Production and Moisture Content Optimization of Cellulase and Xylanase by Newly Isolated *Rhizopus oryzae*UC2 using Raw Oil Palm Frond Leaves as Substrate in Solid State Fermentation

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Abstract—Oil palm frond (OPF) is the most abundant waste biomass produced in major oil palm producing countries such as Malaysia. By virtue of being a lignocellulosic biomass, contains cellulose and hemicellulose that stimulate the production of cellulase and xylanase enzymes by certain fungi. Moisture content, being one of the major factors affecting fungal growth and enzyme production in solid state fermentation (SSF), was optimized using the newly isolated cellulolytic and xylanolytic zygomycetes strain- Rhizopus oryzaeUC2 and raw oil palm frond leaves (OPFL) as growth substrate. Maximum cellulase and xylanase activities were recorded at optimum moisture content of 40 % (v/w). Under SSF conditions of using dried and grinded raw oil palm frond leaves of size 1-3 mm as growth substrate, moisture content (40 %), initial pH (5.2), temperature (30° C) and inoculum size of 2.0 × 10^8 Spores/g, the activities of CMCase (endoglucanase), FPase (exoglucanase), β-glucosidase and xylanase reached their maximum of 112 U/g, 13 U/g, 145 U/g and 205 U/g respectively, all on the 5th day except β-glucosidase which was on the 6th day. These results show that relatively, low moisture SSF conditions are suitable for efficient cellulase and xylanase production by R.oryzae using OPFL as growth substrate.

Keyword— Moisture content optimization; oil palm frond; fungal enzymes; solid state fermentation; lignocellulosic biomass

1 INTRODUCTION

The lignocellulosic biomass constitutes the most abundant renewable biopolymer on earth, having cellulose, hemicellulose, lignin and in some cases pectin as the major components. A large share of this waste biomass is produced as post-harvest plant biomass by the agro-industry. In the palm oil mill, palm oil makes up only 10% of the total biomass. The rest, 90% biomass are discarded as wastes [95]. During oil palm fruit harvesting the pruning of oil palm frond (OPF) produces approximately 44 million tonnes dry weight of OPF annually. In 2012, an estimated 83 million tonnes (dry weight) of oil palm biomass wastes were available throughout the country [96], [97]. It is projected that these wastes will keep increasing, up to 100 million tonnes dry weight by 2020 [98]. Globally, over 190 million tonnes of solid and liquid residues are generated from the palm oil industries [99].

In 2009, it was recorded that about 77.3 million tonnes of oil palm biomass wastes was generated by the Malaysian oil palm industry [100]. The different types of oil palm biomass (OPB) waste generated and their respective quantities based on relative percentage dry weight is as follows; oil palm fronds contributed the highest- 44.8 million tonnes (57.96%), making it the largest biomass source in the oil palm industry [101], trunk forms the second largest -13.97 (17.98%) million tonnes followed by fibres and shells (15.01%) and empty fruit bunch (9.06%). These excess OPB are left in the plantations, some of which are used for nutrient replacement or mulching purposes [97], while most of them lie in waste and left to decompose naturally. This process takes a considerable length of time due to the recalcitrance of the lignocellulosic contents. OPF which translates to OPFL are available throughout the year, when the palms are pruned or during the fruit harvest [102]. There are high potentials for the biomass waste to be converted to value-added products such as enzymes, biofuel, compost, among others, thus could generate income for the nation.

Bacteria and fungi has the ability to degrade amorphous cellulose component of lignocellulosic biomass, rapidly and efficiently [103], but the ability to degrade crystalline cellulose is restricted to very specialized cellulose degrading microorganisms [104].

Fungi are the best lignocellulosic degraders and among microorganisms, they are of great significance in enzyme production as they secrete their enzymes extracellularly [105]. They belong to soft rot fungi

(Ascomycetes, Zygomycetes) and brown or white rot fungi (Basidiomycetes) groups. Each group produces a collection of enzymes dedicated to the degradation of a specific plant polysaccharide; including many families of glycoside hydrolases, esterases, and lyases. Many of these families are characterized by multiple catalytic activities, many of which show complementary activity over the same substrates [106], [107]. *R. oryzae* is a soft rot Zygomycetes which have been used in previous works in food fermentation and enhancement of food tastes [108] , production of ethanol and organic acids such as lactic acid, succinic acid etc [109]–[111], but, none has assessed its use for the production of cellulolytic and xylanolytic enzymes using raw oil palm frond leaves as growth substrates.

