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Cloning Cellulase Genes from Victoria Falls Rainforest Decaying Logs Metagenome

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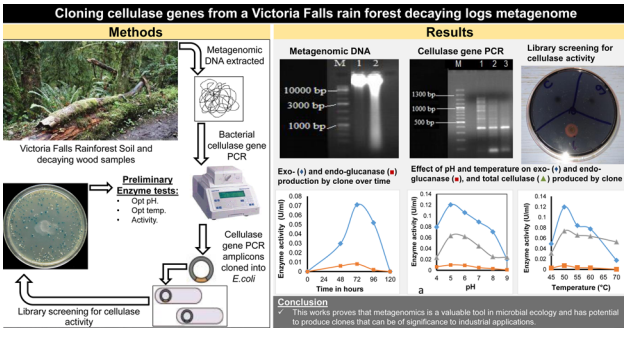
Abstract

The Victoria Falls rainforest is a protected site whose forest floors harbor a host of cellulolytic microorganisms involved in biomass degradation. This study collected decaying logs and soil from the rainforest for bioprospecting cellulases from their metagenomes. Metagenomic DNA was isolated from the compound sample. Degenerate cellulase primers were used to amplify cellulase genes in the metagenome. The resulting amplicons cloned into Z-competent *Escherichia coli* DH5α were analyzed by functional screening for the production of cellulase extracellularly. Functional screening of the clones resulted in one clone (Clone-i) testing positive for extracellular cellulase production. Submerged fermentation of Clone-i was carried out for cellulase production. The cellulases were characterized to determine their activity's optimum pH and temperature. The diversity of the cellulases produced by Clone-i was determined. Clone-i's optimum enzyme activity was observed after 72 hours of incubation at 50°C and pH 5. Clone-i produced 80% more exoglucanases as compared to endoglucanases. The cellulolytic Clone-i' isolate shows Victoria Falls rainforest's potential as an enzyme bioprospecting site, reflecting that metagenomics is a valuable tool in microbial ecology.



K e y w o r d s: Victoria Falls rain forest, decaying logs, cellulases, metagenomics, bioprospecting

Introduction

The Victoria Falls rainforest is an undisturbed environment protected by the Zimbabwean National Conservation Act (UNESCO 1989). The forest's high humidity and the fact that it is undisturbed may contribute to great microbial diversity. The decomposition of the varied plant biomass in the rain forest is being carried out by a community of microorganisms whose cellulolytic potential could be of value to research and industrial applications.

Cellulases are of interest due to their diverse applicability for bio-polishing fabrics in the textile industry, improving the digestibility of animal feeds, and as clarifiers in fruit juice processing in the food industry (Li et al. 2009; Dhillion 2011). Cellulases are inducible enzymes which hydrolyze cellulose to low molecular weight compounds, such as hexoses, pentoses, cellobiose, and finally to glucose (Lynd et al. 2002). Cellulose is a crystalline polymer composed of D-glucose residues connected by β-1,4-glycosidic bonds (Sukumaran et al. 2005). The polymer is the primary structural material of plant cell walls. It is the most abundant biopolymer on earth, continuously synthesized in photosynthesis (Monserrate et al. 2001).

Cellulases are synthesized by various microorganisms either cell bound or extracellularly as the microorganisms grow on cellulosic substrates. Various microorganisms, such as fungi and bacteria, produce cellulases that decompose cellulosic biomass. Industrial cellulases

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are mainly produced by fungi in submerged fermentation because of the ease of handling and greater control of environmental factors such as temperature and pH (Mrudula and Murugammal 2011). Cultural methods are generally applied to isolate the enzyme-producing microorganisms. However, cultivation-dependent approaches are limited because the overwhelming majority of natural microorganisms cannot be cultured under laboratory conditions.

To expand the range of natural product discovery, culture-based methods may be complemented or replaced by culture-independent metagenomic approaches. Metagenomics is the genomic analysis of microorganisms by directly extracting and cloning of deoxyribonucleic acid (DNA) from an assemblage of microorganisms (Rolf 2005). Metagenomics can be employed in the search for enzymes and biomolecules. The not-yet-cultivated microbes represent an unlimited resource for discovering genes, enzymes, and chemical compounds for use in biotechnology.

This study sought to explore the Victoria Falls rainforest microbial community in search of cellulase-producing isolates using metagenomics and to partially characterize their cellulases.

