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CELLULASE PRODUCTION BY IMMOBILIZED CELLS OF *CANDIDA TROPICALIS* ISOLATED FROM GRASSHOPPER *ZONOCERUS VARIEGATUS* IN SAW DUST AND RICE HUSK MEDIUM

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Abstract

This study investigated the isolation of yeast from insect gut *Zonocerus variegatus*, screening and production of cellulase enzyme by immobilized yeast in saw dust and rice husk medium. Yeasts were isolated from the gut of grasshopper and were screened for cellulase production using Carboxyl Methyl Cellulose agar. Immobilization was carried out using *Irvingia gabonensis.* Effect of bead size, bead number, inoculum load and bead reusability were investigated. *Candida tropicalis* had the highest cellulase production, cellulase enzyme production was optimum at 72 h, 6 numbers of bead, 4 mm bead size, 6% gel concentration and 4% inoculum size. There was no obvious loss of activity with re-use of immobilized *Candida tropicalis*. This study shows that *C. tropicalis* isolated from *Zonocerus variegatus* can be immobilized on *I. gabonensis* and produce cellululase enzyme from agricultural waste.

Introduction

Enzymes are important products obtained for human needs through microbial sources. Large number of industrial processes in the areas of industrial, environmental and food biotechnology use enzymes during production (REHMAN and ELAHI 2018). Example of such enzyme are cellulase enzyme which hydrolyze cellulose to simple sugar. These enzymes are mainly produced by microorganisms (TECHAPARIN et al. 2017).

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Cellulases are used in the textile industry, pulp and paper industry, in the production of detergents, for improving digestibility of animal feeds, in food industry and cellulase enzyme account for a significant share of the world enzyme market (BAEZA et al. 2016). The growing concerns about decrease in petroleum supplies and increase in environmental pollution from emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel has resulted in an increased focus on production of bioethanol from lignocellulosics and the possibility of using cellulases to perform enzymatic hydrolysis of the lignocellulosic material (THONGEKKAEW and KONGSANTHIA 2016).

Different microorganisms had been identified as cellulase producers; only a few yeast strains have been seen as producers of cellulase enzyme. For this reason, the search of new strains of yeast from nature is a method for discovering new enzymes which may permit the production of cellulase enzyme at an industrial scale (SHIL et al. 2014). Recently, yeasts have been developed as host organisms for the production of cellulase enzyme (TECHAPARIN et al. 2017). *Trichosporon laibachii*, *Phodotorula glacialis*, *Tetracladium* spp. and *Mrakia blollopis* are known to use both pentose and hexose sugars (CARRASCO et al. 2016). Cellulase production had been reported in several yeast specie; *Saccharomyces diastaticus, Aureobasidium* pullulans and *Wikerhamomyces* spp. (ADELABU et al. 2018, THONGEKKAEW and KONGSANTHIA 2016).

Grasshopper are common insect in the forest regions of West and Equatorial Africa (ADEMOLU and IDOWU 2011b). It has been reported that that gut sections of *Z. variegatus* harbored various microorganisms; bacteria, mould and yeast (JING et al. 2020). These 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). 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). Insects *such as Zonocerus variegatus* rely on microorganisms present in their guts to digest plant materials (JING et al. 2020).

Lignocellulosic materials such as corn straw, wheat straw, sorghum straw, rice straw, and sugarcane baggase are alternative materials for cellulase production. These cellulosic materials are cost effective, environmental friendly, readily available and are renewable (REHMAN and ELAHI 2018).

Immobilization is a technique of confining cells or enzymes on organic, inorganic, or hybrid carriers (ZDARTA et al. 2017). Cell immobilization enables easy separation of products from production medium (TECHAPARIN

et al. 2017). An advantage of cell immobilization is the possibility to reuse the cell and thereby reduce production costs (RODRIGUES et al. 2019). Another advantage is increased in stability of the immobilized cell compared to free cell (MUHAMMAD et al. 2019). Cell immobilization can be accomplished by cell support interaction through adsorption, affinity binding, covalent coupling or by entrapment of the cell (ZDARTA et al. 2017). Immobilization materials should have high mechanical strength, resistance to microbial attack, large surface area, many surface groups promoting interaction with the enzyme and should preferably be cheap to produce.

