

Optimization of cellulase enzyme from sorghum straw by yeasts isolated from plant feeding–termite *Zonocerus variegatus*

Adelabu Blessing Adebola^{1,*}, Kareem Olateju Sarafadeen², Adeogun Abideen Idowu³ and Wakil Sherifah Monilola⁴

Abstract

The effective fermentation of cellulose remains an intractable challenge in food industry. The relevant cellulase enzyme is also in a high demand from industry for several biotechnological applications that inevitably in recent times led to many efforts for screening some yeasts for better cellulase production and fermentation performance. Plant–feeding termites can truly be considered as highly efficient natural bioreactors. The highly specialized gut systems of such insects are not yet fully realized, particularly, in cellulose fermentation and cellulase production for industrial applications as well as industrial bioethanol technology. Two yeasts (*Wickerhamomyces chambardii* and *Saccharomyces diaststicus*) were used to produce cellulase using sorghum straw (2.5–15% (w/v)) at pH (4–7) and were incubated at (30–60°C) for 96 h. The yeasts utilize sorghum straw and highest cellulase was recorded at 72 h of fermentation. Highest enzyme activity was achieved with 7.5% (w/v) sorghum straw and 7.5% (v/v) inoculum concentration. Optimization of growth parameters showed that *W. chambardii* produced more cellulase enzyme ($223 \pm 18.34 \mu\text{mL}$) than *S. diaststicus* ($211 \pm 25.80 \mu\text{mL}$), except optimization of inoculum concentration where *S. diaststicus* produced more ($231 \pm 6.27 \mu\text{mL}$) than *W. chambardii* ($211 \pm 33.32 \mu\text{mL}$). Optimum pH and temperature for *W. chambardii* was 4.5 and 45°C respectively while *S. diaststicus* was 5.0 and 40°C respectively. This study suggests cellulolytic yeasts such as *W. chambardii* and *S. diaststicus* for cellulase production from lignocellulosic materials.

Key words: Cellulase, Cellulolytic yeast, Insect gut, Lignocellulosic, *Wickerhamomyces* sp

¹ Department of Microbiology, Chrisland University, Idi-Aba, Abeokuta, Ogun State

² Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State

³ Department of Chemistry, Federal University of Agriculture, Abeokuta, Ogun State

⁴ Department of Microbiology, University of Ibadan, Oyo State

*Corresponding author e-mail: bola.blessing@yahoo.com

1. Introduction

Grasshoppers are a group of insects belonging to the suborder Caelifera. Insects are efficient in converting cellulose present in plant materials into glucose with their highly efficient gut systems which can be considered as natural bioreactors (Irene, 2018). Grasshoppers, *Zonocerus variegatus* are group of insects belonging to the order Orthopoda and suborder Caelifera that are found in a variety of habitats. Grasshoppers occur in greatest numbers in lowland tropical forests, semiarid regions, and grasslands (Zhand *et al.*, 2013). They are polyphagous species capable of consuming most of the plant species in its surroundings (Ademolu and Idowu, 2011a), and is reported to consume more than 250 plant species among 71 families (Ademolu and Idowu, 2011b), among which are many crops and agricultural wastes, including citrus, cocoa, banana, vegetables, wheat straw, sorghum straw and corn straw. Cellulolytic activity has been reported in the gut fluids in different insect species (Rojas-Jiménez and Hernández, 2015). The cellulase activity of these insects has been attributed to symbiotic gut microorganisms. Herbivorous insects often rely on microorganisms present in their guts to digest plant materials such as cellulose and lignin (Shil *et al.*, 2014). Hence, the guts of insects could be a potential source of microorganisms with novel cellulolytic activities for biofuel production.

Several microorganisms can produce cellulase enzyme (Carrasco *et al.*, 2016). Fungi secrete higher amounts of cellulases enzyme than bacteria (Saravanakumar *et al.*, 2013; Tao *et al.*, 2010), molds have long growth cycle, therefore a huge spore formation, and this limits their performance in terms of cellulase production and its safe utilization. The most commonly used cellulases in industry are produced by molds, which have optimum temperature at 50°C (Juturu and Wu, 2014). Hydrolysis of cellulose had also been reported in several yeast species, such as, *Debaryomyces hansenii*, *Meyerozyma guilliermondii*, *Candida parapsilosis* and *Saccharomyces* species (Saravanakumar *et al.*, 2013). *Wickerhamomyces* species had just been recently added to group of yeasts that produce ethanol from lignocellulosic materials (Kurtzman, 2011). Although cellulases from some psychrophilic yeast have been described, within cold active enzymes, cellulase production from yeast remain less studied (Juturu and Wu, 2014). Given the benefits of yeast stress tolerance, which can significantly enhance cellulase production costs, the search for cellulase producing yeasts is a major challenge in achieving high cellulase enzyme.