SSF is generally defined as the growth of microorganisms on solid material in the absence or near absence of free water [112]. It has been well established from previous studies that enzymes produced in SSF systems are several folds higher than in submerged fermentation (SmF) systems [113]. Enzyme production through SSF has advantages such as process is simple, it is cost effective, high concentration of products, reduced energy requirement, less effluent released thus reduces pollution, aeration process is easier, resembles the natural habitat of some fungi and bacteria and easier downstream processing [114].

In line with the basic requirement for all living creatures, fungi require three essential components to sustain life, which includes air (for aerobic fungi), water and nutrition (food). Most fungi are aerobes and digest a vast array of organic matter. The availability of water is one of the major factors that can be controlled to either prevent or enhance fungal growth and involved in primary metabolic processes such as secretion of enzymes, sustained enzyme activity and subsequent breakdown of complex substances, dissolution and absorption of nutrient solutes as well as in secondary metabolic processes. A certain level of "free" water (moisture) which is unique to various fungi is required for fungal growth to occur. Free means not bound to other molecules or to the cell wall. Cellulolytic and enzyme production depends greatly on the moisture to substrate ratio and the moisture requirement varies with microorganisms and type of substrate. In light of this, moisture content is therefore an essential SSF parameter and has become imperative to optimize the culture conditions for this newly isolated *R.oryzae*UC2 towards the enhancement of cellulase and xylanase productions forming the main focus of this study.

2 MATERIALS AND METHODS

2.1. Materials

All chemicals used in this work were produced by Sigma-Aldrich, USA and EMD Chemicals Germany. The fungal strain used was isolated from decaying OPEFB and molecularly identified as *Rhizopus oryzae*UC2.

2.2. Preparation of Inoculum

The inoculum was prepared by maintaining the fungus on PDA plates at 30°C for 7 days. *R.oryzae*UC2 spores were harvested according to the method of Ang et al. 2013.

The spores were then diluted to obtain the spore inoculum of 2.0×10^8 spores/g of ground OPFL.

2.3. Production of Cellulolytic and Xylanolytic Enzymes and Optimization of Moisture Content in Solid-State Fermentation

The OPFL was ground into smaller particle sizes using a table blender (NL9206AD-4, Philips UK); particles size of 1-3 mm. The solid-state fermentation (SSF) was prepared by moistening the sieved OPFL with appropriate volumes of the production medium and spore suspension of *R.oryzae*UC2 (2.0×10^8 spores/g of OPFL) until final moisture levels of 40 %, 60 %, 70 %, 80 %, 90 % was achieved. The final moisture levels of the OPFL substrates were determined using a moisture analyser (MX50, A&D Weighing Co., Ltd., Japan). The flasks containing the OPFL substrates plus the production medium were autoclaved at 121 °C and 20 psi for 20 mins followed by inoculation with the fungal spores. The production medium consisted of a modified Mendel medium [115] with adjustments made with the increase in yeast 1.25g/L, peptone 1.0 g/L and 2mL of Tween 80. Production media set at an initial pH 5.0 using 1M NaOH and 1M HCl. All inoculated flasks were incubated at 30 °C for 7 days and 4 g of the fermented OPFL substrates were drawn every 24 h intervals for cellulases and xylanase analysis.

2.4. Extraction of Crude Enzymes and Analysis of Cellulase and Xylanase Activities

Fermented substrate of approximately 2 g was transferred into a 250 mL Erlenmeyer flask containing 50 mL of cold 0.05M sodium acetate buffer. The suspension was vortexed at maximum speed for 1 min to extract the cellulases and xylanase enzymes. The mixture was centrifuged at 4000 rpm for 20 min and the supernatant used as crude enzymes source was decanted. The crude enzyme cocktails containing cellulases and xylanase were stored at -20°C prior to assay to prevent enzyme degradation.The EnG, ExGl and BGL activities were considered to represent the cellulase activity of the crude enzyme. A glucose calibration curve was used to estimate the (carboxymethylcellulase, CMCase), and ExG (filter paper, FPase) activities, a ϱ-nitrophenol (ϱNP) calibration curve was used to estimate the βGL activity while a xylose calibration curve was used to estimate xylanase activity. All calibration curves were determined using a set of triplicate preparations to minimize error in the respective assays. The enzyme activities were assayed in triplicates and the activity of each type of enzyme was estimated based on standard procedure recommended by IUPAC [116]. Xylanase activity was assayed under the same

