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Amylase Production and Optimisation of Culture Conditions for Some Ericoid Mycorrhizal Fungi

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Abstract

Keywords:

Amylase,
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Leohumicola incrustata,
Culture conditions.

Enzymes occur in all living things, hence in ericoid mycorrhizal fungi (ERM). Every species of microorganisms produce enzymes capable of hydrolyzing various substrates. Amylases are known to play a significant role in the food, brewing, detergent and pharmaceutical industries. *Leohumicola incrustata*, and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083) and Leotiomycetes (EdRU002) were tested for their ability to produce amylases under different culture conditions that included various temperature, pH, metal ions, carbon sources and nitrogen sources in a modified Melin-Norkrans (MMN) liquid medium. *Leohumicola incrustata* produced 1.16 U/mg protein while EdRU083 and EdRU002 had activity of 0.81 and 0.91 U/mg protein at the same pH with corresponding biomass yields of 112.67, 125.00 and 95.33 mg/50 mL respectively. Improved enzyme activities and biomass yields were noticed when supplemented with potassium, sodium, glucose, maltose, cellobiose, tryptone and peptone while NaFe-EDTA and cobalt inhibited enzyme activity. Therefore, this study opens a new opportunity for exploring ERM fungi as potential sources of microbial enzymes for commercial purposes.

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INTRODUCTION

Hydrolases belong to a class of enzymes which are widely distributed in nature. They can be found within a range of microbes ranging from bacteria to higher eukaryotes (Cairney and Burke, 1998; Moreno *et al.*, 2013). Scientists are now developing methods to isolate, produce, and characterise novel hydrolytic enzymes with unique properties. Extracellular proteins secreted by mycorrhizal fungi can degrade components of starch. Starchy materials of plant origin are one of the most abundant resources on earth. Amylases such as those obtained from microorganisms help to degrade starch (Hur *et al.*, 2001). Extracellular enzymes from microbes can break down large complex polysaccharides to smaller units.

The soil of temperate, boreal forests and heathlands is characteristically enriched with Ericoid

mycorrhizal (ERM) fungi (Smith and Read, 2008). The roots of ericaceous plants harbour these fungi to confer eco-physiological benefits on the host (Bizabani and Dames, 2016). The primary function of mycorrhizal fungi is to ease the breakdown of complex substrates into utilizable nutrients while the fungi acquire photosynthetic carbon supply as either glucose or sucrose from the host to complete their life cycles (Burke *et al.*, 2011; Pritsch and Garbaye, 2011).

Hymenoscyphus ericae (ERM) has been widely investigated for its ability to grow on a variety of complex organic substrates such as starch (Cairney and Burke, 1998). Assessing production of amylase *in vitro* involves the use of mineral salt medium supplemented with 1% soluble starch as a carbon source. The idea is to mimic the natural pattern where hydrolysis of starch takes place due to action of amylolytic microorganisms in soil or plants

(Kusuda et al., 2007). The saprophytic ability of ERM fungi has been confirmed by culture-based method (Cairney and Meharg, 2003) as well as in soils, where these biomolecules unlock organic complexes to release mineral nutrients for plant uptake.

Amylases of microbial origin are preferred choice for the industrial production due to their advantages such as specificity, stability, consistency, cost effectiveness, less processing time and ease of optimisation (Burhan et al., 2003; Satyanarayana et al., 2005). Amylases have several industrial applications ranging from conversion of starch to sugar syrups, to production of cyclodextrins for the pharmaceutical industry (Sunitha et al., 2012). Amylases are broadly classified into α , β , and γ subtypes. α -amylase cleaves at random locations on the starch chain, to yield maltotriose, maltose and glucose from amylose and amylopectin (Gopinath et al., 2017). β -amylase catalyses the hydrolysis of α -1,4 glycosidic bond from the non-reducing end to yield two glucose units (maltose) (Das et al., 2011). γ -amylase cleaves α -1,6 glycosidic linkages, as well as the last α -1,4 glycosidic linkages at the nonreducing ends of amylose and amylopectin to produce glucose (Saranraj and Stella, 2013).

In this study, *Leohumicola incrustata*, and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083) and Leotiomyces (EdRU002) were investigated for their ability to produce amylase under various temperature, pH, metal ions, carbon sources and nitrogen sources in a modified Melin-Norkrans (MMN) liquid medium.

