microbial biotechnology

Enzyme biotechnology development for treating polymers in hydraulic fracturing operations

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Summary

Carboxymethyl cellulose (CMC) is a polymer used in many different industrial sectors. In the oil and gas industry, CMC is often used during hydraulic fracturing (fracking) operations as a thickening agent for effective proppant delivery. Accumulations of CMC at fracture faces (known as filter cakes) can impede oil and gas recovery. Although chemical oxidizers are added to disrupt these accumulations, there is industrial interest in developing alternative, enzyme-based treatments. Little is known about CMC biodegradation under fracking conditions. Here, we enriched a methanogenic CMC-degrading culture and demonstrated its ability to enzymatically utilize CMC under the conditions that typify oil fields. Using the extracellular enzyme fraction from the culture, significant CMC viscosity reduction was observed between 50 and 80°C, at salinities up to 20% (w/v) and at pH 5–8 compared to controls. Similar levels of viscosity reduction by extracellular enzymes were observed under oxic and anoxic conditions. This proof-of-concept study demonstrates that enzyme biotechnology holds great promise as a viable approach to treating CMC filter cakes under oilfield conditions.

Introduction

Cellulose is a versatile polymer that is widely used for a variety of applications in multiple sectors including in the

food, pharmaceutical, biomedical and alternative energy (e.g. biofuel) industries (Lavanya *et al.*, 2011; Weiner and Kotkoskie, 1999). Cellulose is insoluble in water due to the strong inter- and intramolecular interactions of the hydrogen bonds between the polymer chains (Hinterstoisser and Salmén, 1999). Many chemical modifications of cellulose have been investigated for their abilities to create new cellulose derivatives with properties suitable for different industrial applications (Lavanya *et al.*, 2011). One of these cellulose derivatives is carboxymethyl cellulose (CMC), a polymer wherein the hydroxyl groups on the glucopyranose units of cellulose are randomly replaced by carboxymethyl groups that render CMC water-soluble and more chemically reactive (Lavanya *et al.*, 2011).

In the oil and gas industry, CMC is used as a gelling or thickening agent (also known as a viscosifier) that is added to water-based fracturing fluids to optimize proppant delivery into fractures created during hydraulic fracturing processes to keep the fissures open (Harris, 1988; Fink, 2013; Barbati *et al.*, 2016). Over the years, different types of gelling agents, including guar gum and CMC, have been used by the energy industry depending on their availability and market pricing (Fink, 2013; Azizov *et al.*, 2015). A recent study highlighted the sustainability of using CMC as the polymer of choice (compared to guar gum, for example) to deliver proppant to the fractures (Azizov *et al.*, 2015). CMC has also been proven to be an effective substitute to guar gum in fractured wells due to its better stability at high temperature (up to 90°C), a better regain of conductivity after its degradation, and for superior proppant suspension (Aliu, 2017).

Though useful as gelling agents, polymers such as CMC can accumulate on the face of a rock fracture, eventually creating a filter cake that can impede oil and gas flow, thus reducing recovery if left untreated (Prud'homme and Wang, 1993). One subsurface phenomenon leading to filter cake formation is leakoff, wherein water from the fracturing fluid adsorbs into the surrounding rock matrix. This process can shear the polymer into smaller molecular weight molecules, allowing them to absorb partially into the rock and also allowing for polymer accumulation at the rock surface, resulting in fracture face blockage (Charoenwongsa et al., 2013).

The hydraulic fracturing industry frequently relies on chemical oxidizers to degrade filter cakes (Smith and Montgomery, 2015). Although highly efficient, the use of
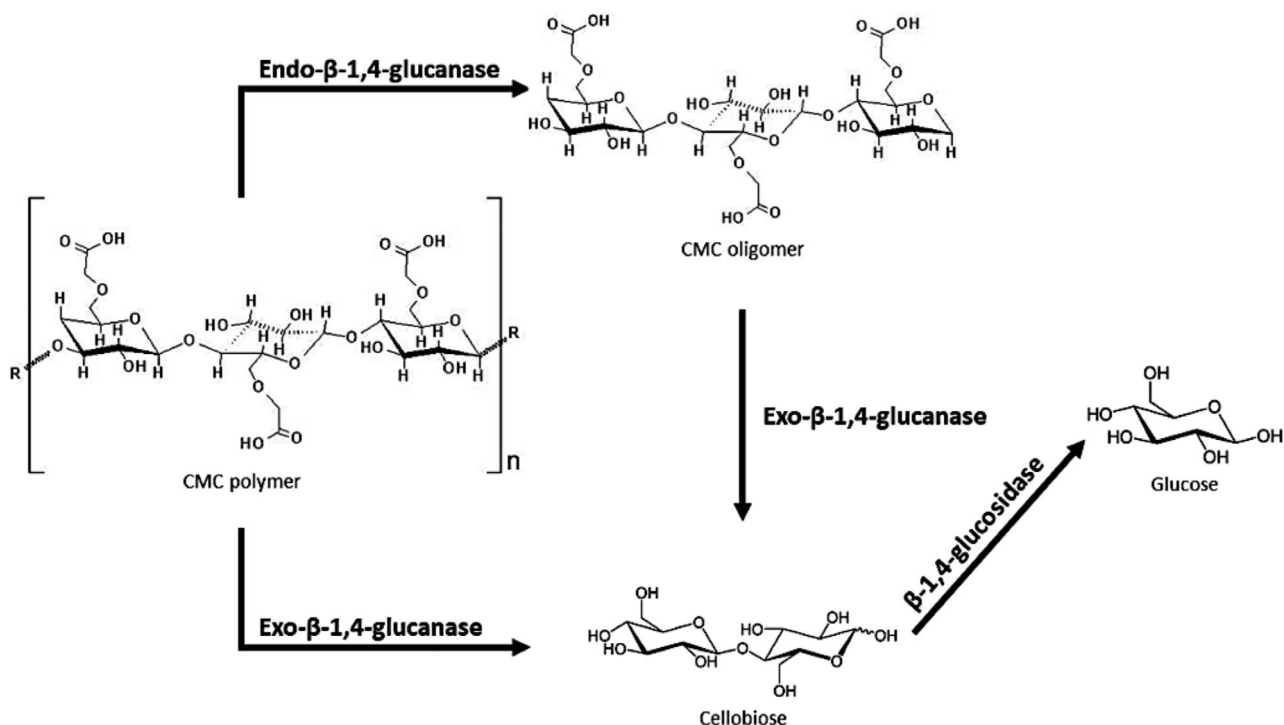
Fig. 1. Biodegradation pathway of carboxymethyl cellulose.

chemical oxidizers has several drawbacks. First, the addition of oxidizers can be coupled with the addition of acids such as hydrochloric acid which can lead to corrosion of oilfield equipment (Freeman *et al.*, 2005). Second, oxidizers do not react with specific substrates, potentially leading to undesirable reactions such as with iron to create equipment-damaging iron oxides (Chopade et al., 2015; Freeman *et al.*, 2005). Third, high concentrations of chemical oxidizers are necessary to ensure treatment efficacy, and these high chemical concentrations are poorly soluble in solution (Chopade et al., 2015; Freeman *et al.*, 2005).

