Using 16S rRNA Identification of an Endo-β-1,4-Glucanase Producing Endophyte from *Brachytrupes membranaceus* Gut

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ABSTRACT---- ***This work was focusing on bioprospecting for cellulase producing endophytes from the gut of* Brachytrupes membranaceus*. Identification of bacterial strains that have the capability of efficiently and effectively hydrolyzing the cellulosic macromolecular structure has realized a microbe that is a mesophilic. The microbe was characterized using 16S rDNA gene amplification and sequencing for phylogenetic relatedness. The 16SrRNA gene amplimer was sequenced using nucleotide sequencing on ABI 3100 DNA Analyser capillary electrophoresis sequencing system. The sequence was used for BLASTN search bioinformatics tools and noted that the sequence had 97 % homology with the* Enterobacter asburiae*. Such phylogenetic relatedness has subspecies differences that can be identified at the genomic level. The organism can grow optimally at pH 6.0 and temperature 40 ºC. Crude cellulase activity showed an optimum enzymatic activity conditions which were exactly the same to growth conditions. The enzymes will be characterized and evaluated for their potential application.***

Keywords— Brachytrupes membranaceus, gut, cellulase, Enterobacter asburiae, 16S rDNA

1. INTRODUCTION

Quite a number of cellulases for the past centuries have been investigated on from several sources that include plants (Hayashi, 1989; Harpster, 2002; Hartati, 2008), microorganisms (Gilkes *et al*., 1991; Zvauya, & Zvidzai, 1995; Ito, 1997; Horikoshi, 1999; Mawadza *et al*., 2002), animals (Bahramian *et al*., 2011) and insects (Cho *et al*., 2010; Willis *et al*., 2010). Historical profiling on cellulase research started from plants, bacteria, and fungi, recent trends in couple of decades ago has shifted towards endogenous insect cellulolytic activity (Watanabe & Tokuda, 2010.

It is of importance that we reflect on the insects as a source of such enzymes because of their commensalism relationship establishment such that they feed on plant, agricultural wastes and grass material. Similar rumen microbial fauna is known to possess major cellulolytic bacterial populations, highly fibrolytic aerobic and anaerobic fungi and protozoa that furnish cellulolytic, hemicellulolytic, xylolytic and glycosidic activity (Ekinci *et al*., 2001; Srinivasan *et al*., 2001; Zhang *et al*., 2009). It is interesting to note that although insects feed on foliages and other green materials of cellulosic and non-cellulosic composition in their diets it has earlier on been reported elsewhere that evidence for low significant cellulolytic activity digestion by foliage-feeding of Blattaria, Hymenoptera, Hemiptera, Phthiraptera, Plecoptera, Diptera, Orthoptera, Coleoptera, Trichoptera or Lepidoptera, has been recorded from the gut fluids of these species particularly of locusts (Morgan, 1975; Martin, 1983; Kim *et al*., 2008; Oppert *et al*., 2010; Watanabe & Tokuda, 2010; Watanabe & Tokuda, 2001; Willis *et al*., 2010; Willis *et al*., 2011; Huang, 2012). These wide spectrums of insects traditionally have been thought that they derive cellulolytic activity from symbiotic bacteria and fungi. The grasshopper digestion of cell wall materials of bean seedlings has shown no evidence to indicate that cellulose is among the cell wall constituents digested (Talmadge & Albersheim.1969). Current research developments have reported of insect-producing cellulases. These insect cellulases have been identified and cloned as well, just like most of the studied ones in the past decades (Zhang *et al*., 2009; Willis *et al*., 2011).

However other latest reports have shown high cellulase activity involving the termite being cited as a good model of symbiosis between microbes and hosts and possesses effective cellulose digestive system from aerobic and anaerobic endophytes. Oxygen-tolerant bacteria, such as *Dyella* sp., *Chryseobacterium* sp., and *Bacillus* sp., were isolated from *Reticulitermes speratus* and *Dissosteira carolina* gut. The endo-β-1,4-glucanase and β-glycosidase activity from other microbes isolated were however found to be quite low.

Increased bioprospecting in an attempt to discover novel enzymes is still an on-going exercise searching for effective and efficient cellulase sources. Attempts using molecular tools have been employed to improve expression and novelty of cellulase catalytic efficiency through genetic engineering (Willis *et al*., 2011). Still these biomolecular engineering techniques have not realized efficient cellulases that hydrolyse effectively on natural and commercially processed cellulose.