MATERIALS AND METHODS

Culture

Fungal isolates used for this study were cultured from roots of ericaceous plants (*Erica demissa* and *Erica chamissonis*). *Leohumicola incrustata* (Isolate code ChemRU330 / Genbank Accession Number MF374380 / The South African National Collection of Fungi Accession Number PPRI 17268), and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083 / KF225587 / PPRI17261) and Leotiomyces (EdRU002 / KF225581 / PPRI17284) were obtained from Mycorrhizal Research Laboratory, Rhodes University, Grahamstown. These isolates were preserved on PDA for further studies.

Enzyme production

Soluble starch (1%, w/v) was added to 150 mL Erlenmeyer flask for amylase production in a salt solution composed of: (g L⁻¹): malt extract 3.0; (NH₄)₂HPO₄ 0.25; MgSO₄·7H₂O 0.15; CaCl₂ 0.05; NaCl 0.025; ZnSO₄·7H₂O 0.003; thiamine-HCl 100

µg L⁻¹ and 1.2 mL of FeCl₃ (1%, w/v). Production medium of 50 mL was distributed into each 150 mL shake flask; the contents were thoroughly mixed and sterilized at 121°C for 15 min. The sterilized medium was inoculated with two discs of 5 mm mycelial plugs of each ERM fungus. The non-inoculated medium was used as a control. Growth was allowed to proceed at 28°C in the dark for three weeks in a rotary incubator shaker at 150 rpm. After 21 d of growth, the cultures were homogenized using IKA's ULTRA-TURRAX homogenizer (20,000 rpm), and centrifugation was performed at 10,000 x g for 15 min to get crude enzyme filtrates (supernatant) using Beckman Coulter Avanti-J high-speed centrifuge. The pellet containing the mycelia was resuspended in 10 mL water and filtered using vacuum suction filtering system to recoup the mycelia on a filter paper (Whatman no 1) (Adeoyo et al., 2018).

Determination of fungal biomass

The mycelial biomass of culture was expressed as dry weight by drying in hot air oven at 80°C for 16 h (Sunitha et al., 2012).

Enzyme assay

The enzyme assay was conducted using dinitrosalicylic acid (DNSA) assay method described by Miller (1959). Soluble starch (1%, w/v) was added to a 10 mL buffer (pH 5.0) of sodium acetate in a Schott bottle. The mixture was stirred with a magnetic stirrer until completely dissolved. A 100 µL each of the enzyme with controls (substrate and enzyme) was added to separate 300 µL of the corresponding substrate in triplicate while blank sample contained 400 µL buffer. All samples were incubated at 37°C for 30 min, followed by centrifugation at 6000 × g for 2 min. After centrifugation, a 150 µL supernatant was each withdrawn and transferred into a new Eppendorf tube, and 300 µL DNS reagent was added. This was followed by boiling on a heating block at 100°C for 5 min and cooled on ice for 5 min. A 250 µL each of blank, controls and samples were dispensed into a 96-well plate and read with the aid of a spectrophotometry machine (BioTek's Synergy™ Mx Microplate Reader) at a wavelength of 540 nm. A unit of enzyme activity was expressed as 1 µmol of glucose (amylase) equivalent released per minute per millilitre of enzyme solution.

Effect of temperature manipulation on enzyme and biomass production

The mineral salt medium (50 mL) was adjusted to pH of 5.0 using HCl and NaOH. The medium was sterilized at 121°C for 15 min and was inoculated with two discs of 5 mm mycelial plugs. The

medium was incubated at 20°C and agitated at 150 rpm in rotary shaker incubator, and the non-inoculated medium was used as a control for 21 d. The experiment was repeated at 24, 28 and 32°C. Enzyme activity was determined after obtaining a cell-free filtrate (Jonathan and Adeoyo, 2011).

Effect of pH manipulation on enzyme and biomass production

The pH of the mineral salt medium was adjusted to 3.0 using HCl and NaOH. The medium was sterilized at 121°C for 15 min and was inoculated with two discs of 5 mm mycelial plugs. The medium was incubated at 28°C with an agitation speed of 150 rpm in rotary shaker incubator, and the non-inoculated medium was used as a control for 21 d. The experiment was repeated at pH 4.0, 5.0, 6.0, 7.0 and 8.0. Enzyme activity was determined after obtaining a cell-free filtrate (Jonathan and Adeoyo, 2011).