These potential drawbacks can be addressed by using enzymes as biotechnological replacements for chemical oxidizers during the filter cake removal process. Enzymes do not require the addition of strong acids to be efficient, therefore minimizing corrosion. Also, they are highly specific and will react only with the substrate in question. Enzymes are highly active, and thus, significantly smaller quantities are required compared to chemical oxidizers for comparable levels of filter cake removal (Chopade et al., 2015; Freeman *et al.*, 2005; Gupta and Prasek, 1995). The enzymatic treatment of guar gum-based filter cakes has found success in the hydraulic fracturing industry (Politz *et al.*, 2000).

Cellulose (and related derivatives such as CMC) is hydrolysed by three different enzymes: (i) endo-β-1, 4-glucanase, (ii) exo-β-1,4-glucanase and (iii) β-1,

4-glucosidase (Fig. 1). The endoglucanase randomly hydrolyses the backbone of the polymer, resulting in the formation of cellobiose, cellotriose and other oligomers.

The rapid decrease in polymer chain length is responsible for the concurrent reduction in viscosity observed in these systems (Martinez-Richa, 1998; Walker and Wilson, 2003). The exoglucanase hydrolyses the ends of the oligomer polymer chains, resulting in the formation of cellobiose, which can then be further hydrolysed by the β-1,4-glucosidase to form glucose (Walker and Wilson, 2003).

Although cellulose metabolism has been widely studied in the context of biofuel production (Brown, 2015; Yang *et al.*, 2015; Lusk *et al.*, 2017), and enzymes targeting other gelling agents (guar gum) have been developed (Tjon-Joe-Pin, 1993; Cheng and Prud'homme, 2000; Politz *et al.*, 2000; You *et al.*, 2016), little information exists for the breakdown of CMC in the context of filter cake removal. As hydraulic fracturing is widely used for oil and gas recovery from tight subsurface reservoirs, and CMC can be used as a gelling agent in this process (Trabelsi and Kakadjian, 2013; Azizov *et al.*, 2015), we aimed here to develop an enzyme-based solution for treating CMC filter cakes that may form during hydraulic fracturing. Following the establishment of a CMC-degrading microbial consortium and confirmation of its enzymatic activity, we tested the properties of the CMC-utilizing enzymes under different pH, temperature and

salinity conditions that can characterize produced oilfield fluids associated with hydraulic fracturing operations. This proof-of-concept study shows strong potential for the use of enzyme-based breaker technology to treat CMC filter cakes.

Results and discussion

CMC-degrading methanogenic enrichment

After testing the different environmental inocula and isolates for their ability to metabolize CMC as a sole carbon source under oxic and/or anoxic conditions at 50°C, the methanogenic cattle manure enrichment showed the most significant degradation of CMC in the shortest amount of time compared to sterile and CMC-free controls (results not shown). For this reason, this enrichment culture was selected for all further work described herein to study the degradation of CMC under thermophilic conditions and to characterize enzymatic activity.

Due to the presence of numerous carbon sources presumed to be present in the initial manure sample, seven successive transfers were required to establish a methanogenic microbial community wherein CMC was the sole carbon source. CMC-utilizing cultures were established under methanogenic conditions because deep subsurface oil and gas reservoirs are anoxic and dominated by methanogenic microbial communities (Head *et al.*, 2014); thus, developing enzyme technology that can be applied under these conditions was crucial. Manure was chosen as the environmental source for establishing the CMC-degrading culture because there is a known symbiotic activity between cellulose degraders and methanogens in cattle rumen (Hook *et al.*, 2010) and, hence, in manure. The initial culture, where manure was added directly to minimal medium, reached its maximum methane production after 8 days with a production of 4862 μM CH_4 (Fig. S1). The corresponding CMC-free control also showed high initial methane values, reaching 2387 μM CH_4, suggesting the methanogenic conversion of other carbon sources present in the initial inoculum. Following the seven transfers, methane production was no longer evident from the CMC-free controls compared to the CMC-amended enrichment which produced 155.5 ± 9.2 μM CH_4 after 70 days of incubation. Stoichiometric predictions using the Buswell equation (Symons and Buswell, 1933) showed that 7.3% of CMC was converted to methane as one of the products formed during CMC hydrolysis. This calculation was done using the molecular weight value of CMC from a commercially available source (Sigma-Aldrich, Oakville, ON, Canada) since the molecular weight of the industrially sourced CMC could not be divulged for confidentiality reasons. A study done by Miller et al. (2002) showed that cellulose addition to cattle manure enrichments

resulted in a high production of volatile fatty acids in addition to methane. In our methanogenic consortium, we speculate that CMC degradation resulted in similar products, with only a small amount of the CMC carbon being converted to methane by methanogens. Regardless of the products formed by the methanogenic community, significant viscosity reduction was observed compared to sterile controls, suggesting a high level of CMC hydrolysis (see results below).

Once a CMC-degrading culture was established, several tests were done to definitively demonstrate CMC biodegradation and enzyme activity by the culture. Trypan blue staining was first carried out to indicate CMC hydrolysis. Within the first 6 days of incubation of the CMC-degrading culture, cultures changed from a dark purple/blue colour to completely clear, while sterile and CMC-free controls remained stained (Fig. S2). The control results indicated that Trypan blue was not abiotically degraded and that the microbial community was not metabolizing the Trypan blue itself, suggesting that all observed colour changes were due solely to enzymatic CMC hydrolysis. Thus, this staining result indicated that the culture hydrolysed CMC within 6 days to form smaller intermediates.

As a second line of evidence for CMC metabolism by the enrichment culture, enzyme activities for the endo-, exo-β-1,4-glucanases and β-1,4-glucosidases were measured using p-4-nitrophenyl-β-D-cellobioside, known as pNPC (for glucanases), and p-4-nitrophenyl-β-D-glucopyranoside, known as pNPG (for glucosidase) assays. Even though cultures were incubated under methanogenic conditions, our preliminary assay showed similar enzyme activity under oxic and anoxic conditions therefore, these assays were done on the bench in the presence of air. As shown in Fig. 2, the highest observed glucanase activity (0.34 ± 0.17 U ml^{-1}) was recorded within the initial 2 days of incubation. This result was expected as glucanases act directly on the CMC backbone (Fig. 1) to form smaller carbohydrate chains, including cellobiose, that can then be further hydrolysed by β-1,4-glucosidase. The enzymatic activity for the β-1,4-glucosidases was at its highest activity later in the incubation, reaching 0.95 ± 0.13 U ml^{-1} after 28 days. By this point, most of the CMC has been hydrolysed to cellobiose and other products, explaining why the β-1,4-glucosidases are active later in the CMC degradation process (Fig. 1). These enzyme activities are similar to those reported in the literature for *Clostridium* (Tuka *et al.*, 1990; Benoit *et al.*, 1995), a member of the *Firmicutes* related to *Ruminococcaceae* which was the dominant family found in the enrichment (see Fig. 4).

