Characterization of cellulolytic activity in the gut of the terrestrial land slug *Arion ater*

Ryan Joynson[a], Arvind Swamy[a], Paz Aranega Bou[a], Ambre Chapuis[a], Natalie Ferry[a]

[a]*School of environment and life science, University of Salford, United Kingdom M5 4WT*

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[*]Corresponding author

Dr Natalie Ferry

School of Environment and Life Science, University of Salford

Cockcroft building, 43 The Crescent

Salford, Greater Manchester M5 4WT

T: +44 (0)161 295 3886

n.ferry@salford.ac.uk

Abstract:

The level of cellulolytic activity in different areas of the gut of the terrestrial slug *Arion ater* was assayed at different temperatures and pH values. To do this, crude gut proteins were isolated and assayed using modified dinitrosalicylic acid reducing sugar assay. Crude proteins sample were also separated and cellulolytic activity identified using in gel CMC zymography and esculin hydrate activity gel assays. pH and temperature profiling revealed optimum cellulolytic activity between pH 5.0 and 6.0 for different gut regions and retention of up to 90% of activity at temperatures up to 50 °C . Zymograms and activity gels revealed multiple endoglucanase and β-glucosidase enzymes. To further investigate the source of this cellulolytic activity bacterial isolates from the gut were tested for carboxymethylcellulase and β-glucosidase activity using growth plate assays. 12 cellulolytic microbes were identified using 16s rDNA gene sequencing. These include members of the genera *Buttiauxella, Enterobacter, Citrobacter, Serratia, Klebsiella*. Gut metagenomic DNA was then subjected to PCR, targeting a 400 bp region of the 16srDNA gene which was subsequently separated and individuals identified using DGGE. This identified members of the genera Citrobacter, Serratia, Pectobacterium, Acinetobacter, Mycoplasma, Pantoea and *Erwinia*. In summary multiple glycoside hydrolase enzymes active over a broad range of temperature and pH values in a relatively under studied organism were detected, Indicating that the gut of *Arion ater is* a viable target for intensive study to identify novel carbohydrate active enzymes that may be used in the biofuel industry.

Key words:

Glycoside hydrolase, Slug, Cellulose degradation, Digestive fluids, Cellulolytic activity, Biofuel, Lignocellulose

1. Introduction

Lignocellulose derived from plant cell walls is one of the most abundant organic materials on the planet. The most abundant carbohydrate component it contains is cellulose, made solely of 1 β(1→4) linked D-glucose units. Three enzymes act sequentially to degrade cellulose into simple sugars, endo-β-1,4-glucanases (endocellulases; EC. 3.2.1.4), exo-β-1,4-cellobiohydrolases (exocellulases; EC. 3.2.1.91), and β-glucosidases (EC.3.2.1.21). The glucose monosaccharides produced can then be fermented to produce bioethanol. Use of lignocellulose as a bioethanol feedstock has the potential to overcome many of the economic and environmental consequences of using food crops but lignocellose has an inherent resistance to degradation due to the complexity of the plant cell wall superstructure; current methods require expensive pre-treatments making its use economically unattractive (Cao et al., 2012; Ibrahim et al., 2011). The most promising method for production of bioethanol from lignocellose is the simultaneous sacchirification and fermentation (SSR) method. This method incorporates lignocellulose degrading enzyme cocktails and fermenting microorganisms or fermenting bacteria metabolically engineered to produce high numbers of lignocellulose degrading enzymes, which are used to produce ethanol from lignocellulose feedstocks. These enzyme cocktails produce monosaccharides which are fermented into ethanol by bacteria such as *Escherichia coli* recombinant strains (Cotta, 2012). Many of these modified strains have been engineered to express highly active cellulase enzymes found in other species. A study by Edwards et al. (2011) showed the benefits of introducing a highly active cellobiase enzyme found in *Klebsiella oxytoca* to *Escherichia coli* strain KO11, which resulted in a 30% increase in enthanol production.

Furthermore, cellulase enzymes are also of great importance in the textile industry, in the food industry and as components of detergents, resulting in a high global demand.

