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Full Length Research Article

ENZYME ASSAY, CLONING AND SEQUENCING OF NOVEL β-GLUCOSIDASE GENE FROM *ASPERGILLUS NIGER* F321 (UNIDENTIFIED NIGERIAN STRAIN)

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ABSTRACT
β-glucosidase is a cellulase enzyme under intense investigation for its potential role in cellulose degradation for the generation of fermentable sugars used in biofuels production. Ten catalytic sites have been identified that are conserved in β- glucosidases from a range of prokaryotes and eukaryotes. NCBI Primer BLAST was used in this study to design primers that successfully clone a partial β-glucosidase gene from an uncharacterised Nigerian strain of the filamentous fungi *Aspergillus niger* F321 (*A. niger* F321). Two β-glucosidase genes from *A. niger* F321 denoted as ANRA12.6 and ANRA12.9 were amplified from genomic DNA using PCR techniques and the amplicons gave estimated PCR products of 1,190 bp and 1,950 bp respectively. Subsequent cloning into *E. coli* produced positive results for blue/white screening of transformed colonies while the colony PCR of their pDNA gave estimated sizes of 860 bp and 1,600 bp respectively. DNA sequencing confirmed that the chosen *A. niger* F321 partial β-glucosidase sequences had been successfully cloned. Bioinformatics studies also suggested that the cloned β-glucosidases share some characteristics with their bacterial counterparts. The findings in this study highlight the increasing need for more information on β-glucosidase structure and function.

Keywords: *Aspergillus niger*, β-glucosidase, cellulase, PCR, sequencing, Bioinformatics

INTRODUCTION

With an increasing need to explore renewable energy resources to preserve our planet, studies into biofuel production are well underway. First generation methods of bioethanol production involving the use of simple sugar fermentation are ineffective at producing a substantial amount of energy, as they result in a great deal of carbon waste within the lignocellulosic substrate that cannot be accessed (Colabardini *et al.* 2014). Second generation biofuel production involves the use of fungi to degrade an energy source i.e. lignocellulosic biomass, into fermentable sugars for industrial use (Ries *et al.* 2013). Some of the most commonly used fungi in this process are species from the genus *Aspergillus*, specifically *Aspergillus niger*.

Aspergilli are filamentous fungi that are used in a wide range of industrial settings and are increasingly important in areas of basic genetic research, so up to until 2010, whole genome sequencing of 4 species of *Aspergillus* had been completed (Bennett 2010). In terms of comparative genomics, sequencing more species of *Aspergillus* will be greatly beneficial in giving insight into some of the reasons why there are such vast differences in levels of pathogenicity in each (Bennett 2010).

Aspergilli encode a wide range of enzymes that allow them to break down complex lignocellulosic biomass into glucose for their own benefit within a natural setting. These enzymes are ordinarily referred to as cellulase. The extent of cellulase production by *Aspergillus* species depends on the environment of the organism; noticeably, media containing higher levels of cellulose will induce better cellulase production (Bansal *et al.* 2012). Although cellulases are produced by a range of organisms including bacteria, fungi are of significant importance as they possess the ability to do so extracellularly (Bansal *et al*. 2012). One of the cellulases produced in this fashion by species of *Aspergillus* is β-glucosidase.

The conversion of cellulolytic materials into fermentable sugars requires the action of endoglucanase [EC 3.2.1.4], exoglucanase [EC 3.2.1.91] and β-glucosidase [EC 3.2.1.21] which work in concert to hydrolyse cellulose (Onyike *et al*. 2008; Zafar *et al*. 2011; Del Pozo *et al*. 2012). Synergistically, these three enzymes work towards full saccharification of cellulose into the valuable end product, glucose (Horn *et al.* 2012) and the schematic process is represented in Figure 1.

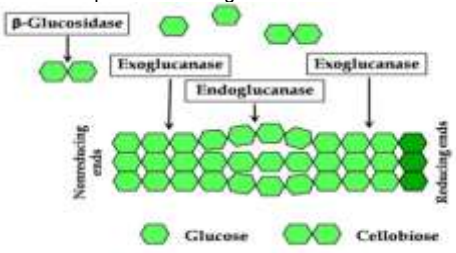

Fig. 1: Schematic presentation of the conversion of cellulose to fermentable sugars (Ratanakhanokchai *et al*. 2013)

The endoglucanases cleave internal β-1, 4-glycosidic bonds randomly, leaving a range of cellulose chains differing in length while exoglucanases act on either the reducing or non-reducing end of these exposed cellulose chains, which releases molecules of cellobiose (Strakowska *et al.* 2013). Finally, β-glucosidase prevents the accumulation of cellobiose at the final rate limiting step by catalysing its conversion to glucose (Strakowska *et al.* 2013).

β-glucosidases are essential components of the cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. The catalysis of cellobiose is important since the accumulation of cellobiose creates feed-back inhibition. β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al.* 2002).

Agricultural residues such as grasses, tree wastes and many other green plants whose disposal is considered as an environmental problem in Nigeria can represent large renewable resources for enzyme production by fungi (Anwar *et al*. 2014). However, the cost of obtaining sugars from lignocellulose biomass for fermentation is still high, mostly due to low enzyme yields of producing microorganisms (Onyike *et al*. 2008).