The groups of hydrolytic enzymes implicated in the bioconversion of celluloses are known as cellulases or cellulase-systems. The cellulase-system comprises three major highly specific enzymes namely; the endo-glucanases, the exo-glucanases and β -glucosidases. These enzymes are non-constitutive and are produced by many microorganisms such as

bacteria, actinomycetes and fungi (Juturu and Wu, 2014). The cellulase-systems of fungal origin are widely studied. Among the fungi, *Trichoderma*, a softwood rotting fungus, is the most potent cellulase-system producer (Carrasco *et al.*, 2016). The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyses the conversion of cellulose to glucose in an enzymatic hydrolysis (Techaparin *et al.*, 2017; Thongekkaew and Kongsanthia, 2016).

Agricultural residues are a great source of lignocellulosic biomass; this is renewable, readily available, and inexpensive. Such resources include: leaves, stems, and stalks from sources like corn cob, corn straw, sorghum straw, sugarcane bagasse, rice hulls, woody crops, and forest residues. Other lignocellulosic waste from industrial and agricultural processes include citrus peel waste, industrial waste, municipal solid waste, paper mill sludge and sawdust, paper pulp. However, the recalcitrant nature of cellulosic biomass and the high cost of cellulase are the major obstacles to utilize cellulosic biomass (Maryam *et al.*, 2018; Rehman and Elahi, 2018).

Huge volumes of cellulosic materials, such as sorghum straw, are renewable resources being generated as waste from various agro allied industries (Szambelan, 2019). These can be exploited as sustainable resource for production of many organic fuels and bioenergy. They can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose problematic solid wastes, and improve air quality (Srivastva, 2018) This study reports isolation and screening for cellulolytic yeasts from gut of grasshopper *Zonocerus variegatus* and production and optimization of cellulase enzyme from sorghum straw by yeasts.

2. Materials and Methods

2.1 Insect and Gut fluid collection

Adult variegated grasshopper, *Zonocerus variegatus* were collected from a farm at Osiele (latitude 7° 10–59' N and longitude 3° 27' 0" E) in Odeda Local Government; Ogun State, Nigeria. They were maintained in clean containers and transported immediately to the laboratory. The external surfaces of the insects were surface sterilized with commercial bleach diluted at 1% for 30 seconds and rinsed for 1 min in three successive baths to ensure that the gut contents were not contaminated by the surface microflora of the body during dissection (Irene, 2018). The insects were then transferred and fixed on to a disinfected dissection board, the intestine were exposed from the ventral side with the aid of sterile dissection scissors and fine –tipped forceps and the gut was exposed (Rojas–Jiménez and Hernández 2015). The guts were transferred into a sterile 1.5 mL Eppendorf tube containing 0.5 mL of 0.7% (v/v) sterile saline solution. They were crushed with a pipette tip, and all the solution (including gut pieces) were used for isolation (Shil *et al.*, 2014).

2.2. Isolation and Characterization of yeast

Macerated guts of the insects were serially diluted and Yeast Extract Peptone Dextrose Agar (YEPDA) containing Yeast Extract; 5 g, Peptone; 10 g, Dextrose; 5 g and Agar; 20 g was used for isolation of yeasts (Carrasco *et al.*, 2016). In this method, sterile media were poured into Petri dishes aseptically and allowed to solidify. One milliliter of the serially diluted macerated insect gut was pipetted out into sterile Petri-dishes. It was spread on the plate with a “L” rod. The plates were incubated in an inverted position at 30°C for 72 h. Yeast colonies were characterized based on size, shape and colour. Colonies were subcultured on YEPDA by streak plate technique. Colony characteristics and cell morphology of the purified yeast were studied. Fermentation test using different sugars were also carried out for classification (Barnett *et al.*, 2000).

2.3 Screening for Cellulolytic Yeast

Qualitative and quantitative screenings were carried out on yeast cultures. Qualitatively screening was carried out with the use of congo red. Purified yeast isolates were grown on YEPDA plates containing 1% (w/v) carboxyl methyl cellulose (CMC). Medium was distributed into Petri dishes. Each plate was inoculated with yeast isolate from 72 h old culture by streaking once across the middle of the agar medium. Plates were incubated at 30°C for 3 days. Cellulolytic isolates were detected after growth, by flooding the plates with 1% congo red solution for 30 min. The dye was drained and plates were flooded with 1 M sodium chloride solution for another 20 min. Clearance around growth of isolate represents cellulase production (Saliu, 2012). Quantitative screening was carried out using Mineral Salt Medium which consist of NH_4NO_3 : 0.2 g, KH_2PO_4 : 0.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.03 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.03 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.16 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.14 g, Tween-80 : 0.1 g, 1% CMC, The medium (100 mL) was inoculated with the pure yeast isolates and were incubated on a gyratory shaker (150 rpm) at 30°C for 3 days and cellulase production was quantified (Amaeze *et al.*, 2015).

2.4 Selection of starters

Two yeasts with best cellulolytic producing abilities were subjected to optimization studies and were used for cellulase production in submerged fermentation.

2.5 Molecular characterization

2.5.1 Genomic DNA extraction

DNA of the yeasts were extracted using Zymo-Fungal/Bacteria DNA extraction kit (Zymo Research, USA). The extraction was carried out according to manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA) (Apiradee, 2006).