Experimental

Materials and Methods

Sampling. Sampling was carried out at the Victoria Falls rain forest (17°55′28″S; 25°51′19″E). Logs with visible signs of decomposition were collected by breaking, using gloved hands, into sterile sampling bags along with soil from underneath the logs. The sampling was not selective of wood type; both hard and soft woods were included. A compound sample was prepared by grinding the various logs and soils into fine particles using a sterile pestle and mortar. The logs were further grounded using a Waring® laboratory blender 7010S (Waring, USA). A 20 g portion was suspended in 180 ml sterile distilled water and used to extract metagenomic deoxyribonucleic acid (DNA).

Metagenomic analysis of the Victoria Falls log samples. The Epicentre Meta-G-Nome DNA isolation kit (Epicentre Biotechnologies, USA) was used to extract metagenomic DNA from 100 ml of the compound sample in distilled water, following the manufacturer's instructions. Gel electrophoresis on a 1% agarose gel was used to validate the quality of the isolated DNA by comparison to the Fosmid Control DNA (40 kb; 100 ng/µl) provided in the kit. Universal degenerate cellulase primers: primer 1 5'-CATATGGCAAGTCCGTCCGTGACGAA-3' and primer 2 5'-CTCCAGTTTCTGATACCCCATTCACC-3' (Liu et al. 2011) were used in PCR reactions with the metagenomic DNA as the template. The PCR reactions were carried out in 25 µl volume, which comprised of 0.2 mM dNTPs, 10 X Dream Taq buffer, 1 µM primer 1, 1 µM primer 2, 1 µg DNA sample, and 1.25 U Dream Taq polymerase enzyme. A titration of $MgCl_2$ of concentrations 2 mM, 3 mM, and 4 mM was carried out, and the reaction made up to 25 µl accordingly with nuclease-free water. The PCR reactions were carried out in the steps of primary denaturation at 95°C for 2.5 minutes, followed by 30 amplification cycles of denaturation at 95°C for 0.5 minutes, annealing at 60°C for 30 seconds, extension at 72°C for 1.5 minutes, and a final extension step of 10 minutes at 72°C.

Cloning of cellulase amplicons from metagenomic DNA. The amplicons from the cellulase PCR reaction using primer 1 and primer 2 were cleaned and concentrated using the Zymoresearch Clean and Concentrate Kit according to the manufacturer's instructions (Zymo Research, USA). The purified amplicons of sizes 400 base pairs to 1,200 base pairs were treated with the blunting enzyme mix from the Thermo Scientific pJet cloning kit (Thermo Fischer Scientific, USA) and subsequently ligated using T4 ligase into pUC19 that had been cut with *Sma*I. The ligation reaction mixture was kept at –20°C and used directly for transformation. For transformation, 3 µl of the ligation reaction mixture was added to 100 µl of Z competent *Escherichia coli* DH5α cells thawed on ice. The transformation reaction mixture was gently mixed while on ice, 50 µl of the reaction was spread on a pre-warmed (37°C) LB agar plate (yeast extract 10 g/l; tryptone 10 g/l; NaCl 10 g/l; Agar 15 g/l) plate supplemented with 50 mg/ml ampicillin, 0.1 M IPTG and 50 mg/ml X-Gal (LB-AMP-IPTG-X-Gal) and incubated at 37°C for 18 to 24 hours according to the method by Maniatis et al. (1982). White colonies were picked and enriched in LB broth with ampicillin for 24 hours at 37°C with vigorous shaking. The clone cultures were stored in 15% glycerol at –20°C for further analysis.

Plasmid extractions from isolated clones. The alkaline lysis method (Birnboim and Doly 1979) was used to extract plasmids from the selected white colonies. The presence and quality of plasmid DNA were validated by gel electrophoresis on a 1% agarose gel.

Functional screening of the isolated clones. The clones were examined for cellulase activity by drop inoculating 5 µl of overnight cultures in (LB-AMP-IPTG-X-Gal) broth on Mandel's agar plates (CMC 10 g/l; NH_4SO_4 1.4 g/l; KH_2PO_4 2 g/l; Urea 0.3 g/l; $CaCl_2$ 0.4 g/l; $MgSO_4$ 0.6 g/l and trace elements $MnSO_4$ 1 mg; $ZnSO_4$ 1.4 mg; $CoCl_2$ 3.7 mg) (Acharya et al. 2008) enriched with ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), and X-Gal. After incubation at 37°C for 24 hours, the plates were flooded with Gram's iodine,

according to Kasana et al. (2008). The clones showing cellulase activity were noted by the presence of clearance zones and were selected for further analysis.