There are so many instances in the literature on the cloning of β-glucosidases from bacterial and fungal sources (Nair *et al*. 2013; Karnaouri *et al*. 2013; Li *et al*. 2014). A β-glucosidase-like enzyme encoding gene (*bglH*) of *Bacillus pumilus* (CL16) was cloned by Bogas *et al*. (2007) in *Escherichia coli*. The complete nucleotide sequence open reading frame of 1419 bp had 472 amino acid residues without a characteristic signal peptide sequence suggesting that the enzyme is intracellular. Karnaouri *et al*. (2013) cloned a β-glucosidase gene (*bgl3a*) from *Myceliophthora thermophila* and were expressed in *Pichia pastoris*. The recombinant β-glucosidase with a molecular weight of 90 kDa was purified and characterized. The optimal pH and temperature of the enzyme on pNPG was 5.0 and 70 ^{o}C respectively, with a Km of 0.39 mM on pNPG and 2.64 mM on cellobiose. The use of *Pichia pastoris* system in the expression of recombinant β-glucosidases from *Chaetomium thermophilum* CT2 is reported by Xu *et al*. (2011). The full-length cDNA of the β-glucosidase contained an open reading frame of 2604 bp nucleotides and encoded 867 amino acid residues with a potential secretion signal peptide.

This research work focuses on the Cloning of β-glucosidase gene from *Aspergillus niger* F321for later expression and characterization

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MATERIALS AND METHODS

Organisms and plasmid

Aspergillus niger F321 (*A. niger* F321) (an uncharacterised Nigerian strain) was a kind gift from Mallam Sani Mohammed of Microbiology department, Kaduna State University, Kaduna. The organism was maintained on malt extract agar (MEA) composed of 1.5% (w/v) agar no.2 and 2% (w/v) malt extract in 100ml of distilled water. The solution was adjusted to pH 7 and autoclaved at 121°C for 15 minutes before use.

Genomic DNA Mini kits and were products of Invitrogen UK. PCR product purification kits, QIA quick Gel Extraction kits and Plasmid DNA extraction kits were all supplied by QIAGEN UK. PCR Master Mix, pGEM^{R}-T Easy vector system II and JM109 Competent cells (> 10^{8} cfu/µg) were obtained from Promega UK. Midori Green Advance stain, High Ranger 1 kb DNA ladder and PCR products ladder were obtained from Geneflow, UK. Primers were designed using the NCBI Primer BLAST and were ordered from Sigma (www.sigmaaldrich.com/united-kingdom.html). Presumptive cloned genes were sent to Source BioScience LifeSciences (UK) using the same forward primers used for PCR amplification. The entire reagents used in this study were of analytical grade.

Rapid method for determining *A. niger* F321 cellulase activity

A simple, rapid and sensitive cellulase assay procedure based on the Congo red clearing zone assay method by Sazci *et al*. (1986) was used to determine cellulase activity. This method allows the simultaneous enzyme comparison for many samples and has been developed to allow the detection of cellulase/β-glucosidase activity under a wide range of conditions for a spectrum of strains. *A. niger* F321 strains were initially grown on Malt Extract Agar (MEA - Malt extract - 30 g, agar - 15 g, per liter) plates. Cultures were stored at 4^{o}C. Spores were aseptically picked from plates and inoculated into 5 ml sterile nutrient broth in a McCartney bottle. The bottles were incubated at 30^{o}C in an orbital shaker (Model G25, S/No. 390534557 U/K) at 150 rev/min for 2 days. After the incubation period, the samples were centrifuged at 3500 rpm (MSE MISTRAL 1000 centrifuge S/No M1000 240-V) to remove mycelia. The supernatant (crude enzyme) was filter sterilized into sterile Bijoux bottles using a sterile MILLEX^{o} GP Syringe Filter Unit (0.22 µm). Sterile petri dishes were prepared and each Petri dish was made up of 15 ml Carboxymethylcellulose (CMC) agar, made with 0.5 g CMC (a soluble form of cellulose); 0.1 g $NaNo_3$; 0.1 g K_2HPO_4; 0.1 g KCl; 0.05 g $MgSO_4$; 0.05 g yeast extract; and 0.1 g glucose in 100 ml of distilled water. The medium was solidified using 1.7% w/v agar no 2. Into the appropriate wells on the CMC agar plates, 40 µl of the crude enzyme (sample) or nutrient broth (Control) was placed and incubated for 2 days. Incubation was carried out at 5^{o}C intervals in the range of 25 - 50^{o}C. After incubation each plate was flooded with 0.1% Congo red solution, and shaken at 50 rev/min for 15 minutes on a shaker (Model R100 rotatest shaker Luckham, S/No. R/2268/B). The plate was also de-stained (flooded) with 1 M NaCl salt solution by shaking again at 50 rev/min for 10 – 15 minutes. The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity. Enzyme activity was stopped by flooding the plates with 1 M NaOH (pH 13.3) which slightly changed the dye color to brownish-red. The diameter of the clear zone was measured to provide qualitative cellulolytic activity. The assay was carried out in triplicate for each sample and mean value was used as a measure of enzyme activity.

Genomic DNA Extraction

Genomic DNA was extracted and purified using EZNA Omega fungal DNA extraction kit following the manufacturer's instructions. In order to amplify the relevant β-glucosidase genes, primers were designed using the NCBI primer design tool. The *Aspergillus* Comparative Database was used to confirm and obtain a full *A. niger* β-glucosidase (EHA19734.1) sequence (http:broadinstitute.org/annotation/genome/). This sequence was inputted to NCBI Primer BLAST, producing 10 possible primers for use, of which primer pairs 6 and 9 were selected (Table 1).

