyrate concentrations (Table 1). The presence of complementary activities in exogenous fibrolytic enzyme (EFE) that are absent, inhibited, or inactivated in the rumen has been previously proposed as a mechanism for the increase in the extent of digestion by EFE (Adesogan et al., 2014). Viscozyme L® is a complex enzyme mixture produced by a selected strain of *Aspergillus aculeatus* that produces a range of CAZymes including cellulases, xylanases, arabanases, beta-glucanases, and pectinases (Gama et al., 2015). In order to identify hydrolases within Viscozyme® that interact with RMEs, Viscozyme® was fractionated using BN PAGE. BN PAGE in combination with LC-MS/MS has been reported to be fast, sensitive and efficient method to fractionate fungal crude enzymes in their native configuration and to identify enzyme actives within individual bands (Silva et al., 2012; Badhan et al., 2017).

3.3. Blue native PAGE.

The BN fractionated protein profile of Viscozyme® indicated the presence of putative high molecular weight multi-enzyme complexes (M wt. range 150 kDa to 480 kDa) as well as low molecular weight non complexed free enzymes (100 kDa-20kDa) (Figure 3A). Identical band patterns across different dilutions of Viscozyme® (data not shown) suggested high affinity and strong structural association between components within the enzyme complex. SDS-PAGE of the high molecular fraction resolved multiple proteins. The cellulase and xylanase profile of BN fractionated Viscozyme® showed CMCase and xylanase activity between regions 7-9, although regions between bands 14 and 15 also showed high CMCase activity (Figure 3B). Microassay screening of enzyme extracts from BN fractions suggested that peak 5, 10, and 13 (region between band 12 and14) contained enzyme components that could enhance the ability of RMEs to saccharify barley straw (Figure 3C). Protein extracted from BN fractionated protein bands (7-9) exhibited maximum CMCase and xylanase activity, but supported nominal glucose release from barley straw when added to RMEs in the microassay (Figure 3). Such results suggest a redundancy of CMCase and xylanase activity in this fraction of Viscozyme® with enzymes that were already present in RME.

3.4. Protein identification

Protein bands showing high saccharification yield (band 5, 10 and 13) were excised from the gel, digested with trypsin and subjected to LC-MS/MS analysis for protein identification. LC-MS/MS analysis identified an oligo-xyloglucan reducing end-specific cellobiohydrolase (Aspac1_36855) and a α-1,3-glucanase (Aspac1_26674) as principal enzyme components of band 5 (Table 2). Whereas pectin lyase (Aspac1_48262, Aspac1_29881, Aspac1_34742 and Aspac1_30695), esterase (Aspac1_54785 and Aspac1_40343), GH5 (Aspac1_32288) and GH7 (Aspac1_50410) proteins were identified as major enzyme activities of band 10. Likewise band 13 showed GH7 (Aspac1_50410), pectin lyase (Aspac1_54671 and Aspac1_46746), esterase (Aspac1_48443) and GH5 (Aspac1_32288) as principal enzyme activities.

Endoglucanase GH74 (XEG) has been shown to be active against diverse xyloglucans from various sources and reported to be a xyloglucan-specific endo-beta-1,4-glucanohydrolase (Pauly et al., 1999). Xyloglucan is known to hinder the accessibility of enzymes to core cellulose as it is attached to cellulose by hydrogen bonds and also covalently linked to pectin and xylan (Hayashi, 1989). Therefore, digestion of crosslinked xyloglucan is considered a perquisite for efficient hydrolysis of cell wall embedded cellulose (Zsuzsa 2008; Harris and Stone 2008). The observation that xyloglucan specific GH 74 endoglucanase and GH 71 α-1, 3-glucanase improved the ability of RME to release glucose suggests positive impact of disrupting cross-linking cell wall constituents like xyloglucans and 1-3 linked glucan for ruminal fiber digestion. Similarly, band 10 and 13 exhibited cellobiohydrolase GH7, mannanase GH5, pectinase and esterase activity (Table 2). Although bands 10 and 13 shared a similar activity profile, the molar ratios of the enzymes they contained differed. Some studies suggested that the molar ratios of enzyme activities within complex enzyme mixtures have a significant impact on the saccharification of lignocellulose (Banerjee et al., 2010; Gao et al., 2010). In view of the absence of GH7s within the rumen (Dai et al., 2015), supplementation with Aspac1_50410 (cellobiohydrolase) belonging to GH7 could increase fermentable sugar release. Similarly a need for esterase activities for ruminal digestion is in accordance with previous reports that identified ester cross-linkages of xylan and pectin to cellulose as major factors that contribute to the recalcitrance of plant cell wall digestion in the rumen (Wang et al., 2004; Jung et al., 2012). Similarly total tract indigestible barley straw residues recovered from bovine feces were shown to be rich in undigested esterified hemicellulose (Badhan et al., 2014; 2015), evidence that further suggest that a deficiency of enzymatic activities that attack the ester cross-linkages within the plant cell wall are limiting ruminal fiber digestion.