With this preview of information on low cellulolytic activity toward cellulose, hence the research was carried out to investigate on bioprospecting for cellulase producing endophytes from *Brachytrupes membranaceus* gut. This insect is a delicacy to the majority of the rural community of Zimbabweans during the mid rainy season, although the insect is found in most parts of Southern Africa and Madagascar. In Zimbabwe, the insect is rendered a pest to crops such as tobacco and maize.

2. MATERIALS AND METHODS

2.1. Screening and isolation of Brachytrupes membranaceus *gut microbes*

A size of five *B. membranaceus* adults of approximately 5.0 cm in length were dug from their burrows located in Lions den, Makonde district, Mashonaland West Province, Zimbabwe which is 17°16′12″S 30°01′30″E. Individual insects were allowed to feed on fresh grass for two days in dark boxes. Then the individual crickets were then killed slowly by subjecting them to 4 ^{o}C before being aseptically dissected. Dissections were carried out on slabs of ice to facilitate easier handling (Shankar et *al*., 2011). The gut tissue from the head to the abdomen was crushed using a sterile glass rod, washed with distilled water into a test tube and then pipetted into 100 ml Erlenmeyer flask with 10 ml of Ringer solution.

2.2. Enrichment for increasing microbial load

For enrichment of cellulases producing bacteria obtained from the insect gut, 5mL of supernatant of sediment's suspension were inoculated in the 250 ml Erlenmeyer flasks which contained 1.0 % (w/v) carboxymethyl cellulose (CMC) mixed with M162 broth medium (Zvidzai *et al*., 2003). The cultures were incubated for 48-72 h at 37 °C on a rotary shaker at 150 rev/min after which centrifugation was performed at 3 000 g to collect cells. The cells were then plated on M162 agar for further screening and obtaining discrete colonies of the cells from the broth medium.

2.3. Screening of cellulase colonies

A qualitative analysis for selecting cellulolytic activity was performed using carboxymethyl cellulose and Congo red (Teather & Wood, 1982). The initial pH of the M162 medium was adjusted to 7.0 with 1.0 M NaOH. The CMC agar plates were then incubated at 37 °C for 5 days to allow for the extracellular release of cellulase enzymes. After the incubation period, the visualization of β-D-glucan hydrolysis zone was done by flooding the agar medium with an aqueous solution of Congo red (1.0 mg/ml) allowed to react for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1.0 M NaCl to obtain the clear zones. The plates were washed with 5.0 % (v/v) acetic acid for 1.0 min to improve the contrast of the clear zones and then finally washed with distilled water. To evaluate for high cellulase activity from each microorganism, diameters of clear zones around each colony was measured. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was noted to contain the highest activity. The colonies that had clear zones were constantly sub-cultured in different Petri dishes containing CMC agar as a method of obtaining pure cultures.

2.4. 3.4. Identification of Bacterium

The bacterium possessing the highest cellulosic activity using the Congo red technique was identified by means of morphological examination, biochemical, physiological and molecular techniques characterization. Physiological and biochemical and fermentation characteristics of the microbe that were investigated are as shown in Table 1. These results were further used to compare with Bergey's Manual of Determinative Bacteria in identification of the microbe (Cowan & Steel, 1993).

2.5. Analysis of 16S rDNA Gene Sequence

2.5.1. DNA Extraction

An overnight culture was prepared by inoculating a single colony of bacteria into a sterile test tube containing LB broth in a 50.0 ml Teflon tube. After this, 1.5 ml of overnight culture was transferred into an Eppendorf tube and centrifuged for 2 minutes at 10 000 rpm. The supernatant was removed so as to obtain a pellet which was then dried by inverting the tubes on multiwipe paper. The pellet was re-suspended in 467.0 µl TE buffer by repeated pippeting. Followed by adding 30.0 µl of 10 % SDS (w/v) and 3.0 µl of 20.0 mg/ml Proteinase K. After mixing the tubes were then incubated for 1 hr at 37 ^{o}C. An equal volume of phenol/chloroform mixture was added and mixed well until the phases had completely mixed. The mixture was centrifuged for 2 min at 10 000 rpm. The upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube and an equal amount of phenol/chloroform was added to the mixture followed by centrifuging for 2 min at10 000 rpm. The upper aqueous phase was transferred to a new tube in which 1/10th sodium acetate was added. A volume of 0.6 times of isopropanol alcohol was added followed by mixing gently until DNA precipitated. The tubes were centrifuged at 10 000 rpm for 3 min. The supernatant was removed so as to obtain a DNA pellet which was then dried by inverting the tubes on multiwipe paper. The pellet was suspended in 1.0 ml absolute ethanol after which was then put in a freezer at -80 ^{o}C for 5 min. The tube was centrifuged for 3 min at 10 000 rev/min followed by washing with 70 % (v/v) ethanol and air dried in a vacuum drier (Sambrook *et al*., 1989). Finally the DNA pellet was resuspended by adding 50 µl of TE buffer. This was then used as DNA template for 16S rRNA amplification.