Natural support materials are matrices used for cross-linking of cells or enzymes; this is one of the varieties of materials used for immobilization. Some of the natural support materials used are *Detarium microcarpum*, (KAREEM et al. 2014), *Mucuna urens* (ADELABU et al. 2019), and Vegetable Sponge (OSHO et al. 2014). *Irvingi gbonensis* produce fruits which are mango-like in nature. Research shows that *Irvingi gabonensis* bears nuts which are rich in fat and protein.

Materials and Methods

Insect and gut fluid collection

Adult variegated grasshopper, *Zonocerus variegatus* were collected from a farm at Osiele (latitude 70 10−59 N and longtitude 30 27”0” E) in Odeda Local Governement area of Ogun State, Nigeria. They were collected in clean plastic containers and transported to the laboratory immediately. Diluted bleach (1%) was used for surface sterilization of the insect. This was carried for 30 seconds and rinsed for 1 min in three successive baths. This is to ensure that the gut contents were not contaminated by the surface microflora of the body during dissection (IRENE 2018). The insects were fixed on to a disinfected dissection board and the intestine were exposed from the ventral side with the aid of sterile dissection scissors and fine-tipped forceps to expose the gut (ROJAS-JIMÉNEZ and HERNÁNDEZ 2015). Guts were then transferred into a sterile 1.5 mL Eppendorf tube which contains 0.5 mL of 0.7% (v/v) sterile saline solution, crushed with a pipette tip and all solution (including gut pieces) were used for isolation (SHIL et al. 2014).

Isolation and characterization of yeast

Yeasts present in the gut were isolated by serial dilution of macerated guts of the grasshopper using 1 mL of the preparation. Modified Yeast Extract Peptone Dextrose-Carboxyl Methyl Cellulose (YEPD-CMC) agar containing Yeast Extract; 5 g, Peptone; 10 g, Dextrose; 5 g, NH_4NO_3; 0.2 g, KH_2PO_4; 0.5 g, $CaCl_2.2H_2O$; 0.03 g, $MgSO_4.7H_2O$; 0.03 g, $FeSO_4.7H_2O$; 0.5 g, $MnSO_4.7H_2O$; 0.16 g, $ZnSO_4.7H_2O$; 0.14 g, 1% CMC and Agar; 20 g was used for isolation of yeasts (CARRASCO et al. 2016). Plates were incubated at 30°C for 72 h. Characterization of yeast isolates was carried out based on size, shape and colour. Cell morphology of the purified yeast was studied. Fermentation test using different sugars were also carried out for classification (BARNETT et al. 2000).

Screening of yeast for cellulase production

Qualitative screening

The yeasts were screened for their ability to grow on YEPDA containing Carboxyl Methyl Cellulose (CMC). YEPDA-CMC plates were inoculated with yeast isolates at 30°C for 3 days. Agar plates were flooded with congo red and allowed to stand for 15 min at room temperature, it was de-stained with 1 M NaCl solution for 15 min. The plates that showed zone of clearance around the line of growth indicated cellulose hydrolysis (AMAEZE et al. 2015).

Quantitative screening

Yeasts were assessed for their ability to grow and produce cellulase enzyme. Screening was carried out in Erlenmeyer 250 mL flask containing 100 mL 1% CMC, NH_4NO_3, 0.2 g; KH_2PO_4, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g; Tween-80, 0.1 g. The flasks were inoculated with yeasts and incubated in a rotary (200 rpm) for 120 h at 30°C (AMAEZE et al. 2015).

Selection of starter

Yeast with the best cellulolytic potential was selected, immobilized and used for submerged fermentation of rice husk and saw dust.

Collection and preparation of substrates

Rice husk and sawdust were used for enzyme production. Rice husk was collected from a farm site while sawdust was collected from wood processing industry both in Abeokuta in Ogun State, Nigeria. The substrates collected were oven dried in electric oven at 65ºC and made to powder by grinding in an electric grinder and sieved through a mesh of 4 mm pore size. *Irvingia gabonensis* was obtained from a market in Abeokuta, Ogun State, Nigeria. The seeds of *Irvingia gabonensis* were blended into powder, defatted using Soxhlet extractor and dried in a hot air oven (Oso et al. 2014).