Finally, viscosity measurements were performed to indicate the activity of the endo-β-1,4-glucanase. This enzyme, which randomly hydrolyses internal β-1,4 bonds
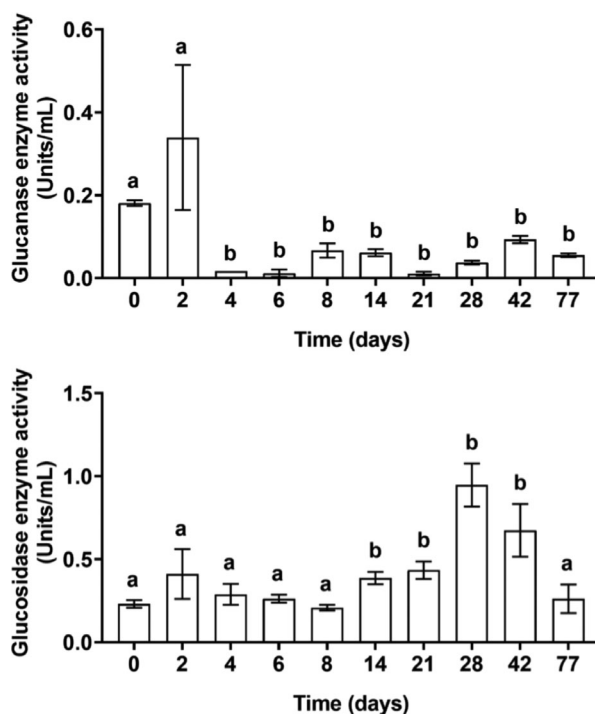
Fig. 2. Extracellular enzyme activity from the cell-free extract of the methanogenic enrichment by hydrolysis of A) pNPC to measure the glucanases activity and hydrolysis of B) pNPG to measure the glucosidases activity. One unit is referred as one micromole of *p*-nitrophenol released per minute under the conditions of the experiment conducted. Background noise from sterile control has been removed from the experimental replicates. Letters (a, b) represent groups that have a statistically significant difference; error bars indicate standard deviations ($p < 0.05$), n = 3.

between the glucose monomers in the CMC backbone (Fig. 1), is the key enzyme responsible for reducing CMC solution viscosity. Previous research has noted that the direct relationship between substrate hydrolysis rate, enzyme activity and polymer chain size can explain viscosity reduction, a key parameter that can be used to assess the success of a filter cake breaker (Martinez-Richa, 1998).

Initial viscosity measurements showed that the CMC-degrading culture amended with 0.5% CMC reached 7.36 ± 2.17 cP at 75 rpm (Fig. 3). Within 4 days of incubation, viscosity dropped to 2.98 ± 0.55 cP. After 14 days, the viscosity reached its lowest value at 1.86 ± 0.21 cP, staying around this value until the end of the experiment (77 days) and nearing the viscosity value for cultures incubated in the absence of CMC. By comparison, the viscosity did not significantly decrease in the sterile controls over time (Fig. 3). These viscosity measurements thus confirmed the Trypan blue test and the enzyme assays showing CMC degradation by the enrichment culture.

Microbial community analysis

Figure 4 shows the results from 16S rRNA gene sequencing of the initially established culture and the

final enrichment after seven transfers wherein CMC was present as the only carbon and energy source for the methanogenic community. The initial culture was composed of 17 different families at > 1% amplicon sequence variant (ASV) and 10.2% of the ASV were of < 1% relative abundance, showing greater diversity than the established culture using CMC as a sole carbon source. After seven transfers, the enrichment culture cultivated solely on CMC became less diverse. The established culture was comprised of seven families, and only 2.4% ASV were of < 1% relative abundance. The enriched culture was dominated by methanogenic archaea (*Methanomicrobiaceae* and *Methanosarcinaeae* at 51.1% and 2.2% relative abundance, respectively) that were presumably responsible for the observed methane production, along with several bacterial taxa known to be associated with the degradation of cellulose and/or its metabolites, including (in % relative abundance) *Ruminococcaceae* (29.4%), *Solibacteraceae* (7.8%), T*hermoanaerobacteraceae* (1.8%), *Caldicoprobacteraceae* (1.7%) and *Limnochordaceae* (0.5%) (Fig. 4). Members of the *Ruminococcaceae* family are well known for their ability to ferment cellulose or its byproducts (Flint *et al.*, 2012) as are members of the *Caldicoprobacteraceae* and *Limnochordaceae* families (Watanabe *et al.*, 2015; Lusk *et al.*, 2017). Thus, one or
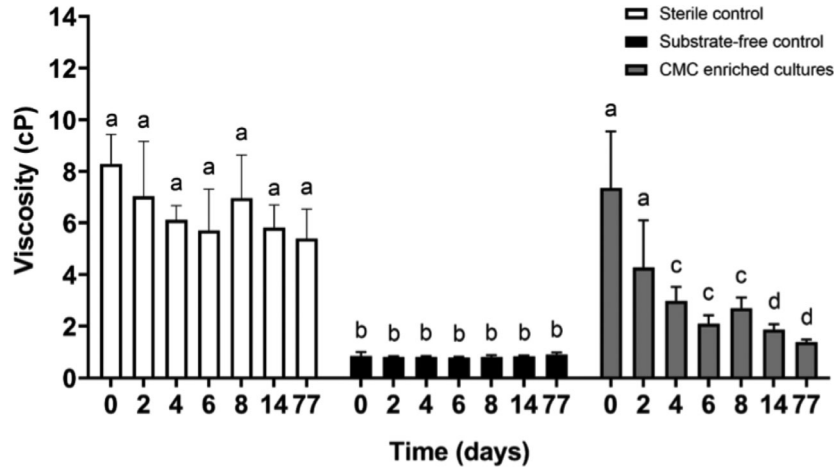
Fig. 3. Viscosity measurements for the established methanogenic enrichment in presence of 0.5% CMC. Viscosity was measured at 75 rpm. Letters (a, b, c, d) represent groups that have a statistically significant difference despite standard deviation (shown as error bars) ($p < 0.05$), n = 3.
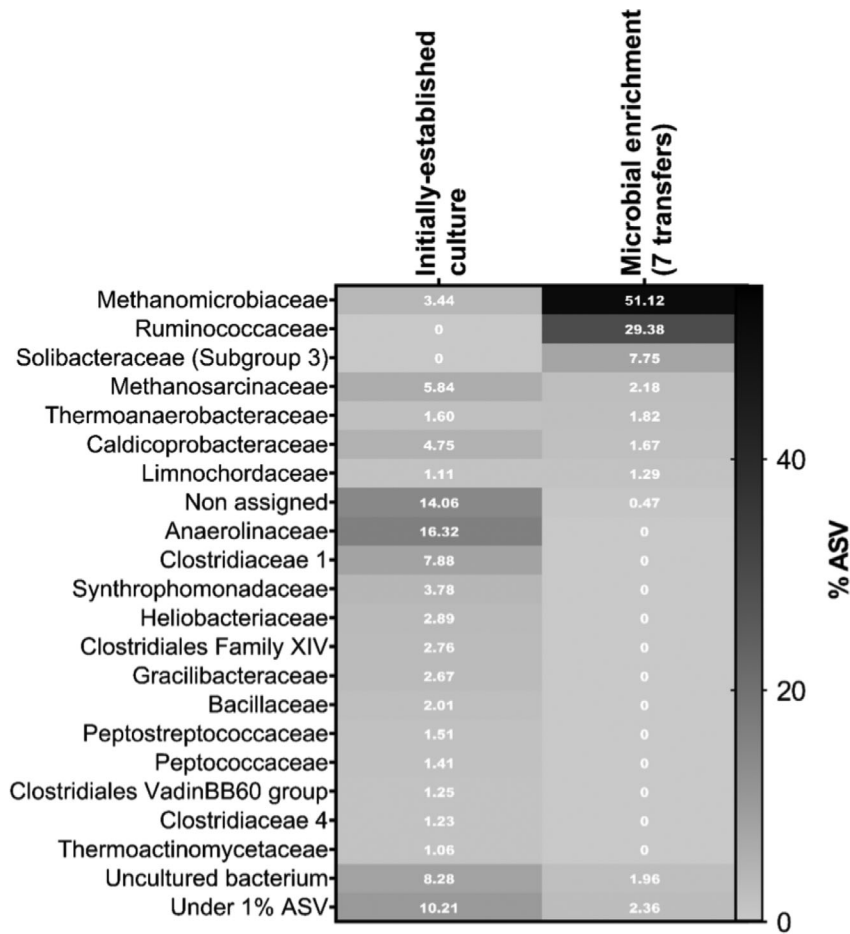
Fig. 4. Microbial community compositions (based on amplicon sequencing of the 16S rRNA gene) for the initial cattle manure enrichment and for the established CMC-degrading culture, n = 2.