Production of cellulases by the selected clones. The selected clones were pre-cultured overnight in LB broth formulated following Maniatis et al. (1982), enriched with IPTG, X-Gal, and ampicillin, and incubated at 37°C with shaking at 160 rpm (Liang et al. 2014). After incubation, 2 ml of the culture was inoculated into 50 ml of Mandel's broth enriched with IPTG, X-Gal, ampicillin, and 1% CMC as the sole carbon source in 250 ml conical flasks. Incubation was done at 37°C for 72 hours with shaking at 160 rpm. The cultures were then centrifuged at 5,000 × *g* for 15 minutes at 4°C, and the supernatants (crude enzyme) were collected for the detection of cellulases (Sethi et al. 2013).

Assaying of cellulase produced by the selected clones. Cellulase activity was measured by using 3,5-dinitrosalicyclic acid (DNS) (Miller 1959) by determining the reducing sugars liberated from substrates in 50 mM citrate buffer at pH 5. The method was scaled down to micro-titre volumes, which were 25 µl substrate, 25 µl of buffer, and 25 µl of crude enzyme, according to King et al. (2009). For the endoglucanase assay, the mixture containing carboxymethylcellulose as substrate was incubated at 50°C for 30 minutes. For the exoglucanase assay, the mixture had Avicel as substrate, and incubation time was 1 hour at the same temperature as for the endoglucanase detection. The micro-titre-based filter paper assay was carried out for total cellulases by incubating a 7 mm disc of Whatman No. 1 filter paper in crude enzyme and 40 µl of 50 mM citrate buffer pH 5 for 1 hour at 50°C (Xiao et al. 2004). The reactions were stopped by the addition of DNS reagent and boiling for 5 minutes at 100°C for color development. The absorbance of the reaction mixtures was read at 540 nm. A calibration curve of glucose standard solutions was used to determine the concentration of glucose produced. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

Determination of enzyme production by the Clone-i over time. The selected clones were cultured in Mandel's media broth enriched with IPTG, X-Gal, and ampicillin at 37°C with agitation at 160 rpm for five days. At 24-hour intervals, 2 ml of broth was aseptically withdrawn from the cultures and centrifuged at 3,300 × *g* for 5 minutes. The obtained supernatants were assayed for cellulase activity over time, as previously described.

Determination of the optimal temperature for cellulase activity. The optimum temperature for enzyme activity was determined by incubating the crude enzyme in 1% CMC, 1% Avicel, or 7 mm filter paper discs substrates at temperatures 45, 50, 55, 60, and 70°C for 5 minutes (Quiroz-Castañeda et al. 2009). Production of reducing sugars was then determined using DNS as previously described for cellulase assessment.

Determination of optimum pH for cellulase activity. To determine the optimum pH for cellulase activity, 1% CMC, 1% Avicel or a 7 mm filter paper disc were prepared in 10 mM buffer solutions in the 4–9 pH range (acetate buffer for pH 4 and 5, phosphate buffer for pH 6, and sodium for 7 and TRIS-HCl for pH 8 and 9). The substrates were then incubated with 0.1 ml of the crude enzymes at their optimum temperatures. Production of reducing sugar was determined using the DNS as previously described for cellulase.

Determination of the diversity of cellulases produced by Clone-i. Crude enzymes sampled at optimum production time for each of the clones were used to determine the diversity of cellulases produced by the bacterial isolates. Enzyme assays were carried out at optimum temperatures and pH for the isolate, as predetermined in the sections above. Production of reducing sugars from the exoglucanase, endoglucanase, and total cellulases were determined using the DNS, as previously described.

Results

Extraction of metagenomic DNA from the Victoria Falls logs compound sample. The isolated metagenomic DNA was fragmented, as indicated by the smear in the agarose gel electrophoresis (Fig. 1). Cellulase primers, primer 1 and primer 2 were used to amplify the isolated high molecular weight metagenomic DNA at

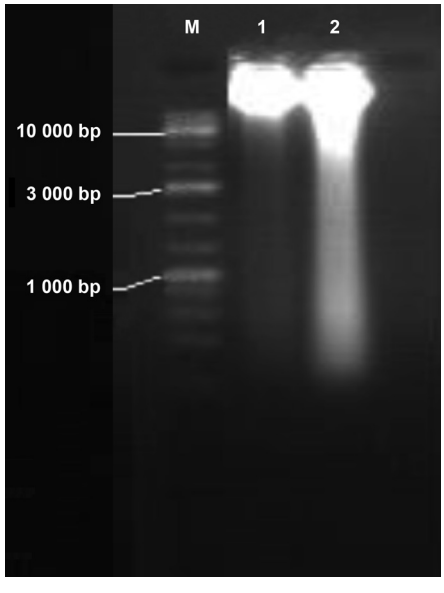

Fig. 1. Gel electrophoresis of the metagenomic DNA isolated from the Victoria Falls decaying compound logs sample.
M – the 1 Kb gene ladder (Thermo Fisher Scientific, USA), lane 1 – the Fosmid Control DNA, lane 2 – the metagenomic DNA isolated from the Victoria Falls compound logs sample