To that end there is considerable interest in the potential for microbial enzymes (cellulases, hemicellulases and lignases) to bring about the biological breakdown of lignocellulose. Of particular interest is the scope for degradation by the symbiont microbiota in wood/plant feeding invertebrates. Mutualisms between microbes and insects have been widely studied and are found in almost every case, they facilitate exploitation of many different food sources by host insects, including plant cell walls which are difficult and sometimes impossible for most animals to digest (Watanabe and Tokuda, 2010).However the of the enzymatic contributions of microbes to insect hebivory is still unclear. Some herbivorous insects possess genes encoding plant cell wall degrading enzymes including a termite which produces its own cellulase (Watanabe et al., 1998), but the overall structural complexity of the plant cell wall superstructure requires a multitude of enzyme classes which gut microbes contribute to. It is therefore thought that the interactions of host and microbe has had a direct impact on the evolutionary transitions in diet in many herbivorous eukaryotes , including insects (Hansen and Moran, 2014). Enzymatic activity has been studied extensively in the digestive fluid of various insects including members of the orders *Isoptera (*Konig *et al., 2013), Coleoptera (*Dojnov *et al., 2013) and Othoptera (*Shi *et al., 2011),* all of which have a high lignoceullose diet. However, this focus on arthropods has been at the expense of other groups such as gastropods. Specifically, there has not yet been a definitive characterisation of the origin of cellulolytic activity in the gut of the common garden slug, *Arion ater,* a significant pest throughout Europe. The diet of the slug is extremely varied depending on location and food availability, including fungi, earthworms, leaves, plant

stems along with dead plant material with a preference for young leaf/stem plants. *A. ater* uses its barbed tongue like appenditure called the radula, which contains up to 27,000 teeth, to shred its food. This increases the surface area of its food for enzymatic degradation. The radula also allows the slug to eat even the toughest plant material in times where food is scarce. Due to the large portion of plant material in its diet, it is logical that the gut contains multiple enzymes which allow it to digest plant cell wall material into utilizable simple sugars. The *A.Ater* gut is particularly interesting as a potential source of active enzymes given the variation in pH along its digestive tract and its ability to eat twice its body weight in vegetation per day. This efficiency in crop degradation has led to more than £30 million pounds a year being spent on slug pellets in the UK alone and a ~70 fold increase in utilization of molluscicides over 3 decades (Agular and Wink, 2005). Consequently, we have carried out in-depth analysis of the cellulolytic activity and associated microbial community of the terrestrial gastropod *A. ater*.

2. Materials and methods

2.1 Slug collection and dissection

Slugs were collected from a suburban area in North Cheshire (53.391463 N, 2.211214 W) 2 hours after last light. Individuals were allowed to feed on celery/lettuce cores for 12 hours. Individuals were cooled to 4 °C prior to dissection to reduce metabolism and spontaneous mucus production during dissection. Whole gut tracts were removed, avoiding rupture that would result in loss or contamination of gut juices. Mucus that might interfere with the assays was removed by blotting. Total guts were further separated into ‘crop’ which denotes the region from the mouth up to and including the digestive gland and the ‘gut’ which corresponds to the gut after the stomach/digestive gland up to the anus (Fig. 1).

2.2 Initial detection of total cellulolytic activity

Gut samples were cut up using a scalpel in a petri dish and then homogenised with a sterile glass rod in a 1.5 mL tube containing 200 µL of 0.2 M sodium acetate buffer (pH 5.2) followed by vigorous vortexing,. To clear cell debris and food matter, samples were centrifuged at 13.3 Krpm for 5 minutes. Supernatants were extracted, pooled (subsequently referred to as 'crude protein samples') and stored at -80 °C. Protein content of the crude samples was estimated using a standard Bradford assay (Bradford, 1976) using BSA to construct the standard curve. Total cellulase activity was measured using the dinitro salysilic acid (DNSA) cellulase assay of (Ghose, 1987) with slight adjustments. This assay allows the detection of cellulolytic enzymes which hydrolyse cellulose internally or externally along with the breakdown of cellobiose, each of these actions produces reducing sugar free carbonyl groups which are measured in this assay. The cellulolytic activity of 50µl of crop and gut samples were tested by mixing 1% carboxymethyl cellulose (CMC) (Sigma Aldrich) in a 100mM sodium citrate buffer (pH 4.5). Samples were incubated at 50 °C for 30 minutes. Reactions were terminated by placing samples on ice, adding DNS reagent and heating to 95 °C for 10 minutes to allow colour development. All samples were tested and boiled simultaneously. Samples were cooled to room temperature and absorbance read at 540 nm using a CMC control sample as a blank. Correction for background sugars in the sample was undertaken by subtracting a time 0 duplicate sample absorbance from the final result. All activities in this paper are given in enzyme units, where 1 U is equal to 1 µM glucose released per minute per mg of protein.