more of these taxa were presumably involved in the initial hydrolysis of CMC. Members of the *Thermoanaerobacteraceae* are thermophilic anaerobic bacteria known to ferment cellobiose, glucose and other small compounds, but not cellulose (Kim *et al.*, 2001), and thus, likely utilized the smaller CMC degradation products. All

Fig. 5. Viscosity measurements for the different cell fractions from the methanogenic enrichment degrading CMC. Viscosity was measured at 75 rpm, n = 3.

of these taxa are affiliated within the Firmicutes, a phylum primarily composed of endospore-forming anaerobic bacteria. Interestingly, the second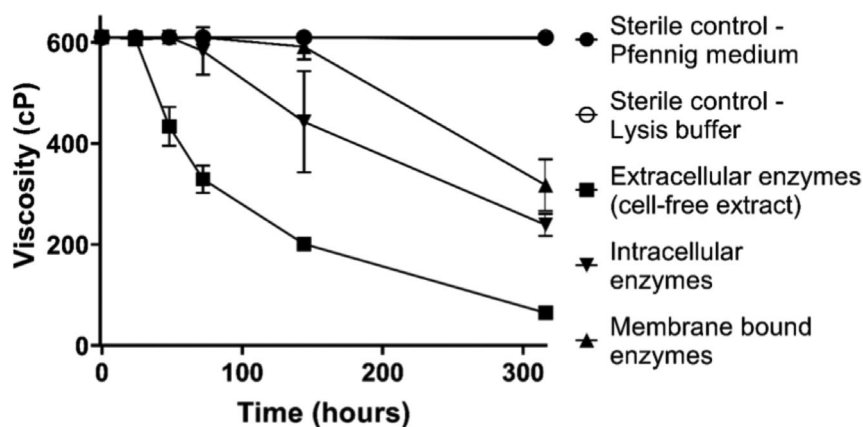 most dominant bacterial taxon in the CMC enrichment culture is affiliated with the *Solibacteraceae* family (7.75% ASV), within the phylum *Acidobacteria*. To date, there is only one isolate cultured from the *Solibacteraceae*, known as 'Candidatus Solibacter usitatus' Ellin6076 (Ward *et al.*, 2009). Genome analysis of this isolate showed that it harbours genes for cellulose metabolism (Ward *et al.*, 2009); thus, members of the *Solibacteraceae* family may also be involved in CMC hydrolysis. Overall, the microbial community composition of the CMC-degrading methanogenic enrichment reflects that which would be expected for the anaerobic digestion of polymers such as cellulose or CMC in that it is composed of cellulose degraders, glucose and small molecule fermenters and methanogens (Zieminski and Frac, 2012).

Enzyme efficacy from different cell fractions

Different cell fractions from the CMC-degrading enrichment culture were obtained in order to pinpoint the location of the greatest CMC-degrading enzymatic activity. Figure 5 shows the viscosity measurements taken during incubation of 2% (w/v) CMC with intracellular, extracellular and cell membrane fractions over the course of a 316 h incubation. A significant decrease in viscosity was observed within the initial 48 h of incubation for the extracellular fraction, with a decrease from 610.89 ± 0.01 cP to 433.61 ± 38.45 cP. The other fractions took a longer amount of time before a decrease in viscosity was detected; 70 h for the intracellular fraction and 140 h for the cell membrane fraction. After 316 h, incubations with the extracellular fraction reached the lowest viscosity, at 56.88 ± 7.39 cP (Fig. 5). Based on

this result, the extracellular fraction was selected for further experiments since it showed the best potential to hydrolyse viscous CMC solutions.