Fermentation of substrates by the yeast

Fermentation of rice husk and saw dust by free yeast was carried out in separate Erlenmeryer flask (250 mL) which contained 100 mL fermentation medium containing NH_4NO_3, 0.2 g; KH_2PO_4, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g; Tween-80, 0.1 g, 10% rice husk and saw dust. Each flask was inoculated with 5% yeast. The fermentation medium was incubated at 30ºC for 120 h. Cellulase activity was measured every 24 h (Carrasco et al. 2016)

Extraction of Enzyme

Crude enzyme was recovered by taking 5 mL from the fermentation medium and centrifuged at 10000 rpm with 7 cm radius, with centrifugal force of 7,826x g for 10 min in a table top high speed centrifuge (HI850R) and the supernatant were taken as the crude enzyme (Thongekkaew and Kongsanthia 2016).

Enzyme Assay

Cellulase assay was carried out using a modified method of Mandels. 0.5 mL CMC (1%) in 0.2 M phosphate buffer (pH 5.0) and 0.5 mL crude enzyme in test tubes. The reaction mixture was 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 5min and then cooled at room temperature. 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 (THONGEKKAEW and KONGSANTHIA 2016). The amount of reducing sugar produced by the action of crude enzyme was read off from the curve. One unit of cellulase activity (U) was defined as the amount of enzyme that liberated 1.0 μmole of D-glucose from substrates (CMC) in 1.0 μL reaction mixture under the assay conditions.

Immobilization of yeast

Immobilization of yeast was carried out with *Irvingia gabonensis* Glutaraldehyde (2.5%) v/v was used to cross-linked 5 g of *Irvingia gabonensis* and the mixture was stirred for 10 min. Yeast suspension was mixed with the slurry obtained and the slurry was used to form spherical beads using a syringe. The slurry was dropped into ethanolic formaldehyde (50 : 50 v/v) for 24 h (KAREEM et al. 2014).

Optimization studies of cellulase production by immobilized yeast cell

Cellulase production from the immobilized yeast was carried out using Mineral Salt Medium which consist of KH_2PO_4 0.8 g, $MgSO_4.7H_2O$ 0.3 g; NH_4NO_3 1.2 g; $FeSO_4.7H_2O$ 0.4 g; $MnSO_4.H_2O$ 1.5 g; $CaCl_2.2H_2O$ 0.3 g; $ZnSO_4.7H_2O$ 1.3 g; Tween-80 0.15 g; peptone 0.75 g, yeast extract 0.3 g; glucose 5 g and 10% each of rice husk and saw dust. The media were incubated at 30°C for 72 h. Samples were taken and analyzed after 72 h of fermentation (KAREEM et al. 2014). Optimization of cellulase production from immobilized yeast cell was carried out considering different parameters; bead size, bead number, *Irvingia gabonensis* concentration, inoculum load and bead reusability. Cellulase production from free and immobilized yeast was also compared.

Statistical analysis

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

Results

Isolation of yeasts

A total of seven (7) yeasts were isolated from the gut of *Zonocerus variegatus*. Sugar fermentation test showed that all the isolates ferment glu-

cose and sucrose while only one isolate ferment urease and lactose. Biochemical and physiological characteristics of the yeasts identified them as *Trichosporon beemeri, Saccharomyces cerevisiae* 1, *S. cerevisiae* 2, *Candida shehatea, Candida tropicalis, Candida krusei* and *Debaryomyces hansenii.*

Screening for cellulase enzyme

Screening of yeasts revealed that the yeasts hydrolyze cellulose, showing zones of clearance on CMC agar plates. The yeast isolates produced zones of clearance from 22.0 to 35.0 mm in diameter. The highest halo zone was observed with *Candida tropicalis* (35.0 mm) while *Saccharomyces cerevisiae* showed the least halo zone (22.0 mm) (Data not shown). Table 1 showed that the tested yeasts grew and produced cellulase enzyme in mineral medium where CMC was the carbon source. Among the yeasts, *Candida tropicalis* had the highest activity (174.67 U/ml), followed by *Candida shehatae and Debaryomyces hansenii,* which had 160 U/ml. *T. beemeri* had the lowest cellulase activity (94.86 U/ml). Statistical analysis showed that cellulase activity by the yeasts were significantly different ($p \geq 0.05$). *Candida tropicalis* was selected for cellulase production in submerged fermentation.