2.3 pH and temperature profiling of crude protein cellulolytic activity

The cellulase detection assay previously described was modified to measure the pH profile of the crude protein cellulolytic activity against CMC, replacing the pH 4.5 buffer with 100mM sodium citrate buffers ranging between pH 4-9 while all other conditions remained the same. To determine the temperature profile of the crude protein sample, the assay was modified by varying incubation temperature between 20 °C and 70 °C.

2.4 Identification of endocellulases using CMC SDS PAGE zymography

CMC Zymography was carried out following the procedure of Schwatz (1987) and Willis *et al* (2010). Samples were ran using a 12% acrylamide SDS gel containing 0.2% CMC as a substrate for activity staining. Before polymerisation was induced, solutions were heated to 30 °C and CMC was added slowly to the resolving gel mixture. Gels were allowed to polymerize for 2 hours and used the same day. Crop and gut crude protein samples were thawed on ice followed by addition of a modified Laemmli loading buffer (minus denaturants). Samples were then heated to 80 °C for 10 minutes followed by pulse centrifugation to denature proteins and prevent substrate digestion during electrophoresis. Size determination and separation was conducted by using 50 µg of each crude extract along with 15 µL of SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). Gels were run at a constant 100 V for 4 hours 30 minutes. For size estimation, the distances travelled by the pre-stained standard bands were measured prior to incubation/staining steps which cause the standards to become difficult to visualise, estimated Mw of bands is indicated on gels by an arrow at appropriate position. The CMC gel was washed in a 5% tritron X-100 solution for 30 minutes (repeated 5 times) to remove SDS. The gel was then rinsed with distilled water, placed in sodium phosphate buffer (50 mM, pH 6.5) and incubated for 2 hours at 4 °C to exchange the buffer system and allow renaturation of proteins in the gel. Phosphate buffer

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was refreshed and the gel was then incubated at 37 °C overnight. Following incubation, the gels were stained with 0.1% (w/v) Congo red for 1 hour, and then destained with a 1 M sodium chloride solution for 3 hours. To enhance visualisation of clear zones acetic acid was added drop wise to the NaCl solution containing the gel, turning the Congo Red from red to a deep purple.

2.5 Identification of β-glucosidase enzymes using esculin hydrate – ferric ammonium citrate Native PAGE activity gel

A 12% native tris-glycine PAGE gel was created using a standard protocol. Native loading buffer was added to crop and gut crude protein extracts and 50 µg of each was loaded on the gel. Gels were run at 100 V for 4 hours. The gel was then placed in a 0.2 M sodium acetate buffer (pH 5.5) for 10 minutes to exchange the buffer system, then the gel was placed in a 0.2 M sodium acetate buffer (pH 5.5) containing 0.1% (w/v) esculin hydrate (Sigma) and 0.03% (w/v) ferric ammonium citrate (Sigma) and incubated for 3 hours at 37 °C to allow in gel hydrolytic activity. Where β-glucosidase enzymes are present esculin is cleaved producing esculitin which goes onto react with ferric iron to produce a black precipitate. To stop the reaction, the gel was placed into a 10% glucose solution.

2.6 Identification of culturable cellulolytic microbes using esculin and CMC LB agar plate assays

Whole guts were extracted as previously described and homogenised in 500 µL of 1 quarter strength Ringer solution. A range of dilutions was placed on LB agar plates containing 0.5% CMC and grown overnight at 25 °C. Replica plates were created and incubated for a further 24 hours. This prevents false identification of cellulolytic bacteria through clearance zones

caused by extracellular endoglucanase enzymes in the plated gut fluid. Replica plates were stained with a 0.1% Congo red solution for 1 hour, followed by destaining with 1 M NaCl for a further hour. Colonies corresponding with zones of clearance were isolated from replica plates, grown overnight in lb broth. Isolates were then plated onto lb agar containing 0.1% esculin and 0.03% ferric ammonium citrate and incubated at 25 °C for 3 hours to confirm β-glucosidase activity. Isolates were identified using 16s rDNA PCR using primers 8F (5'-AGAGTTTGATCCTGGCTC-3') and 1512R (5'-ACGGCTACCTTGTTACGA-3'). Each amplified PCR product was sequenced using Sanger sequencing system big dye v3.1. Sequences were searched using BLASTn for matches in the 16s rDNA database.