Enzyme efficacy under different conditions and in oilfield produced waters

The extracellular fraction containing CMC-degrading enzymes was evaluated for their efficacy across ranges of temperature, pH levels and salinities found in typical hydraulic fracturing operations. Viscosity reduction was used to determine enzyme efficacies. Since temperatures within wells experiencing hydraulic fracturing will typically reach between 50 and 80°C (Marina *et al.*, 2015), enzymes used for treating CMC filter cakes should be thermostable at these temperatures. Figure 6A shows enzyme efficacy under different temperatures ranging between 50 and 80°C. The incubation at 50°C revealed the fastest and greatest viscosity reduction of all the temperatures tested, a result that was expected since this is the temperature at which the CMC-degrading enrichment was maintained. This temperature is also the average temperature at which hydraulically fractured wells are operated (Trican Well Services, personal communication). At 50°C, the viscosity reduction decreased the most within the initial 48 h of incubation, starting at 611.47 ± 0.00 cP and reaching 121.87 ± 54.11 cP. After 312 h, the lowest viscosity value was reached (10.17 ± 0.41 cP). Surprisingly, the incubation at 80°C yielded the second largest viscosity reduction, achieving a viscosity of 76.14 ± 12.85 cP after 312 h. Controls with the autoclaved culture supernatant confirmed that these results are due to the enzymatic activity itself and not by-products of growth within the medium (results not shown). Enzymatic degradation of CMC also occurred at 60 and 70°C but not as effectively as observed at 50 or
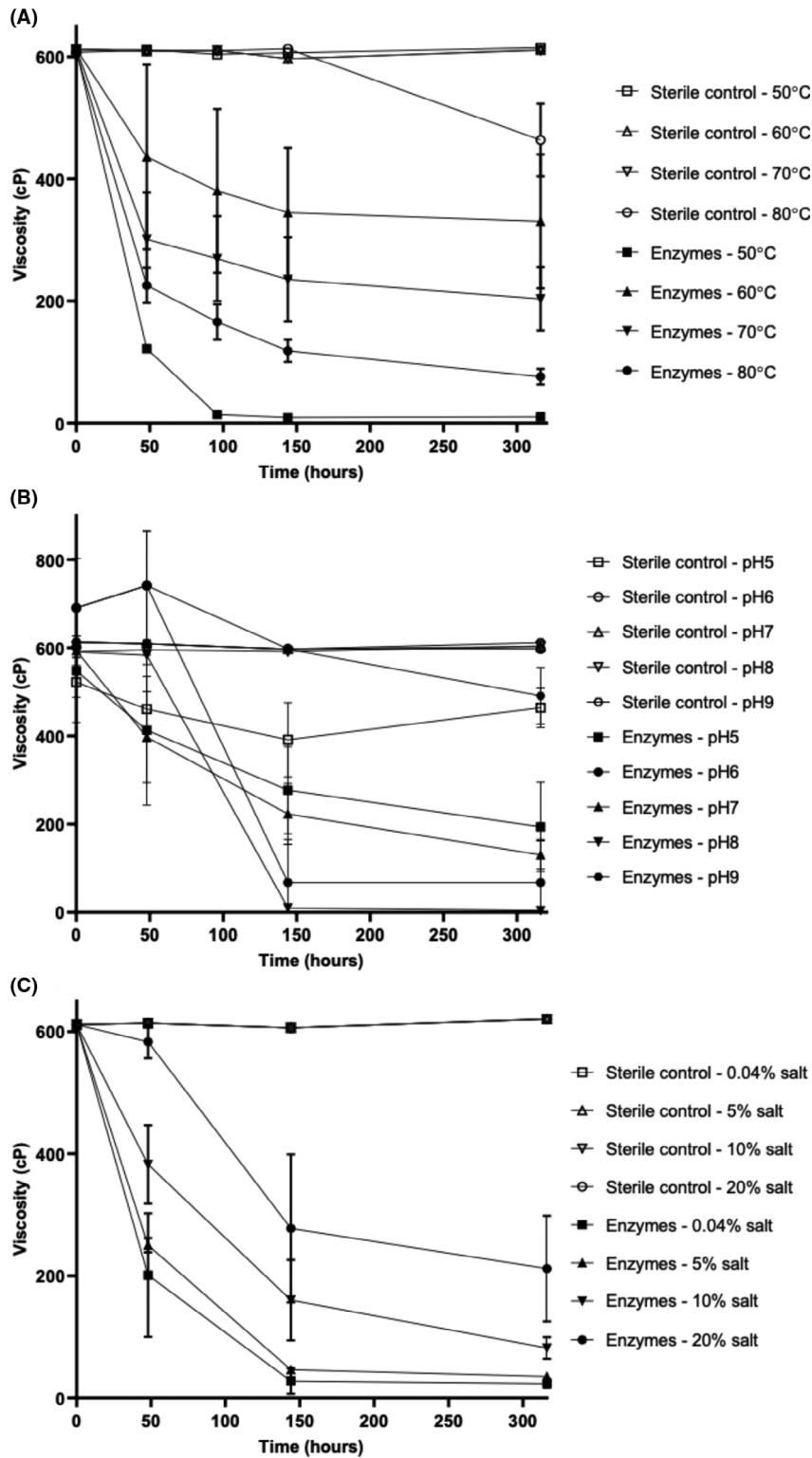
Fig. 6. Viscosity measurements comparing the efficacy of the extracellular enzymes from the methanogenic enrichment under different A) temperatures, B) pH values, and C) salt concentrations (% w/v). Speed = 16 rpm, n = 3.
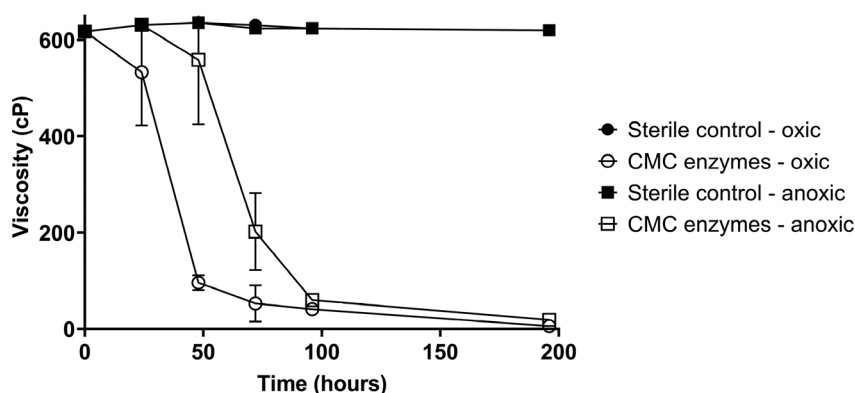
Fig. 7. Viscosity measurements for the extracellular enzymes in presence or absence of oxygen. Viscosity was measured at 75 rpm, n = 3.

80°C. Tests showed that the microorganisms cannot grow at 80°C, yet clear enzymatic activity was observed. Literature supports this trend as thermophiles (microorganisms that grow between 50 and 80°C) express enzymes that are active in this same range, between 60 and 80°C (Vieille and Zeikus, 2001). Another explanation for apparent viscosity reduction at 80°C could be due to a structural change of the polymer at this temperature that makes the polymer backbone more accessible for the enzyme activity compared to other temperatures. As this was the only temperature where abiotic degradation of the polymer was observed (Fig. 6A), is it possible that such a structural change occurred.