Table 1

Screening for cellulase enzyme

Yeast isolates	Cellulase activity [µ/mL]
Candida tropicalis	174.67 ± 23.21^{d}
C. shehatae	160.66 ± 10.34^{c}
C. krusei	$143.81 \pm 11.76b^{c}$
Debaryomyces hansenii	160.43 ± 31.68^{c}
Saccharomyces cerevisiae	127.02 ± 13.28^{b}
S. cerevisiae 2	105.86 ± 20.62^{a}
Trichosporon beemeri	94.86 ± 19.17^{a}

Each value is a mean of 3 readings ± 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

Cellulase production from rice husk and saw dust

Cellulase production from rice husk and saw dust by immobilized *C. tropicalis* is shown in Figure 1. The figure showed that cellulase activity increased from 24 hours to 72 hours of fermentation for both rice husk and saw dust; highest cellulase activity was achieved at 72 hour with the

immobilized yeast (234 U/ml) and thereafter decreased for both substrates (Fig. 1). Sharp decrease in cellulase activity was observed in fermented rice husk from 72 hour (198 U/ml) to 96 hour (142 U/ml) – Figure 1. Fermentation of substrates with immobilized *C. tropicalis* showed that saw dust produced the highest cellulase activity (234 U/ml), while rice husk had 198 U/ml cellulase activity (Fig. 1).

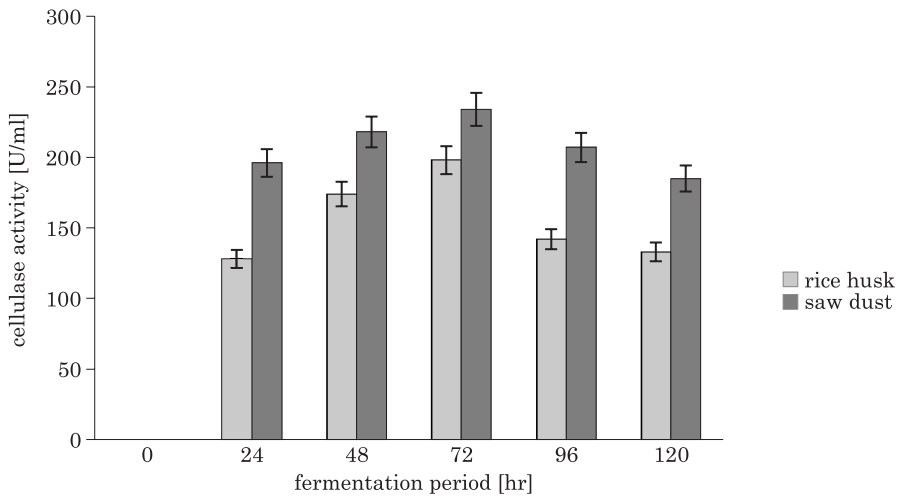

Fig. 1. Effect of fermentation period on cellulase production

Effect of bead size on cellulase production

Figure 2 shows that bead size had significant effect on cellulase production from rice husk and saw dust. Cellulase activity increased with bead size 3 mm to 4 mm and decreased with further increase in bead size. Use of 4 mm bead size produced the highest cellulase enzyme (218.49 U/ml) while fermentation with immobilized *C. tropicalis* of bead size 6 mm had the least cellulase activity (108 U/ml) for both substrates (Fig. 2). Saw dust medium fermented with *Irvingia gabonensis* immobilized *C. tropicalis* showed highest cellulase activity (229.49 U/ml) while rice husk medium had 113.74 U/ml cellulase activity (Fig. 2).