Effect of metal ion manipulation on enzyme and biomass production

The mycelia of collected fungi were grown in a liquid medium similar to that used in the previous experiment. The liquid medium was supplemented with 0.1% (w/v) each of K⁺, Na⁺, Mn⁺², Zn⁺², or Co⁺² supplied in sulphate form while FeNa-EDTA (ferric EDTA) was provided as ethylenediaminetetraacetic acid iron (III) sodium salt to investigate the effect of metal ions. The incubation was carried out at 28°C for 21 d, and enzyme activity was determined (Gautam *et al.*, 2011).

Effect of carbon source manipulation on enzyme and biomass production

The effect of carbon sources on the enzyme production by the fungi was carried out using glucose, maltose, sucrose, cellobiose and starch as supplements. The fungi were incubated at 28°C for 21 d, and their enzyme activities were determined (Gautam *et al.*, 2011).

Effect of nitrogen source manipulation on enzyme and biomass production

The mycelia of collected fungi were cultured in a liquid medium similar to that used in the previous experiment. Nitrogen sources (1.0% w/v) supplemented into the liquid medium included either peptone, yeast extract, malt extract, tryptone or sodium nitrate (NaNO₃). The fungi were incubated at 28°C for 21 d, and their enzyme activities were determined (Gautam *et al.*, 2011).

Protein estimation

Analysis of protein concentration was carried out according to the method of (Bradford, 1976) using a standard consisting of bovine serum albumin (BSA, Sigma-Aldrich). This method analyses the interaction between the proteins' amino acids and the colouring Coomassie Blue G-250 in an acid solution. The reading was done at 595 nm on a UV/Visible spectrophotometry machine (BioTek's Synergy™ Mx microplate reader). The values of absorbance were compared with a standard curve (0.1–1.4 mg/mL protein BSA standard). The protein standards (5 µL each) were added to separate wells in the 96 well plate and to the blank, 5 µL of buffer was added. A 250 µL Bradford's reagent was added to each well and mixed on a shaker for 20 s. Samples were incubated at room temperature for 30 min. After that, the absorbance was read at 595 nm. A graph of the net absorbance versus the protein concentration was plotted, and the unknown sample concentration was extrapolated from the standard curve (Bradford, 1976).

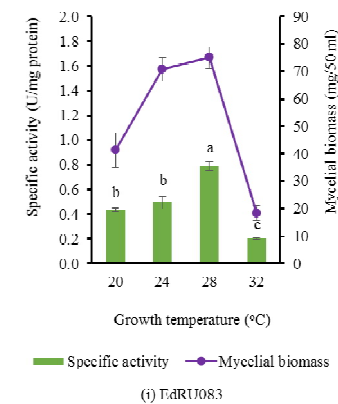
Data Analysis

All experiments were conducted in triplicate and analysed using ANOVA. A significant difference between the means of each treatment was determined using least significant difference (LSD) test at the 0.05 level of significance. Least significant difference analysis was conducted using software by Assaad *et al.* (2014), and error bars were represented as the standard errors of the means (SEM).

RESULTS

Effect of temperature on amylase and biomass production

A range of temperature between 20 and 32°C was used to study the effect of temperature on the hydrolytic enzyme and mycelial biomass production (Figure 1). The best temperature was found at 28°C, followed by 24°C and 20°C, while least amylase production was observed at 32°C. No amylase activity was detected with isolate EdRU002 at 32°C.



Further increase or decrease in pH beyond this resulted in a significant decline in amylase production and biomass yield for all isolates.