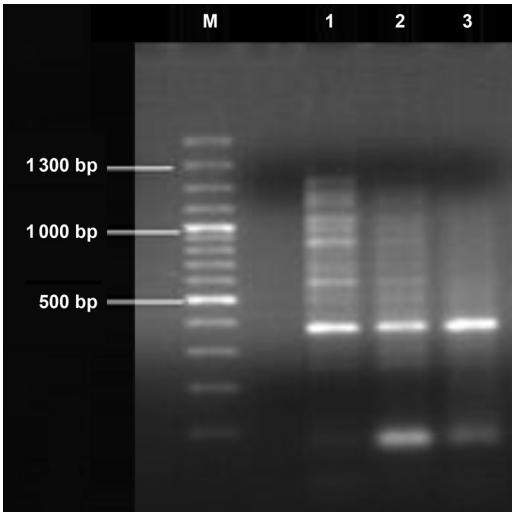

Fig. 2. Gel electrophoresis of cellulase Primer 1 and Primer 2 PCR products.

M – the 100 bp molecular weight marker (Thermo Fischer Scientific), lanes 1, 2, and 3 – the reactions with $MgCl_2$ concentrations of 2 mM, 3 mM, and 4 mM, respectively. Amplicons from Lane 1 of fragment size 400 bp to 1,200 bp were subsequently used for cloning.

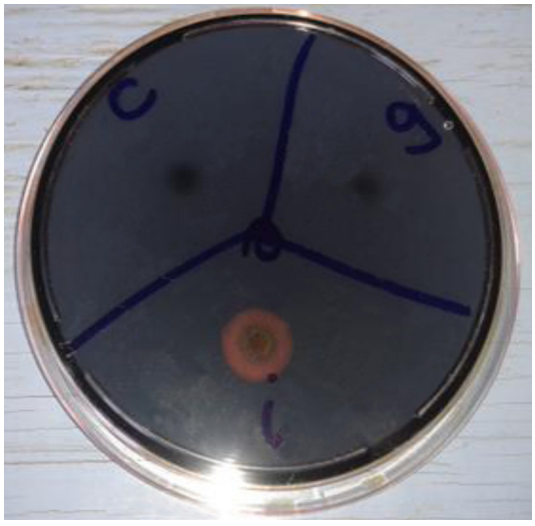

Fig. 3. Functional screening of the Clones-i, -c, and -g on Mandel's media after 18 hours of incubation followed by flooding with Gram's iodine. Clone-i had a zone of clearance indicating the production of extracellular cellulases.

three different concentrations of $MgCl_2$. The results of the agarose gel electrophoresis are shown in Fig. 2.

Cloning of cellulase amplicons from metagenomic DNA PCR. Amplicons from the PCR using cellulase primer 1 and primer 2 on metagenomic DNA isolated from the Victoria Falls decaying compound logs were ligated into PUC19 and transformed into Z competent *E. coli* DH5α cells. Fifteen white colonies were picked from Mandel's agar plates enriched with ampicillin, IPTG, and X-Gal. Functional screening of the selected clones resulted in 1 clone (Clone-i), giving a positive result for extracellular cellulase production. It was indicated by a zone of clearance on Mandel's media

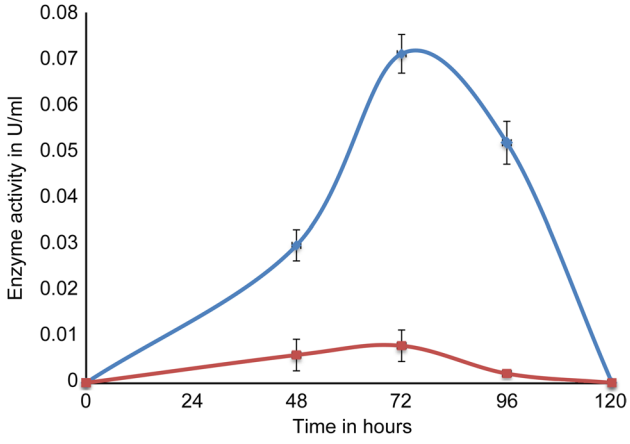

Fig. 4. Production of exoglucanase (♦) endoglucanase (■) by Clone-i over time. Enzyme production was carried out over 120 hours at 37°C.