2.5.2. DNA Amplification

Two primers of the 16S rDNA were, 5´AGA GTT TGA TCC TGG CTC AG3´ (forward primer) and 5´AAG GAG GTG ATC CAG CC3´ (reverse primer) respectively were used for amplification. PCR amplification was carried out in a final reaction volume of 25 µl, and the reaction mixture contained 1.5 µl of each primer, 2.5 µl Tris buffer, 2.0 µl of 10 mM dNTPs, 2.0 µl of genomic DNA, 0.5 µl of Dream Taq^{TM}, 13 µl nuclease free water and 2.0 µl of 25 mM $MgCl_2$. The PCR reaction was run for 40 cycles. The following thermal profile was used: denaturation at 95 ˚C for 1 min, annealing at 60 ˚C for 1 min, extension at 72 ˚C for 2 min. The final cycle included extension for 15 min at 72 ˚C to ensure full extension of the products (Zvidzai *et al*, 2003). The amplified PCR products were then analyzed on a 1.0 % (w/v) agarose gel; required fragments were excised from the gel and purified using a Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research). Analyses were done using the gel documentation. The 16S rDNA sequence of the isolate was analyzed using the BLASTN algorithm available on National Centre for Biotechnology Information (NCBI) platform on the internet.

2.6. Reducing Sugars and endoglucanase assays

The DNS method (Ghose, 1987) was used to determine the amount of reducing sugars released by the cellulolytic bacterial organism. The DNS reagent was prepared as follows; 5.0 g NaOH and 100g of sodium potassium tartrate (Rochelle's salt) were dissolved in 500 ml of distilled water followed by the addition of 1.0 g phenol and 5.0 g DNS reagent. Standard glucose solutions (0.07 % w/v) together with sodium sulphite (5.0 % w/v) were prepared and 1.0 ml of each was added to 100 ml of DNS solution before use. The standard Glucose Calibration Curve (absorbance versus glucose concentration) was produced according to a method described by Ghose (1987. Glucose standard mixtures were serially diluted to give 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml with distilled water. Each standard glucose dilution was then treated by making a further half dilution with phosphate-citrate buffer pH 7.0. An amount of 3.0 ml of DNS solution was added and the mixture was immersed in boiling water for 5 minutes, cooled and the absorbance read at 540 nm. The glucose calibration curve was used for extrapolation of unknowns.

Endoglucanase activity was determined by using 0.2 ml of culture supernatant added to 1.8 ml of 1 % (w/v) hydroxyethylcellulose (Fluka product 54290, DP 4540, DS 0.9-1.0) in 0.05 M citrate-phosphate buffer at pH 6.0. The mixture was incubated at 50 °C for 10 min. The reducing sugars produced by the endoglucanase activity were measured using 3,5-dinitrosalicylic acid (DNS) method above. The enzyme blank tube was incubated with 1.8 ml of substrate only, after which 0.2 ml of the enzyme sample was then added immediately followed by pipetting DNS reagent. The reducing sugars (glucose equivalents) were used to calculate the units of enzyme expressed in nanokatal ml^{-1} where 1 kat = 1 mol s^{-1}, glucose equivalents released (Ghose, 1987).

2.7. Effect of initial pH on growth and endoglucanase production

Stock solutions of 2.0 M buffers (Gerhardt *et al.*, 1994) were used to determine effect of initial pH on growth and endoglucanase production by the *Brachytrupes membranaceus*. The buffers were prepared as follows: pH 4.0 (acetate), 5.0 (citrate-phosphate), 6.0 and 7.0 (phosphate), and 8.0 and 9.0 (Tris-HCl). Separately autoclaved 10 ml volumes of 2.0 M of the buffer solutions were added into a 500-ml shake flask with 190 ml of M162 medium in different flasks to introduce different

initial pH values in the medium. A 5 % (v/v) overnight pre-culture was prepared with a 10 ml of the medium for each flask (). The shake flasks were then incubated at 45°C and shaken at 200 rpm in an Innova 4000 Applikon Shaker (New Brunswick Scientific). Samples (5.0 ml) were collected at intervals and cell density of the culture was determined on a Shimadzu UV-120-02 spectrophotometer (OD_{600nm}). Changes in pH during cultivation were monitored. After cell density determination, the samples were centrifuged using a bench centrifuge to get a cell-free supernatant and these samples were kept frozen at –20°C, until endoglucanase analysis. The endoglucanase assay is described in the enzyme assay procedure**.**