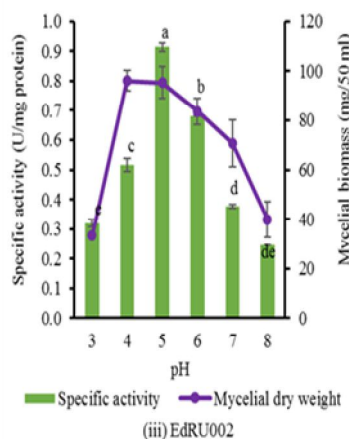
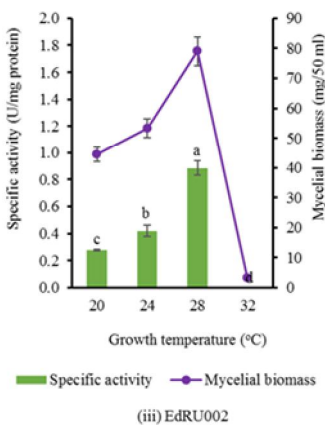
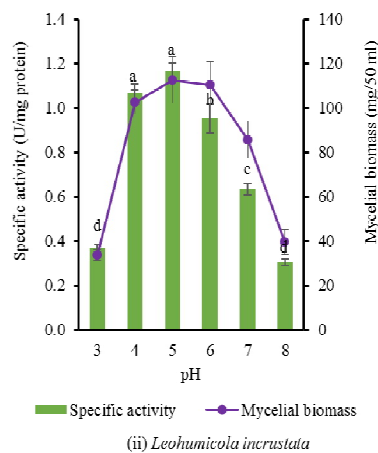
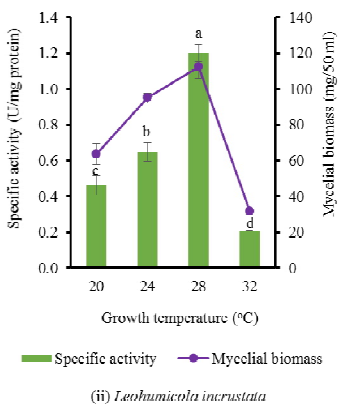
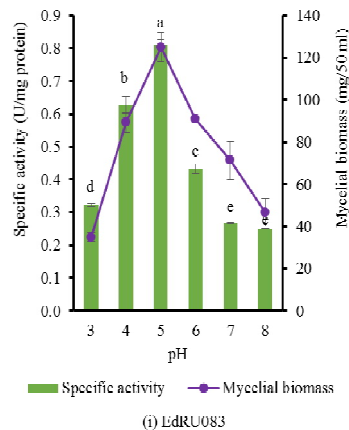


Figure 1: Effect of temperature manipulation on amylase and biomass production (i, ii, and iii).

Figure 2: Effect of pH manipulation on amylase and biomass production (i, ii, and iii).

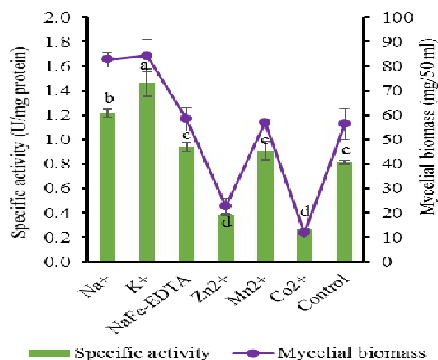
Effect of pH on amylase and biomass production

Effect of metal ions on amylase and biomass production

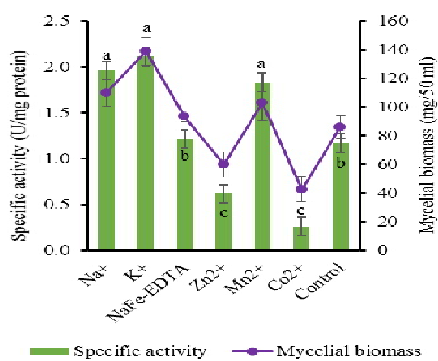
Enzyme activity was determined using sodium acetate buffer (pH 5.0). Maximum enzyme activity and biomass yield by the fungi were obtained between pH 4.0–6.0 (Figure 2). *Leohumicola incrustata* had the highest activity (1.16 U/mg protein), followed by EdRU002 (0.91 U/mg protein) and EdRU083 (0.81 U/mg protein) at pH of 5.0 with corresponding biomass yields of 112.67, 95.33 and 125.00 mg/50 mL respectively.

The effect of metal ions on the enzyme secretion and mycelial biomass was significant when either 0.1% Na⁺, K⁺, Mn²⁺, Zn²⁺, NaFe-EDTA or Co²⁺

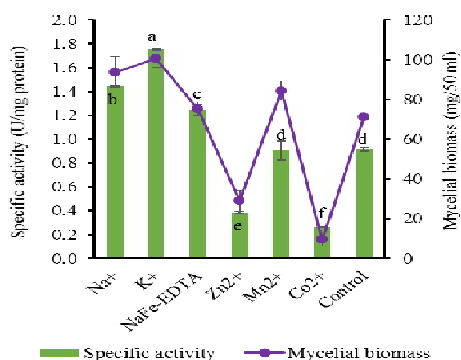
was added to the liquid medium (Figure 3). The metal ions used in this experiment were so selected to complement those already present in the basal mineral salt medium. An improved activity was observed when the medium was supplemented with either potassium (2.11 U/mg protein for *L. incrustata*) or sodium (2.11 U/mg protein for *L. incrustata*) or sodium (1.96 U/mg protein for *L. incrustata*), while a low activity was observed when cobalt was added to the liquid medium (Figure 3).