3.5. Phylogenetic analysis

Enzymes identified by LC-MS/MS from the fractionated Viscozyme® mixture, belong to CAZyme families which contain multiple activities and are active on a variety of substrates. To inform what specific activities and potential substrates the identified enzymes may act upon and identify candidate CAZymes for future applications, a phylogenetic analysis was conducted using the SACCHARIS pipeline (Jones et al., 2018). Examining fraction 13 that exhibited high glucose yield from barley straw when combined with RME, phylogenetic trees were generated corresponding to the CAZyme families; GH5, GH7, GH28, and PL1 (Figure 4). Aspac1_32288, Aspac1_50410, and Aspac1_46746 were closely related to enzymes of fungal origin (Table 3). Aspac1_32288 partitioned with a group of fungal mannanases belonging to GH5 subfamily 7, Aspac1_50410 was closely related to a GH7 cellobiohydrolase from *Aspergillus niger* CBS 513.88, while Aspac1_46746 grouped with a collection of GH28 polygalacturonases from members of the *Aspergillus* genus. Interestingly, the closest characterized enzyme activity to Aspac1_54671 was not of fungal origin, but was a bacterial pectate lyase belonging to PL1 subfamily 2 from the plant pathogen *Dickeya dadantii* (Table 3).

It is interesting to note the positive effect of mannanase (Aspac1_32288) and pectinase (Aspac1_46746 and Aspac1_54671) activity on glucose release from ruminant feedstocks. Mannanases are important enzymes for digesting the polysaccharide β-mannan in hemicellulose (Tewoldebrahan et al., 2017). A few studies have been conducted on the effectiveness of mannanase supplementation to ruminants and they reported beneficial nutritional effects in goats, beef and lactating cows (Lee et al., 2014; Seo et al., 2016; Tewoldebrahan et al., 2017). Likewise, pectinase supplementation could potentially increase absorption of nutrients by depolymerisation of non-biodegradable fibers, therefore liberating nutrients occluded by pectic fibers and improving overall feed digestibility (Hoondal et al., 2002). It should be noted that GH families like 7, 74, 71, 28 and PL1 that enhanced saccharification yield of RME have been shown to be either absent or of low abundance in rumen based on metagenomic and metatranscriptomic analysis of the rumen microbiome in ruminants fed forage (Dai et al., 2015, Qi et al., 2011). Although functional aspects of these enzymes may be complemented by other enzymes in the rumen, it would be worth investigating if the addition of fungal enzymes from these families to the diet results in significant improvement in rumen fiber digestion. The association of these activities with improvements in RME performance presents an interesting future opportunity to investigate the role these activities may play in the bioconversion of agricultural feedstocks. Phylogenetic analysis also provided a set of candidate CAZymes that are related to LC-MS/MS identified Viscozyme® fractions with high synergistic potential (Table 3). Future cloning, expression and characterization of candidate CAZymes identified in this study with respect to their abilities to improve ruminal fiber digestion may yield a set of efficient enzymes for constructing enzyme formulations as feed additives.

4. Conclusion

The strategy described here incorporates a microassay to screen exogenous complexes or recombinant enzymes in the presence of RMEs to identify improvements in saccharification yield. Follow-up *in-vitro* batch cultures validated the merit of identified enzymes. Identifying those fractionated enzymes that synergistically enhanced saccharification by RMEs, led to identification of enzymes that may limit the rate of ruminal fiber digestion. Using xyloglucan specific GH74 endoglucanase, GH71 α-1,3-glucanase, GH5 endo-β-1,4-mannanase, GH28 polygalacturonase and esterase activities in a synergistic manner with RME presents an opportunity to explore the ability of this approach to improve the efficiency of ruminal fiber digestion. Genes coding for some of these selected candidates have been cloned into a protein expression system for scale up production of recombinant enzymes for assessment in *in vivo* metabolism and growth performance studies using sheep and beef cattle fed high fiber diets.

E-supplementary data for this work can be found in e-version of this paper online.

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Figure Captions

Figure 1: Graphical representation of experimental work flow adopted to identify enzyme activities with potential to enhance ruminal digestion of barley straw. Different stages of experimental work flow included screening, enzyme discovery and selection of candidate enzymes for future formulations are shown in Red, yellow and green boxes, respectively.