Table 1: List of primers designed for PCR amplification of β-glucosidase genomic DNA from *A. niger* F321

Primer pairs	Sequence (5' – 3')	T_m (°C)
6 -F	GGATTGATCTGGACCCCGTC	59.89
6 - R	ACACATAGCTCAACACCTGC	58.19
9 - F	AGCGGCGTGGTCAATCAATA	60.11
9 - R	AGCGCTCTTGTCACAAACACA	61.01

F: Forward primer; R: Reverse primer

PCR reaction conditions
In order to amplify the relevant β-glucosidase genes, PCR reactions were carried out using a Prime Techne thermocycler from Geneflow. A Promega PCR Master Mix containing 50 units/ml of Taq polymerase [pH 8.5], 400µl of dATP, 400µl of dGTP, 400µl of dCTP, 400µl of dTTP, 3mM $MgCl_2$ was ordered from Promega (www.promega.co.uk). PCR reaction solutions were made up to a volume of 25µl per tube with nuclease-free water. Thermo-cycling conditions for the PCR reactions included an initial denaturation step at 95°C for 5 minutes. This was then followed by 30 cycles of denaturation at 95°C for 1 minute. The primer annealing temperature for each primer was set based on its melting temperature (T_m) value -5°C for 2 minutes. This was superseded by an extending temperature step of 72°C for 2 minutes and a final extension temperature of 72°C for 5 minutes. For analysis, 10 µl of the reaction mixture was electrophoresed on a 1.2% agarose gel and stained with 0.5µL web green (Syngene Geneflow - serial No.: SYGV/4688), a nucleic acid dye that stains DNA to aid its visualization under UV light. 10 µl of sample was loaded with 2 µl of 6x loading buffer per well with appropriate marker and run at 40V for 3 - 4 hours.

Cloning and sequencing of the β-glucosidase gene
Fragments were isolated and ligated to pGEM-T vector. The plasmid mixture was introduced into *E. coli* JM109, which was then plated onto LB medium containing ampicillin (100 µg/ml). Three and two clones were obtained for primer set 6 and primer set 9 respectively. Each clone was transferred to two plates (plate for plasmid preparations and master plate). Plasmid DNA were isolated from *E. coli* using EZNA Plasmid DNA Mini Kit I (D6945-00) following the manufacturer's instructions. Candidate genes were selected using amplification of β-glucosidase gene fragments by colony PCR using primer 6 and primer 9.

Sequencing of plasmid insert DNA was carried out at Source BioScience Life Sciences laboratory in Rochdale United Kingdom using Next-generation DNA sequencing (Shendure and Ji 2008). The sequences were read using Chromas Lite 2.1.1 sequence converter (Free Technelysium Software package for DNA sequencing – http://technelysium.com.au/?page_id=13).

Bioinformatics and Phylogenetic Tree construction
The sequence reads of appropriate quality obtained for each gene were compared against the non-redundant nucleotide sequence collection at NCBI Genbank using the web interface of NCBI-BLAST. EMBOSS (2000) Sixpack (http://www.ebi.ac.uk/Tools/st/emboss_sixpack/) and NADV (http://nadv.herokuapp.com/sequence/new_results) were used to display DNA along with translation in all 6 reading frames. Multiple Sequence Alignments (MSA) were performed on the sequenced gene to identify regions of similarity, structure and function using a standard β-glucosidase gene (Lima *et al*. 2013) with TCoffee advanced (Notredame *et al*. 2000), SignalP 4.1 (to avoid similarities within the signal sequence alone) and ClustalX 2.1 (http://www.clustal.org/clustal2/) (Page 1996).

RESULTS

Expression and assay of β-glucosidase activity
The effects of temperature on *A. niger* F321 cellulase production were investigated in these studies. Figure 2 showed that the expression of cellulases for hydrolysis of the CMC supplemented plates were stable at temperature of 25 – 40 °C with highest cleared zone of hydrolysis at 40 °C, with no hydrolysis at 50 °C. The controls (without enzyme) showed the absence of hydrolysis. The halo produced by hydrolysis of CMC is directly related to the region of action of cellulase activity, since the Congo dye only remain attached to regions where there are β-1,4-D-glucose bonds (Florencio *et al*., 2012). The diameter of the halo showed the presence or absence of the cellulase production. Extracellular cellulase activity of *A. niger* F321 had highest activity on CMC with 2.22 ± 0.04 mm zone of hydrolysis (Figure 3).

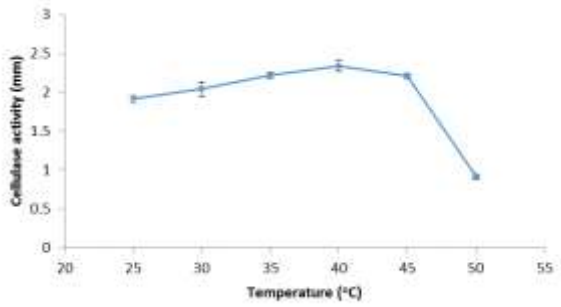

Fig. 2: Effect of temperature on *A. niger* F321 cellulase activity (mm) after 48 hours of incubation on CMC

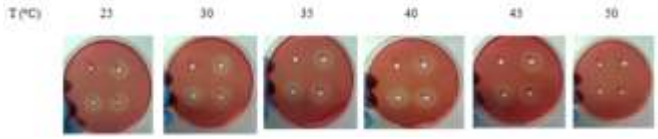

Fig. 3: Zone of hydrolysis of *A. niger* F321 cellulase. Pictures were taken after 2 days of incubation

Amplification and cloning of *A. niger* F321 β-glucosidase gene
Two β-glucosidase genes from *A. niger* F321 genomic DNA were amplified using PCR techniques with two set of primers. The PCR products of primer set 6 and primer set 9 gave estimated PCR products of 1,190 bp and 1,950 bp respectively. Cloning of these

genes gave 3 and 2 clones for primer set 6 and primer set 9 respectively and amplification of an appropriate DNA fragment by PCR was detected in one colony. A plasmid was prepared from a cultured cell originating from the selected colony. The Electrophoretic gel images of the plasmid DNA is shown on Figure 4. A 0.86 Kb amplicon was obtained by colony PCR for primer 6 set (designated as ANRA12.6) while 1.5 Kb amplicon was obtained for primer 9 set (designated as ANRA12.9) (Figure 5). Sequencing results confirmed integration of the recombinant gene into the pGEM-T vector.