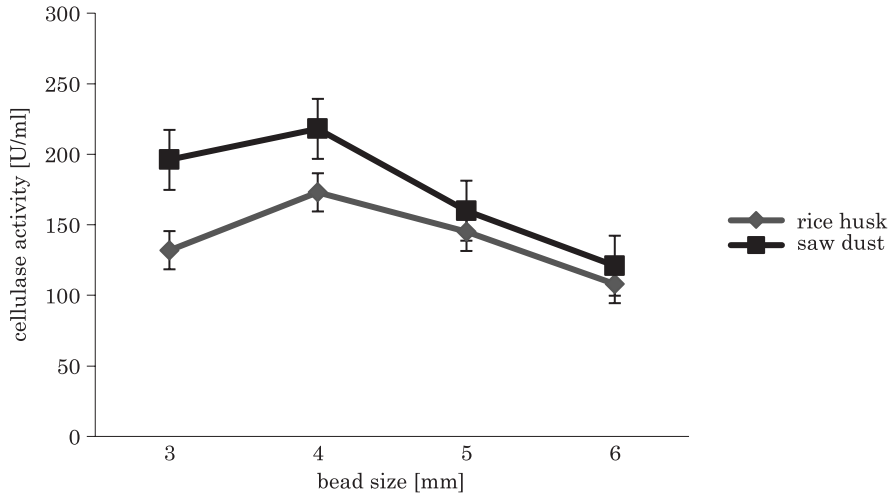

Fig. 2. Effect of bead size on cellulase production

Effect of bead number on cellulase production

Cellulase production from fermentation of saw dust and rice husk by different bead sizes of immobilized *C. tropicalis* is shown in Figure 3. Data illustrated showed that cellulase increased gradually with lower bead number and optimum production was achieved with 6 beads (268.21 U/ml) while fermentation with 10 beads had least cellulase activity (190.47 U/ml) for both substrates (Fig. 3). Maximum production of cellulase enzyme was achieved with fermentation of rice husk by immobilized *C. tropicalis* (268.21 U/ml).

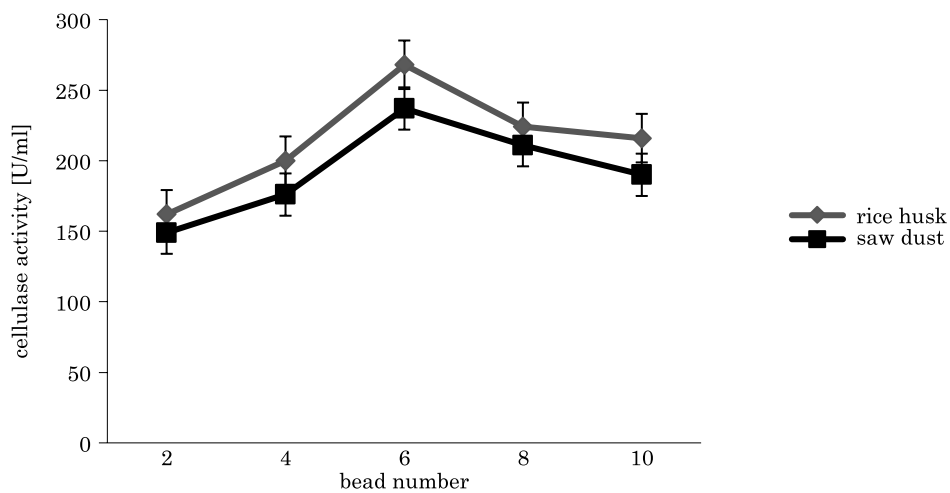

Fig. 3. Effect of bead number on cellulase production

Fermentation of saw dust by immobilized *C. tropicalis* had 237.90 U/ml as its highest cellulase activity and 190.63 U/ml as its least cellulase activity (Fig. 3).

Effect of *Irvingia gabonensis* concentration on cellulase production

Effect of different concentration of *Irvingia gabonensis* on cellulase production was carried out using concentration which ranges from 2–10%. Result presented in Figure 4 showed that fermentation of rice husk and saw dust with immobilized *C. tropicalis* of 6% gel concentration had the highest cellulase activity (246 U/ml) while a decrease in enzyme activity was observed at higher gel concentration (Fig. 4). It was observed from Figure 4 that rice husk fermented with 6% immobilized *C. tropicalis* had the highest cellulase activity (264 U/ml) and (172 U/ml) as its least cellulase activity with 10% *Irvingia gabonensis* concentration. Fermentation of saw dust also had its highest cellulase activity (206 U/ml) with 6% gel concentration while its least activity was 141 U/ml at 10% concentration (Fig. 4).