Figure 2: (A) Microassay screening of the commercial enzymes Viscozyme® (VIS) and Dyadic® -xylanase (DYD), and the secretome from *Aspergillus niger* (ASP) and *Mycothermus thermophilus* (MYC) for their ability to enhance glucose yield from barley straw incubated with rumen mixed enzymes (RME). The protein load was 10 mg/g DM of barley straw. RME and enzyme additives were mixed in 50:50 ratios. (B) Glucose yield at different protein loads of RME and Viscozyme®. Grey and black bars represent glucose yield of RME and Viscozyme®, respectively.

Figure 3: (A) Blue native fractionation of Viscsozyme®, (L1; 90µg protein load, L2; protein marker comprised of IgM hexamer (1236 k Da), IgM pentamer (1048 k Da), apoferritin band 1 (720 k Da), apoferritin band 2 (480 k Da), B-phycoerythrin (242 k Da), lactate dehydrogenase (146 k Da), bovine serum albumin (66 k Da) and sobean trypsin inhibitor (20 k Da). Location of fraction 1-15 is shown as blue grid. **(B)** CMCase (blue) and xylanase (red) activity profile of BN fractionated Viscozyme®. **(C)** Saccharification yield of Viscozyme® in combination with RME in terms of glucose release (g/Lt) from barley straw. Location of BN PAGE protein bands with respect to CMCase/Xylanase activity or peak glucose released and their respective molecular weights are shown by BN gel images on the X axis.

Figure 3: Phylogenetic trees of candidate enzyme activities from BN fractionated Viscozyme® band 13. Identified enzymes with high saccharification yield (red star) are aligned with characterized sequences from related GH5, GH7, GH28, and PL1 families. Phylogenic tree members are colored based on characterized activity or substrate. Close-up view of clusters

containing characterized CAZymes closely related to query sequences displayed in bottom right inserts for GH5 and GH28. Scale bars represent a genetic distance of 0.5 for each tree. Details of characterized enzymes within each cluster are shown in Table 3.

Tables

Table 1. Effect of Viscozyme® on DM, NDF, ADF disappearance, ammonia-N and volatile fatty acid (VFA) profiles after 12, 24 and 48 h of incubation of barley straw in ruminal batch cultures.

Table 2: Composition of BN fractionated Viscozyme® bands with high glucose yield as identified by mass spectroscopy.

Table 3: List of characterized CAZymes clustering with **c**andidate enzyme activities identified from Viscozyme® fractions with high saccharification yield. CAZymes are grouped by family and list characterized enzyme activity and EC number. Percent identity is calculated based on full length gene sequences.