2.5.2 PCR amplification

The genotypic identification of the yeasts were conducted by reading of the nucleotide sequence of DNA encoding 18S rRNA (rDNA). The rDNA was amplified using universal fungal primers (Apiradee, 2006), forward primer, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and reverse primer, NS2 (5'-GGCTGCTGGCAC CAGACTTGC-3') (Apiradee, 2006). The amplification result was detected on 1% agarose gel electrophoresis staining with 10 mg/mL of ethidium bromide. Before being sequenced, the amplified fragment was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega, USA) from agarose gel. Nucleotide sequences were analyzed by Macrogen (Macrogen, Inc., Korea). All the sequences were compared for their similarity with reference yeast strains by a BLAST search.

2.5.3 Sequence analysis

The sequence data was compared to gene libraries (GenBank) with BLAST and FASTA programs. Multiple sequence alignments of translated gene sequence were carried out with the program CLUSTALW (version 1.83). Genetic distances were estimated by using the Maximum parsimony with bootstrap method employed by PHYLIP (Apiradee, 2006).

2.6. Collection and processing of substrate

Sorghum straws were collected from a farm at Kishi in Oyo State, Nigeria. The straws were oven dried at 70°C for 2 h and grounded into powdered using an electric blender (Philips INO23) and was sieved using 40 mm mesh. 10% (w/v) of the straw was used for fermentation.

2.7 Inoculum Preparation

A loopful of culture from 48 h old YEPDA plate was inoculated into sterile 100 mL of YEPD broth containing nutrient required for yeast growth, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.03 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.16 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.14 g in 250 mL Erlenmeyer flasks. Inoculum was grown aerobically at 30°C on a rotary shaker at 150 rpm for 48 h (Amaeze *et al.*, 2015).

2.8 Fermentation of sorghum straw

Mineral Salt Medium which are chemically defined for cellulase production was used for fermentation. This comprises of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 1.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.3 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.4 g, NH_4NO_3 : 1.2 g, KH_2PO_4 : 0.8 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.3 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.3 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 1.3 g, Tween-80 : 0.15 g, peptone : 0.75 g, yeast extract : 0.3 g, glucose : 5 g was prepared and used for the production of cellulase 100 mL of the prepared media was dispense into 250 mL conical flasks using a measuring cylinder and 10 g of sorghum straw was added into the conical flasks and labeled properly. pH of the media was adjusted to 5.5 Experiments were carried out in triplicates and the media were sterilized in an autoclave at 121°C for 15 min. The flask was inoculated with 5% yeast suspension and incubated at 30°C for 96 h in an

orbital shaker which was set at 150 rpm (Thongekkaew and Kongsanthia, 2016). Fermented sorghum straw was analyzed for cellulase production at 24, 48, 72 and 96 h to determine the effect of fermentation time.

2.9 Extraction of Enzyme

Yeast cells were harvested by centrifugation at 10,000 rpm with 7 cm radius, with centrifugal force of $7,826 \times g$ for 10 min in a table top high speed centrifuge (Clifton, Nikel, England) (HI850R) and the supernatant were taken as the crude enzyme (Thongekkaew and Kongsanthia 2016).

2.10 Enzyme Assay

Cellulase assay was carried out according to the method of Rai *et al.*, (2012) 0.5 mL of CMC (1% (w/v)) in 0.2 M sodium acetate buffer, pKa 4.76 (pH 5.0) and 0.5 mL crude enzyme in test tubes. Control tube was made by adding 1 mL of 1% cellulose solution and 2 mL of distilled water. The reaction mixture in all test tubes were incubated at 50°C for 30 min and the reaction was terminated by adding 1 mL of 3, 5–dinitrosalicylic acid (DNSA) reagent. The tubes were heated at 100°C in boiling water bath for 5 min and then cooled at room temperature. The absorbance was read at 540 nm using a spectrophotometer. Glucose standard was prepared with varying concentration of glucose ranging from 0.1 to 2.0 mg mL⁻¹ and treated the same way. Absorbance was plotted against concentration of glucose to obtain a calibration curve (Carrasco *et al.*, 2016). The amount of reducing sugar produced by the action of crude enzyme was read off from the curve. One unit of cellulase activity (μ) was defined as the amount of enzyme that liberated 1.0 μ mol of substrate per min at 5.0 pH and 50°C.

2.11 Optimization the fermentation conditions of cellulase production

2.11.1 Effect of pH

Effect of pH on the rate of cellulase production using the selected yeast strains were studied by conducting experiments at different pH (4.0, 4.5, 5.5, 6.0) 10 g of sorghum straw was added to 100 mL of each fermentation media in conical flasks. Flasks were inoculated with 5% (v/v) yeast suspension and incubated at 30°C for 72 h (Rai *et al.*, 2012). Cellulase productions by yeast strains were determined as previously described

2.11.2 Effect of temperature

Cellulase was produced from the substrates in flasks inoculated with yeast cells 10 g of sorghum straw was added to 100 mL of each fermentation media in conical flasks and pH were adjusted to 5.5 and inoculated with 5% (v/v) yeast suspension. The flasks were incubated at different temperature to determine if the yeasts are thermotolerant or not (30°C, 35°C, 40°C,

45°C, 50°C and 60°C) (Rai *et al.*, 2012). Flasks were incubated for 72 h and cellulase productions by yeast strains were determined as previously described.