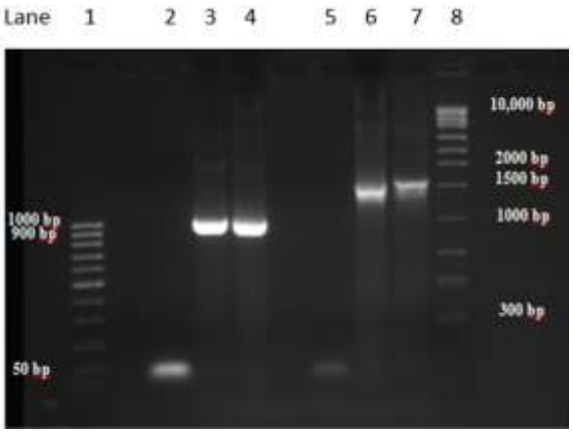

Fig. 4: Electrophoresis gel images of ANRA12.6 and ANRA12.9 Plasmid DNA**.**

[Lane 1: PCR product ladder, 2: ANRA12.6 plasmid DNA, 3: ANRA12.9 plasmid DNA, 4: High ranger ladder.]

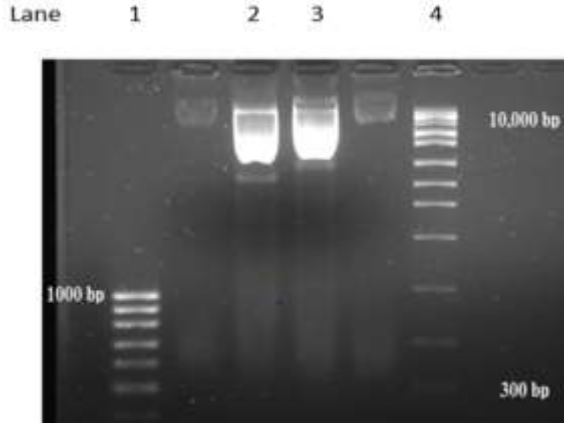

Fig. 5: Electrophoresis analysis of presumed ANRA12.6 and ANRA12.9 colony PCR products.

[Lane 1: PCR product ladder, 2: negative control (no DNA), 3 and 4: ANRA12.6 amplicon, 5: negative control (no DNA), 6 and 7: ANRA12.9 amplicon, 8: High range ladder.]

Bioinformatics

When the sequences were analysed by the SignalP 4.1 program (Petersen *et al*. 2011), it was predicted that the encoded ANRA12.6 and ANRA12.9 β-glucosidase enzyme could be secreted because it had a signal peptide. The open reading frame (ORF) using JavaScript DNA translator 1.1 (Perry 2002) of ANRA12.6 (frame 1) and ANRA12.9 (frame 3) encoded a protein of 255 amino acids and 405 amino acids with a predicted molecular mass of 29.23 kDa and 47.95 kDa respectively (http://www.bioinformatics.org/sms/prot_mw.html).

BLASTx analysis of ANRA12.6 sequence indicated that the sequence has 99% identity with 84% coverage to *Aspergillus niger* 513.88 (accession number - XP_001394592.2) β-glucosidase protein with an E-value of $1e^{-97}$. The sequence also had close similarity with *A. niger* ATCC1015 hypothetical protein (accession number - EHA19734.1) with 99% identity, 84% coverage and E-value of $1e^{-96}$. The results indicated the existence of ANRA12.6 insert in the clone. BLASTx analysis of ANRA12.9 sequence indicated that the sequence has 96% identity with 93% coverage to *Aspergillus niger* ATCC 1015 (accession number – EHA19734.1) hypothetical protein with an E-value of 0.0; and also with β-glucosidase from *Aspergillus kawachii* IFO 4308 (accession number – GAA83698.1) with identity of 95%, coverage of 93% and E-value of 0.0. The result suggests the existence of ANRA12.9 insert in the clone.

Figure 6 shows the deduced amino acid sequence open reading frame (ORF) using JavaScript DNA translator 1.1 (Perry, 2002) of ANRA12.6 (frame 1) and ANRA12.9 (frame 3). The deduced amino acid sequence was used for multiple sequence alignment.

