Zimbabwe Journal of Science & Technology pp 34 – 47 Vol.15 [2020]
e-ISSN 2409-0360
Zimbabwej.sci.technol

Optimization of solid state fermentation conditions for improved production of cellulases by *didymella phacea*

S. Nkala, S. L. Rabi and T. Ncube

National university of Science and Technology, Department of Applied Biology and Biochemistry, P O Box AC 939, Ascot, Bulawayo, Zimbabwe.
thembekile.ncube@nust.ac.zw

Abstract
Enzyme production by microorganisms may be improved by mutagenesis of the producing strains and/or the optimization of conditions for production. The effective and efficient use of cellulases in industrial processes has been hampered by the high cost of the enzymes which in consequence lead to high overall production costs. This study aimed at optimizing solid state fermentation conditions using affordable susbtrates and sustainable additives for improved cellulase production by *Didymella phacea.* Cellulase production during solid state fermentation of different lignocellulosic substrates and the effect of varying physical parameters including temperature, pH, additional supplements like nitrogen source and lactose were investigated. A 188% increase in cellulase production by *D. phacea* was achieved using solid state fermentation of Jatropha seed cake, incubated at 30 °C for a period of 72 hours, with an initial pH of 6, peptone at a concentration of 1 g/L, lactose at a concentration of 3.5 g/L and an additional 10% thatch grass as an additional carbon source. There was a significant difference in the increase of cellulase production before and after the optimization process ($p< 0.05$). Optimization of culture conditions may be used as a quick, cheap and effective alternative to increase enzyme production.
Keywords
Cellulase, *Didymella phacea*, Solid state fermentation, Optimisation, Lignocellulosic
Received: 14.08.20 **Accepted:** 17.12.20

1. INTRODUCTION

Cellulose is the most abundant renewable biological resource (Akula and Golla 2018) and a low-cost energy source based on energy content. The production of bio-based products and bioenergy from less costly renewable lignocellulosic materials is beneficial to local economies, environment, and the national energy security.

The bioconversion of cellulosic residues to value added products requires four steps for processing which are pre-treatment, de-polymerization (saccharification) of cellulose, fermentation, plus separation and purification of the products. The first three steps are bio-related processes whilst the fourth step is a chemical engineering process. Pre-treatment makes cellulose more accessible to the cellulolytic enzymes, which in turn reduces enzyme requirements and thus cutting down the costs of the bio-based products (Mosier *et al*., 2005; Maryam *et al*., 2018). After pre-treatment, cellulose is hydrolysed to soluble monomeric sugars using cellulases (Adnan, 2010).

Cellulases are a group of hydrolytic enzymes capable of degrading lignocellulosic materials (Singh *et al*.,2019). The enzymes are widely distributed throughout the biosphere. The enzymes hydrolyse β-1,4 linkages in cellulose chains. Cellulases thus provide a key to utilization of biomass with numerous benefits. Considerable economic interest has risen to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. However, high costs of cellulases remain one of the largest obstacles for commercialization of biomass biorefineries due to the large amount of cellulase consumed for biomass saccharification, for example, approximately 100 g enzymes are required to per gallon of bioethanol produced (Zhang and Zhang, 2013; Akula and Golla, 2018) Despite the cost of these enzymes, developing the know-how for enzyme production processes remains a

strategic issue especially to effective cost-reduction (Zhang and Lynd, 2004).

Cellulases are produced by a variety of life forms (Sing *et al*., 2019a) but are most abundant in fungi and bacteria (Zhang and Zhang, 2013; Elsebaay *et al*., 2018). Amongst the prominent microorganisms are the filamentous fungi such as *Trichoderma, Didymella, Penicillium* and *Aspergillus spp* that have been reported to be good producers of these enzymes (Elsebaay *et al*., 2018; Darabzadeh *et al*., 2018; Singh *et al*., 2019). The fungi are preferred in cellulase production because of their ability to colonize and penetrate the substrates used in the fermentation and the extracelluar excretion of the desired enzymes (Mosier *et al*., 2005). Fermentation methods employed in cellulase production are solid state fermentation and submerged fermentation (Singh *et al*.,2019). Solid-state fermentation (SSF) is a process in which an insoluble substrate with sufficient moisture is fermented by microorganisms (Farinas *et al*., 2010; Sukumaran *et al*., 2005; Ramesh and Bohero, 2017). Microbial enzyme production through a solid state fermentation (SSF) process has been described as a better option in terms of product stability when compared to submerged fermentation (Singh *et al*.,2019). SSF is an environmentally friendly process with low waste water production and low energy consumption. The process is an economical technology in synthesizing cellulase enzyme as compared to submerged fermentation.

Substrates for cellulase activity assays can be divided into two categories based on their solubility in water. Soluble substrates include cellodextrins from two to six sugar units and their derivatives. Insoluble cellulose-containing substrates for cellulase activity assays include nearly pure celluloses such as cotton linter, Whatman No.1 filter paper, bacterial cellulose, microcrystalline cellulose and amorphous cellulose and impure cellulose-containing substrates including dyed cellulose, α-cellulose, and pre-treated lignocellulose (Ozioki, 2013). The majority of commercial cellulase fermentations use cellulosic biomass including straw, spent hulls of cereals, wheat bran, saw dust, paper industry waste, maize cobs and several other lignocellulosic residues (Ahmed and Bibi, 2018). Cow dung is also an interesting lignocellulosic material since the cow consumes grass that contains cellulose. As the grass goes through the rumen, the accessibility of the cellulose to the enzymes may increase due to acid digestion of lignin. Jatropha seed cake is also an emerging carbon source in cellulase production (Dave *et al*., 2012; Ncube *et al*., 2012).

Cellulase production in cultures is associated with growth of the organism and is influenced by various physical and chemical parameters (Singh *et al*.,2019). The interaction of these factors affects cellulase productivity. Physical process parameters such as initial pH of the culture and temperature also play a vital role for the cellulase production efficiency of microorganisms. Medium additives or supplements also affect cellulase production, for example the addition of organic or inorganic nitrogenous compounds (Sukumaran, 2005; Ahmed and Bibi, 2018) Organic additives such as lactose, a well-known inducer of cellulase genes has been studied. Lactose is a soluble and economic carbon source for the industrial production of cellulases or recombinant proteins (Karaffa, 2006).