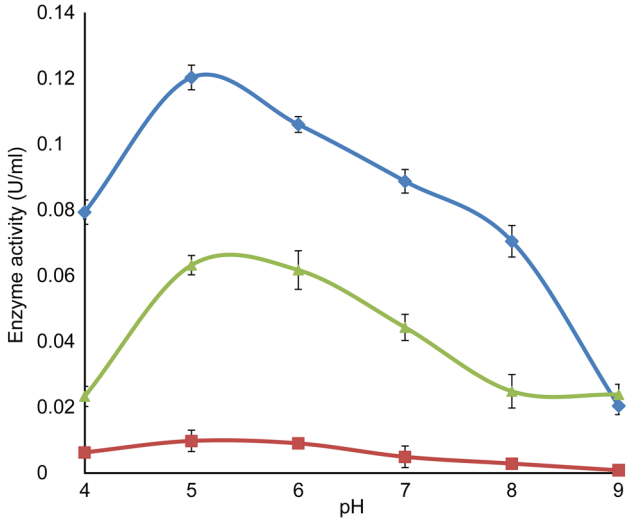

Fig. 5. Effect of pH on exoglucanase (♦), endoglucanase (■), and total cellulase activity (▲) produced by Clone-i grown at 37°C for 72 hours at pH 4 to 9.

after 18 hours of incubation followed by flooding with Gram's iodine, as seen in Fig. 3.

Assay for production of cellulases by Clone-i over time. The cellulolytic Clone-i produced cellulases in submerged fermentation at 37°C with the highest exoglucanase activity of 0.071 U/ml and endoglucanase activity of 0.008 U/ml, both observed after 72 hours of fermentation. The trend of Clone-i's enzyme activities over time is shown in Fig. 4.

Determination of the optimum pH for the activity of cellulases. The endoglucanase produced by Clone-i showed activity in the pH range of 5 to 8 (Fig. 5). Exoglucanases produced by Clone-i exhibited optimum activity at pH 5.

Determination of optimum temperature for cellulase activity. The total cellulase activity produced by Clone-i dropped by 10.8% with the increase in temperature from 50°C to 55°C. After that, enzyme activity was

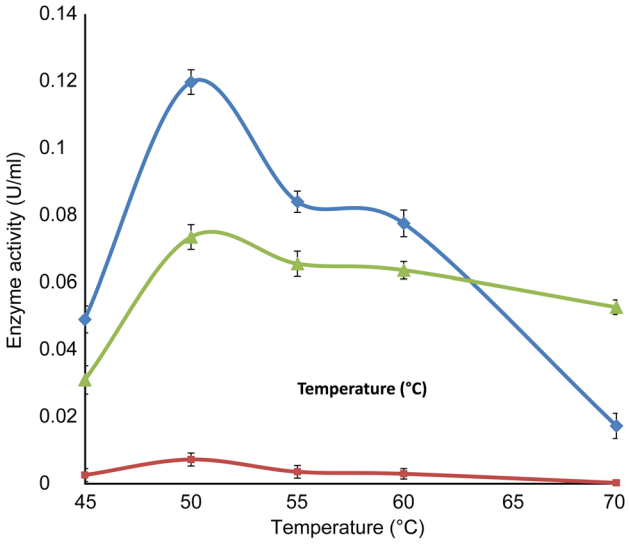

Fig. 6. Effect of temperature on exoglucanase (♦), endoglucanase (■), and total cellulase activity (▲) of enzyme produced by Clone-i grown at 37°C for 72 hours.