(i) EdRU083



(ii) *Leohumicola incrustata*



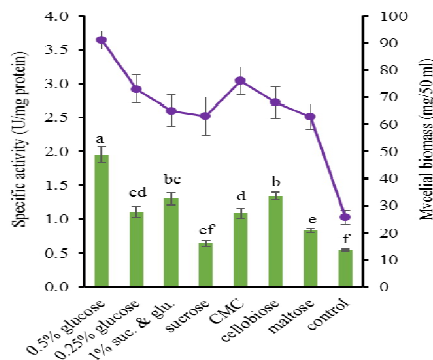
(iii) EdRU002

Figure 3: Effect of metal ion manipulation on amylase and biomass production (i, ii, and iii).

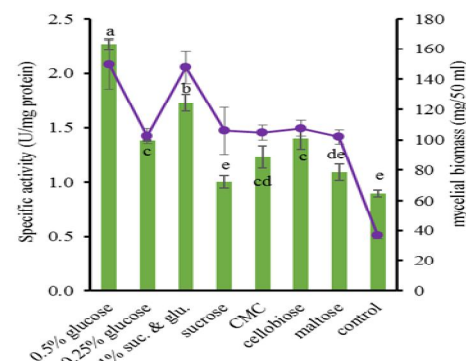
Effect of carbon source on amylase and biomass production

The fungi were able to grow moderately in all the tested carbon sources, and increased enzyme

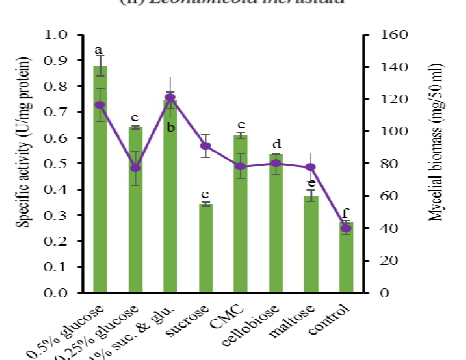
production and yield of biomass were observed. Glucose (0.5%) gave the highest enzyme activity (2.27 U/mg protein for *L. incrustata*) followed by 1.0% cellobiose, 1.0% maltose, 1.0% sucrose/glucose (ratio 1:1), 0.25% glucose, 1.0% sucrose and 1.0% starch (Figure 4). There was a significant difference in both enzyme activity and mycelial biomass yield when the production medium was supplemented with 0.5% glucose.



(iii) EdRU002



(ii) *Leohumicola incrustata*



(i) EdRU083

Figure 4: Effect of carbon source manipulation on amylase and biomass production (i, ii, and iii).

Effect of nitrogen source on amylase and biomass production

The highest enzyme activity was observed when the production medium was supplemented with yeast extract (2.16 U/mg protein) followed by malt extract for *L. incrustata* and EdRU002 (2.03 U/mg protein), while yeast extract (1.78 U/mg protein) was followed by peptone (1.41 U/mg protein), malt extract (1.29 U/mg protein) and tryptone (1.21 U/mg protein) for isolate EdRU083. Sodium nitrate had no noticeable effect across all the isolates tested (Figure 5).