The search to further improve the properties of fungi as protein producers has seen many strategies such as signal sequence optimization, use of strong promoters, co-expression of chaperones and foldases, genetic modifications which improve secretion capabilities (such as the knockout of specific genes or random mutagenesis), reduction of proteolytic activity,and optimization of fermentation conditions, among others. Improvement of the secretion of extracellular enzymes by methods such as random mutagenesis and screening has been successfully performed in various filamentous fungi though they are known to be expensive (Ribeiro *et al*., 2013). Also many of the mutations may be deleterious making the organism less biologically adaptable and some may even be lethal. Some mutations may have neutral effects and may not confer immediate benefits

There is a critical need to produce cellulases for industrial process using cost effective production systems (Elsebaay *et al*., 2018). In

this study we report the optimization of conditions for the production of cellulases by *D. phacea* using low cost lignocellulosic substrates and low cost addivities to boost the enzyme titres. This may possibly lead to effective increase in cellulose titres at reduced costs thus maintaining sustainability of industrial applications that use the enzyme.

2. MATERIALS AND METHODS

2.1 Preparation of the lignocellulosic Substrates

Sawdust, cow dung, *Hyparrhenia* species(common thatch grass), *Jatropha* seed cake, banana pseudo stem and maize cobs were collected from areas in and around Bulawayo (Zimbabwe). The substrates were dried in an oven at 70°C to constant mass.. The substrates were then ground into fine textured powders using a commercial blender after which they were sieved using a 1 mm mesh. The sieved substrates were then transferred into a sealed container and autoclaved for 15 minutes at 121°C and 1 atmosphere. The autoclaved substrates were stored in dark bottles at 4°C until used.

2.2 Culturing of the organism

Didymella phacea was grown on malt extract agar plates (MEA) for 5 days at a temperature of 30°C. Spores were harvested from 5 day old cultures by adding 10 ml sterile distilled water onto the culture plates. A sterile swab was used to gently dislodge the spores from the agar plate. A standard spore count was performed on a Neubauer bright lined counting chamber. The spore suspension was adjusted to 1 x 10^6 spores/ml to obtain the inoculum and stored at room temperature.

2.3 Production of the cellulolytic enzymes by solid state fermentation

The fungal isolates were cultured in 250 ml Erlenmeyer flasks containing 5 g of each of the substrates The substrates were hydrated with nutrient mixture as described by Mandel and Weber (Oberoi et al, 2008) to attain a 70% moisture content. The nutrient mixture contained; Urea 0.3 g, $(NH4)_2SO4$ 1.4 g, K_2HPO4 2.0 g, $CaCl_2$ 0.4 g, $MgSO4.\ 7H_2O$ 0.3 g, peptone 1.0 g, $FeSO4.\ 7H_2O$ 5.0 mg, $MnSO4.\ 7H_2O$ 1.04 mg, $ZnSO4.\ 7H_2O$ 1.4 mg, $COCl_2$ 2.0 mg and yeast extract 0.5 g (per litre of distilled) water). The pH of media was adjusted to 5.0 ± 0.2. The media was sterilized by autoclaving at 121°C for 15 min. After cooling, inoculation was done using *D. phacea* spores to attain a final concentration of 1 x 10^6 spores per gram of substrate used. Fermentation was carried out at 25°C over a period of 120 hours.

2.3 Extraction of enzymes from SSF fungal Cultures

After the fermentation period, the crude enzyme was extracted by adding 5ml of 0.05 M acetate buffer (pH 5.0) per gram of fermented substrate followed by shaking on an orbital shaker set at 150 rpm. Shaking was conducted for 2 hours after which the medium was centrifuged at 1 500 g for 20 minutes and aliquoted into 1.5 ml Eppendorff tubes which were stored at -20°C and thawed at room temperature when required.

2.4 Preparation of standard curve for glucose determination

The glucose stock solution was prepared by weighing 0.18 g of the sugar into 100 ml of 0.05M acetate buffer pH 5. The standards were prepared by diluting the sugar stock solution with 0.05 M acetate buffer pH 5 as follows: 1:1, 1:2 and 1:4 dilutions giving 5, 3.33 and 2 µmol/ml of the substrate respectively. The diluted sugar standards (100 µl) were placed in test tubes containing 0.9 ml acetate buffer and reacted with 1.5 ml 3,5-dinitrosalicyclic acid (DNS). The solutions were placed in a boiling water bath for 15 minutes and then cooled on ice. The intensity of colour development from the reaction of the reducing sugars and DNS was read as absorbance at 540 nm and a standard curve was plotted against the concentration of the sugars. The slope of the variable was calculated from the curve and used for determination of the reducing sugar content in the enzyme samples reacted with carboxymethylcellulose (CMC).

2.5 Assaying for cellulase activity

Cellulase activity was determined by mixing 0.9 ml of 1% CMC prepared in 50 mM acetate buffer pH 5 with 0.1 ml of the enzyme solution appropriately diluted to give an absorbance reading below 0.7 on assay. The enzyme-substrate mixture was incubated at 50 °C for 5 minutes.The released reducing sugars were determined by the use of DNS and glucose

was used as a standard for cellulase activities. One unit of cellulase activity is defined as the amount of enzyme that liberates 1 µmol of xylose or glucose equivalents per minute, respectively.

2.6 Time course analysis for the different cellulosic substrates

Didymella phacea was cultured in 250 ml conical flasks containing Jatropha seedcake at 25°C over 216 hours to select the optimum incubation period for the production of cellulases. The cellulase activity was monitored every 24 hours by taking out the entire culture flask, extracting and quantifying the enzyme.

2.7 Optimum temperature for cellulase production

Optimum temperature for the production of cellulases by *D.* phacea was investigated by incubating cultures at 25°C, 30°C, 35°C and 40°C over 120 hours. Cellulase activity was monitored every 24 hours by quantification of the reducing sugars produced as described above.

2.8 Effect of initial pH on cellulase production

D. phacea was cultured in Jatropha seed cake with a 70% moisture content (as described above) in the following buffers: 50 mM acetate buffer (pH 3-5), 50 mM sodium phosphate buffer (pH 6-7), and 50 mM Tris-HCl (pH 8-10) (Ncube, 2013). The flasks were incubated at 30°C for 120 hours. The cellulase activity was monitored every 24 hours.

2.9 Effect of nitrogen source on cellulase production

The fermentation medium for production of cellulases was supplemented with organic and inorganic nitrogenous compounds namely; Urea, mixture of peptone, malt extract and yeast extract, peptone alone, ammonium chloride and sodium nitrate at a concentration of 1 g/L. Controls without nitrogen supplementation were also prepared. The fermentation was carried out at 30°C for 96 hours at an initial pH of 6. The cellulase activity was monitored every 24 hours using reducing sugars.