2.8. Effect of temperature on growth and endoglucanase production

The effect of temperature on growth of the *Brachytrupes membranaceus* and endoglucanase production was determined in the temperature range 30 to 80°C. A 10 ml overnight pre-culture of the strain was prepared to inoculate 190 ml of the M162 medium with an initial pH value of 6.0 in 500 ml Erlenmeyer flask. The shake flasks were then incubated in Innova 4000 Applikon Shakers set at different temperatures. Samples (5.0 ml) were collected at 2 h intervals and cell density of the culture was determined at 600 nm on a Shimadzu UV-120-02 spectrophotometer (Zvidzai *et al*., 2003). After cell density determination, the samples were centrifuged using a bench centrifuge to have cell-free supernatants and then the samples were kept frozen at –20°C, until endoglucanase analysis as described in the enzyme assay procedure.

3. RESULTS

3.1 Congo Red cellulase enzymatic assaying

The method was very effective in screening for the bacterium that had the highest cellulolytic activity. The ratio of the clear zone diameter to the colony diameter (thus the hydrolysis capacity value) proved to be a very simple quantitative measure for the level of cellulolytic activity. Figure 4 illustrates the clear zones that were observed after flooding bacteria growing on CMC agar with Congo red for 15 min followed by 1.0 M sodium chloride. The bacteria that had the highest cellulolytic activity had a clearing zone of 3.0 mm which was observed after 72 hrs of incubating the plate and performing the Congo Red staining. From figure 2 below, coincidentally the second row shows the clear zones of endo-β-1,4-glucanase activity.

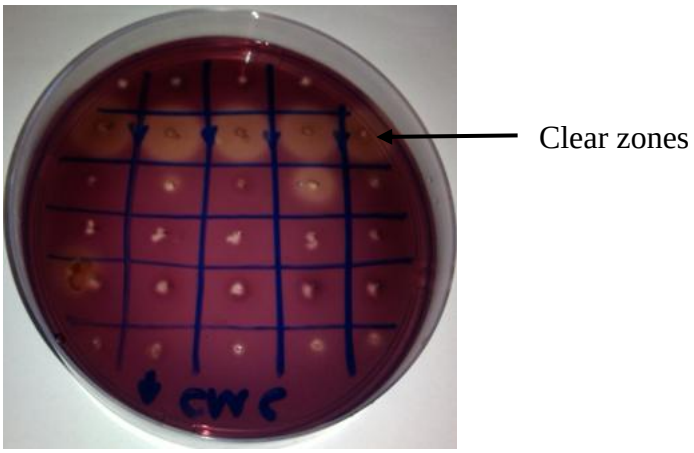

Figure 2: Congo Red staining of a plate of bacterial colonies from the gut of *Brachytrupes membranaceus* grown on CMC agar expressing cellulase activity.

3.2. Physiological and biochemical tests for cellulase expressing colonies

Table 1: Biochemical reaction and characteristics of the isolate from *Brachytrupes membranaceus.*

Characteristics and Biochemical tests	Result
Cell shape	Rod
Gram reaction	-
Indole production test	-
Motility	+
Catalase	+
Oxidase	-
Voges Proskauer reaction	+
Use of citrate	+
Hydrolysis of gelatin	+
Reduction of NO_3^- to NO_2^-	+
Arabinose fermentation	+
Glucose fermentation	+
Mannitol fermentation	+
Presence of endospore	+

Key: + positive reaction, **-** negative reaction of the test performed.

This rod shaped microbe was Gram negative and produced a cylindrical subterminal endospore which was swollen. The cells exhibited high motility, however it's colonies on a Petri dish they formed rough-surfaced, flat and opaque and which were off-white coloured. The microbe showed fermentation of basic sugars like glucose, mannitol, arabinose and cellulose derivative (CMC) which is quite common with Gram positive microbes. It was noted that the microbe could hydrolyze a number of organic sugars as well as citric acit and gelatin. Hence it can grow on a wide array of sources of material for its energy and cellular requirements.

3.3. Analysis of 16S rDNA Amplification & Sequencing

The 16S rDNA sequenced after amplification was obtained from the electrophoretogram shown in figure 2 below.