2.7 Culture independent microbe identification using DGGE analysis

Other members of the *A. ater* gut community were identified using denaturing gradient gel electrophoresis (DGGE). Metagenomic DNA was extracted from a whole gut using a modified version of the Meta-G-nome DNA isolation kit protocol (Epicentre) and extracted DNA was subjected to PCR targeting a 400 bp region of the 16s rDNA using primers F984GC and R1378 according to Heuer *et al* (18). PCR products were separated by sequence variation using a 30-60% gradient of urea and formamide in a polyacrylamide gel, using the protean 2 system run at a constant 100 V for 16 hours at 60 °C. Gels were stained with Gel Red™ (Biotium, Inc.) and individual bands were excised and placed into wells of a 1% agarose gel and electrophoresed into agarose. Bands were then extracted using the Wizard gel extraction kit (Promega) and sequenced using big dye v3.1. Sequences were submitted to BLASTn for bacterial identification against the 16s rDNA database.

3. Results

3.1 Measurement of cellulolytic activity in *A. ater* gut samples

Total cellulase activity in the crop and gut regions (Fig. 1) of *A. ater* were assayed (Fig. 2A). Cellulase activity was observed in both the gut and crop with the crop portion showing the highest activity at 1.57 U/mg of protein and the gut showing 1.11U/mg of protein.

3.2 Temperature and pH profiling of total gut cellulolytic activity

Both gut and crop samples showed resilience to heat up to around 50 °C at which point activity begins to decline, with both crude samples showing greatest activity at 30-35 °C (Fig 3A). The pH profiles for the two samples were however quite distinct, with the crop samples showing greatest activity at pH 5 and gut at pH 6 (Fig. 3B). At pH values higher than 6.5 the activity of both samples begins to decline up to pH 9 at which point activity is ~4 fold lower than at optimum pH for each sample.

3.3 CMC zymography and esculin hydrate activity gel assays

Due to the differences seen in the crop and gut cellulolytic activity profiles, CMC zymography (Fig. 2B) and esculin hydrate activity gel assays (Fig 2C) were carried out in order to identify whether or not similar enzyme systems were being incorporated in the crop and gut digestive juices. In CMC zymograms we observed almost identical cellulose activity patterns. We observed 3 main bands in both crude samples, corresponding to proteins of approximately 103, 58 and 22 kDa in size. The β-glucosidase activity gels showed three bands at positions 1, 2 and 3 (indicated with black arrows) which appear to be at identical locations in the gel for both the gut and crop samples.

3.4 Identification of cellulolytic microorganisms

To gain an understanding of the origin of at least a portion of the cellulolytic activity seen in this study, gut microorganisms were isolated and tested for cellulolytic activity. Microbial isolates were grown on agar containing CMC and on agar containing ferric ammonium citrate and esculin hydrate to identify endoglucanase (Fig 4B) and β-glucosidase (Fig. 4A) respectively. 12 isolates showed both endoglucanase and β-glucosidase activity, including members of *Aeromonas, Acinetobacter, Buttiauxella, Citrobacter, Enterobacter, Klebsiella, Kluyvera, Salmonella* and *Serratia* (Table 1). Only 4 of these microbes could be identified to within 97% similarity of bacterial 16s rDNA genes in the NBCI 16s rDNA and NR databases while the remaining 8 were seen to have between 96-79% similarity to database entries. Subsequently, a DGGE study was carried out to identify microbes that might be present but which may be less easy to culture, using metagenomic DNA samples as templates for 16s rDNA targeted PCR (Fig. 5). This revealed multiple bands from which DNA was extracted and sequenced. Nine further microbes were identified, from the genera *Citrobacter*, *Serratia*, *Pectobacterium*, *Acinetobacter*, *Mycoplasma*, *Pantoea* and *Erwina* (Table 1). Sequences for cultured and uncultured 16s rDNA studies can be seen in supplementary file 1.