2.10 Effect of lactose as an inducer for cellulase production

The effect of lactose was investigated using concentrations varying between 0-3.5 g/L in Mandel's fermentation medium using Jatropha as a carbon source. The fermentation was carried out at 30°C for 96 hours with an initial pH of 6 and peptone as a nitrogen supplement. The cellulase activity was monitored every 24 hours using reducing sugars as indication of cellulase activity.

2.11 Effect of an additional carbon source on cellulase production

To find out the effect of an additional carbon source, conical flasks containing Jatropha seed cake plus 10% (w/w) of each of the following; cow dung, maize cobs, cooked maize cobs, saw dust, banana pseudo stem or thatch grass were prepared and incubated at 30°C for 96 hours at an initial pH 6, peptone as a nitrogen supplement and lactose at 3.5g/L concentration. The cellulase activity was monitored every 24 hours using reducing sugars as indication of cellulase activity.

2.12 Protein estimation for the crude enzyme extracts

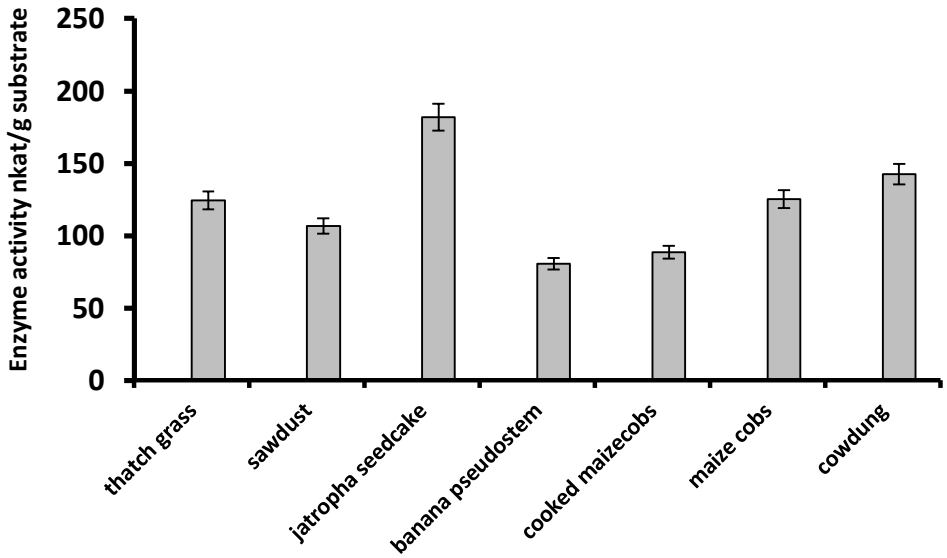
Protein content in the crude enzyme extracts was determined using Lowry et al (1951) method. Bovine serum was used to prepare the standard curve. Six millilitres of the alkaline copper reagent was added to 900 µl of sample solution in each test tube and was left to stand for 10 minutes at room temperature. After this incubation period, 600 µl of Folin-Ciocalteau reagent was added and immediately mixed and the tubes were allowed to stand for 30 minutes at room temperature. After the incubation time had elapsed, the absorbances were read at 750 nm. The total protein was estimated using the standard curve.

3 RESULTS

3.1 Effect of different carbon sources on the production of cellulases by *D. phacea*

The use of different lignocellulosic substrates in solid state fermentation resulted in different cellulase production, as shown in Fig. 1.

The highest cellulase activity of 181.9 nkat/g substrate was observed when the organism was grown on Jatropha seedcake. The least cellulase activity of 80.6 nkat/g substrate was observed when the organism was grown on banana pseudo-stem. The incubation time used was pre-determined in previous studies (not published)



. 258

Figure 1. Cellulase production by D. phacea cultured on different cellulosic substrates over 144 hours at 25°C.

3.2 Time course analysis for the production of cellulase by *D. phacea*

A time course analysis for cellulase production yielded the results shown in Fig. 2. Cellulase production increased with an increase in the incubation period. The highest cellulase activity of 309.9 nkat/g substrate was observed after 120 hours of incubation, thereafter a steady decrease in enzyme production was observed.

The time course analysis was used to ascertain the time needed for incubation of cellulase before the titres decreased as a result of factors such as breakdown by proteases (Ncube, 2013).

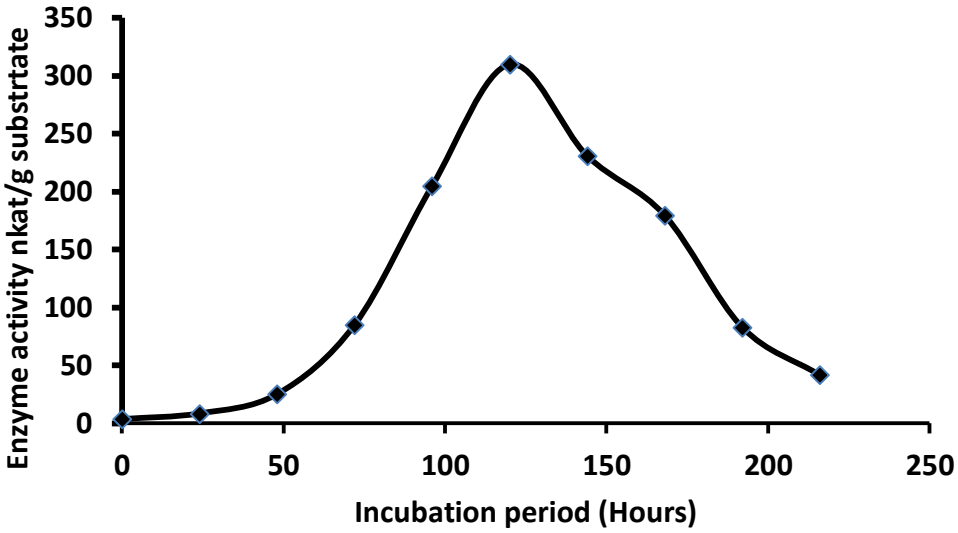


Figure 2. Time course analysis for cellulase production by *D. phacea* during solid state fermentation of Jatropha seed cake at 25 °C over a period of 216 hours

3.3 Optimum temperature determination for cellulase production by *D. phacea*

Solid state fermentation of Jatropha seed cake at temperatures 25°C, 30°C, 35°C and 40°C yielded different amounts of cellulase as shown by Figure 3. Maximum cellulase activity of 98.5 nkat/g substrate was recorded when incubation was carried out at 30°C after a period of 96 hours of incubation. The least cellulase production was observed at 40°C. Cellulase activity increased with an increase in the incubation period, a decrease was observed after 120 hours of incubation (as pre-determined by the time course analysis).