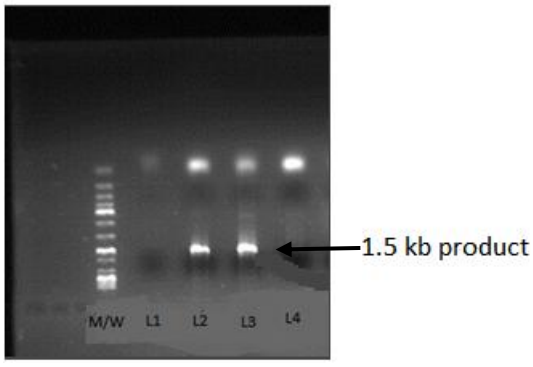

Figure 2: 16S rRNA PCR amplification from the bacterium isolated from *Brachytrupes membranaceus*. **Lane M:** DNA molecular weight marker; **Lane 1:** Control PCR amplification run and **Lane 2 to 3:** PCR amplification run with bacterium genomic DNA with 2, 3, and 4 µl of 25 mM $MgCl_2$ concentration.

The 1.5 kb band labeled in lanes 3 and 4 were more distinct as compared to those in other lanes on the electrophoretogram. The bands were excised from the gel followed by purification then sequenced. After sequencing of the 16S rRNA amplified PCR product, the sequence was subjected to reconstructing and analyzing phylogenetic relationships between molecular sequences using BLASTN algorithm tools for the bacterial strain. The microbial sequence data showed that the query had a 96 % similarity to *Enterobacter asburiae*. This strain had also strong Blast hits with other microbes and was quite closely related to the following microbes in decreasing order; *Enterobacter hormaechei*, *Enterobacter cloacae*, *Enterobacter hormaechei* subsp. *steigawaltii*, an unculturable *Enterobacter* sp. and

Enterobacter aerogens. The figure 3 below shows the BLAST tree view phylogenetic relatedness analysis. It is interesting to note that the microbe that had highest zone of clearance had a score of 0.01 hence had a difference of 1% between two sequences of the query and that of *Enterobacter asburiae* using 250 nucleotides per change sites. The number of nucleotides substitution per site for each branch length is quite high and it shows that hence there is much difference between the microbe from *Brachytrupes membranaceus* and *Enterobacter asburiae*. Hence we named this microbe *Enterobacter asburiae* subsp.

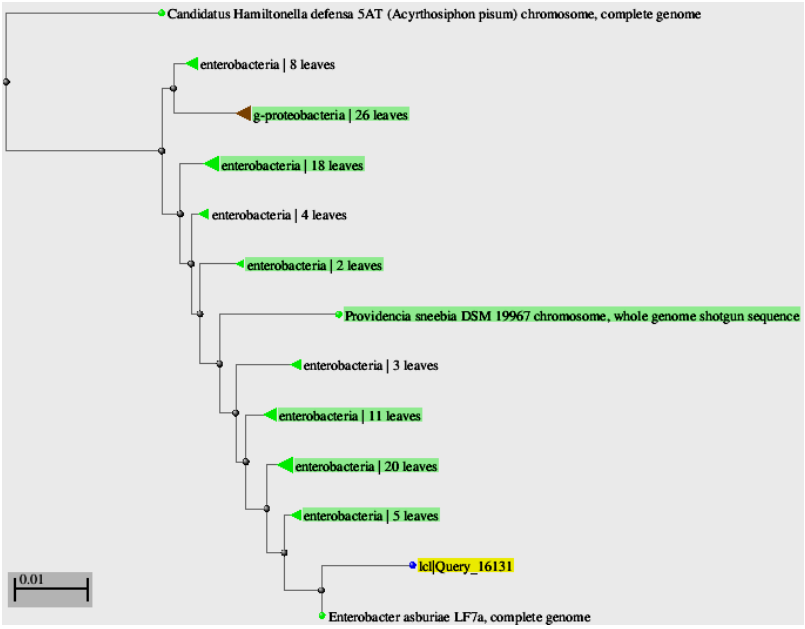

Figure 3: BLAST tree view phylogenetic relatedness analysis of the 16S rRNA sequence of the highest endo-β-1,4-glucanase producer from *Brachytrupes membranaceus* noted as Query_16131.