Discussion:

This study has further characterized the cellulolytic activity in the gut of *A. ater* through biochemical testing of different portions of the gut, along with identification of multiple cellulolytic microorganisms and thus we begin to characterize the *A. ater* gut microbiome. Cellulase activity assays showed the overall cellulolytic activity in the gut of *A. ater* found in the North of England to be greater than that of many insects (Oppert et al., 2010), including members of the genera Coleoptera, Isoptera, Orthoptera and Diptera. We also demonstrate relative stability across a wide pH and temperature range, with optimal activity at pH values

that would be feasible for use in modern industrial lignocellulose degradation methods. A separate investigation of the cellulolytic activity of *A. ater* of North American origin by James et al. (1997) showed higher overall cellulolytic activity than in this study, but with an optimal pH of 7 as opposed to the crop optimum of pH 5 observed here. A possible reason for this observed difference in optimal pH is the native environment from which individuals were taken, with the average soil pH for the area of North Cheshire being <5.0, whereas in Bellingham WA, the soil is at a pH of between 6-6.6, each correlating with the optimal pH values observed. Acidic environments have been observed in multiple land Pulmonates such as *Helix aspersa*, (6.1-7.4) *Helix pomatia* (5.5-6.4), *Elona quimperiana* (5.3-6.6) (Charrier and Brune, 2003) and *Pomacea canaliculata* (6.0-7.4) (Godoy et al., 2013) which would suggests that members of this class harbour dietary enzymes that can function in acidic environments, including *A. ater*, as we have observed. Also, the cellulolytic systems appear to have varying temperature profiles, with our study showing crop and gut samples retaining 90% and 85% activity respectively at 50 °C while the study of the North American species shows practically no activity against CMC in the same conditions. It is also important to note that the gut microbiome is a very dynamic environment which can be heavily altered by living in a different habitat, this has been demonstrated not only in humans (Huttenhower et al., 2012), but also in insects (Dillon and Dillon, 2004). The temperature profile we observed shows the crude enzyme extracts retain much of their activity even at 50 °C and demonstrates no clear optimum temperature. However this is not surprising when the complexity of the crude mixture is taken into account, as having multiple enzymes of different microbial origin would cause there to be variation in optimum temperatures for activity for cellulase enzymes of different glycoside hydrolase groups and, furthermore even within groups.

Using modified cellulase zymograms and esculin hydrate activity gel assays we have also identified three highly abundant individual endocellulase and β-glucosidase enzymes present in both the crop and gut juices, thereby demonstrating that a very similar cellulolytic system throughout the gut and therefore suggesting little activity compartmentalization throughout the gut regions. It is also important to take into consideration that the minimum detectable amount of active enzyme in the esculin hydrate activity gel assay is relatively low at >10 ng (Kwon et al., 1994). Our discovery of multiple endoglucanase and β-glucosidase producing bacteria suggests that there are much greater number of individual cellulolytic enzymes present than we observed in our gel methods. The individual microbes isolated may not make up a high enough proportion of the gut microbiome to produce their enzymes in sufficient abundances to be detectable using in gel separation methods.

Our study also confirmed that at least a portion the cellulolytic activity seen in the gut of *A. ater* is due to symbiotic activity of gut microbes and, for the first time, isolated and identified individual cellulolytic microbes. Many studies have carried out growth plate assays successfully, quickly and accurately isolating gut cellulolytic microbes from gastropods (Antonio et al., 2010), insects *(Huang et al., 2012)* and mammals *(Ruijssenaars and Hartmans, 2001)*. CMC and esculin hydrate activity growth plate assays allowed us to identify 12 cellulolytic gut microbes, only 4 of which could be identified with great confidence (>97% similarity). This strongly suggests that the A. *ater* gut microbiome contains uncharacterized microbes with uncharacterized cellulolytic systems that we have shown to have robust pH and temperature activity profiles. In the non-culture based DGGE study we identified 9 further microbes of which *Pectobacterium carotovorum, Erwinia amylovora* and