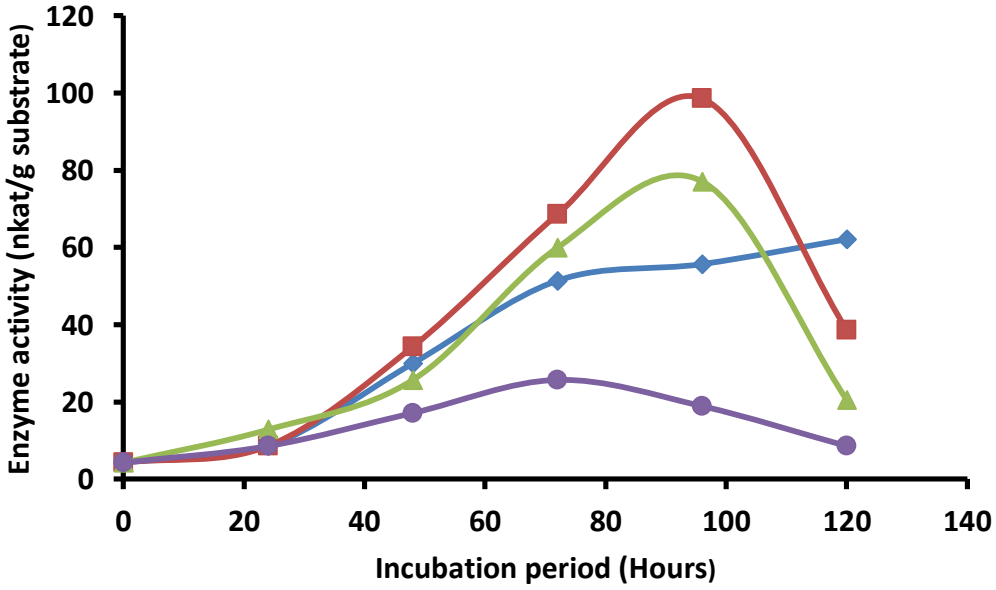


Figure 3. Effect of temperature on cellulase production by *D. phacea* during solid state fermentation of Jatropha seed cake at 25 °C (♦), 30 °C (■), 35 °C (▲) and 40 °C (●) over a period of 120 hours

3.4 Effect of initial pH on cellulase production by *D. phacea*

The best initial pH for cellulase production was pH 6. As shown in Figure 4 the highest cellulase activity of 301 nkat/g substrate observed at pH 6 after 72 hours of incubation. The least activities

were observed at pH 4.

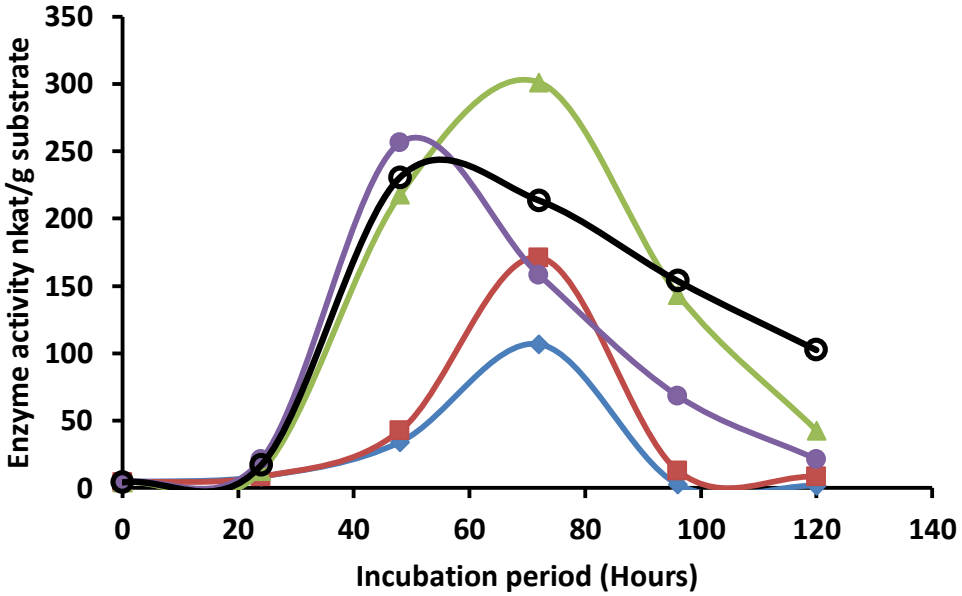
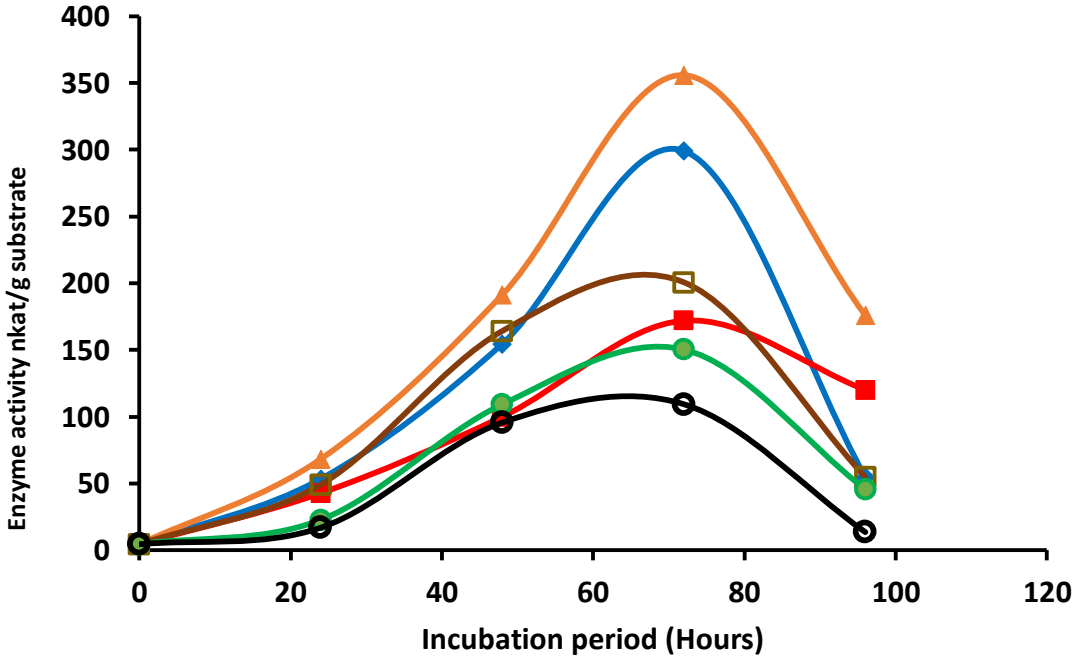