2.11.3 Effect of inoculum concentration

Effect of inoculum concentration on cellulase production by selected yeast strains were carried out using YEPDA medium incorporated with 10% sorghum straw. pH of the media were adjusted to 5.5 and each medium was sterilized and inoculated with varying yeast suspension of 5%, 7.5%, 10%, 12.5% and 15% (v/v) (Rai *et al.*, 2012). Media were incubated at 30°C for 72 h. Cellulase productions by yeast strains were determined as previously described.

2.11.4 Effect of substrate concentration

Effect of substrate concentration on cellulase production by selected yeast strains were carried out using YEPDA medium incorporated with various sorghum straw concentrations (5%, 7.5%, 10% and 12.5%) w/v. pH of the media were adjusted to 5.5 and each medium was sterilized and inoculated with 5% (v/v) yeast suspension (Rai *et al.*, 2012). Media were incubated at 30°C for 72 h. Cellulase productions by yeast strains were determined as previously described.

2.12 Statistical Analysis

All the experiments were performed in triplicates and the results were presented as mean \pm standard deviation and were also analyzed by ANOVA using statistical software SPSS version 17.0

3. Results and discussion

3.1 Isolation of yeast

A total of thirteen yeasts were isolated. All the isolates had earthy smells with smooth surfaces colour ranged from cream white to colourless. Yeasts were oval and round shape, occurring singly and had raised elevation. All yeast isolates grew well at 10% NaCl combined with 5% glucose while only two grew at 50% glucose. Four of the yeasts assimilate glucose, raffinose and maltose while two isolates assimilate urease and xylose. Four isolates do not ferment Inositol. Four isolates do not ferment sorbose (Table 1). Among the yeast isolated, 10% belonged to the genus *Saccharomyces*, 38% to *Candida*, 32% to *Trichosporon*, 10% to *Debaryomyces*, and 10% to *Wickerhamomyces*.

The yeasts isolated represented five different genera. Physiological and biochemical tests identified the yeasts as *S.cerevisiae*, *S.diastaticus*, *C.chauliodes*, *C.tropicalis*, *C.krusei*, *C.corydali*, *T.beemeri*, *T.lutetiae*, *T.beigelil*, *D.hansenii*, *Debaryomyces spp* *W.chambardii* and *W.subpelliculosus*.

Candida spp were the predominant yeast isolated, they are known to adapt best in high temperature and low pH (4–5) (Moubasher *et al.*, 2017). This is in agreement with the report of Adelabu *et al.* (2019) where *Candida* species were isolated from compost piles. Rojas–Jiménez and Hernández (2015) reported the isolation of ascomyceteous and basidiomyceteous yeasts from the gut of the 16 species of passalid beetles collected in nine localities in Guatemala with the xylose–fermenting (X–F) yeasts were the most abundant taxa isolated. *Candida* species and *Saccharomyces cerevisiae*, had also been isolated from gut of black beetle (Moubasher *et al.*, 2017). Reports had also shown that *Wickerhamomyces* spp are yeast which is associated with insect gut (Papagianni and Papamichael, 2017). Novel species of *Candida* (Hui *et al.*, 2012) and *Wickerhamomyces* (Hui *et al.*, 2013) were described from guts of wood–boring insect larvae or ambrosia beetle.

Table 1: Biochemical characteristics and clear zones radii of yeast isolates

Isolate Code	Inositol	Arabinose	Raffinose	Sucrose	Galactose	Maltose	Lactose	Glucose	Acid from glucose	Urease	10%/NaCL/5% glucose	50% glucose	Xylose	Growth in starch	Cellobiose	Ethanol growth	Probable organism	Clear zone Radi (mm)
Y1	+	-	+	+	-	+	-	+G	+	-	+	-	-	+	-	-	<i>S. cerevisiae</i> 1	12.81±1.85 ^{bc}
Y2	+	-	+	+	-	+	-	+G	+	-	+	-	+	+	-	-	<i>S. cerevisiae</i> 2	15.50±2.64 ^{ef}
Y3	+	-	+	+	+	-	+	+	+	-	+	-	-	+	-	-	<i>C.chauliodes</i>	12.21±1.08 ^{bc}
Y4	+	-	+	+	-	-	-	+G	+	+	+	-	-	+	-	-	<i>C. tropicalis</i>	14.15±2.21 ^{de}
Y5	-	-	-	+	+	+	+	+	+	-	+	-	-	+	-	-	<i>C. krusei</i>	14.93±2.51 ^{de}
Y6	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	<i>C. corydalisi</i>	13.22±1.100 ^c
Y7	+	+	-	+	+	+	-	-	+	-	+	-	-	+	-	-	<i>T. beemerii</i>	10.0±1.52 ^a
Y8	+	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	<i>T. lutetiae</i>	12.45±1.81 ^{bc}
Y9	+	-	+	+	-	-	-	-	+	+	+	-	-	+	-	-	<i>T. beigeli</i>	13.75±1.62 ^c
Y10	+	+	-	+	+	+	-	+	+	-	+	-	-	+	-	+	<i>D. hansenii</i> 1	12.57±1.52 ^{bc}
Y11	+	-	+	+	-	-	-	+	+	-	+	-	-	+	-	-	<i>D. hansenii</i> 2	11.6±2.00 ^{ab}
Y12	-	+	+	+	+	+	-	-	+	-	+	+	+	+	-	+	<i>P. chambardii</i>	18.00±1.52 ^f
Y13	-	+	+	+	+	+	-	+	+	-	+	+	-	+	-	+	<i>P. subpelliculosus</i>	11.7±1.51 ^{ab}