Erwinia tasmaniensis species all have cellulolytic enzymes linked to their species in the NCBI database (http://www.ncbi.nlm.nih.gov/). In this study we have identified a high number of memebers of the gut belong to the G*ammaproteobacteria* class, with only two *Mycoplasma* species being from outside that class. The microbes *Klebsiella pneumonia*, *Citrobacter freundii* and *Serratia liquefaciens* have also been identified in the gut of the *Bombyx mori* larvae (silk worm) and their cellulolytic activity was also observed (Anand et al., 2010). Multiple *Enterobacter* species, the species Salmonella enterica and serratia marcescens have also been identified in the gut of beetle larvae during their development (Azambuja et al., 2004; Butera et al., 2012). Further to this, a metagenomic study into the gut microbiome of the giant African Snail interestingly shares all but one of the microbial species identified here (Cardoso et al., 2012), this suggests that there may be a set of gut microbes on which multiple land gastropods rely to aid their digestion of lignocellulose. This also indicates that the gut microbe host interaction could have played an important role in the evolutionary dietary transitions of land gastropods as it is thought to have in insects (Hansen and Moran, 2014).

Gastropods have not been the main focus of recent cellulase prospecting using modern methods due to the initial successes with the insect families, specifically in termites (Tokuda and Watanabe, 2007) but also in beetles (Wei et al., 2006b) (Wei et al., 2006a) and grasshoppers (Oppert et al., 2010) (Willis et al., 2010). However the recent study into the microbiome of the giant African snail has identified thousands of glycoside hydrolase enzymes and carbohydrate binding modules of microbial origin (Cardoso et al., 2012). Our findings and these promising results from related species give a strong indication that the gut of *A. ater* is a viable target for more intense study to identify individual novel, plant cell

wall degrading enzymes which may be key to improving contemporary biochemical methods in the biofuel industry. In addition, further understanding of the essential biochemical pathways involved in slug feeding could be used to develop more target-specific pest control measures for slugs. Here for example, the identification of these different classes of enzymes demonstrates that the slug gut has the capability to digest the cellulose portion of its diet from long polymer cellulose to individual, utilizable, glucose monosaccharides. This therefore confirms that the slug has the ability to efficiently utlilize the cellulose portion of plant matter it consumes as a source of carbon and we have also identified that gut microbes play a significant role in making this glucose accessible. Increases in physiological understanding are especially important given the detection of high levels of the generic slug pellet poison metaldehyde in water in the UK (Kay and Grayson, 2013) and the recent European Union regulation , which imposes a complete ban on sales of traditional slug pellets by 19th September 2014 (Commission Implementing Regulation 187/2014).

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Figures and tables

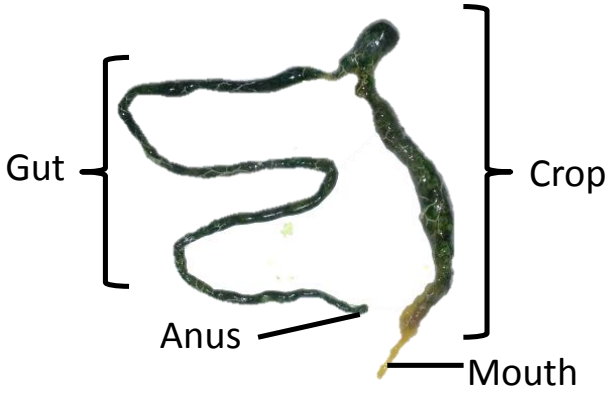
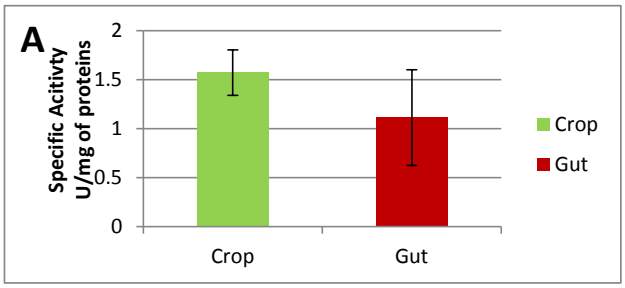