Figure 4. Effect of pH on cellulase production by *D. phacea* during solid state fermentation of Jatropha seed cake at pH 4 (♦), pH 5 (■), pH 6 (▲), pH 7 (●) and pH 8 (○) at 30 °C over a period of 120 hours

3.5 Effect of nitrogen supplementation on cellulase production by *D. phacea*

The addition of 1 g/L of peptone as a nitrogen source for *D. phacea* during the solid state fermentation of Jatropha seed cake resulted in an activity of 356 nkat/g substrate Whereas cellulase activity from cultures without a nitrogen supplement was 109.4 nkat/g substrate after 72 hours of incubation (Fig 5



.

Figure 5. Effect of nitrogen source on cellulase production by *D. phacea* during solid state fermentation of Jatropha seed cake at 30 °C and initial pH 6 over a period of 96 hours. Urea (♦), peptone, malt extract and yeast extract (■), peptone (▲), ammonium chloride (□), sodium nitrate (●), no nitrogen (●)

3.6 Effect of Lactose as an Inducer for cellulase Production

The presence of lactose increased cellulase production(Fig 6). As lactose concentration increased, cellulase production also increased. The highest activity of 248 nkat/g substrate was observed at a lactose concentration of 3.5 g/L. The least cellulase activities were observed when lactose was not added to the media.

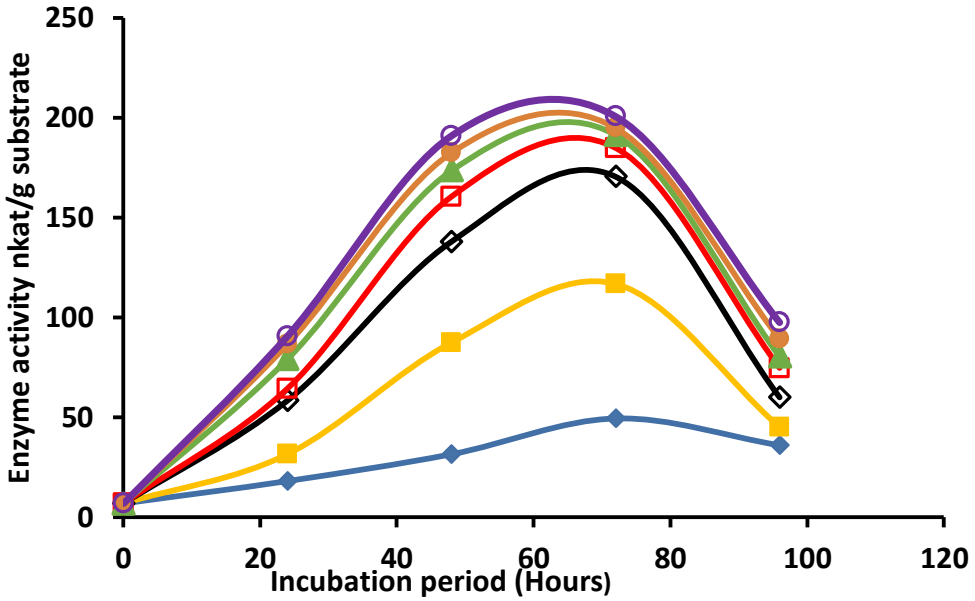


Figure 6. Effect of lactose on the production of cellulases by *D. phacea* during solid state fermentation of Jatropha seed cake with peptone as the nitrogen source, at 30 °C and initial pH 6 over a period of 96 hours. No lactose (♦), 0.5 g /L lactose, (■), 1.0 g/L lactose (◊), 1.5 g/L lactose (□), 2.0 g/L lactose (▲), 2.5 g /L lactose (●), 3.0 g/L lactose (○)

3.7 Effect of an Additional Carbon Source on Cellulase Production

Cellulase production was shown to increase upon addition of other carbon sources to Jatropha seed cake (Fig 7). The highest activity obtained was 65.1 nkat/g substrate after 72 hours of incubation observed when 10% of thatch grass was added to Jatropha seed cake. The least cellulase activities

were observed Jatropha only was used as the sole substrate.

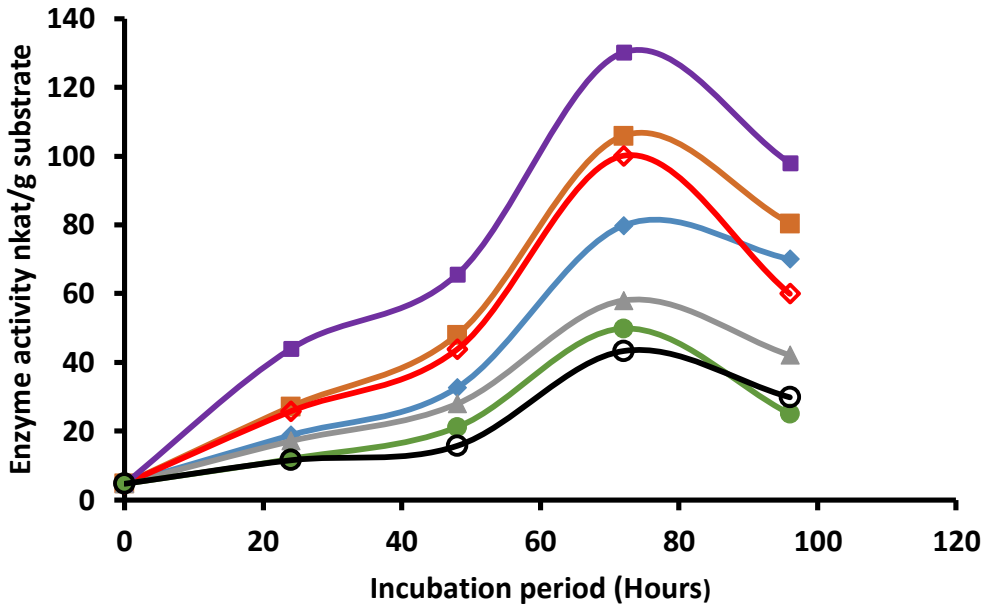


Figure 7. Effect of an additional carbon source on cellulase production by *D. phacea* during solid state fermentation of Jatropha seed cake over a period of 96 hours. Jatropha and 10% saw dust (♦), Jatropha and 10% cow dung (■), Jatropha and 10% cooked maize cobs (▲), Jatropha and 10% uncooked maize cobs (◊), Jatropha and 10% thatch grass (■), Jatropha and 10% banana pseudostem (●) Jatropha only (○).