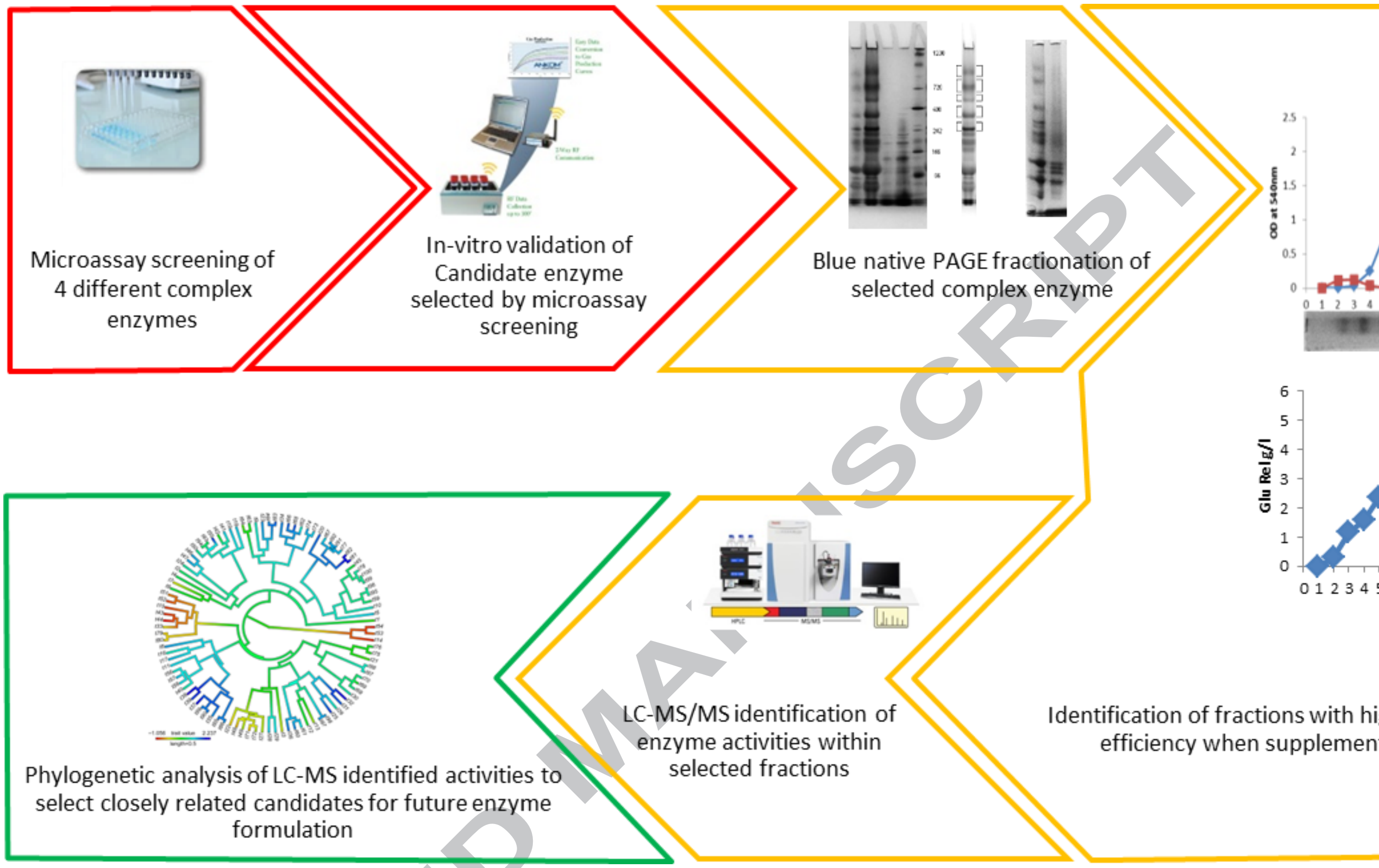
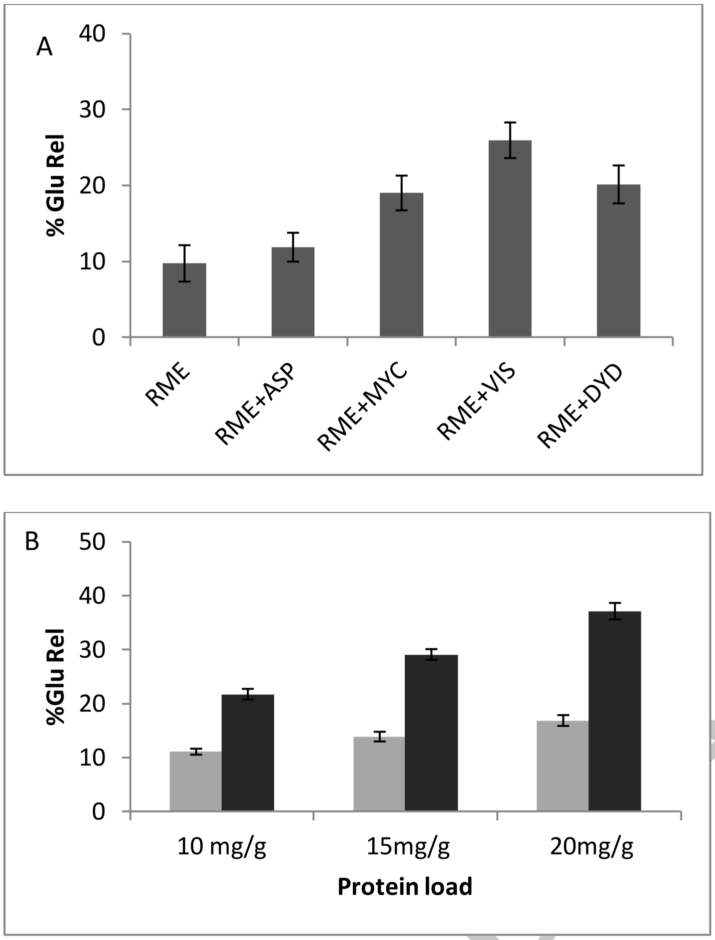

Microassay screening of 4 different complex enzymes
In-vitro validation of Candidate enzyme selected by microassay screening
Blue native PAGE fractionation of selected complex enzyme
OD at 540nm
2.5
2
1.5
1
0.5
0
0 1 2 3 4
Glu Rel g/l
6
5
4
3
2
1
0
0 1 2 3 4 5
Identification of fractions with hi
efficiency when supplemen
LC-MS/MS identification of enzyme activities within selected fractions
HPLC
MS/MS
Phylogenetic analysis of LC-MS identified activities to select closely related candidates for future enzyme formulation

Figure 1: Graphical representation of experimental work flow adopted to identify enzyme activities with potential to enhance ruminal digestion of barley straw. Different stages of experimental work flow i.e., screening, enzyme discovery and selection of candidate enzymes for future formulations are shown in Red, yellow and green boxes respectively.