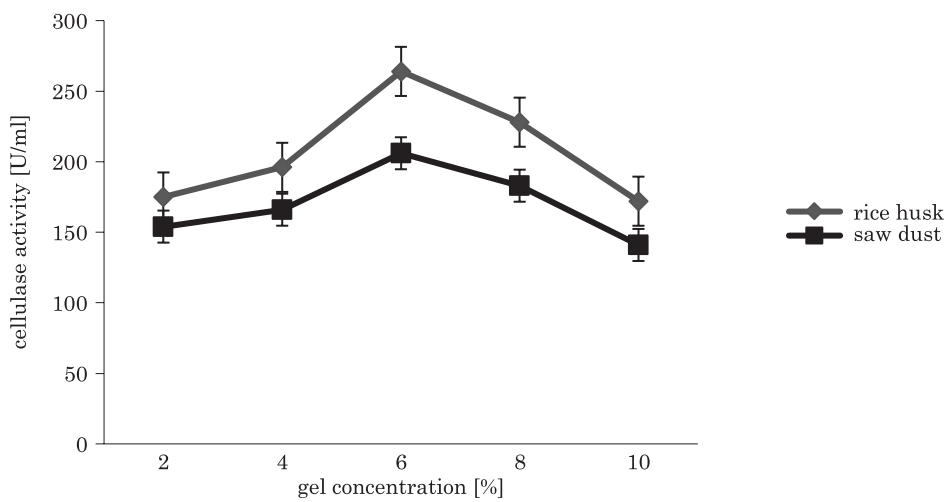

Fig. 4. Effect of *Irvingia gabonensis* concentration on cellulase production

Effect of inoculum concentration on cellulase production

Effect of inoculum load showed that cell load had a very strong influence on cellulase production. Immobilized *C. tropicalis* had maximum enzyme activity at inoculum load of 8% v/v (Fig. 5). Highest cellulase activity observed with use of 8% v/v inoculum in saw dust and rice husk medium

was 172.08 U/ml and 206.5 U/ml respectively. However, higher concentration of yeast did not lead to improved cellulase activity (Fig. 5). Least cellulase activity of 105.03 and 139.01 U/ml was observed for saw dust and rice husk respectively with the use of 10% inoculum concentration.

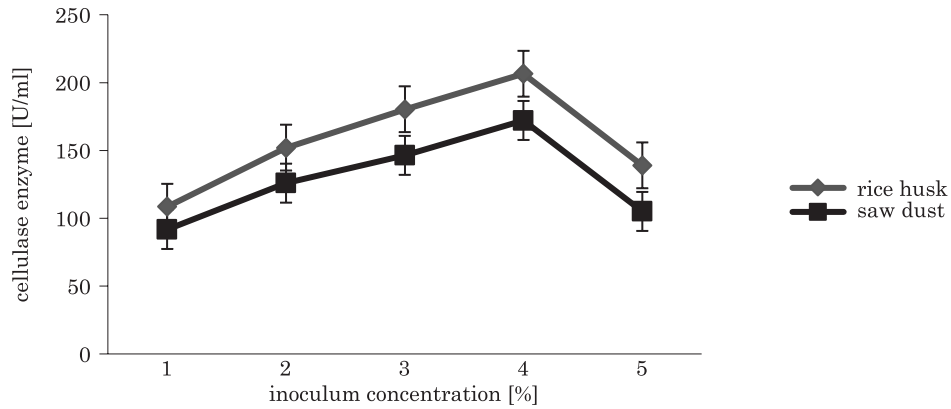

Fig. 5. Effect of inoculum concentration on cellulase production

Effect of reused cross-linked *C. tropicalis* on cellulase production

Potential use of *I. gabonensis* – immobilized *C. tropicalis* on saw dust and rice husk in different cycles during cellulase production was described in Table 2. The results in Table 2 showed that reusing the entrapped cells result in an increase in the enzyme activity during cell re-use for both substrates. Rice husk retained 91, 84 and 75% cellulase enzyme yield for second, third and fourth cycle while saw dust retained 86, 72 and 61% cellulase enzyme yield. Further re-use of yeast cells during the 5th cycle showed a sharp decrease in cellulase enzyme yield (Table 2).

Table 2

Reusability of immobilized *C. tropicalis* on cellulase production

Number of cycle	Cellulase enzyme yield [%]	
	rice husk	saw dust
1	91	86
2	84	72
3	75	61
4	63	58
5	35	20

Each value is a mean of 3 readings ± standard deviation

Comparative cellulase production by free and immobilized cells of *C. tropicalis*

Cellulase enzyme production by free and immobilized cells of *C. tropicalis* was shown in Table 3. Higher cellulase activity was observed in immobilized cells compared to free cells for both substrates (Table 3). The table shows that fermentation with immobilized cells of *C. tropicalis* had higher cellulase activity (132.11 U/ml) compared to its free cells (128.64 U/ml). Fermentation of saw dust with immobilized cells of *C. tropicalis* also had higher cellulase activity (125.31 U/ml) compared to fermentation with free cells (122.91 U/ml) – Table 3.