Key + = Positive, - = Negative, G = Gas production

3.2 Screening of yeasts

The yeasts obtained were positive for cellulase production, they all showed clear zones on carboxyl methyl cellulose (CMC) agar. The yeasts revealed that they have pathway for the conversion of complex polysaccharide (Waghmare *et al.*, 2014). Zones of clearance from 6.0 mm to 18.0 mm in diameter were produced by the yeasts. Highest halo zone (18.0 mm) was produced by *P.chambardii*, followed by *S.cerevisiae* 2 (15.5 mm), while the least was observed in *T.beemerii* (10.0 mm). Table 2 showed the result of the quantitative screening. Highest cellulase activity was produced by *P.chambardii* (174.67 μ mL), followed by *S.cerevisiae* 2 (161.22 μ mL), *Candida tropicalis* had 135.00 μ mL cellulase activity while *D.hansenii* had

132.87 μmL . The least cellulase activity was observed in *T.beemeri* (115.66 μmL) (Table 2). Different researchers had referred *Pichia* spp as cellulose-consuming yeast (Díaz-Rincón *et al.*, 2017; Fang and Xia 2015; Carrasco *et al.*, 2016). This observation is in agreement with an earlier study of Adelabu *et al.* (2018a), which states that yeast species such as *C.shehatae*, *C.tropicalis*, *S.diastaticus* and *W.chambardii* assimilated cellulose and ferment it to ethanol. In addition, *P.spp* had been added to group of yeasts that produce cellulase from lignocellulosic materials (Kurtzman, 2011). The differences in the amount of enzyme produced by each of the isolate suggest that production rate depends on the genetic composition of the microorganisms (Biswas *et al.*, 2017). Two yeasts (*Pichia chambardii* and *Saccharomyces cerevisiae*) which were later identified through molecular methods as *Wicherhamomyces chambardii* and *Saccharomuyces diastaticus* displayed the best cellulolytic producing abilities. Statistical analysis showed that cellulase activity by the two yeasts were significantly different ($P \geq 0.05$) and were significantly different and were selected for cellulase production in submerged fermentation.

Table 2 Screening for cellulase production in yeasts

Yeast Isolates	Cellulase activity (μmL)
<i>S. cerevisiae</i> 1	125.70 \pm 7.26 ^{bc}
<i>S. cerevisiae</i> 2	161.22 \pm 20.21 ^{ef}
<i>C. chauliodes</i>	132.67 \pm 21.40 ^{cd}
<i>C tropicalis</i>	135.00 \pm 26.43 ^{cd}
<i>C. krusei</i>	128.94 \pm 16.00 ^{bcd}
<i>C. corydali</i> ,	130.36 \pm 13.81 ^{bcd}
<i>T. beemeri</i>	115.66 \pm 10.34 ^a
<i>T. lutetiae</i>	119.61 \pm 5.28 ^{ab}
<i>T. beigelil</i>	123.47 \pm 34.16 ^{abc}
<i>D. hansenii</i>	132.87 \pm 22.43 ^{cd}
<i>Debaryomyces spp</i>	120.39 \pm 27.20 ^{abc}
<i>P. chambardii</i>	174.67 \pm 23.21 ^{gh}
<i>P. subpelliculosus</i>	142.50 \pm 18.03 ^d

Key: Each value is a mean of 3 readings \pm standard deviation. Values in the same column followed by the same letter (or subscripts) are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test

3.3 Molecular identification

Polymerase Chain Reaction (PCR)-based method is described as effective in the differentiation of yeast strains of different species (Apiradee, 2006). The yeasts were genetically characterized using 18S rDNA analysis. Genomic DNA of these yeasts were extracted and used as DNA template for PCR amplification. The PCR reaction was performed by NS1 and NS2 primers. The amplified DNA Fragment of approximate size 500 bp was generated for the two yeasts. The distribution of organisms identified in the library of DNA yeast isolates based on the analysis by sequence of the PCR product in the non-redundant nucleotide database from National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) (NCBI) was presented Table 2. The two yeasts with NCBI Accession number X02649.4 and EF550482.1 were identified as *Saccharomyces diastaticus* and *Wickerhamomyces chambardii* which were identified biochemically as *Saccharomyces cerevisiae* and *Pichia chambardii* respectively. Their maximum percentage identity was 100 and 99% respectively (Fig 1).