The second parameter tested was pH, as diverse pH values can characterize different production well fluids. Different pH levels were tested (3–9), but results at pH 3 and 4 are not presented because these lower pH values influence the stability of the polymer, and it is impossible to discern whether viscosity is due to chemical or enzymatic hydrolysis (Esmaeilirad *et al.*, 2016). As shown in Fig. 6B, the most effective viscosity reduction was observed at pH 8 with a viscosity decrease from 590.54 ± 0.00 cP to 10.27 ± 6.98 cP in 140 h. Viscosity reduction was also obtained when enzymes were at pH 7, with viscosity decreasing from 593.26 ± 33.56 cP to 130.96 ± 31.96 in 312 h. While little or no enzymatic activity was recorded at pH 9, there was still activity detected at pH 5 with a decrease from 547.53 ± 60.13 cP to 193.75 ± 101. 05 cP within 312 h. Similar results were obtained at pH 6 with a decrease from 690.26 ± 0.00 cP to 67.81 ± 0.00 cP in 140 h. These results suggest that the enzymes are active between pH 5 and 8, which was expected since the medium that was used to enrich the microbial culture is between 7.1 and 7.3.

The last parameter tested was salinity. The salt concentrations tested were chosen to be similar to those of

Table 1. Characteristics of the produced water samples (as analyzed by Trican Well Services) used in this study to test for enzymatic CMC viscosity reduction.

Produced Water Sample	Salt concentration (% w/v)	pH	Viscosity reduction (%)
1	20.00	5.76	0.00
2	18.00	6.39	67.38
3	7.90	7.30	98.32
4	0.87	8.39	96.84

produced water associated with oilfields (Table 1). Results were as expected: the viscosity from the solution with the lowest salt concentration (0.04%, the salinity of the medium used to cultivate the CMC enrichment) decreased to the greatest extent, from 611.47 cP to 23.64 ± 7.27 cP in 312 h (Fig. 6C). However, the extracellular enzyme fraction was also effective under the 5 and 10% salt concentrations tested, reaching 35.07 ± 0.34 cP and 81.76 ± 17.86 cP, respectively, after 312 h of incubation. Although not as effective, extracellular enzymes were also active at 20% (w/v) salt concentration and were able to decrease viscosity from 611.47 cP to 211.76 ± 86.68 cP after 312 h. In all, the CMC-utilizing enzymes derived from the enrichment culture were found to be effective in reducing viscosity up to 80°C, within a pH range of 5-8, and up to 20% salinity, conditions that can be present in oilfield produced waters.

Additionally, the enzymes were tested for their ability to degrade CMC under oxic and anoxic conditions since the oilfield environment to which they would be applied is anoxic. As the CMC-degrading enzymes were derived from a methanogenic culture, it was expected that they would be active under anoxic conditions. Figure 7 shows the viscosity reduction by the extracellular enzymes in the presence and absence of oxygen. Although the initial

rate of viscosity reduction was higher under oxic conditions, incubations under both conditions converged to the same viscosity values after 96 h. Under anoxic conditions, viscosity was reduced to 59.67 ± 7.93 cP, while viscosity was reduced to 40.98 ± 11.09 cP under oxic conditions. These results demonstrate that the enzymes are metabolically flexible and are efficient at hydrolysing CMC under either oxic or anoxic conditions.

Produced water samples from hydraulically fractured wells were tested to determine whether the extracellular enzyme fraction could degrade CMC in the presence of real-world produced water chemistries. Figure S3 shows the viscosity measurements for four different produced water samples (see Table 1 for chemical characteristics). Due to the different speeds at which the samples were analysed in the viscometer, samples cannot be compared with one another. Ions have an effect on the polymer rheology by preventing cross-linking in many polymers; therefore, lower pH levels will result in lower viscosities (Esmaeilirad *et al.*, 2016). For this reason, different rheometer programmes were used with different speeds to reach optimal measurements. Of the four samples tested, produced water sample 1 (20% salinity, pH 5.76) was the only sample that did not show a significant viscosity reduction, likely due to the combination of high salinity and lower pH. Enzymes tested against produced water samples 3 (7.9% salinity, pH 7.3) and 4 (0.87% salinity, pH 8.4) resulted in the largest decrease in viscosity, from 1077.75 ± 0.00 cP to 62.34 ± 12.63 cP for sample 3 and from 324.34 ± 17.20 cP to 40.30 ± 7.49 cP for sample 4, in only 24 h. Produced water sample 2 (18% salinity, pH 6.39) also showed a significant decrease in viscosity, from 353.39 ± 75.45 cP to 128.82 ± 75.78 cP in 144 h. Although the effect was not as pronounced as samples 3 and 4, the results showed that the extracellular enzyme fraction was capable of significant CMC degradation in comparatively high salinity produced waters. These results showed that the extracellular enzymes are able to degrade CMC concentrations that may be found in wells where filter cakes are formed and in chemical environments that are similar to those found in fractured wells and under similar temperatures (50°C).

Overall, we demonstrated that extracellular enzymes derived from a CMC-degrading methanogenic culture significantly reduced CMC viscosity under the environmental conditions often found in hydraulically fractured wells (50 and 80°C, pH from 5 to 8 and at 0.04% and 20% (w/v) salinities), providing a proof-of-concept for the application of such biotechnology to treat CMC filter cakes. Although the enzymes used in this study were not derived from microorganisms inhabiting oilfield fluids, our results demonstrating effective CMC viscosity reduction under oilfield conditions and in oilfield samples indicated that effective filter cake breaker enzymes can be derived from other sources. Similarly, effective guar-breakage enzymes for use in oilfield applications have been derived from soil-associated microorganisms such as *Aspergillus niger* (Tjon-Joe-Pin, 1993) and *Enterobacter* (You *et al.*, 2016) rather than oilfield fluids. Since enzyme breaker technology would involve the use of specific enzymes rather than whole cells, the source of the enzymes would not factor into enzyme performance providing that they function under field-relevant conditions. As such, we showed that extracellular enzymes derived from cattle manure were able to effectively reduce CMC viscosity in the presence of real-world, produced water samples containing a variety of oilfield chemistries. Future work to characterize the enzyme biotechnology to treat CMC filter cakes using a laboratory-scale high pressure chamber will help to assess the feasibility of field application.