Figure 1 Dissected whole gut tract of *Arion ater*



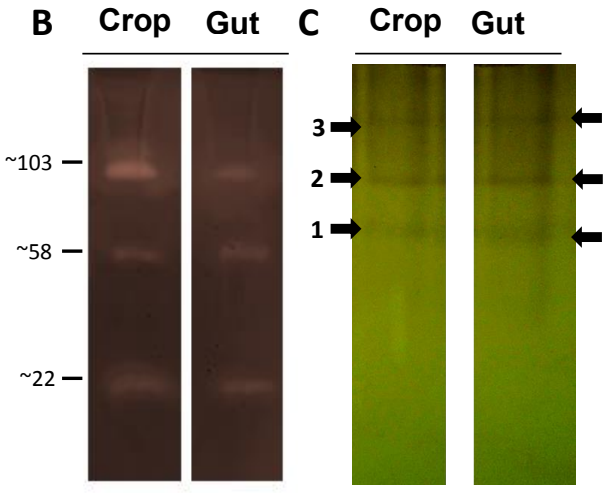


Figure 2 (A) Total specific cellulolytic activity seen in the gut fluids from *Arion ater* against CMC at 50°C and pH 5.0 using the DNSA cellulase activity assay. (B) A 12% SDS PAGE 0.2% CMC zymogram using 50ug of crude gut and crop protein per lane. Gel stained with Congo red to allow activity visualisation. (C) A 12% native PAGE gel containing 100ug of protein per lane, gels were incubated in a 0.2m sodium acetate activity buffer containing 0.1% (w/v)

esculin and 0.03% w/v ferric ammonium citrate for one hour. Black precipitate show areas of activity, indicated by black arrows.

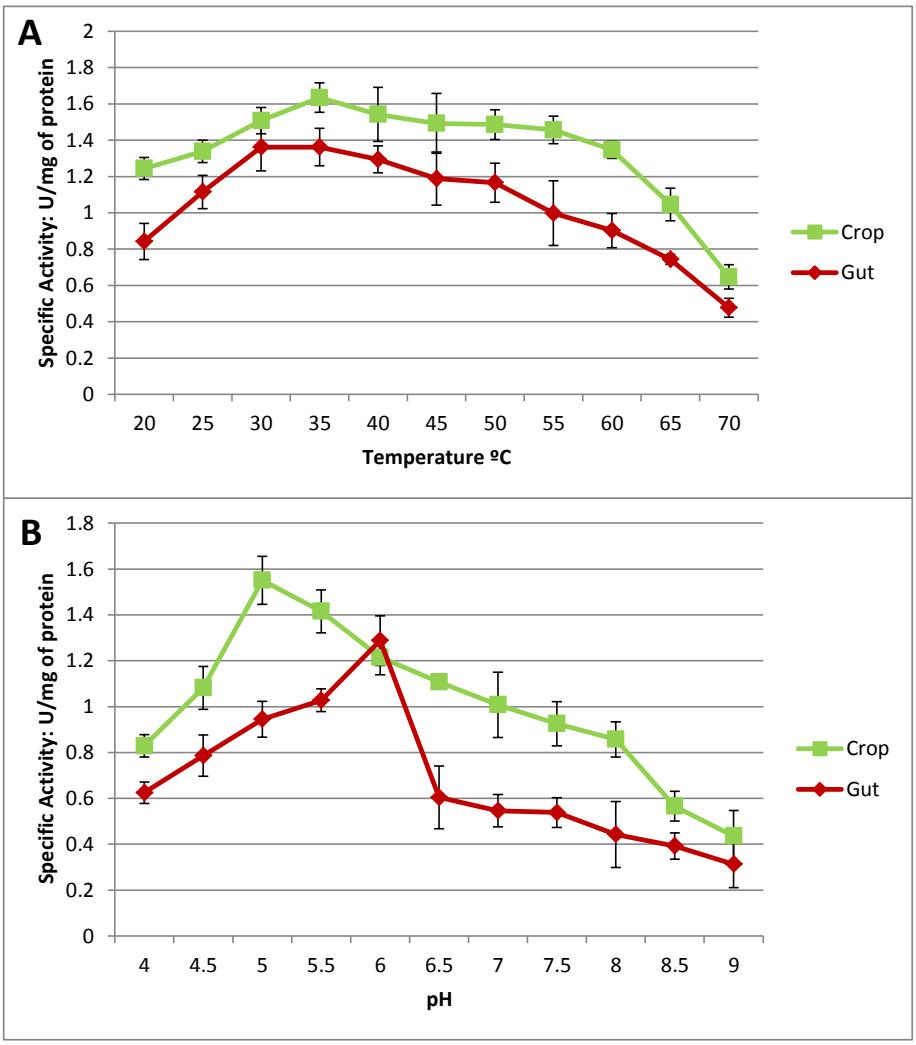


Figure 3 The temperature profiles (A) and the pH profiles (B) of the two crude gut protein isolations showing the total cellulolytic activity of each sample against a CMC substrate.. Temperature and pH profiles were obtained using a modified cellulase assay with incubation steps at temperatures between 20-70ºC and at pH values 4-9 respectively. Specific activity shown as enzyme units (U) where 1 U is equal to 1 µM glucose released per minute per mg of protein.