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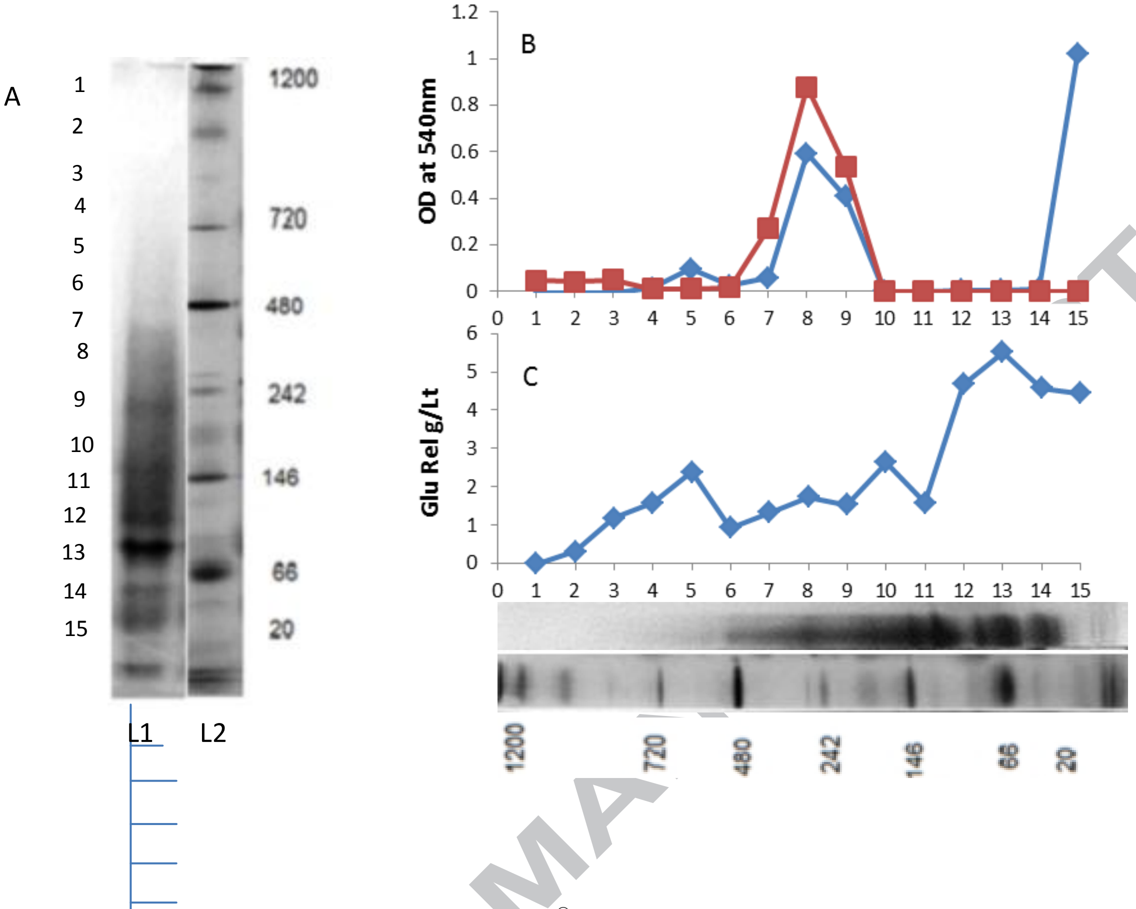