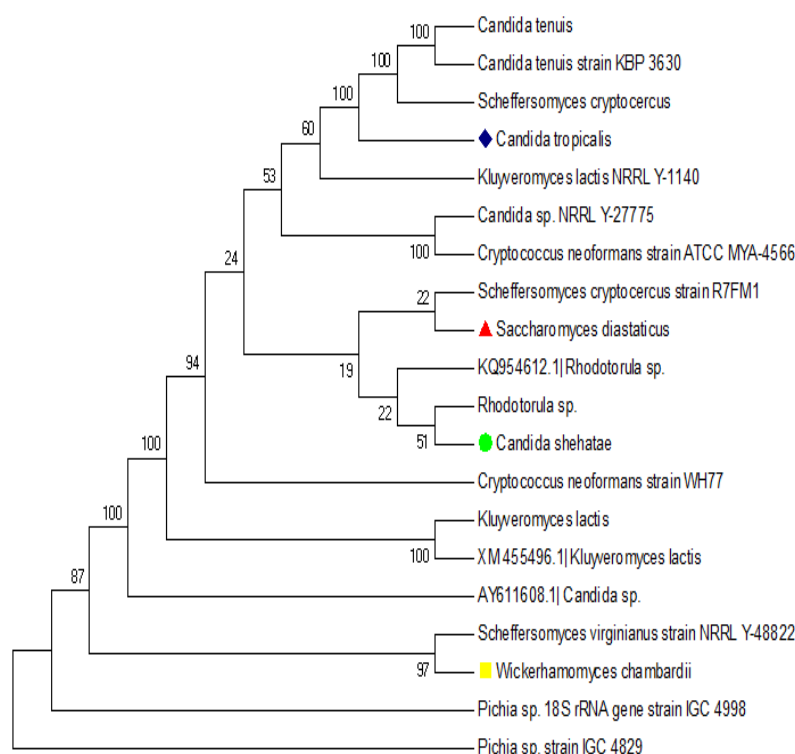


Fig 1 Phylogenetic relationship of obtained yeast isolates.

Note: Branch lengths are proportional to the numbers of nucleotide and amino acid changes and the numerals given on the branches are the frequencies with which a given branch appeared in 100 bootstrap replications. Reference sequences were retrieved from GenBank under the accession numbers in parentheses.

3.4 Cellulase production from sorghum straw

The result of cellulase production of the two selected yeasts is shown in Fig 2. The result reveals that the yeasts produced cellulase and cellulase activity increased with increase in fermentation time till 72 h and decreased when incubation was extended (Fig 2). The enzyme levels found to evaluated yeasts are in according to the cellulolytic activities described in literature for yeasts (Giese *et al.*, 2017). *Wickerhamomyces chambardii* had maximum cellulase activity of 238 μmL at 72 h while *S. diastaticus* had of 195 μmL . This may be due to the fact that this yeast has more cellulolytic enzyme (xylose isomerase) which is responsible for the breakdown of lignocellulolytic materials to glucose (Pothiraj *et al.*, 2015). This result showed the efficient conversion of cellulose and hemicellulose into the corresponding monosaccharides (Tofighi *et al.*, 2014). This is similar to earlier report of Damisa *et al.*, (2012), where cellulase production increased with fermentation time. Substantial decrease in enzyme production level was detected at 96 h by the two yeasts. According to Damisa *et al.* (2012), accumulation of cellobiose in the medium could result into end product inhibition, thus inhibiting the endoglucanase and Beta-glucosidase enzymes of the organism. Cellulase production in this study was two-fold higher than those obtained in earlier report of Rojas-Jiménez and Hernández (2015) where cellulase was produced by yeast isolated from rongkho forest.

Enzyme activity at time zero was not measured because reaction had not been initiated at this fermentation time and no glucose had been liberated from the cellulolytic substrate (Schreiber *et al.*, 2017; Xie *et al.*, 2017). Enzyme activity is a measure of how much enzyme is present in a reaction. This could be measure in two ways; monitoring the decrease in substrate concentration in a period of time and the other is to measure the increase in concentration of a product after a period of time (Schreiber *et al.*, 2017), thus initial enzyme activity from the inoculum was not measured.