>ANRA12.6 frame1

GKQATPPTPLVILPKWTPPLHHPLQGIRLPIQRGPPRPSLRTNPRRFHRGPIHRLPPLPQSQHHSPLPVWSRPLLHH
LHLLHSIPHHRHRPRHCLPPSSLIQRPNPIVQHRHPKPIRGRLAILLLSYLALLVPISRQPAICHQLLFIFIPLSIRLLH
HSETCTPSWWWRRWKSRPVGCCLRGGRNHHKQWERRSRCGPAVCRATDRYTGGRCAIQTVTVVENGDPGAR
ESQTVVLNVTRKDVSFMFSFFFFF

>ANRA12.9 frame3

SALPCSRRHHPSQERKYSPFVDNRLTKDLWHRRPQLQRAQLVHRPRLRQRRPHHGLGQWHVSSPISCNPPTSHR
QSLFLRRILHHRLIPVRPQPQCLHRARLHQRRLRRKLHHRRKQPRPHHRRPLRVAQRRRSRQSRSRRLLHRHRD
CPHRRPYSPRELDFGPRQSRPHRPSPRSRSRLLPNRHPLRLILPKWTPPLHHPLQGIRLPIQRGPPRPSLRTNPRRFH
RGPIHRLPPLPQSQHHSPLPVWSRPLLHHLHLLHSIPHHRHRPRHCLPPSSLIQRPNPIVQHRHPKPIRGRLAILLLS
YLALLVPISRQPAICHQLLFIFIPLSIRLLHHSETCTPSWWWRRWKSRPVGCLRGGRNHHKQWNVSARWPSCMS
SYRPITGVDAHPTYGIKGLRD

Fig. 6: Deduced amino acid sequences of ANRA12.6 and ANRA12.9 (Perry 2002)

Figure 7 shows the multiple sequence alignment of β-glucosidases from *Aspergillus niger* AnBg11 (Lima *et al*. 2013), and the deduced amino acid sequence of ANRA12.6 and ANRA12.9. A complete sequence alignment was carried out on the alignment program TCoffee (Notredame *et al*. 2000) to generate a multiple sequence alignment. The results as indicated in Figure 7 shows core catalytic active sites of ANRA12.6 and ANRA12.9 not to be aligned with those of AnBgl1 as described by Lima *et al*. (2013). The carbohydrate-binding PA-14 domain (Yoshida *et al*. 2010) was absent in both ANRA12.6 and ANRA12.9 sequences. Also, the N-terminal domain, which is suggested to act as solubility enhancers for the folding C-terminal domains *in vivo* (Kim *et al*. 2007), was missing in ANRA12.6.

H

Fig. 7: Multiple sequence alignment of β-glucosidases amino acids from *Aspergillus niger* AnBg11, ANRA12.6 and ANRA12.9.

[Symbols: Catalytic site ( ); helix –A and –B (green box), intermediate amino acids from linker 2 and N-terminal domain (black boxes) (Lima *et al*. 2013)].

Figure 8 shows the phylogenetic trees (neighbour joining) of β-glucosidase sequences which were constructed using ClustalX and Treeview (Larkin *et al*. 2007) with the bootstrap value set at 2000. Phylogenetic tree was constructed to compare *Aspergillus* enzymes (AN12.6 and ANRA12.9) to bacterial counterparts (from *Gluconacetobacter xylinus, Bacillus* subtilis) and with *A. niger* (AnBg11), Afu6g12010 and NFIA_027390 enzymes. Characterized bacterial enzymes were included in tree drawing so as to root the trees in order to identify whether ANRA12.6 and ANRA12.9 enzymes resembled bacterial enzymes. However, it became clear that examples of bacterial β-glucosidases in some cases were grouped with ANRA12.6 and ANRA12.9 enzymes (Figure 8).

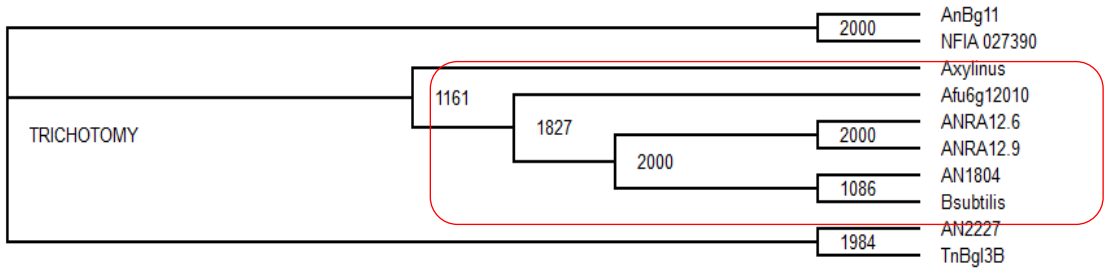

Fig. 8: Treeview analysis of β-glucosidase genes.

[*A. niger* (AnBg11, ANRA12.6, ANRA12.9), *Thermotoga neapolitana* (TnBgl3B) NFIA_027390, *A. nidulans* (AN1804.2), and bacterial enzymes from *Gluconacetobacter xylinus* (*G. xylinus*) (WP_007399076) and *Bacillus subtilis* (YP_003864539.1) (shown with values out of 2000 bootstraps for branch points). Red rounded rectangle: an example of fungal and bacterial proteins forming a clade].

DISCUSSION

Cellulose is the most abundant organic substance in the world and comprises of glucose which is a major component of biomass energy. Cellulolytic microorganisms can be isolated from soil, agricultural wastes and animal dung. For example, Saxena *et al*. (1993) isolated cellulose degrading bacteria from soil and termite gut. Teunissen *et al*. (1992) isolated an anaerobic fungus *Piromyces sp*. from the droppings of an elephant and found that the fungus was able to use cellobiose, cellulose, glucose, starch, wheat straw and wheat bran as carbon source. Temperature plays an important role in expressing the activity of biological system and has great influence on the production of end product (Chandel *et al*. 2013). Figure 3 showed optimum temperature of *A. niger* F321 cellulase hydrolysis to be at 40 ^{o}C. Previous research work (Murao *et al*. 1988; Lu *et al*. 2003) have reported different optimum temperatures for cellulase enzyme production suggesting that maximum/optimal temperature for cellulase production depends on the strain of microorganism. The fungal cellulase optimum temperature of 40 ^{o}C in this study tend to agree with the result of Shaikh *et al*. (2013) where the optimum activity of CDB30 cellulase enzyme from *Bacillus* species was reported to be highest at 50^{o}C. Pardo and Forchiassin (1999) also reported the optimal temperature for the activity of cellulase system from *Nectria catalinensis* to range between 50 ^{o}C to 55^{o}C. The optimum of 40^{o}C temperature for cellulase produced by *A. niger* has also been reported by Gautam *et al.* (2011). Cellulases have novel applications particularly in the production and processing valuable components from plant materials.