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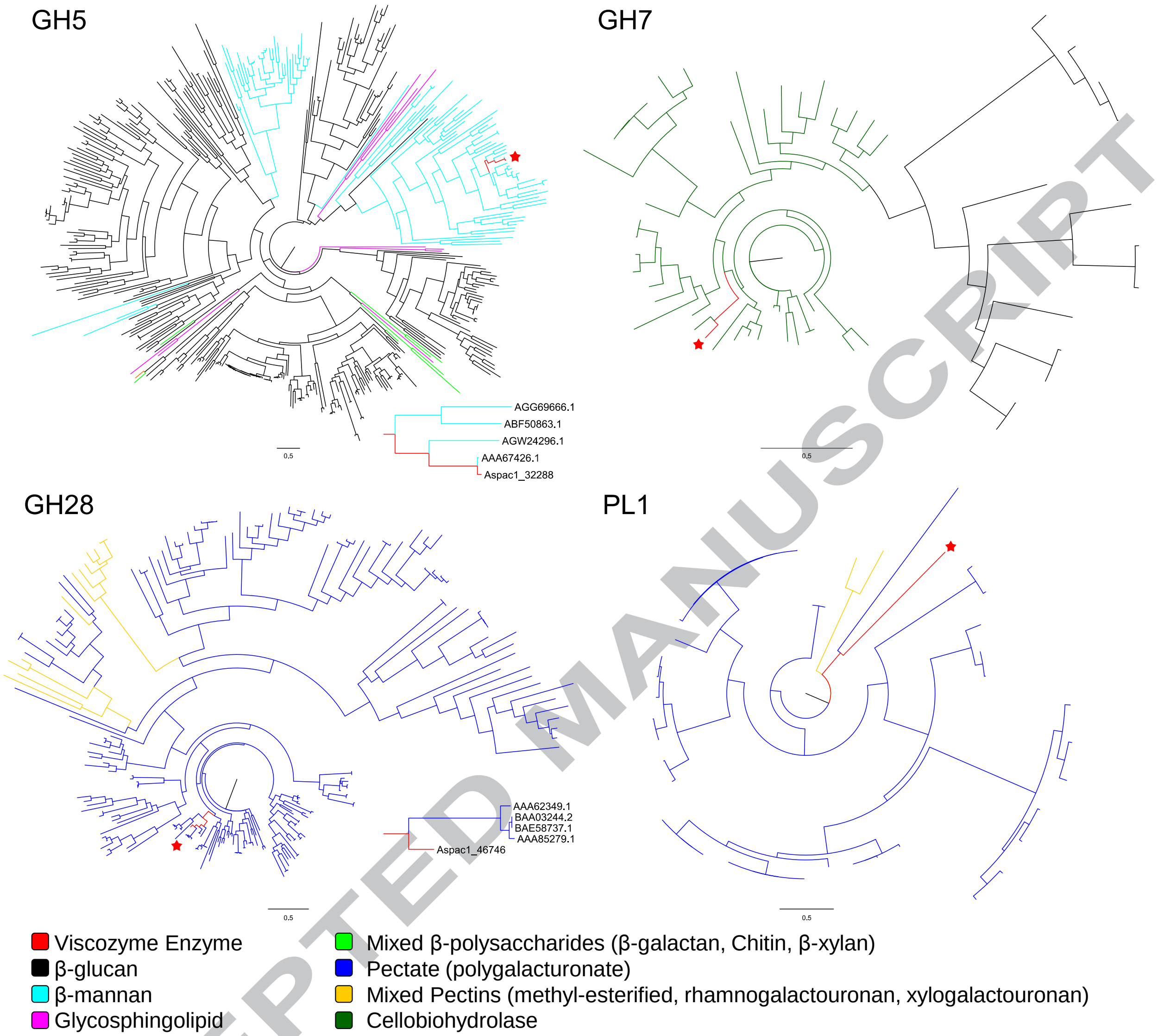
GH5
GH7
GH28
PL1
AGG69666.1
ABF50863.1
AGW24296.1
AAA67426.1
Aspac1_32288
AAA62349.1
BAA03244.2
BAE58737.1
AAA85279.1
Aspac1_46746
0.5
0.5
0.5
0.5
🟥 Viscozyme Enzyme
⬛ β-glucan
■ β-mannan
🟪 Glycosphingolipid
🟩 Mixed β-polysaccharides (β-galactan, Chitin, β-xylan)
🟦 Pectate (polygalacturonate)
🟨 Mixed Pectins (methyl-esterified, rhamnogalactouronan, xylogalactouronan)
🟩 Cellobiohydrolase

Figure 3: Phylogenetic trees of candidate enzyme activities from BN fractionated Viscozyme® band 13. Identified enzymes with high saccharification yield (red star) are aligned with characterized sequences from related GH5, GH7, GH28, and PL1 families. Phylogenic tree members are colored based on characterized activity or substrate. Close-up view of clusters containing characterized CAZymes closely related to query sequences displayed in bottom right inserts for GH5 and GH28. Scale bars represent a genetic distance of 0.5 for each tree. Details of characterized enzymes within each cluster are shown in Table 3.

Table 1. Effect of Viscozyme® on DM, NDF, ADF disappearance, ammonia-N and volatile fatty acid (VFA) profiles after 12, 24 and 48 h of incubation of barley straw in ruminal batch cultures.

	Control			Viscozyme (0.01%/g of DM)			Viscozyme (0.05%/g of DM)			SE
	12h	**24h**	**48h**	**12h**	**24h**	**48h**	**12h**	**24h**	**48h**	
TGP,mL/g of DM	31.8	62.3	98.1	33.9	64.3	91.6	36.3	69.1	102.8	2.
DMD%	19.5	31.5	40.7	20.8	33.0	41.2	20.7	33.6	41.5	0.
NDFD%	10.0	24.7	35.5	10.9	26.0	35.6	11.2	26.6	36.2	0.
ADFD%	7.4	21.9	32.5	5.9	23.9	33.2	8.6	22.9	34.3	0.
Ammonia-N, mM	8.7	10.2	14.2	8.8	9.8	14.8	9.5	10.6	14.2	0.
Total VFA,mM	26.5	40.1	52.8	27.0	41.0	53.5	27.8	43.0	56.3	0.
Acetate, mM	17.1	24.3	31.5	17.6	25.1	32.4	18.2	26.4	34.0	0.
Propionate, mM	4.6	8.1	11.5	4.6	8.2	11.3	4.7	8.6	12.1	0.
Butyrate, mM	1.9	2.9	3.8	1.9	2.9	3.8	2.0	3.1	4.1	0.
Acetate:Propionate	3.7	3.0	2.7	3.7	3.0	2.8	3.8	3.0	2.8	0.