3.8 Effect of optimization on cellulase production

Figure 8 shows specific activities of cellulase yielded before and after optimization of solid state fermentation conditions. Before optimization the specific activity was 15.1 nkat/mg protein), and after optimization there was a188% increase in cellulase specific activity (43.3 nkat/mg protein).
).

The results were subjected to statistical analysis and there was a significant difference in the increase of cellulase production before and after the optimization process ($p < 0.05$

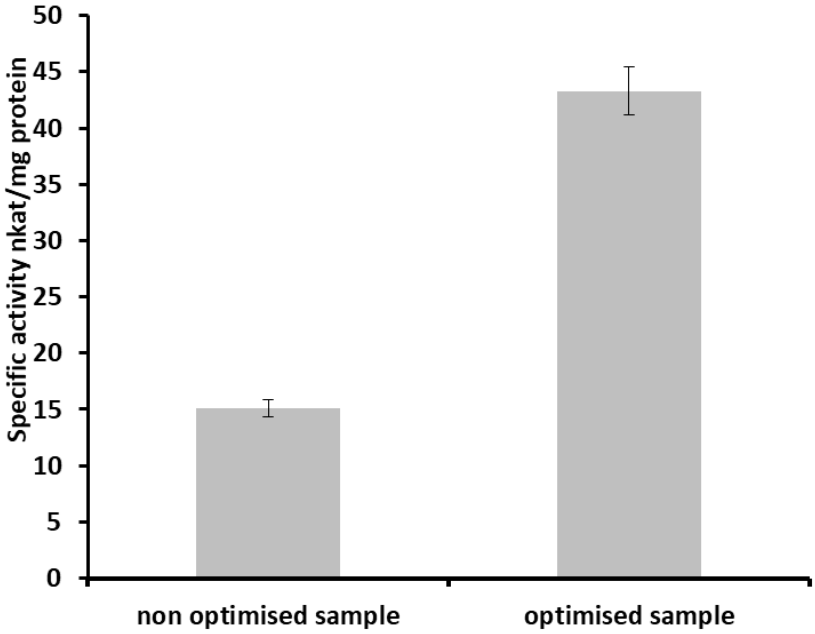


Figure 8. Effect of optimizing conditions of solid state fermentation of Jatropha seed cake on cellulase production by *D. phacea*

4 DISCUSSION

The production of cellulases by the fungi is induced by the cellulose present in the substrates. Different substrates induced production of different levels of cellulases. Cellulases induced by Jatropha seedcake yielded an enzyme activity of 181.9 nkat/g substrate whilst those induced by thatch grass yielded an enzyme activity of 124.4 nkat/g substrate (Fig 1). The difference could be due to the accessibility of cellulose in the substrate matrix. Cellulose fibres are embedded in a matrix of other biopolymers primarily hemicellulose and lignin (Sukumaran *et al*., 2005). Lignin acts as a barrier to any solutions or enzymes that degrade cellulose and hemicelluloses (Sweeney and Xu, 2012). Owing to a strong association between lignin and cellulose, it has been shown that the rate of biological degradation of the cellulose fraction of lignocellulosic materials is inversely proportional to the extent of lignification (Huttermann *et al*. 2000, Bosembes and Mazeau, 2005). The banana pseudo stem contains 8.9% lignin (Li *et al*.,2010) whilst thatch grass contains 3.1-5.6% lignin (Wongwatanapaiboon *et al*., 2012). The banana pseudo stem has a higher lignin content thus explaining the low cellulase production compared to cellulase production during solid state fermentation of thatch grass. Other factors which may contribute to different cellulase production during solid state fermentation of different substrates include the level of crystallinity which is known to inhibit cellulose bioconversion (Sukumaran *et al*., 2005).

Soft wood and hard wood substrates have different accessibility levels. Generally soft wood are more accessible than hard wood. However soft wood substrates might have low cellulose content. In this study sawdust, a product of hardwood processing, enzyme activity showed a cellulase activity of 106.6 nkat/g substrate and banana pseudo stem yielded an activity of 80.6 nkat/ g substrate. Although the banana pseudo stem is soft and its cellulose is easily accessible, the pseudo-stem contains a lower cellulose content of 39%, whilst saw dust contains 50% cellulose (Adnan, 2010). The banana pseudo stem contains a higher lignin content of 8.9% compared to saw dust with 3.1-5.6 % lignin (Adnan, 2010). Thus higher cellulase production could have been limited by the cellulose content in the banana pseudo-stem. Jatropha seed cake was the best substrate for cellulase production, as shown in Fig 1. However, Jatropha seed cake has a high lignin content of 41.4% (Dos Santo *set al*., 2014). The interactions of lignin with cellulose differ from plant to plant. In some cell walls the sugar chains are densely cross linked with lignin forming a tough, water proof resin (Yanamura *et al*., 2012). In some plants the interaction is minor; this may be the case with Jatropha seed cake hence explaining the high cellulase yield. Moreover, many different lignin compounds are found in plants, some less degradable than others. Pre-treatment generates intermediates that are easy to process, therefore the physical/ mechanical method used to grind the samples proved to be efficient for lignin degradation in Jatropha seed cake.

Determination of the optimum incubation period is essential so as to harvest cellulases at the peak of their production. The production of cellulases by *D. phacea*, using Jatropha seedcake was highest after 120 hours of incubation, thereafter a decrease was observed. The decrease in enzyme activity upon prolonged incubation may be due to the irreversible adsorption of enzyme to the substrate, denaturation of the enzyme, exhaustion of nutrients, decrease in moisture content with increased incubation times and the increased production of extracellular proteases by the fungi (Nochur *et al*., 1993; Haq *et al*., 2005) and also possibly product inhibition. Temperature is a crucial parameter that affects both growth of the fungus and cellulase production. Temperature requirements vary from organism to organism, the optimal temperature for cellulase production depends on the strain variation of the microorganism. There was a 2-fold increase in cellulase production by *D. phacea* after the optimum temperature of 30°C was employed compared to 25°C which was initially used (Fig3). At 40°C, cellulase yields were much lower because the enzyme may be undergoing denaturation.