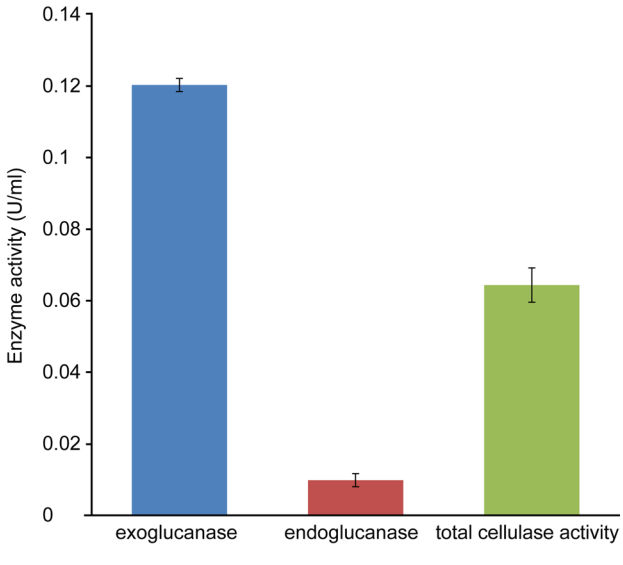

Fig. 7. Activities of the diverse cellulases produced by Clone-i.

relatively stable up to 70°C (Fig. 6). The profile of the activities of the different cellulases is shown in Fig. 6.

Determination of the diversity of Clone's-i cellulases. The diversity of cellulases produced by Clone-i is shown in Fig. 7. Assaying of the different cellulases was carried out at optimum pH and temperature. Clone-i had a much higher (80%) exoglucanase activity than endoglucanase activity. Under the optimum conditions, Clone-i's exoglucanase activity was 0.12 U/ml (Fig. 7).

Discussion

The epicentre metagenomic DNA isolation kit proved efficient as high molecular weight DNA was isolated free of impurities. The amplification of the Victoria Falls decaying logs' metagenomic DNA by PCR indicates that the DNA extraction method successfully eliminated humic acids. Rolf (2005) stated in his review that humic acids present in environmental samples are often co-extracted with metagenomic DNA and interfere with downstream processes such as PCR amplification, cloning, and transformation. PCR amplification and cloning of metagenomic DNA are critical for analyzing a metagenome by sequence-based and functional screenings. Inhibition of these processes would mean the metagenomic DNA cannot be analyzed further.

Primer 1 and 2, encoding the endoglucanase gene, successfully amplified the metagenomic DNA by PCR. PCR amplicons from primers 1 and 2 had fragment sizes ranging from 400 base pairs to 1,200 base pairs, suitable for cloning into a plasmid vector pUC19.

The efficiency of an efficient transformation is indicated by the number of colonies; hence ten white colonies were picked from the cloning of primer 1 and primer 2 PCR amplicons. Functional screening of the clones resulted in one clone showing cellulase activity on Mandel's agar. There are several possible reasons why the other clones did not show cellulase activity. Metagenomic DNA is made up of fungi, archaea, and bacterial DNA (Wang et al. 2008); if the cloned amplicons were from fungal DNA, their expression in the host (*E. coli* DH5α) could have been a challenge. According to Wang et al. (2008), fungal genes contain introns, and hence *E. coli* cannot process messenger ribonucleic acid (mRNA) transcripts of such genes. The glycosylation of fungal proteins is vital for enzyme secretion and activity, but *E. coli*, being prokaryotic, do not glycosylate proteins. According to Uchiyama and Miziyaki (2009) using a different expression system such as yeast could have increased the chances of obtaining positive clones in the functional screening as it would appropriately express amplicons of fungal origin.

The sensitivity of the screening method contributes to the success of functional analysis of the clones (Kennedy et al. 2011). The sensitivity of our plate screening method could have been increased by using the cell lysates of the clones to analyze the intracellular accumulation of the enzyme (Kennedy et al. 2011). The analysis of intracellular enzymes was not done in this work because the application of intracellular enzymes is faced with challenges of purification of the enzyme from different cell proteins and other components (Aehle 2007).

According to Bai et al. (2012), temperature and pH are essential parameters for cellulase activity. The findings of Clone-i's activity profile are similar to Nacke et al. (2011) cellulolytic clone Cel01, which showed optimal activity at 50°C and was highly active from pH range of 5 to 9. Under optimum conditions, Clone-i's exoglucanase activity at pH 5 and 50°C was 0.12 U/ml. The endoglucanase activity of Clone-i was found to be 0.009 U/ml making it 8.25% of the exoglucanase activity.

Conclusions

Clone-i, which was engineered from amplicons from the Victoria Falls metagenomic DNA, showed extracellular cellulase activity. Clone-i's exoglucanase activity was over a broad temperature range of 50°C–70°C, and a pH range of 5–8, respectively. Clone-i's ability to produce extracellular cellulases and the pH and temperature conditions of the activity of the enzyme makes it suitable for application in industry. The successful cloning of Victoria Falls metagenomic DNA cellulase amplicons indicates the great potential metagenomics has in bioprospecting of industrial importance.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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