Table 2: Composition of BN fractionated Viscozyme® bands with high glucose yield as identified by mass spectroscopy.

Band#	**Accession and Description**	**Merged_IPR_desc**	**Score**	**Coverag**
V5	Aspac1_36855|e_gw1.22.30.1	Cellulose-binding region, fungal ; BNR repeat GH 74 endo-1,4-beta-glucanase	22.32	8.19
	Aspac1_26674|e_gw1.5.1170.1	Glycoside hydrolase, family 71	16.62	9.34
V10	Aspac1_48262|Genemark1.10438_g	Glycoside hydrolase, family 28 ; Pectin lyase fold/virulence factor	86.70	25.56
	Aspac1_29881|e_gw1.9.111.1	Pectinesterase, catalytic ; Pectin lyase fold/virulence factor	83.37	34.85
	Aspac1_54785|fgenesh1_pm.18_#_52	Carboxylesterase, type B	47.79	22.19
	Aspac1_32288|e_gw1.13.515.1	Glycoside hydrolase, family 5 ; Glycoside hydrolase, catalytic core	38.76	31.03
	Aspac1_34742|e_gw1.17.128.1	Glycoside hydrolase, family 28 ; Pectin lyase fold/virulence factor	38.32	17.5
	Aspac1_30695|e_gw1.10.837.1	Pectinesterase, catalytic ; Pectin lyase fold/virulence factor	30.31	22.32

	Aspac1_40343|Genemark1.2519_g	Lipase, GDSL ; Esterase, SGNH hydrolase-type	29.12	21.74
	Aspac1_50410|fgenesh1_pm.3_#_264	Glycoside hydrolase, family 7 ; Concanavalin A-like lectin/glucanase	23.54	16.44
	Aspac1_56395|fgenesh1_pg.1_#_622	Aldose 1-epimerase ; Glycoside hydrolase-type carbohydrate-binding	21.96	9.16
V13	Aspac1_50410|fgenesh1_pm.3_#_264	Glycoside hydrolase, family 7 ; Concanavalin A-like lectin/glucanase	53.45	25.78
	Aspac1_54671|fgenesh1_pm.17_#_135	Pectate lyase/Amb allergen ; Pectin lyase fold/virulence factor	37.57	21.37
	Aspac1_48443|Genemark1.10619_g	Lipase, GDSL ; Lipocalin ; Esterase, SGNH hydrolase-type	29.08	18.9
	Aspac1_32288|e_gw1.13.515.1	Glycoside hydrolase, family 5 ; Glycoside hydrolase, catalytic core (mannanase)	24.13	11.94
	Aspac1_46746|Genemark1.8922_g	Glycoside hydrolase, family 28 ; Parallel beta-helix repeat ; Pectin lyase fold/virulence factor	23.40	13.22

CAZyme Family	**GenBank ID**	**Organism**	**Percent Identity to Identified Sequence**	**Characterized Activity**
GH5_7	AAA67426.1	*Aspergillus aculeatus* KSM 510 / MRC11624	100.00%	endo-β-1,4-mannanase
GH5_7	AGW24296.1	*Penicillium oxalicum* GZ-2	69.00%	endo-β-1,4-mannanase
GH5_7	ABF50863.1	*Aspergillus nidulans* FGSC A4	66.31%	endo-β-1,4-mannanase
GH5_7	AGG69666.1	*Aspergillus nidulans* XZ3	65.83%	endo-β-1,4-mannanase
GH7	AAF04491.1	*Aspergillus niger* CBS 513.88	82.67%	cellobiohydrolase
GH28	AAA85279.1	*Aspergillus flavus* 70	83.47%	polygalacturonase
GH28	BAE58737.1	*Aspergillus oryzae* RIB40	83.47%	polygalacturonase
GH28	BAA03244.2	*Aspergillus oryzae* KBN616	83.47%	polygalacturonase
GH28	AAA62349.1	*Aspergillus parasiticus* SU1 / ATCC 163	84.30%	polygalacturonase
PL1_2	ADN00347.1	*Dickeya dadantii* 3937	21.35%	pectate lyase