The interaction of temperature and the initial pH may increase or decrease cellulase production. Although the pH might change during incubation, the initial pH may boost cellulase production at the beginning of incubation. At an initial pH of 6, *Didymella phacea* produced a 2-fold increase in cellulase production compared to the initially used pH of 5 (Fig 4). *D. phacea* seemed to thrive in almost neutral conditions and less in acidic conditions. The results obtained are in agreement with the findings of Okoye and co-workers (2013). who observed high cellulase production at pH 6 using *Aspergillus fumigatus* and corn cobs as the carbon source.

The addition of organic or inorganic nitrogenous compounds enhanced cellulase production. Nitrogen is a main component of proteins necessary in cell metabolism. Organic nitrogenous compounds seem to induce higher cellulase production than inorganic nitrogenous compounds; this is because they are easily metabolizable. Peptone, an organic nitrogenous source, induced a high cellulase activity of 356 nkat/g substrate, however a combination of peptone, malt extract and yeast extract, although organic, lowered the enzyme activity by 51.7%. This could be because of the reduction of ease of metabolism because of the complexity of the mixture. Thus the enzyme production became lower than the production observed when some inorganic compounds were used, for example urea and ammonium chloride, which yielded enzyme activities of 299 nkat/g substrate and 200.6 nkat/g substrate respectively. These results are consistent with the findings of Bai and co-workers (2012), who observed high cellulase activity with peptone as a nitrogen supplement using *Bacillus subtilis*. Optimizing the nitrogen source produced an enzyme yield three fold higher than when there was no nitrogen supplement.

Lactose is a well-known inducer of the cellulase genes. As lactose concentration increased, cellulase production also increased (Fig 6). This result was expected since lactose has become the only currently available soluble inducer for commercial cellulase production. Lactose has been adopted industrially because it is cheap and is readily available as it is a by-product of cheese manufacture (Zhang and Zhang, 2013). Lactose however can induce cellulase production to a certain point, a further increase in lactose concentration would not increase production any further. There was a significant difference in the increase of cellulase production when lactose was added up to a concentration of 1.5 g/L ($p<0.001$). Thereafter there was no significance difference in the increase of cellulase production when lactose concentrations were increased up to 3.5 g/L ($p> 0.01$). However, a lactose concentration of 3.5 g/L produced a four-fold increase in cellulase production compared to the enzyme yield without lactose.

Addition of another carbon source to Jatropha in solid state fermentation increased cellulase production. These results were expected since the fungi would have been provided with extra substrate to act on and hence produce more cellulases, since the presence of cellulose induces cellulase production by the microorganism. However different additional substrates induced cellulase production up to different levels, even individually the different substrates induce cellulase production up to different levels (Fig 1). The highest increase was observed during solid state fermentation of Jatropha plus 10% thatch grass. Thatch grass has a low lignin content of 3.1-5.6% and high cellulose content of 31.6 -38.5% (Wongwatanapaiboon *et al*., 2012). Due to the low extent of lignification and the high cellulose content, cellulose degradation is faster and easier when thatch grass is the additional carbon source. There was a two-fold increase in cellulase production by *D. phacea* fermenting Jatropha seed cake plus 10% thatch grass compared to fermentation of the Jatropha seed cake without a supplementary carbon source. This is in agreement with work by Ncube *et al*.,(2012), who found that supplementation of Jatropha seed cake with grass increase cellulase yields using *Aspergillus niger* FAGSC 733.

There was a significant difference in the increase of cellulase production before and after optimization ($p<0.05$). The specific activity of the enzyme produced after optimization was 43.3 nkat/mg protein compared to the initial 17.1 nkat/ mg protein. This is almost a 153% increase in enzyme production. Higher cellulase production can be achieved if the substrate concentration

and the inoculum size are optimized, as carried out by Acharya *et al*.(2008).

5 CONCLUSION

From the findings of this study, it can be concluded that optimization for culture conditions for an organism may significantly improve enzyme secretion by organism. In this case the yield for cellulase production by *D. phacea* was improved by 188%. The conditions used in the final production were use of solid state fermentation of Jatropha seed cake, at 30°C for a period of 72 hours, initial pH of 6, peptone at a concentration of 1 g/L as a nitrogen supplement, lactose at a concentration of 3.5 g/L and an additional 10% thatch grass as an additional carbon source. Enzyme titres can improve with improved culture conditions and optimized substrates since organisms show preferences in lignocellulosic substrate use. In addition, low cost substrates may be used for production of high value enzymes such as cellulase at a low cost under optimised conditions. This may be a quick, sustainable and significant alternative in the reduction of costs where cellulases are used for industrial products.

ACKNOWLEDGEMENTS

The project was funded by the Research Board funds, National University of Science and Technology (NUST), Zimbabwe

REFERENCES

Adnan, M.F.B. (2010) Recovery and separation of cellulose from saw dust wood hydrolysates using membrane reactor: Effect of trans-membrane pressure (TMP) and cross-flow velocity (CFV).Bachelor of Chemical Engineering Research project, University of Malaysia Pahang.

Ahmed, A. and Bibi, A. (2018) Fungal cellulose; Production and application: Minireview. *International Journal of Health and Life Sciences* 1(4): 19-36.

Akula, S. and Golla, N. (2018) Optimisation of cellulose production by Aspergillus niger isolated from forest soil. *Open Biotechnology Journal*. 12, 256-269. DOI:10.2174.

Bai, S., Kumar, M.R., Mukesh, D.J., Balashanmugam, P., Bala-kumaran, M.D. and Kalaichelvan, P.T. (2014) Cellulase production by *Bacillus subtilis* isolated from cow dung. *Archives of Applied Science Research* 4 (1):269-279.

Besombes, S and Mazeau, K. (2005). The cellulose/lignin assembly assessed by molecular modelling. Part 1: adsorption of a threo guaiacyl β-O-4 dimer onto a β-cellulose whisker. *Plant Physiology and Biochemistry*.,43: 299-308.