Figure 7 shows both ANRA12.6 and ANRA12.9 to have good alignment with AnBgl1. Most interestingly, multiple sequence alignments also predicted an unexpected similarity of fungal proteins to those of enzymes from bacteria (result not shown), suggesting possible horizontal gene transfer events from bacteria. For example, Figure 8 highlights the unexpected association in phylogenetic trees of ANRA12.6 and ANRA12.9 proteins grouping with bacterial proteins (*G. xylinus and B. subtilis*) with over 50 % bootstrap value (red rounded rectangle) with bacterial counterparts. The presence of the acquired genes could be associated with evolutionary adaptations for nutrition to allow the colonization of new environments (Hakkinen *et al*. 2012). Horizontal gene transfer (HGT) in microbes has played an important role in their evolution and in the generation of genes involved in the synthesis of cellulase enzymes which degrade cellulose. The process and mechanisms involved in the exchange of DNA between distant species is unknown (Mallet *et al*. 2010) but horizontal gene transfer between bacteria and fungi in nature is well documented (Schmitt and Lumbsch 2009).

Generally, *Aspergillus* species are known as a useful source of β-glucosidases (Dan *et al*. 2000; Kamaruddin *et al*. 2015). Kamaruddin *et al*. (2015) have described a β-glucosidase from *A. niger* which is active at low pH values (pH 3.0 – 6.0) as well as high temperature (60 ^{o}C) and the enzyme effectively hydrolyses pNPG with a specific activity of 347.62 U/mg. Baba *et al*. (2015) also characterized *Aspergillus aculeatus* β-glucosidase 1 (AaBGL1) and compared its properties to a commercially supplied orthologue in *Aspergillus niger* (AnBGL). Their results showed recombinant AaBGL1 to be more insensitive to glucose inhibition and more efficient at hydrolyzing one of major transglycosylation products, gentiobiose than AnBGL. The recombinant AaBGL1 also completely hydrolysed 5 % cellobiose to glucose faster than AnBGL. In this study, a novel β-glucosidase gene was successfully cloned from *A. niger*, a Nigerian strain, thus achieving the first step towards developing recombinant β-glucosidases capable of degrading cellulosic material.

There are several reports on the molecular cloning and expression of β-glucosidase genes from *Aspergillus niger* (Meko'o *et al*. 2010; Kamarudin *et al*. 2015) and *Trichoderma reesei* (Sarah *et al*. 2007; Dashtban and Qin 2012). Meko'o *et al*. (2010) synthesized a 2526 bp gene encoding *Aspergillus niger* β-glucosidase for its heterologous expression in *Pichia pastoris* using methanol as an inducer. They reported the enzyme to be a monomer with an apparent weight of 90 kDa. Dashtban and Qin (2012) also successfully engineered a thermostable β-glucosidase gene from the fungus *Periconia sp*. into the genome of *T. reesei* QM9414 strain. The engineered *T. reesei* strain showed higher β-glucosidase activity compared to the parent strain and was thermotolerant at temperatures as high as 60 ^{o}C after two-hour incubation.

CONCLUSION

An *A. niger* F321 strain was isolated from Nigerian soil and two encoding sequences for a β-glucosidase gene were successfully amplified from the genomic DNA. The matching DNA sequence of *Aspergillus niger* ATCC 1015 β-glucosidase is under accession number EHA19734.1. In this study, encoding sequences for the two novel β-glucosidases genes from *A. niger* 321 were successfully cloned into pGEM-T vector. Bioinformatics studies suggested that both ANRA12.6 and ANRA12.9 β-glucosidases share some characteristics with the bacterial proteins. The grouping of ANRA12.6 and ANRA12.9 with the bacterial counterparts could be as a result of convergent evolution where proteins have evolved from different species to fill the same role. If expressed, these potential novel enzymes may find application in the processing of cellulolytic materials for renewable energy.

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Authors' contributions

RA, IR and PH conceived and designed the experiments; RA coordinated the manuscript. RA and AC performed the experiments; RA, IR and PH analysed the data; RA drafted the paper; all authors read and approved the final manuscript.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article

REFERENCES

Anwar, Z., Gulfraz, M. and Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. *Journal of Radiation Research and Applied Sciences*, **7**: 163 – 173.

Baba, Y., Sumitani, J., Tani, S. and Kawaguchi, T. (2015). Characterization of *Aspergillus aculeatus* β-glucosidase 1 accelerating cellulose hydrolysis with *Trichoderma* cellulase system. *Applied Microbiology and Biotechnology Express*, **5**(3): 1 – 9.

Bansal, N., Tewari, R., Soni, S.K., (2012). Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Management*. **32**(7): 1341-1346

Bennett, J.W. (2010). An overview in the genus *Aspergillus*. *Aspergillus: Molecular Biology and Genomics*. Edited by Machida, M., and Gomi, K., Caister Academic Press. 1 – 15.