Table 3

Comparative cellulase production by free and immobilized cells of *C. tropicalis*

Fermentation period	Immobilized yeast		Free yeast	
	rice husk	saw dust	rice husk	saw dust
0	0.00 ± 0.000^{a}	$0\ 0.00\pm0.000^{a}$	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}
24	124.58 ± 1.192^{b}	$24\ 118.71\pm1.126^{b}$	119.64 ± 1.124^{b}	114.00 ± 1.083^{b}
48	127.21 ± 1.141^{c}	121.48 ± 1.121^{c}	123.35 ± 1.231^{c}	119.45 ± 1.254^{c}
72	132.11 ± 1.254^{e}	126.35 ± 1.213^{e}	128.64 ± 1.212^{e}	122.91 ± 1.225^{e}
96	128.00 ± 1.054^{d}	122.94 ± 0.697^{d}	125.11 ± 1.184^{d}	120.13 ± 1.213^{d}

Note: each value is a mean of 3 readings ± standard deviation.Various superscripts in Table 3 indicate significant differences ($p < 0.05$)

Discussion

Immobilization of cells for the production of industrially important enzymes offers various advantages such as reduced cost of production, ease separation of cell from production medium, ability to reuse cell in different cycles and reduced possibility of contamination. The immobilization matrix – *Irvingia gabonensis* was noted to provide stability and strength (both physical and mechanical) to this yeast immobilization system because there is no physical change in the beads during cellulase production. This is an advantage over the use of synthetic matrices where disintegration of immobilizing materials was observed (BRETHAUER and WYMAN 2010).

It has been reported that insects are in a set of diffuse mutualisms with yeasts (MADDEN et al. 2018). From the study, *Candida* species were the most frequent yeast isolated from the gut of *Zonocerus variegatus*, this yeast are known as thermotolerant yeast and are able to survive in environment with high pH (KAUR et al. 2018). This had earlier been reported by ADELABU et al (2018) where *Candida* species were isolated from com-

post piles. *Saccharomyces* species had also been isolated from the gut of black beetle. KAUR et al. (2018), they have been reported to utilize wide range of nutrients and can secret different enzymes (PADILLA 2016). *Debaryomyces hansenii* and *Trichospron beemeri* had been reported by different authors as yeasts isolated from insect gut (ADELABU et al. 2018).

Insects have developed very effective strategies to use lignocellulosic materials as source of energy (WILLIS et al. 2010). *Zonocerus variegatus* had been reported to established symbiotic interaction with different yeasts to carry out key hydrolytic activities (ADEMOLU and IDOWU 2010a). Screening of the yeast isolates confirmed that the yeasts isolated from gut of *Zonocerus variegatus* were able to utilize Carboxyl Methyl Cellulose (CMC), but their ability to degrade CMC differs. Differences in their ability to hydrolyze the substrates could be due to the amount of enzyme excreted into the medium and different growth rates of the yeast strains (PINJARI and KOTARI 2018).

The yeasts were confirmed as cellulose degrading yeast because they all grew on 1% CMC, this is an indication that the yeasts are potential cellulase producers. *Candida tropicalis* had the highest cellulase activity. This specie of *Candida* might possess more exogluconases, endogluconases, and β-glucosidases which are classes of cellulase enzyme than other species isolated in this study. This is confirmed by THONGEKKAEW et al. (2019) who reported that *Candida* species isolated from gut of insect assimilated D-xylose. Least cellulase activity was observed with *Trichospron beemeri,* this yeast might have low xylose reductase (XR) and xylose dehydrogenase (XDH) which are enzymes responsible for xylose and cellulose degradation (KHAN and DWIVEDI 2013). Though the yeast are of the same general, the gene that code for cellulase production in each of them might differs, hence the difference in the amount of cellulase enzyme produced.

Fermentation time is an important factor from an economic point of view in enzyme production. The findings on the effect of fermentation time on enzyme production from rice husk and saw dust revealed that *Candida tropicalis* produced cellulase from both agrowastes. Enzyme production increased with increase in fermentation time with each of the agrowastes, such increase may be due to the gradual breaking down of complex sugars to simple sugar during fermentation (REHMAN and ELAHI 2018). Saw dust fermented with *C. tropicalis* was shown to be the best, yielding a higher amount of cellulase enzyme. When comparing cellulase production in this study to earlier reports, activities of *C. tropicalis* was higher than that of ZDARTA et al. (2017) who reported 164 U/ml as the highest cellulase production from compost piles.