4.4. Effects of initial pH on growth rate of bacterium

At pH 4, the onset of the exponential phase of *Enterobacter asburiae* subsp. was observed to begin after 3 hours of lag phase after which cellular development resumed. However at pH 5, 6 and 7, the exponential growth phase was observed within 2 hours. Although at pH 6 and 7 on the contrary, optical density (OD) readings showed that the exponential growth occurred in the 1 hour. This rapid cell development behaviour was almost similar in the initial 5 hours for pH 6 and 7 after which then there was more growth at pH 6.

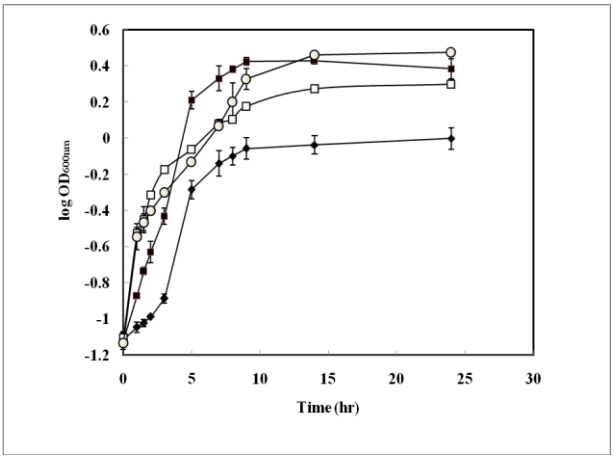

Figure 3: Determination of effect of initial pH of 4 (♦), 5 (■), 6 (□) and 7 (○) on the growth rate of *Enterobacter asburiae* sp. cultured with M162 medium at temperature of 30 C

After evaluation for optimum pH for growth, it was noted that *Enterobacter asburiae* subsup. it was found to be pH 6. In terms of the specific growth rate values, the maximum rate (μ) was 0.16 as shown in Figure 7. The specific growth rate increased linearly between pH 4 to 6 after which then the rate decreased. Hence for a high growth rate when

culturing the bacteria, pH 6 was found to be ideal. This optimum pH value has been recorded from various microbes that include *Bacillus* sp. (Gilkes *et al*., 1991; Horikoshi, 1999; Mawadza *et al*., 2000). Hence the *Enterobacter asburiae* subsps. can be classified as a neutrophile whose optimum pH is in the range 5.5 to 8.0 (Neidhart *et al*., 1992).

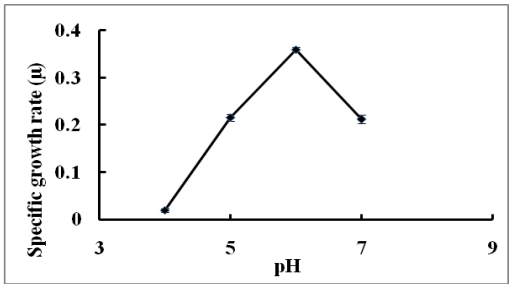

Figure 4: Determination of optimum growth rate of *Enterobacter asburiae* subsp. under different initial pH values from 4 to 7.

3.5. Effects of temperature on growth rate of bacterium

Optimum temperature for growth of *Enterobacter asburiae* subsp. was noted to have a profound effect on the growth as depicted by the log ODs profiles observed with time shown in Figure 8. The values for all the temperatures increased drastically in the first hour of growth between 30 and 40 ^{o}C with optimum growth at 40 °C that declined in the range 45-50 ^{o}C. The microbe had much reduced activity as temperature levels reached thermophilic levels of 50 ^{o}C.

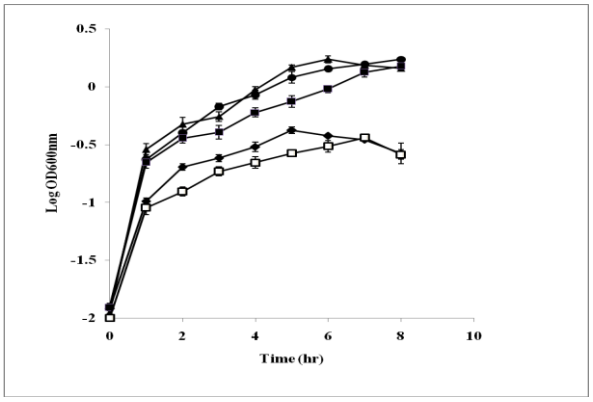

Figure 5: Log OD600nm growth profiles of *Enterobacter asburiae* sp. with respect to temperature of 30 (♦), 35 (●), 40 (▲), 45 (■) and 50 °C (□).