Bogas, A.C., Watanabe, M.A.E., Barbosa, A., Vilas-Boas, L.A., Bonato, A.C., Dekker, R., Souza, E.M. and Fungaro, H.P. (2007) Structural characterization of the *bglH* gene encoding a beta-glucosidase-like enzyme in an endophytic *Bacillus pumilus* strain. *Genetics and Molecular Biology*, **30**(1): 100 – 104.

Chandel, K., Jandaik, S., Kumari, V., Sarwati, S., Sharma, A., Kumar, D. and Kumar, N. (2013). Isolation, purification and screening of cellulolytic fungi from mushroom compost for production of enzyme (cellulase). *International Journal of Current Research*, **5**(1): 222 – 229.

Colabardini, A.C., Ries, L.N.A., Brown, N.A., dos Reis, T.F., Savoldi, M.H.S., Goldman, Menino, J.F., Rodrigues, F. and Goldman, G.H., (2014). Functional characterization of a xylose transporter in *Aspergillus nidulans*. *Biotechnology for Biofuels*. **7**:46.

Dan, S., Marton, I., Dekel, M., Bravdo, B.A., He, S., Withers, G.S. and Shoseyov, O. (2000). Cloning, expression, characterization and nucleophile identification of family 3, *Aspergillus niger*β-glucosidase. *Journal of Biological Chemistry*, **275**: 4973 - 4980.

Dashtban, M. and Qin, W. (2012). Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw. *Microbial Cell Factories*, **11**(63): 1 – 15.

Del Pozo, M.V., Fernandez-Arrojo, L., Gil-Martinez, J., Montesinos, A., Chernicova, T.N., Nechitaylo, T.Y., Waliszek, A., Tortajada, M., Rojas, A., Huws, S.A., Golyshina, O.V., Newbold, C.J., Polaina, J., Ferre, M. and Golyshin, P.N. (2012). Microbial β-glucosidases from cow rumen metagenome enhance the saccharification of lignocellulose in combination with commercial cellulase cocktail. *Biotechnology for Biofuels*, 5(73): 1 – 13.

Florencio, C., Couri, S. and Farinas, C.S. (2012) Correlation between Agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. *Enzyme Research*, 1 – 7.

Gautam, S.P., Bundela, P.S., Pandey, A.K., Khan, J., Awasthi, M.K. and Saraiya, S. (2011). Optimization for the production of cellulase enzyme from municipal; solid waste residue by two novel cellulolytic fungi. *Biotechnology research International*, 1 – 8.

Hakkinen M., Arvas M., Oja M., Aro N., Penttila M., Saloheimo M. and Pakula T.M. (2012). Re-annotation of the CAZy genes of *Trichoderma reesei* and transcription in the presence of lignocellulosic substrates. *Microbial cell Factories*, 11: 134.

Harhangi, H.R., Harry, R., Peter, J.M.S., Akhmanova, A.; Jetten, M.S.M.; Van, D.D.C. and Opdencamp, H.J.M. (2002). A highly expressed family 1 β-glucosidase with transglycosylation capacity from the anaerobic fungus *Piromyces* sp. E2. *Biochimica et Biophysica Acta*, **1574**(3): 293 – 303.

Horn, S.J., Kolstad, G.V., Westereng, B. and Eijsink, V.G.H. (2012). Novel enzymes for the degradation of cellulose. *Biotechnology for biofuel*, **5**(45): 1 – 12.

Kamaruddin, S., Abubakar, F.D., Illias, R.M., Said, M., Hassan, O. and Murad, A.M.A. (2015). Overexpression, purification and characterization of *Aspergillus niger* β-glucosidase in *Pichia pastoris*. *Malaysian Applied Biology Journal*, **44**(1): 7 – 11.

Karnaouri, A., Topakas, E., Paschos, T., Taouki, I. and Christakopoulos, P. (2013) Cloning, expression and characterization of an ethanol tolerant GH3 β-glucosidase from *Myceliophthora thermophila*. *PeerJ*, **46**: 1 – 20.

Kim, C.W., Han, K.S., Ryu, K.S., Kim, B.H., Kim, K.H., Choi, S.I.L. and Seong, B.L. (2007). N-terminal domains of native multidomain proteins have the potential to assist *de novo* folding of their downstream domains in vivo by acting as solubility enhancers. *Protein Science*, **16**:635–643.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., Mcgettigan, P.A., Mcwilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**: 2947-2948.

Li, Y., Liu, N., Yang, H., Zhao, F., Yu, Y., Tian, Y. and Lu, X. (2014) Cloning and characterization of a new β-glucosidase from a metagenomics library of rumen of cattle feeding with *Miscanthus sinensis*. *BMC Biotechnology*, **14**(85): 1 – 9.

Lima, M.A., Neto, M.O., Kadowaki, M.A.S., Rosseto, F.R., Prates, E.T., Squina, F.M., Leme, F.M.P., Skaf, M.S. and Polikarpov, I. (2013). *Aspergillus niger* β-glucosidase has a cellulase-like tadpole molecular shape: Insights into glycoside hydrolase family 3 (GH3) β-glucosidase structure and function. *The Journal of Biological Chemistry*, **288**(46): 32991 – 33005.

Lu, W., Li, D. and Wu, Y. (2003). Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphurous*. *Enzyme and Microbial Technology*, **32**(2): 305 – 311.

Mallet, L.V., Becq, J. and Deschavanne, P. (2010). Whole genome evaluation of horizontal transfers in the pathogenic fungus *Aspergillus fumigatus*. *BMC Genomics*, **11**(171): 1 - 13.