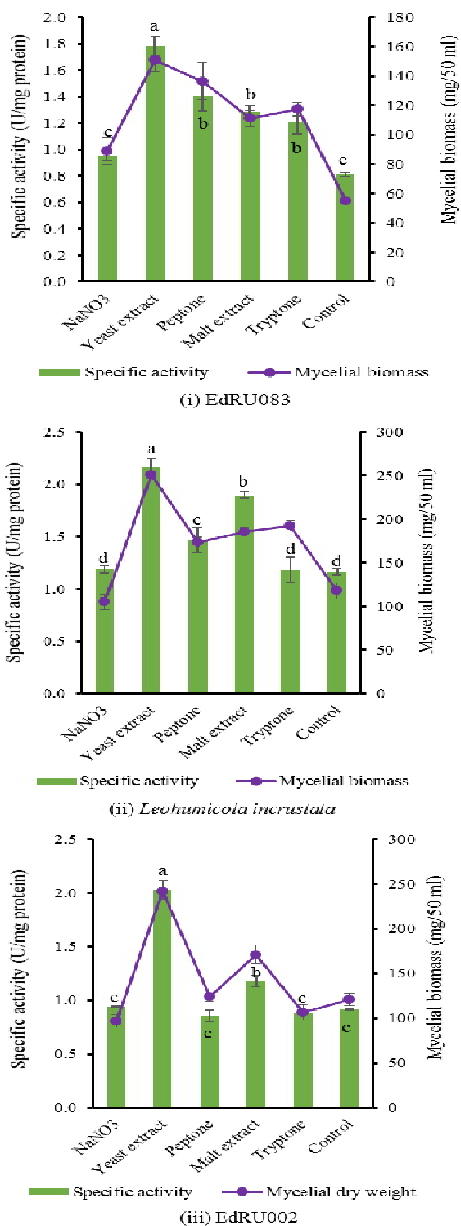


Figure 5. Effect of nitrogen source manipulation on amylase and biomass production (i, ii, and iii).

DISCUSSION

The enzyme production differed for various isolates at a different temperature values. This investigation revealed 28°C as the best temperature for enzyme production. The variation observed with other temperatures could be as a result of the followings: (i) low temperature could not allow enough biomass production and (ii) inactivation at a high temperature that destroys amino acid or hydrolyse the peptide chain. The temperature of incubation played a significant role in the metabolic activities of these microorganisms. Hence, we acknowledged that slight changes in temperature could affect enzyme production. These findings agreed with a report by Norouziyan (2008), who stated that growth and production of microbial enzymes is dependent on the temperature of cultivating medium.

The study showed that isolates EdRU083, *L. incrustata*, and EdRU002 had highest enzyme activities at the pH of 5.0. The pH of a growing medium is one of the most crucial factors influencing microbial growth, biomolecules production and transport of various constituents across the cell membrane (Lynd *et al.*, 2002). Thus, accounts for the rate and measure of growth, reproduction, and morphology of microorganisms in a liquid medium.

The amylase activity increased when either Na⁺, K⁺ or Mn²⁺ was added to the production medium, followed by Zn²⁺, NaFe-EDTA and least when Co²⁺ was used (Figures 3). The least activity and mycelial biomass recorded for both NaFe-EDTA and Co²⁺ could be as a result of their toxic effect on these fungi during assimilation processes. The findings of Baldrian and Gabriel (2002), Baldrian (2003) confirmed that a white-rot fungus (*Pleurotus ostreatus*) needed trace amounts of either Cd, Mn or Zn for enzyme secretion. Based on our findings, cobalt ion and zinc (at 0.1% or more) were inhibitory to the enzyme production.

Enzyme expression was heavily influenced by the substrate used for microbial colonisation, particularly the presence of a specific carbon source. This aligns with the findings of Cao and Crawford (1993), where four strains of *Pisolithus tinctorius* were investigated for carbon nutrition. They revealed that glucose, maltose and cellobiose supported rapid mycelial growth. According to a report by Bizabani and Dames (2016), ericaceous root-associated fungi (*Melinomyces* sp., *Acremonium implicatum*, *Leohumicola* sp., *Cryptosporiopsis erica*, *Oidiodendron maius* and an unidentified Helotiales fungus) grew well on glucose and cellobiose. Thus, we affirmed that either glucose or cellobiose (Figure 4) is good at improving enzyme activity when compared to other

sugars, and their incorporation into right mineral salt medium should be encouraged to improve mycelial biomass and enzyme production.

Organic nitrogen sources have been reported to have an inducing effect on production of amylases (Jonathan and Adeoyo, 2011). The effect of organic nitrogen on enzyme production was more pronounced when the production medium was supplemented with either yeast extract. These observations were similar to the findings of Jonathan and Adeoyo (2011) and corroborated the report of El-Hadi *et al.* (2014) who confirmed the role of some organic nitrogen at increasing enzyme activity during optimisation of cultural and nutritional conditions.

CONCLUSION

The potential of using ERM fungi in producing improved amylases under various pH and nutritional conditions was investigated to maximise production. This study provides useful information that can improve the mycelial biomass yield of any selected starter culture for better mycorrhizal fungus inoculum production, while the amylase produced can help starch bio-conversion in industries. Also, the enzyme obtained from these fungi represent a potential other source of amylase for industrial applications. Therefore, a mineral salt liquid medium (MMN) of 0.1 - 0.5% glucose, 0.3 - 1.0% yeast extract and 0.5 g/L KH_2PO_4 (pH 5.0) is adequate for amylase production at 28°C. Finally, we recommend that further studies should be conducted on *L. incrustata* to underpin its suitability for commercial enzyme production.

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