Experimental procedures

Chemicals

Carboxymethyl cellulose (CMC), chemical oxidizers (proprietary) and a guar-degrading enzyme formulation were provided by Trican Well Services (Calgary, AB, Canada). 4-Nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-β-D-cellobioside (pNPC), D-glucono-1,5-δ-lactone and trypan blue were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). All other chemicals used were obtained from Sigma-Aldrich or VWR (Radnor, PA, USA).

Field samples and isolates

Various environmental samples and isolates were obtained to examine CMC biodegradation. Cattle manure samples were collected at the Crowfoot Creek Ranch (Hussar, AB, Canada), sheep rumen was collected at the W.A. ranch operated by University of Calgary (Calgary, AB, Canada), and sewage sludge samples were collected from a municipal wastewater treatment plant (Calgary, AB, Canada). Based on literature reports describing their cellulose-degrading abilities at higher temperatures, three bacterial isolates were acquired from Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ, Braunschweig, Germany). The strains included *Thermobifida fusca* (DSM43792) (Gomez del Pulgar and Saadeddin, 2013), *Geobacillus stearothermophilus* (DSM22) (Agüloglu & Enez, 2014) and *Aeribacillus pallidus* (DSM3670) (Scholz *et al.*, 1987). Produced water samples with characterized pH levels and salinities (Table 1) were obtained from four different hydraulic fracturing operations (provided by Trican Well Services).

Cultivation methods

Each environmental sample and commercially available bacterial isolate was tested for CMC-degrading ability at 50°C under oxic and/or anoxic conditions.

Isolates were grown by using their respective recommended medium from DSMZ supplemented with 0.5% (w/v) CMC. Cultures were incubated at 50°C until the optical density (600 nm) reached a value of 0.6. Each culture was subsequently transferred into fresh medium (20% v/v) to which all other carbon sources except CMC (0.5% w/v) were removed to assess the capacity of the strain to use CMC as a sole carbon source.

For aerobic cultivation of the environmental samples (sheep rumen, cattle manure, sewage sludge), a minimal salts medium (Bao *et al.*, 2012) was prepared with the addition of 0.2% (w/v) of CMC. Bottles were closed and autoclaved at 121°C for 20 min and then left 12 to 24 h at 50°C to allow for the polymer to fully hydrate in solution. Initial incubations were inoculated with 20% (w/v) of cattle manure or 20% (v/v) sewage sludge samples and incubated at 50°C.

For anaerobic cultivation, a minimal salts medium (McInerney *et al.*, 1979) was prepared under strict anoxic conditions with minor modifications to the original medium composition (no clarified rumen fluid was used, and sodium sulfide instead of cysteine sulfide was used as the reductant). No electron acceptor was added in order to establish methanogenic enrichments. Serum bottle incubations were flushed with a mixture of N_2/CO_2 (90%/10% (v/v)) and sealed with butyl rubber stoppers and aluminium crimps. Carboxymethyl cellulose was added at a final concentration between 0.5 to 2% CMC (w/v), and bottles were sterilized by autoclaving at 121°C for 20 min. Initial incubations were inoculated with 20% (w/v) cattle manure samples and incubated at 50°C. Unless otherwise stated, all incubations and enzyme tests were conducted at 50°C. This temperature was selected based on temperatures frequently found associated with hydraulic fracturing systems (Trican Well Services, personal communication).

Methane measurements

Methane production was monitored using a gas chromatograph equipped with a flame ionization detector (GC-FID) (Fowler *et al.*, 2012) for the anaerobic cultures as a surrogate measurement for growth. Once methane production had reached the plateau phase in a culture, 20% (v/v) inoculum was transferred into fresh minimal medium either in the presence or absence of 2% (w/v) CMC. Transfers were performed several times until methane production ceased from the CMC-free cultures to ensure that CMC was the sole carbon source in the CMC-amended cultures. The same procedure was carried out for the aerobic cultures except that CO_2 was measured as an indication of growth using a gas chromatograph equipped with a thermal conductivity detector (GC-TCD; Agilent 7890A, Santa Clara, CA, USA) and a HP-PLOT/Q capillary column (30 m). For CO_2 analysis, the inlet temperature was set at 250°C, the detector temperature was set at 200°C, and the oven temperature was held at 80°C for 5 min.

Carboxymethyl cellulose staining process

CMC hydrolysis was visualized through the addition of 0.01 % (v/v) trypan blue to the cultures that were prepared as described above. Trypan blue is known to bind to the intact polymer backbone of β-glucans such as CMC (Williams, 1983). Fresh CMC solutions supplemented with trypan blue appear dark purple/blue. However, subsequent enzymatic hydrolysis of the β-1,4 bonds releases the dye (Kim *et al.*, 2012), causing the colour intensity to decrease with an increasing degree of hydrolysis, until the solutions finally appear colourless. Substrate-free and sterile controls were also stained to verify that the culture was metabolizing CMC to glucose and that the CMC was not being transformed abiotically.

Enzyme activity determinations

Enzyme activities for various steps of the CMC degradation process were measured using an assay that involves the hydrolysis of pNPG and pNPC at 50°C as described by Lokapirnasari *et al.* (2015). pNPC is a common reagent used during spectrophotometric assays to measure endo- and exo-β-1,4-glucanase activities, as its hydrolysis releases nitrophenol that then reacts with sodium carbonate under alkaline conditions to develop a yellow colour (Lokapirnasari *et al.*, 2015). D-glucono-1,5-δ-lactone was added to a final concentration of 0.25 mg ml^{-1} to the pNPC solution to inhibit the β-1,4-glucosidase activity (Holtzapple *et al.*, 1990; Mandels and Reese, 1963). This was done to ensure that the pNPC assay measured only activity from the glucanase since glucosidase can also hydrolyse pNPC if not inhibited, resulting in a false positive. pNPG follows the same chemical reaction, but is precisely hydrolysed by β-1,4-glucosidases (Cai *et al.*, 1999; Lokapirnasari *et al.*, 2015). Enzyme activity was measured by measuring yellow colour development by spectrophotometry (405 nm). One unit was defined as one micromole of *p*-nitrophenol released by hydrolysis per minute under the experimental conditions.

Viscosity measurements from different culture fractions

To determine whether the enzymes responsible for CMC degradation were extracellular, intracellular or membrane-bound, CMC-degrading enrichment cultures were

grown until the polymer was hydrolysed (∼ 6 days) and then centrifuged at 6000 rpm for 30 min at 4°C. Although we found that the highest enzyme activity for the glucanase was after the initial 2 days of incubation of the enrichment (as measured using the pNPC hydrolysis assay, Fig. 2), we found that after 2 days of incubation, the viscosity of the medium prevented successful enzyme recovery. Thus, when the enrichment culture was prepared for viscosity assays, the culture was pre-grown for 6 days which we found to be the optimum time for enzyme recovery from the enrichment.

