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Simultaneous production of cellulase and amylase by *Aspergillus fumigatus* IB-A1

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Abstract

Utilization of agricultural wastes for production of useful metabolites requires hydrolysis using both cellulase and amylase enzymes. We isolated *Aspergillus fumigatus* IB-A1 and evaluated its ability to simultaneously produce both cellulase and amylase. Although the isolate could produce both cellulase and amylase from either soluble starch or carboxymethyl cellulose, amylase activity was higher with soluble starch while cellulase activity was higher when carboxymethyl cellulose was used as the sole carbon source. With a mixture of carboxymethyl cellulose and soluble starch, both the amylase and cellulase activities increased with increase in the ratio of soluble starch. The optima ratio of carboxymethyl cellulose to soluble starch for cellulase and amylase activities were 0.7:0.3, and 0.4 to 0.6 respectively. For practical application, the optimum ratio of carboxymethyl cellulose to soluble starch in the production medium depends on the relative composition of cellulose and starch in the substrate to be hydrolyzed. The isolate was also able to efficiently produce both amylase and cellulase from cassava peel. With 10 g/L cassava peel, the cellulase and amylase activities were 6.122 ± 0.320 U/ml/min and 4.342 ± 0.210 U/ml/min respectively. When the cells were immobilized on loofa sponge and subjected to alternating air phase- liquid phase culture, cellulase and amylase production from cassava peel increased to 8.106 ± 0.620 U/ml/min and 5.206 ± 1.24 U/ml/min respectively. The optimum ratio of the air phase to the liquid phase was 3 hours of air phase to 21 hours of liquid phase.

Keywords: *Aspergillus fumigatus* IB-A1; cassava peels; submerged culture, alternating air phase-liquid phase culture; cellulase and amylase.

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Introduction

Majority of the agricultural wastes are lignocellulosic materials which are abundant and cheap biomass resources for production of useful metabolites (Edama et al., 2014; Kongkiattikajorn, 2012; Pooja and Padmaja, 2014). Thus, they have been used as carbon sources for production of various products such as single cell protein, industrial enzyme, and fine chemicals (Mohammed et al., 2013; Ndubuisi et al., 2018; Murata, et al., 2021). However, since cellulose, and starch are the major components of most of these agricultural wastes, their efficient utilization requires the use of both cellulase and amylase enzymes. The costs of these enzymes are usually very high, especially in most developing countries. Thus, sustainable utilization of these agricultural wastes requires that these enzymes must be cheaply produced. The cost of substrates used for production of these enzymes represents a significant percentage of the final production costs. In the case of ethanol, for example, the cost of raw materials can represent 40-75% of the total production cost, depending on the type of feedstock (Arijina et al., 2018).

Thus, the cost of the upstream process can be significantly reduced by using cheap and easily available raw materials. It will be very interesting to develop a system where agricultural waste materials are used to produce enzymes that will again be used for efficient conversion of the agricultural wastes to other useful materials. Furthermore, the production costs can be reduced by using microbial strains that can simultaneously produce both amylase and cellulase, and by improving their enzyme productivities.

Nigeria is the world largest producer of cassava and most of the produced cassava are utilized locally, leading to generation of huge amounts of cassava peels. Presently, the cassava peels pose a disposal problem and would even be more problematic in future with increased industrial production of cassava products such as garri, flour, and starch. It is therefore very important to convert these cassava peels into value-added products. The aim of this work was therefore to use cassava peels to produce both cellulase and amylase enzymes that can be again used for efficient conversion of the cassava peels to other useful metabolites. The potentials of a thermophilic microorganism, *Aspergillus fumigatus* IB-A1 to simultaneously produce cellulase and amylase were first investigated

using cellulose and starch as the carbon sources. Using this microorganism is expected to save costs since the two enzymes are produced simultaneously, the risk of contamination by mesophilic microorganisms, as well as the cost of reactor cooling will be reduced. The advantages of immobilizing microorganisms for various processes have been documented. These include: It allows for re-use of bio-catalyst, it permits continuous cultures at high hydraulic retention time, high cell density can be achieved with consequent increase in productivity, and it makes downstream processing very easy. Cell immobilization techniques using biological materials is eco-friendly and have many advantages over synthetic material (Hideno et al., 2007). It has been reported that loofa sponge (*Luffa cylindrica*) is an excellent carrier for immobilization of aerobic microorganisms (Roble et al., 2003a&b). It has also been demonstrated that alternating air phase-liquid phase system combines the advantages of solid-state cultures and submerged cultures (Roble et al., 2020; Ugwu et al., 2021). Thus, in order to further reduce the production costs, simultaneous production of cellulase and amylase by *Aspergillus fumigatus* in an alternating air phase-liquid phase system was investigated.