It was interesting to note although the source of *Enterobacter asburiae* subsp. was from an insect whose survival temperatures were also mesophilic. After determination of specific growth rate, figure 6 shows that the optimum temperature for culturing *Enterobacter asburiae* subsp. is 40 °C. As the temperature increased from 30 to 40 ^{o}C, there was a hyperbolic relationship in specific growth rate increase. However, from 40 ^{o}C onwards, there was a linear decrease in the specific growth rate. The highest growth rate was however noted at 40 ^{o}C. Such optimum temperature is for mesophilic microbes hence *Enterobacter asburiae* subsp. is a mesophilic microbe.

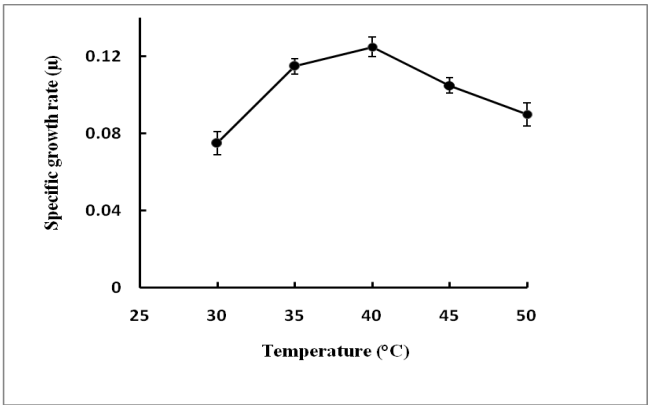

Figure 6: Specific growth rate of *Enterobacter asburiae* subsp. at different temperature values.

3.6. Effects of initial pH on endo-β-1,4-glucanase activity

The *Enterobacter asburiae* subsp. endo-β-1,4-glucanase enzymes were able to hydrolyze CMC optimally at pH 6 liberating as much as 0.32 mg/ml/min of glucose. In the pH range 3 – 6 there was none Arrhenius relation as shown by an increase in glucose concentration in figure 7. At pH 7, there was a decline in enzymatic activity. There was very low enzymatic activity at low pH such as 4 that produced about 0.09 mg/ml/min. The optimum pH for growth was 6 which coincided with the optimum for enzyme activity too.

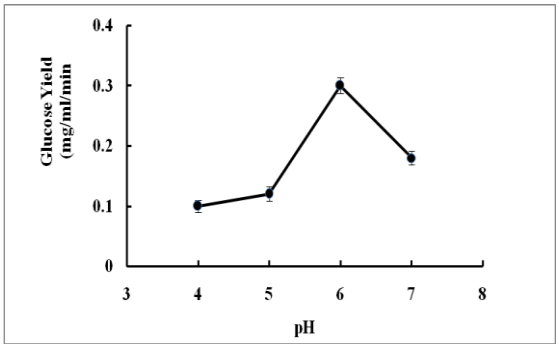

Figure 7: Effect of pH on endo-β-1,4-glucanase activity obtained from *Enterobacter asburiae* subsp.

3.6. Effects of Temperature on Enzyme Activity

The endo-β-1,4-glucanase activity against temperature exhibited Gaussian relationship to activity as noted with a majority of enzymes. However, the highest enzyme activity was observed at 40 ^{o}C with 0.32 mg/ml/min glucose yield. Using temperatures around 30 °C reduces the enzymatic activity greatly, as much as using thermophilic conditions. There was a drastic reduction in endo-β-1,4-glucanase activity using 40 °C, the optimum, compared to 45 °C although 50 °C retained some activity. An unusual observation was a marked enzymatic reduction to lowest levels of hydrolysis was noted at 30 ^{o}C. From figure 8, it was noted that endo-β-1,4-glucanase activity increased from 30 ^{o}C up to an optimum of 40 ^{o}C in a hyperbolic fashion just like its growth curve. It is interesting to note that the optimum growth temperature is exactly the same as the optimum endo-β-1,4-glucanase activity.

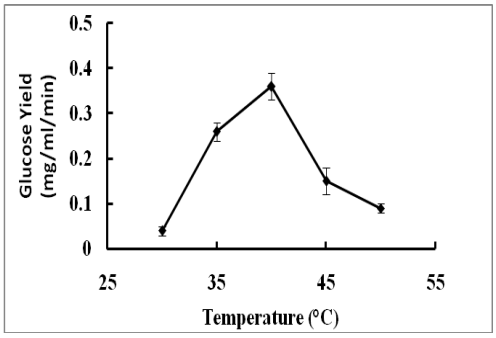

Figure 8: Effects of temperature on endo-β-1,4-glucanase activity obtained from *Enterobacter asburiae* subsp.