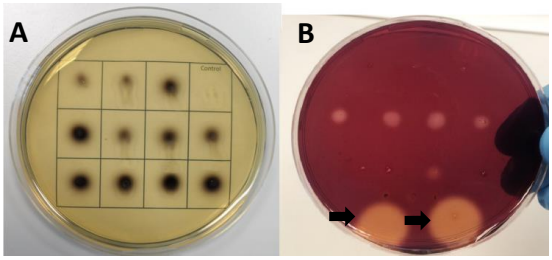


Figure 3~~4~~ (A) An esculin hydrate plate assay demonstrating the β-glucosidase activity of microbial isolates. Isolates were grown on agar plates containing 0.1% (w/v) esculin and 0.03% (w/v) ferric ammonium citrate. A black precipitate indicates β-glucosidase activity. Untransformed top10 *E. coli* (Invotrogen) was used as a negative control. (B) A CMC plate assay showing endoglucanase activity. Bacterial isolates were grown on agar plates containing 0.5% CMC after 16 hour incubation plates were stained with congo red and destained with 1 M NaCl in order to visualise zones of clearing. 5 and 10 µL of 1 mg/mL *A.*

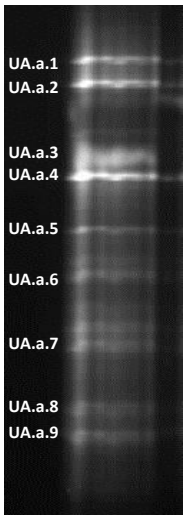


Figure 4~~5~~ Differential gradient gel electrophoresis gel, 30-60% gradient of formamide and urea. Labels show bands from which successful microbial identifications were deduced.

Name	Description	E-value	Identity	Accession
CA.a.1	*Acinetobacter calcoaceticus*	0	92%	NR_042387.1
CA.a.2	*Aeromonas hydrophila*	0	99%	NR_104824.1
CA.a.3	*Buttiauxella agrestis*	0	79%	DQ440549.1
CA.a.4	*Buttiauxella agrestis*	0	99%	NR_041968.1
CA.a.5	*Citrobacter braakii*	0	85%	NR_028687.1
CA.a.6	*Citrobacter freundii*	0	99%	NR_028894.1
CA.a.7	*Enterobacter sp. E6-PCAi*	0	94%	JN853247.1
CA.a.8	*Klebsiella pneumoniae*	0	96%	NR_037084.1
CA.a.9	*Kluyvera intermedia*	0	99%	KF724024.1
CA.a.10	*Salmonella enterica*	0	91%	NR_044371.1
CA.a.11	*Serratia liquefaciens*	0	86%	GU586145.1
CA.a.12	*Serratia marcescens*	0	91%	NR_036886.1
UA.a.1	*Mycoplasma hyorhinis*	1.00E-158	93%	NR_041845.1
UA.a.2	*Mycoplasma iners*	4.00E-158	93%	NR_025064.1
UA.a.3	*Uncultured Citrobacter*	0	99%	AY847172.1
UA.a.4	*Uncultured Serratia*	0	100%	KC253894.1
UA.a.5	*Pectobacterium carotovorum*	0	99%	NR_041971.1
UA.a.6	*Acinetobacter beijerinckii*	0	98%	NR_042234.1
UA.a.7	*Pantoea sp. 57917*	0	99%	DQ094146.1
UA.a.8	*Erwinia amylovora*	0	99%	NR_041970.1
UA.a.9	*Erwinia tasmaniensis*	0	99%	NR_074869.1

Table 1 NCBI BLASTn search results for each amplified 16s rDNA gene from cultured cellulolytic microbes (CA.a.*) and for uncultured microbes from the DGGE study (UA.a.*). Sequences were queried against the NCBI 16s rRNA database or the nr database if no match was found.