Meko'o, D.J.L., Yang, Y., Li, T., Cao, R. and Liu, J. (2010). Molecular cloning and expression of a synthetic gene encoding a β-glucosidase of *Aspergillus niger* in the methylotrophic yeast *Pichia pastoris*. *International Journal of Biology*, **2**(2): 40 – 49.

Murao, S. Sakamoto, R. and Arai, M. (1988). Cellulase of *Aspergillus aculeatus*, in Methods in Enzymology. W.A Wood and S.T. Kellog, Eds. Academic Press London, **160**: 275 – 284.

Nair, A., Kuwahara, A., Nagase, A., Yamaguchi, H., Yamazaki, T., Hosoya, M., Omura, A., Kiyomoto, K., Yamaguchi, M., Shimoyama, T., Takahashi, S. and Nakayama, T. (2013) Purification, gene cloning and biochemical characterization of a β-glucosidase capable of hydrolysing sesaminol triglucoside from *Panibacillus sp*. KB0549. *PLoS ONE*, **8**(4): 12 -23.

Notredame C, Higgins DG, Heringa J (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology*, **302**(1): 205–217.

Onyike, E., Auta, R. and Nok, A.J. (2008). Isolation, partial purification and characterization of endoglucanase (EC.3.2.1.4) from *Aspergillus niger* S.L.1 using corn cobs as carbon source. *Nigerian Journal of Biochemistry and Molecular Biology*, **23**(2): 1-11.

Page, R.M. (1996). Treeview. An application to display phylogenetic trees on personal computers. *Computer Applications in Biosciences*, **12**: 357 – 358.

Pardo, A.G. and Forchiassin, F. (1999). Influence of Temperature and pH on cellulase activity and stability in *Nectria catalinensis*. *Revista Argentina de Microbiología*, **31**(1): 31 – 35.

Perry, W.L. (2002). JavaScript DNA translator: DNA-aligned protein translations. *Biotechniques*, **33**(6): 1318 – 1320

Petersen, T.N. Brunak, S., von Heijne, G. and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, **8**: 785 – 786.

Ratanakhanokchai, K., Waeonukul, R., Pason, P., Tachaapaikoon, C., Kyu, K.L., Sakka, K., Kosugi, A., Mori, Y., (2013). Paenibacillus curdlanolyticus Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization. *Biomass Now – Cultivation and Utilization.* 369-394.

Ries, L, Pullan, S.T., Delmas, S., Malla, S., Blythe, M.J. and Archer, D.B., (2013). Genome-wise transcriptional response of *Trichoderma reesei* to lignocellulose using RNA sequencing and comparison with *Aspergillus niger*. *BMC Genomics*. **14**:541.

Sarah, R., Motallebi, M. and Zamani, M.R. (2007). Cloning and characterization of Beta-1, 4 Glucosidase 2 (*Bgl* 2) genes from high producer cellulolytic enzyme *Trichoderma harzianum* (T7). *World Applied Science Journal*, **2**(4): 315 – 322.

Saxena, S., Bahadier, J. and Varma, A. (1993). Cellulose and hemicellulose degrading bacteria from termite gut and mound soils of India. *Indian Journal of Microbiology*, **33**(1): 55 – 60.

Sazci, A., Radford, A. and Erenler, K. (1986). Detection of cellulolytic fungi by Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. *Journal of Applied Bacteriology*. **61**: 559 – 562.

Schmitt, I. and Lumbsch, H.T. (2009). Ancient horizontal gene transfer from bacteria enhances biosynthetic capabilities of fungi. *PLoS ONE*. **4**(2): 1 - 8.

Shaikh, N.M., Patel, A.A., Mehta, S.A. and Patel, N.D. (2013). Isolation and screening of cellulolytic bacteria inhabiting different environments and optimization of cellulase production. *Universal Journal of Environmental Research and Technology*, **3**(1): 39 – 49.

Shendure, J. and Ji, H. (2008). Next-generation DNA sequencing. *Nature Biotechnology*, **26**: 1135 – 1145.

Strakowska, J., Błaszczyk, L. and Chełkowski, J. (2013). The significance of cellulolytic enzymes produced by *Trichoderma* in opportunistic lifestyle of this fungus. *Journal of Basic Microbiology.* **54**: 2 – 13

Teunissen, M.J., De Kort, G.V., Op den Camp, H.J. and Huisin't Veld, J.H. (1992). Production of cellulolytic and xylanolytic enzymes during growth of the anaerobic fungus *Piromyces sp*. on different substrates. *Journal of General Microbiology*, **138**: 1657 – 1664.

Xu, R., Teng, F., Zhang, C. and Li, D. (2011) Cloning of a gene encoding β-glucosidase from *Chaetomium thermophilum* CT2 and its expression in *Pichia pastoris*. *Journal of Molecular Microbiology and Biotechnology*, **20**(1): 16 – 23.

Yoshida, E., Hidaka, M. Fushinobu, S, Koyanagi, T., Minami, H., Tamaki, H., Kitaoka, M. Katayama, T. and Kumagai, H. (2010). Role of PA14 domain in determining substrate specificity of a glycoside hydrolase family 3 β-glucosidase from *Kluyveromyces marxianus*. *Biochemical Journal*, **431**: 39 – 49.

Zafar, M., Sheik, M.A., Rehman, S.U. and Jamil, A. (2011). Cloning of a cellulase gene from indigenous strain of *Bacillus* species. *Pakistan Journal of Life and Social Sciences*, **9**(2): 116 – 120.