The supernatants were collected and saved as the extracellular fraction. The cell pellets were re-suspended in lysis buffer and were lysed using a FastPrep-24 Grinder (MP Biomedicals, Santa Ana, CA, USA) for 40 s at 6 m/s and placed immediately on ice. After a second centrifugation, the resulting supernatant was collected and saved as the intracellular fraction, while the pellet, re-suspended in lysis buffer, was saved as the cell membrane fraction.

Each saved fraction was then evaluated for its efficacy in degrading CMC. Deionized water (20 ml) containing 2% (w/v) CMC was prepared in serum bottles that were sealed with butyl rubber stoppers and aluminium crimps. The bottles were autoclaved at 121°C for 20 min and agitated (120 rpm) overnight at 50°C to allow for full hydration of the polymer. The serum bottles were divided into 3 groups, with each group being supplemented with 5 ml of a particular saved culture fraction, prior to being incubated. Controls for the extracellular fraction were prepared by adding 5 ml minimal medium, while controls for the intracellular and cellular membrane fractions were prepared by adding 5 ml lysis buffer (50 mM Tris, 150 mM NaCl; pH 7).

Viscosity of the resulting solutions in each group was measured using a Brookfield DV-II + Pro viscometer (Brookfield, Middleboro, MA, USA) with a CP52 type spindle. Viscosity was measured at 40°C using 1 ml of sample. Samples were subjected to a speed range of 2–28 rpm with an increment of 2 rpm every 10 s. Viscosity readings were recorded before each speed change.

Viscosity measurements from the extracellular enzymes under different conditions

All assays to determine CMC enzymatic activity under different pH, salinity and temperature conditions were performed using the extracellular enzyme fraction. Every condition was tested independently. Enzyme performance was assessed using viscosity measurements (as described above). All enzyme tests were carried out in triplicate for 14 days using a 2% CMC solution, during which samples were removed periodically to measure viscosity. Triplicate enzyme-free controls were also prepared for each condition tested.

To determine the salinities at which the enzymes were capable of degrading CMC, various concentrations of NaCl (0.04, 5, 10 and 20% (w/v) NaCl) were added to a 2% (w/v) CMC solution. For the determination of the temperature range for activity, serum bottles were incubated at 50, 60, 70 or 80°C. Enzyme activities were also assessed at pH values of 3 to 9. Two different buffers were used to assess the pH range covered by these buffers. Citrate buffer was used to obtain the pH solutions of 3 to 6, and Tris buffer was used to obtain the pH solution of 7 to 9 (Stoll and Blanchard, 2009). Viscosities were monitored over time for each condition.

To determine the effect of oxygen on extracellular enzyme activity, one set of bottles was prepared with a 2% (w/v) CMC solution with oxygen in the headspace, while a second set was prepared anoxically by boiling the solution, and flushing the headspace was flushed with N_2 to remove any oxygen from the bottles. Triplicates were prepared for each condition. Oxic and anoxic tests were incubated at 50°C, and viscosities were monitored over time.

The effect of the extracellular enzyme fraction on the viscosity of produced water samples (characteristics shown in Table 1) obtained from different oil fields was also evaluated. The assays were performed as described above, except the produced water samples were used to prepare the CMC solution instead of deionized water. Because ions can have a strong effect on polymer rheology by preventing cross-linking, solutions characterized by higher salinities and low pH values can lower viscosity (Esmaeilirad *et al.*, 2016). Thus, due to the various salt concentrations and pH values associated with the different produced water samples (Table 1), different final concentrations of CMC were prepared to be in the same range of viscosity. Produced water samples 1 and 2 were prepared to have a final CMC concentration of 5% (w/v), while the samples 3 and 4 were prepared to have a final CMC concentration of 2% (w/v).

DNA extraction and 16S rRNA gene sequencing

Genomic DNA was extracted both from the initial CMC-degrading methanogenic enrichment and from the established CMC-degrading culture (after seven transfers) using a commercial kit (FastDNA Spin Kit for Soil, MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. Results were quantified by fluorometry (Qubit; Thermo Fisher Scientific, MA, USA). Amplification of the V6-V8 hypervariable region of the 16S rRNA gene was accomplished by following the protocol provided with the KAPA HiFi HotStart ReadyMixPCR (KAPA Biosystems, MA, USA) with 10 ng of template DNA and using the 926F (3'-AAACTYAAAKGAATTCACGG-5') and 1392R (3'- CGGTTACCTTGTTACGACTT-5') primers (Menon and Voordouw, 2016). Initial denaturation was

done at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 65°C for 15 s and extension at 72°C for 15 s. A final extension step was carried out at 72°C for 1 min. PCR products were analyzed on a 1% agarose gel to ensure the presence of a single band and were quantified again after purification. The second PCR to attach the adapters was done with the same PCR mix and same PCR programme as described previously and sequenced on the Illumina MiSeq platform at the University of Calgary.

All data were visually inspected and cleaned of low-quality ends using DADA2 (Callahan *et al.*, 2016). Data were processed with QIIME 2 (Bolyen *et al.*, 2019). Denoising was done by merging a 50 bp overlap with mismatches of ≤ 10% allowed and subjected to additional quality control steps (removal of chimeras, ambiguities and sequences with an average quality lower than 20). Quality-controlled reads were analysed as amplicon sequence variants (ASV) and classified using the SILVA 132 database (Quast *et al.*, 2013). All ASVs over 1% relative abundance were selected for analysis.

Statistical analyses

All statistical analyses were performed doing an unpaired t-test using the *t*-test calculator from GraphPad (Graph pad, 2018). All data considered statistically significant had a *p*-value lower than 0.05. To distinguish significant from non-significant data, groups were formed and designated a, b, c, etc. Groups with the same letter signify that the values within the group are not significantly different (*p*-value higher than 0.5), while groups with different letters signify that the values in that group are significantly different than the values from the other lettered groups (*p*-value lower than 0.5).

Acknowledgements

Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Methane production from A) the initial CMC-degrading culture established with cattle manure as the inoculum in a minimal salts medium, and B) the established CMC-degrading methanogenic enrichment culture after seven transfers of the culture, *n* = 3.

Fig. S2. Established CMC-degrading methanogenic enrichment supplemented in 0.01% (v/v) Trypan Blue as an indicator for CMC biodegradation. 'Sterile Ctl' stands for sterile control. 'CMC-free Ctl' is the control without CMC added to the medium (substrate-free 6#control), *n* = 3.

Fig. S3. Viscosity measurements for the cell-free extract containing extracellular enzymes (empty symbol) compared to sterile controls (full symbol) in different produced water samples (B) sample 1; C) sample 2; D) sample 3; E) sample 4) from fractured wells with different pH and salinities (%) compared to A) a positive control with CMC hydrated in deionized water. Refer to Table 1 for sample details, *n* = 3.