conditions as above except birchwood xylan 1% (w/v) was used as the substrate[117]. One unit of exoglucanase activity is expressed as 1 μmole of glucose liberated per ml of enzymes per minute and one unit of xylanase activity is expressed as 1μmole of xylose liberated per ml of enzyme per minute. All reaction mixtures above were boiled for 5 mins with 1 mL 3,5-dinitrosalicylic acid (DNS) solution and 2 drops of 0.1 M sodium hydroxide to estimate the reducing sugars released [118]. BGL production was done according to a modified method of Takashima *et al.*, 2007 [119]. One unit of β-glucosidase activity is expressed as 1μmole of ϱ-nitrophenol liberated per ml of enzyme, per minute.

3 RESULTS AND DISCUSSION

Filamentous fungi play invaluable role in the earth's carbon cycle. This fit is made possible by the groups of hydrolytic and oxidative enzymes they produce that drives the recycling of carbon through efficient decomposition of plant cell wall materials.

Our results showed that enzyme activity was influenced by moisture content variation which is unique to the fungal species and nature of substrate used. This affects both fungal growth and secondary metabolism [120]. The production of cellulases and xylanase by *R. oryzae*UC2 in SSF for 7 days using OPFL as growth substrates, at 2.0×10^8 spores/g inoculum size and at initial pH of 5.2 was tested in moisture content ranging from 40 – 90 %. 40 % moisture content was best suited for the production of CMCase (Figure 3.1), FPase, β-glucosidase and xylanase revealing yields of 112.13±0.10 U/g, 13.0±0.29 U/g, 145.47±0.32 U/g and 205.05±0.14 U/g respectively. These highest activities were all recorded on the 5th day except β-glucosidase which was on the 6th day. A similar result of SSF moisture content optimization was reported by Battaglino et al 1991 using *Aspergillus oryzae*. The substrates with high moisture may have presented an impediment to oxygen transfer that consequently constituted an adverse environment for fungal growth and enzyme production.

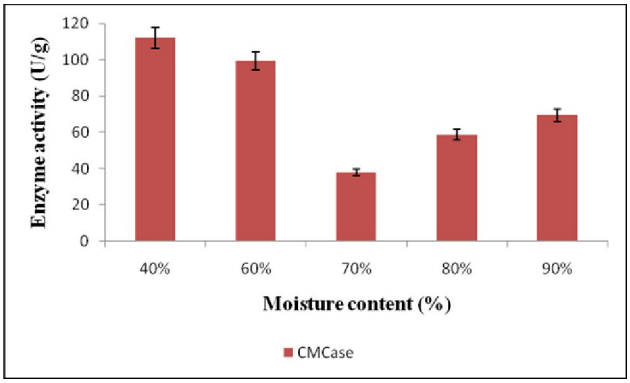

Figure 3.1 Highest CMCase activities on different moisture content (%)

B-glucosidase is the cellulase enzyme that acts directly on the dimeric glucose molecule cellobiose, converting it to monomeric glucose units. In this study B-glucosidase recorded the highest activity among the cellulase enzymes which may imply that *R.oryzae*UC2 is a cellobiose-utilizing fungi [121], [122] which can promote saccharification.

Being the first report on the use of OPFL as growth substrate using *R.oryzae*UC2, we have limited works to compare with since the effect of moisture variations in enzyme production not only vary with microorganism used but also to a large extent the nature of substrate used.

4 CONCLUSION

From the results of the study we conclude that for the production of cellulase and xylanase enzymes using *R.oryzae*UC2 in SSF, a moisture content of 40 % is the optimum and that raw OPFL is a promising growth substrate for the enzymes production. Future works include investigation of other SSF parameters, biochemical characterization of the enzymes, optimization using Response Surface Methodology (RSM) and assessment of degradation/saccharification of OPFL.

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