Growth of yeast is influenced by the availability of nutrient (Rehman and Elahi, 2018). When nutrient are available throughout the fermentation period, cell viability is at its optimum while depletion of nutrient may lead to decrease in cell growth (Rehman and Elahi, 2018). Yeast cells were concluded to be viable during cellulase production, because cellulase production increase with increase in fermentation time till 72 h. This suggests that enzyme production is dependent on biomass. As cellulaase enzyme is part of primary metabolites, they are produced during the exponential phase of growth and at the onset of death phase, enzyme secretion start decreasing (Rai *et al.*, 2012). Fermentation medium supplemented with Mg, Zn, Mn, Mn, NH₄ and K had also been reported to increase cell growth (Damisa *et al.*, 2012)

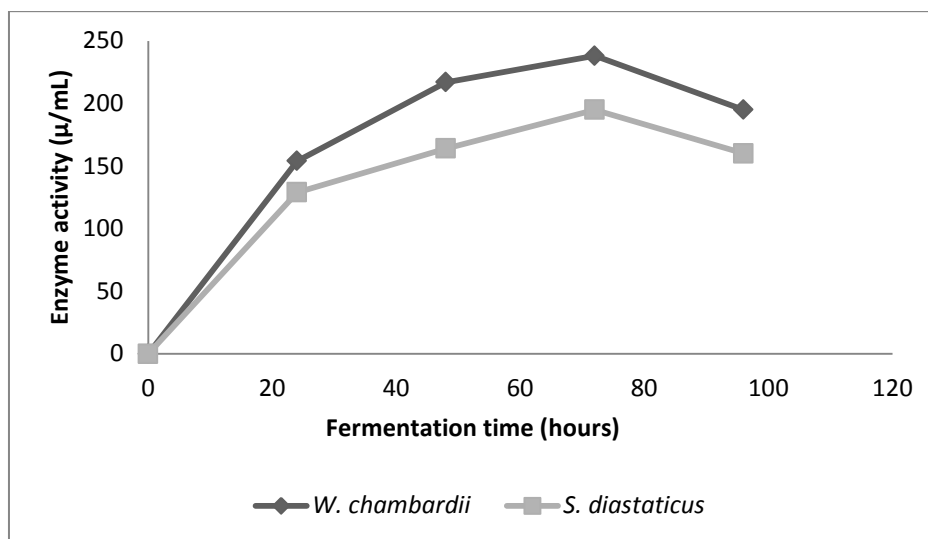


Fig 2 Effect of fermentation period on cellulase production from sorghum straw medium

3.5 Optimization of fermentation conditions of cellulase production

3.5.1 Effect of hydrogen ion concentration (pH)

Effect of initial pH on cellulase production from sorghum straw at different pH ranging from 4–7 is illustrated. The yeasts produced cellulase even at lower pH during fermentation. Report had showed that the influence of pH on enzyme production was found to be an important parameter (Deshmukh *et al.*, 2018). The presence of glucose in the broth could have induced the production of these enzymes by all the yeast (Ahmed and Bibi, 2018). The two yeasts showed a wide range of pH tolerance (pH 4–7) for cellulase production, but maximum cellulase production was achieved at pH 4.5 by *Wickerhamomyces chambardii* (185.17 μ/mL). *Saccharomyces diaststicus* produced highest cellulase enzyme at pH 5.0 (126.22 μ/mL). *Saccharomyces cerevisiae* had also been identified as cellulolytic yeast isolated from rotten iris potato (Amaeze *et al.*, 2015). Further increase in pH reduced cellulase activity of both yeasts. The reason for decreasing production at higher pH was probably due to proteolytic inactivation of cellulase. Hence, it suggested that slightly acidic pH values favored cellulase production, while when further increasing pH, the cellulase activity decreased gradually, which is in agreement with earlier result of other researchers (Deshmukh *et al.*, 2018; Irfan *et al.*, 2017). The hydrogen ion concentration in the fermentation medium thereby had a profound effect on the enzyme production. Ability of two yeasts to have maximum enzyme activities at different pH must have been to the morphological changes in microbes and in enzyme secretion (Rehman and Elahi, 2018). Although *W.chambardii* produced highest cellulase enzyme, there

was a sharp decrease in cellulase production from pH 4.5 to pH 5.0 with this yeast; this could have resulted from a change in pH which affects the ionization of essential active site of amino acid residues that are involved in substrate binding and catalysis (Techaparin *et al.*, 2017). Least enzyme was produced with pH 7. this might be due to the fact that alkaline pH has an inhibitory effect on growth of yeasts (Thongekkaew and Kongsanthia, 2016)