Sizes of beads determine the number of cells available for the production of cellulase enzyme. Optimum cellulase production was achieved with bead size of 4 mm, which indicate that at this size, the number of pore spaces made available is highest and the number of biomass occupying each space is maximum (XING et al. 2015). Smaller bead size exhibited better cellulase production, when compared with large-size beads. This might be due to increased sur-face area of the bead, which enhances the mass transfer.Thus smaller beads (4 mm) have more surface area per unit volume and hence more productivity (AHMED et al. 2019).

It was observed that maximum cellulase production was obtained with 6 beads. Enzyme production decreased at high initial cell loading (10 beads/flask). This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting (DEVI and NAGAMANI 2018). Also high cells loading had been observed to result in low yield, because cells entrapped in *Irvingia gabonensis* may experience diffusional problem due to inability of the immobilized cells to access nutrients in the fermentation broth (RODRIGUES et al. 2019).

Reports had shown that concentration of immobilized material can enhance enzyme production (DEVI and NAGAMANI 2018, DONG et al. 2013, KAREEM et al. 2014). The effect of different gel concentration showed that cellulase enzyme yield was optimum at gel concentration of 6% while a decrease in cellulase enzyme activity at higher gel concentration, this may be due to diffusional limitations imposed by the solid nature of the hardened matrix (OSHO et al. 2014). Increasing in bead concentration did not cause any improvement in cellulase production, but caused some leakage problems. Also, increasing the beads concentrations caused reductions in enzyme production because the beads settled at the bottom of the fermentation medium (FERNANDEZ-LAFUENTE 2019).

Studies on inoculum concentration showed that highest cellulase activity was observed in *C. tropicalis* fermented rice husk. Lower cellulase biosynthesis at lower inoculum size was probably due to lower cell density in the bead at low cell loadings (MAJOLAGBE et al. 2010). The positive effect of increasing cell loading from 3 to 4%, which led to improve cellulase production, was the same results obtained for the production of gluconic acid by *Aspergillus niger* immobilized in Calcium alginate beads (DONG et al. 2013). Decreased yield at higher inoculum size is probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (DEVI and NAGAMANI 2018). This decrease in enzyme production with further increase in inoculum might also be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release

(MUHAMMAD et al. 2019, SIKANDER et al. 2017). Similar observation was also reported by KOURKOUTAS et al. (2004) where maximum ethanol yield was obtained at 4% inoculum size.

During repeated use of immobilized *C. tropicalis* cells, it was observed that increase in cellulase production in the first cycle was considerably lower than those observed in second and third cycles. This might be due to the fact that during the first cycle, cells are in an adaptation phase and they might be suffering stress caused by immobilization (DEVI and NAGAMANI 2018). Decrease in cellulase enzyme was also observed as the re-use number increased. This may be as a result of clogging of yeast cells immobilized within the matrix. IKEDA et al. (2015) reported that accumulation of enzyme inside the cell inhibited activity of endo-glucanases, exo-glucanases and β-glucosidases found in cellulase-system and thus led to decrease cellulase enzyme production.

Comparative cellulase enzyme production by free and immobilized cells of *C. tropicalis* showed that Immobilized cells gave an improved cellulase enzyme yield. This result agreed with KAREEM et al. (2014) who produced citric acid from free and immobilized cells. BAYRAKTAR and MEHMETOGLU (2012) reported that immobilized cells offer several advantages over free cells such as decreased medium viscosity, enhanced oxygen and nutrient transfer, higher productivity, operational stability. Use of immobilized cells also prevent decreased contamination of the product by free cells.

Reports has shown that other methods of immobilization are entrapment, crosslinking encapsulation and covalent bonding. Covalent bonding is widely used for immobilization. This involves formation of covalent bonds between the chemical groups in enzyme and the chemical groups on the support or carrier. Each immobilization technique has its advantages and disadvantages.

Conclusion

Result in this study showed that saw dust and rice husk have desirable properties for cellulase production. Immobilized *C. tropicalis* showed higher cellulase activity compared to its free cells, thus immobilized yeast can be used for cellulase production from cost effective materials.

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