4. DISCUSSION

4.1 Identification of the microorganisms

Although the microbe could be equated to other rod shaped microorganisms, using BLASTN search tools it was concluded that it had 1 % homology to *Enterobacter asburiae* and hence it was named *Enterobacter asburiae* subsp. Using nucleotide gap of 250 nucleotides indicated that there was a slight difference between the queried microorganism and *Enterobacter asburiae*. 16S rRNA phylogenetic relatedness of this microbe to *Enterobacter asburiae* was also substantiated using the traditional microbial techniques in comparing fermentation, hydrolysis and Gram reaction techniques. The epiphytic coexistence of this bacterium (parasitism or commensalism) with the plants and insects by *Enterobacteriaceae* family has been reported elsewhere (Thayer, 1976; Bandi *et al*., 1995). Since host *Enterobacter asburiae* is known to be a Gram-negative, facultative anaerobic, oxidase negative, non-motile and non-pigmented rod-shaped species that have been screened from soil, water and food products (Lau *et al*., 2014). It was however noted that this *Enterobacter asburiae* subsp produced motile rods and endophyte with *Brachytrupes membranaceus* insects. Other sources of *Enterobacter asburiae* species recorded included sweet potato, lettuce leaves and soft tissues (Asi & Adachi, 2009; Koth *et al*., 2012, Lau *et al*., 2013). A number of cellulose-aiding microbes that coexist in symbiotic relationship with their hosts have been screened from several sources including insects too (Martin, 1983; Kim *et al*., 2008; Oppert *et al*., 2010; Huang, 2012). The use of M162 medium has been noted to be restricted to aerobic microbes and anaerobic microbes were missed using this screening technique since termites have been noted to have both aerobic and anaerobic microbes (Cho *et al*., 2010). Although cellulose hydrolyzing microbes of *Enterobacter asburiae* were screened using this medium type. It has been reported that termites *Reticulitermes* sp. of *R. santinesis* and *R. speratus* expressed endoglucanase activity coming from the endophytic microbes (Cho *et al*., 2010; Matteoti *et al*., 2011) and several other insects orders (Martin, 1983; Watanabe & Tokuda, 2001; Willis *et al*., 2010). Huang and co-workers identified a diverse spectrum of predominant groups of Gram-negative bacteria that included *Pseudomonas, Ochrobactrum, Rhizobium, Cellulosimicrobium,* and *Microbacterium* although members of *Bacillus, Dyadobacter, Siphonobacter, Paracoccus, Kaistia, Devosia, Labrys, Ensifer, Variovorax, Shinella, Citrobacter*, and *Stenotrophomonas* were also found using three media types. All the microbes expressed cellulolytic activity.

4.2 Optimum growth and cellulase activity.

The optimum temperature and pH growth conditions and endoglucanase activity expressed by the *Enterobacter asburiae* subsp. was found to be exactly the same at 40 °C and 6.0 respectively. Quite a number of cellulases from insects rather than the symbiotic ones have been noted to have an optimum temperature and pH ranging between 40 – 50 °C and 5.0 – 7.0 (Lee *et al*., 2005; Zhou *et al*., 2007; Kim *et al*., 2008). Equally endophyte microbes that symbiotically coexist with several microbes mentioned earlier on produce enzymes that have been observed to have their optima conditions within the same range. Hence our microbe can be investigated to check on the production of other xylanolytic, amylase, and β-glucosidases production to note the pool enzymes produced from *Enterobacter asburiae* subsp. The quality of cellulolytic systems feed by the insect are of low lignocelluloses biomass, hence *Brachytrupes membranaceus* might produce other enzymes not assayed during this study. However, our results indicate that the endophyte microbe from *Brachytrupes membranaceus* has cellulolytic activity as shown by clear zones of cellulose hydrolysis after Congo Red staining as recorded by other workers (Teather & Wood, 1987, Willis *et al*., 2010). The cellulolytic activity was further verified from the extracellular expression of high endo-β-1,4-glucanase activity yielding 0.3 mg/ml/min of glucose at optimum conditions of pH 6.0 and temperature, 40 °C. Since *Brachytrupes membranaceus* have been noted to be pest to tobacco and maize, their cellulase study have been mentioned to aid in development of insecticidal technologies aimed at inhibiting their vital digestive role (Zhou *et al*., 2008).

Currently, our research work is endeavoring to use more media types to note diversity of microbes from *Brachytrupes membranaceus* which might produce enzymes of more activity and the enzymes will be characterized and evaluated for their potential application.

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