Darabzadeh, N. Hamidi-Esfahani, Z. and Hejazi, P. (2018) Optimisation of cellulase production under solid state fermentation by a new mutant strain of *Trichoderma reesei*. *Journal of Food Science and Nutrition* 7(2):572-578. DOI: 10.1002. PMID: 30847136.

Dave, B. R., Sudhir, A. P., Pansuriya, M., Raykundaliya, D. P and Subramanian. (2012) Utilization of Jatropha deoiled seed cake for production of cellulases under solid state fermentation. *Bioprocess and Biosystems Engineering* 35:1343-1353.

Dos Santos, R.S., De Macedo, A.L.,Pantoja, L.A. and Dos Santos, A.S.(2014) Bioethanol from Jatropha Seed Cakes Produced by Acid Hydrolysis Followed by Fermentation with Baker's Yeast. *International Journal of Applied Science and Technology* 4(4):*111-117*.

Elsebaay, H.H., Shoukry, A.A., Hassan, F.R.H. and El-Shall, H.S. (2018) Optimisation of cellulase enzyme production from *Pleurotus* under submerged fermentation. *Middle East Journal of Agriculture Research* 1(7):50-59. ISSN 2077-4605.

Farinas, C.S., Loyo, M.M., Junior, A., Tardioli, P.W., Neto, V.B. and Couri, S.(2010) Finding stable cellulase and xylanase: evaluation of the synergistic effect of pH and temperature. *New Biotechnology* 27:810-815.

Goutham Rao, H.R.V.N., Naga Shravani, H., Vutukuru, S.S, Subramanyam, K., Rajasekhar, P. (2012) Strain Improvement by classical mutagenesis for L-arinine production. *International Journal of Science & Technology* . 2(4)|:16-26. ISSN (online): 2250-141X.

Haq, I., Javed, M.M., Khan, T.S. and Siddiq, Z. **(**2005) Cotton saccharifying activity of cellulase produced by co-culture of *Aspergillus niger* and *Trichoderma viride*. *Research Journal of Agriculture and Biological Science* 1(3): 241-245.

Huttermann, A., Majcherczyk, A., Braun-Lullemann, A., Mai, C., Fastenrath, M., Kharazipour, A., Huttermann, J and Huttermann, A. H. (2000) Enzymatic activation of lignin leads to an unexpected copolymerization with carbohydrates. *Nat urwissenschaften*, 87:539-541.

Karaffa, L., Fekete, E., Gamauf, C., Szentirmai, A., Kubicek, C.P. and Seiboth, B. (2006) D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates. *Microbiology* 152(5):1507-1514.

Li, K., Fu, S., Zhan, H., Zhan, Y. and Lucia, L.A.(2010) Analysis of the chemical composition and morphological structure of banana pseudo stem. *Bioresources* 5(2):576-585.

Maryam, B., Qadir, A., Zameer, M., Ahmed, S.R., Nelafer, R., Jamil, N., Arzoo, S. and Afzaal, R. (2018) Production of cellulases by *Bacillus cellulosilyticus* using lignocellulosic material. *Polish Journal of Environmental Studies* 6 (27):2659-2667. DOI: 10.15244/pjoes/80867.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Lodish, M.(2005) Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresources Technology* 96: 637-686.

Ncube, T. (2013) Development of a fungal cellulolytic enzyme combination for use in bioethanol production using *Hyparrhenia spp* as a source of fermentable

sugars. PhD Thesis. University of Limpopo (Turfloop campus).

Ncube,T., Howard, R.L., Abotsi,E.K.,Jansen van Rensburg,E.L. and Ncube, I. (2012) Jatropha curcas seed cake as substrate for production of xylanase and cellulase by *Aspergillus niger* FGSCA733 in solid-state fermentation. *Industrial Crops and Products*. 37:118–123.

Nochur,S.V., Roberts, M.F. and Demain, A.L. (1993) True cellulase production by *Clostridium thermocellum* grown on different carbon sources. *Biotechnology Letters* 15 (6): 641– 646.

Oberoi S. H., Chavan, Y., Bansal, S and Dhillon G, S. (2008) Production of cellulases through Solid state fermentation using kinnow pulp as a major substrate. *Food Bioprocess Technology*. DOI 10.1007/s11947-008-0092-8.

Okoye, I.G., Ezugwu, A.L., Udenwobele, D.I., Eze, S.O.O., Anyawu, C.U. and Chilaka, F.C.(2013) Production and Partial Characterization of cellulases from *Apergillus fumigatus* using two distinct parts of corn cob as carbon sources. *Nigerian Journal of Biotechnology* 26:50-59.

Ozioko, P.C.(2013) Review article: Cellulases, their substrates, and Activity and assay methods. The Experiment. *International Journal of Science and Technology* 12(2*)*:778-785.

Ramesh, R.C. and Behero, S.S. (2017) Solid state fermentation for production of Microbial cellulases: An Overview. Chapter 3. *Biotechnology of Microbial Enzymes*. DOI: 10.1016.

Ribeiro, O., Magalhaes, F., Aguiar, T. Q., Wiebe, M.G., Penttila M., Domingues, L., 2013. Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii. Bioengineered* 4:5, 1–10.

Singh, N., Devi, A., Bishnoi, M.B., Jaryal, R., Dahiya, A., Tashyrev, O. and Hovorukha, V.(2019) Overview of the process of enzymatic transformation of biomass. DOI: 10.5772/ *Intechopen* 85036.

Sweeney, M.D. and Xu, F. (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: Recent developments. *Catalysts* 2: 244-263.

Sukumaran, R.K., Singhania, R.R. and Pandey, A.(2005) Microbial cellulases-production, applications and challenges. *Journal of Scientific and Industrial Research* 64:832-844.

Wongwatanapaiboon, J., Kangvansaichol, K., Burapatana, V., Inochanon, R., Winayanuwattikun, P., Yongvanich, T. and Chulalaksananukul, W. (2012) The potential of cellulosic ethanol production from grasses in Thailand. *Journal of Biomedicine and Biotechnology* 12:1-10.

Yanamura, M., Akashi, K., Yokota, A., Hattori, T., Suzuki, S., Shibata, D. and Umezawa, T.(2012) Characterization of *Jatropha curcas* lignin. *Plant Biotechnology* 29:179-183.

Zhang, X. and Zhang, Y.P. (2013) Cellulases: Characteristics, Sources, Production and applications. John Wiley & Sons, Inc.

Zhang, Y.H.P. and Lynd, L.R. (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose:Non-complexed cellulase systems. *Journal of Biotechnology and Bioengineering* 88(7): 797-824.