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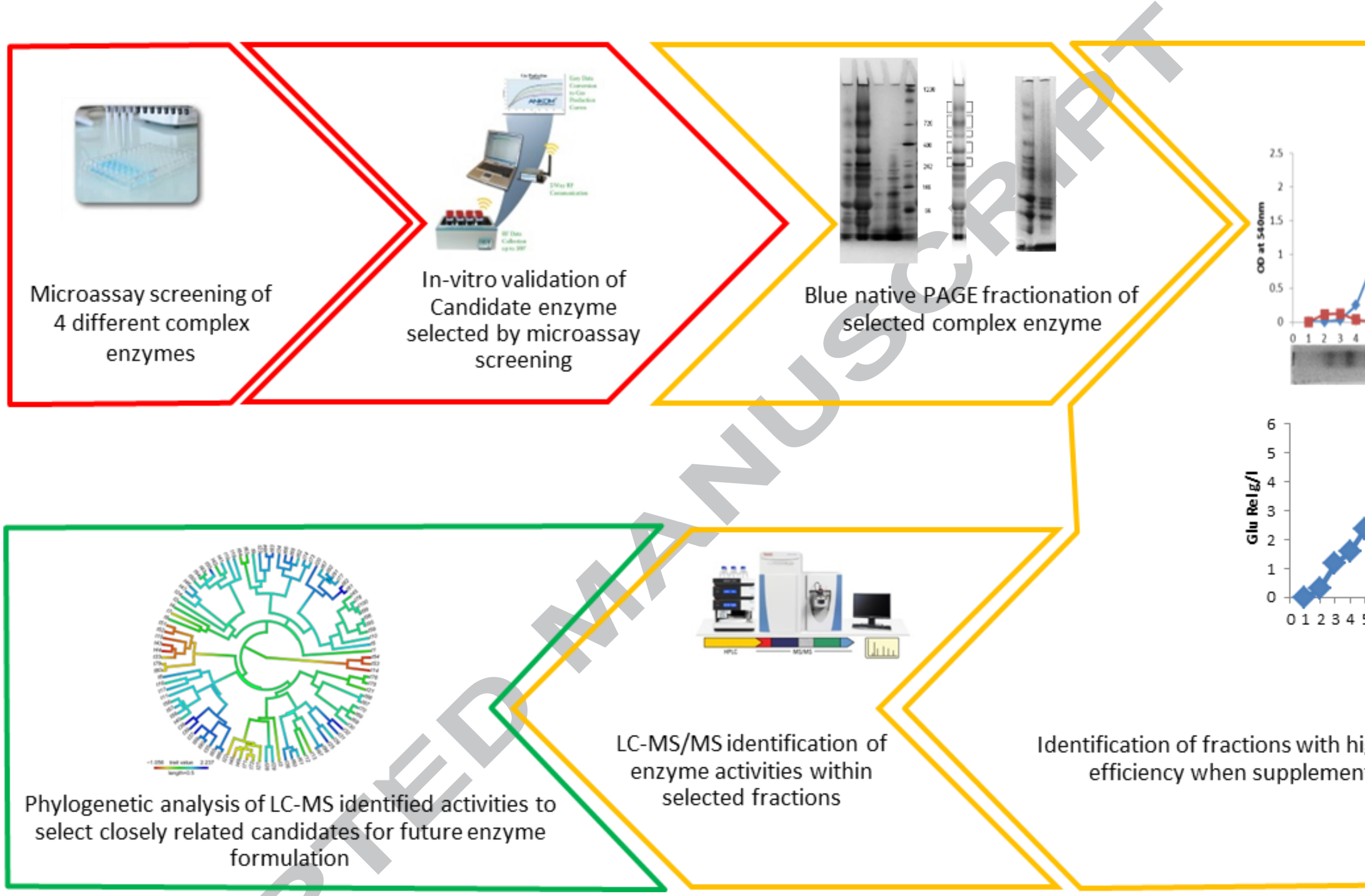
Highlights

- Microassay enabled rapid screening of candidate enzymes.
- *In-vitro* rumen microbial batch cultures validated microassay results.
- BN PAGE fractionation/microassay isolated enzymes linked to enhanced fermentation.
- Mass spectrometric analysis identified enzymes linked to enhanced fermentation.
- Phylogenetic analysis identified candidate enzymes with high additive potential.


Microassay screening of
4 different complex
enzymes
In-vitro validation of
Candidate enzyme
selected by microassay
screening
Blue native PAGE fractionation of
selected complex enzyme
2.5
2
1.5
1
0.5
0
OD at 540nm
0 1 2 3 4
6
5
4
3
2
1
0
Glu Rel g/l
0 1 2 3 4 5
Identification of fractions with hi
efficiency when supplemen
LC-MS/MS identification of
enzyme activities within
selected fractions
Phylogenetic analysis of LC-MS identified activities to
select closely related candidates for future enzyme
formulation

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