3.5.2. Effect of incubation temperature on cellulase production

Effect of different incubation temperature (30–60°C) on cellulase production by the two yeasts grown in sorghum straw medium was carried out. Optimum temperature for cellulase production (202.40 μmL) by *W.chambardii* was 45°C while *S.diaststicus* (104.83 μmL) had its optimum cellulase production at 40°C. One of the advantages of rapid fermentation at high temperature is decrease in the risk of contamination. The results of this experiment clearly the yeast were able to grow and produce high amount of cellulase enzyme at 40 and 45°C. Reports had shown potential applications of thermotolerant yeast strains especially those belonging to *Saccharomyces* spp (Saravanakumar *et al.*, 2013), *Wikerhamomyces* spp (Techaparin *et al.*, 2017). Variation in optimum temperature by the yeasts could be due to genetic composition of the yeasts (Khan and Dwivedi, 2013). Also Yeasts respond to heat and hydrogen ion stresses by synthesizing heat-shock proteins following exposure to temperatures above 35°C (Techaparin *et al.*, 2017). *Wikerhamomyces chambardii* had the lowest cellulase activity of 102.25 μmL while *S.diaststicus* had 63.00 μmL as its least cellulase activity at 60°C. The decrease in cellulase production level might be possibly due to lower transport of substrate across the cells at lower temperature, causing lower yield of the product (Rehman and Elahi, 2018). This is similar to the findings of Touijer *et al.*, (2019) who reported yeast that showed high temperature tolerance level up to 45°C. In addition, Papagianni and Papamichael (2017) reported *Wikerhamomyces* species of yeast that grow at higher temperature. In contrast, Carrasco *et al.* (2016) reported optimum temperature for higher cellulase production was 35°C and the lowest yield was achieved at 22°C.

3.5.3 Effect of inoculum concentration on cellulase production

Result of cellulase production by yeast cells from different amount of standard inoculate (5, 7.5, 10, 12.5, and 15%) v/v from sorghum straw. the yeasts were able to produce cellulase enzyme at different inoculum concentration ranging from 5 to 15% (v/v) during the incubation periods, with 7.5% v/v inoculum having the highest cellulase activity (231.50 μmL). Higher concentration of cell did not lead to high cellulase production; this may be attributed to substrate limitations or product inhibition (Adelabu *et al.*, 2019). The results of Thongekkaew and Kongsanthia (2016) confirmed our results, where maximum cellulase production was obtained at 8% inoculum size. Further increase in inoculum size resulted in gradual decrease

in production of cellulase. The use of low inoculum size (5%), yeast cells was not enough to utilize sorghum straw medium in a better way hence, this result in less growth and cellulase production. On the other hand, at high concentration of inoculum, anaerobic condition of fermentation sorghum straw medium, due to tremendous growth of yeast cells may lead to nutritional imbalance caused by tremendous growth resulting in autolysis of cells in medium which inturn lead to sharp decrease in cellulase production (Saravanakumar *et al.*, 2013). *Saccharomyces cerevisiae* and *W.chambardii* had 231.50 μmL and 211.84 μmL respectively as their highest cellulase activity while 140.21 μmL and 123.57 μmL was observed as the least cellulase enzyme produced by *S.cerevisiae* and *W.chambardii* respectively. Lower inoculum size required longer time for yeast cells to multiply so as to utilize the substrate for enzyme production.

3.5.4 Effect of substrate concentration on cellulase production

Concentration of substrate also affected the cellulase activity of the yeast isolates. Activity increased as the concentration of sorghum straw increased from 2.5% (w/v) up to a maximum of 7.5% (w/v). A drop in activity was obtained at concentration higher than 7.5%. Increase in sorghum straw concentration could have led to high concentration of complex sugars in the fermentation medium and this could have had high inhibitory effect on yeast growth and their capability to produce cellulase enzyme (Mardawati *et al.*, 2017). Also high sugar concentration may exert high toxicity on yeast and the nutrient may be deficient at the final stage of fermentation (Aliberti *et al.*, 2017; Dahnum *et al.*, 2015). High substrate concentration could inhibit the conversion of cellulose. The increasing of viscosity materials could also affect to reduce enzyme mobility. There is a chance of end product inhibition that could prevent hydrolysis (Mardawati *et al.*, 2019). This is in agreement with Michaelis Menten kinetics as rate of enzyme activity increased with increase in substrate concentration and reaches a maximum at 7.5% after 72 h of fermentation (Deshmukh *et al.*, 2018). Thongekkaew and Kongsanthia (2016) also reported that increase in substrate concentration has a dynamic influence on cellulose hydrolysis. *Wikerhamomyces chambardii* had the highest cellulase activity (223.18 μmL), while *S.diastaticus* had 211.82 μmL as its cellulase activity during sorghum straw hydrolysis (Fig 6). *Wikerhamomyces* species formally known as *Pichia* species has been identified as cellulose fermenting yeast (Adelabu *et al.*, 2018b; Techaparin *et al.*, 2017). *Saccharomyces* species had been reported as yeast that can utilize waste materials (Adelabu *et al.*, 2019). The data obtained clearly cellulose production increased by increasing the sugar concentration up to 7.5% by the yeasts. These results are nearly similar to those recorded by Touijer *et al.* (2019) who reported 6% xylose concentration for maximum cellulase productivity of *P.stipitis*, which is comparable with the current study.

4. Conclusions

This result showed that yeasts are found in gut of adult variegated grasshopper, *Zonocerus variegatus* and these yeasts can produce cellulase enzyme from sorghum straw. No initial treatment with chemicals was required because cellulase enzyme was directly produced by the yeasts. The ability of the yeasts to produce cellulase from sorghum straw helps to save cost from purchasing commercial cellulases and can further be applied in direct production of ethanol from lignocellulosic biomass.

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