MATERIALS AND METHODS

Collection and preparation of the substrates

Cassava tubers were obtained from the Faculty of Agriculture, University of Nigeria, Nsukka, Enugu State, Nigeria. The tubers were harvested from variety 98/2101 at the age of 11 months, peeled and the fresh cassava peels were washed and sun dried. The dried cassava peels were ground into a fine powder using a hand grinding machine. The powder was stored in a refrigerator (4°C) and aliquots were taken for each experiment.

Microorganism and culture maintenance

A thermotolerant *Aspergillus fumigatus* IB-A1, previously isolated and maintained at the Department of Microbiology, University of Nigeria, Nsukka was used. The cultures were maintained on potato dextrose agar (PDA) slants at 4 °C and sub-cultured at two weeks intervals. The identification of the isolate has been described (Ezea et al., 2022).

Inoculum preparation

Inoculum was prepared according to Sivaramakrishnan et al., (2007). To a 7-day old culture slant, 10 ml of 0.1 % Tween 80 solution was added and the spores were dislodged using an inoculation needle under sterile condition. A 10 ml of spore suspension containing 2×10^7 spores per ml was used for inoculation. The spore concentration was estimated using haemocytometer.

Evaluation of the ability of *Aspergillus fumigatus* IB-A1 for simultaneous production of cellulase and amylase from a mixture of carboxymethyl cellulose and soluble starch

The basal medium was composed of 1 % carboxymethyl cellulose, 1 % soluble starch, 0.25 % NaNO_3 , 0.1% KH_2PO_4 , 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.06 % CaCl_2 . One hundred (100) mL of the medium was prepared in 250 mL flask, the pH was adjusted to 5.5 using 1 N NaOH and the medium sterilized at 121°C for 15 min. After cooling, the flask was inoculated with 10 mL of *Aspergillus fumigatus* spore suspension, containing 2×10^7 spores per mL and incubated at 45°C for 7 days. Thereafter, the culture broth was centrifuged and the supernatant used as the crude enzyme. The optimum ratio of carboxymethyl cellulose to soluble starch for enzyme production was determined by varying the ratios of carboxymethyl cellulose to soluble starch in the medium, but the total concentration of the two was maintained at 1%.

Production of enzymes from cassava peel in submerged culture

Submerged culture was carried out using the basal medium without soluble starch and carboxymethyl cellulose but containing 1 % of cassava peel as the carbon source. One hundred milliliter of the medium was poured into 250 ml Erlenmeyer flask and sterilized at 121°C for 15 minutes. The pH was adjusted to 5.0 or 6.0 using 1 N NaOH or 1 N HCl. After cooling, 10 ml of the spore suspension containing 2×10^7 spores per ml was inoculated and incubated at 45°C for 5 days.

Alternating air phase – liquid phase culture

The fungal isolates were immobilized on loofa sponge (*Loofa cylindrica*) as previously described (Ogbonna et al., 1994; Roble et al., 2003a&b;

Roble et al., 2020; Ugwu et al., 2021). The dried outer cover of the loofa sponge was removed and the porous loofa sponge fiber was sliced and washed properly followed by drying in air and in a hot oven at 105°C for 1 hour. A 10 ml of the spore suspension (2×10^7 spores per ml) was mixed properly with a sterilized viscous starch medium containing (per L) 50 g starch, 2.5 g NaNO_3 , 1.0 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, and 0.6 g CaCl_2 . The mixture was poured onto the loofa sponge and pre-incubated in air inside a bioreactor for 3 days. After germination of the fungal spores, a medium containing (per L) 10 g sterilized cassava peel, 2.5 g NaNO_3 , 1.0 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, and 0.6 g CaCl_2 was poured into the reactor and cultivated for another 48 hours. The cultivation was then done under alternating air phase-liquid phase. Initially, the ratio of the air-phase to liquid phase was fixed at 6h:18h (Figure 2) and the effects of the ratio of the air-phase to liquid phase was investigated by varying the ratios as described previously (Roble et al., 2020; Ugwu et al., 2021) (Figure 3).

Crude enzyme preparation

Culture broth samples were taken at time intervals and filtered using Whatman number 1 filter paper. The filtrate was used as the crude enzyme for determination of the cellulase and amylase activities.

Determination of cellulase activity

Cellulase activity (CMCase) was assayed using a method of Mendel and Weber (1969) and Ghose (1987). The activity was estimated using 1 % solution of carboxymethyl cellulose (CMC) in 0.05 M citrate buffer (pH 4.5). The reaction mixture contained 1 ml citrate buffer, 0.5 ml of the 1% CMC and 0.5 ml of the crude enzyme. The reaction was carried out at 50°C for 30 min. The reaction was stopped by addition of 3 ml of 3, 5 dinitrosalicylic acid reagents (DNSA) and boiled for 10 minutes followed by addition of 3 ml of distilled water.

Thereafter, the absorbance was taken at 540 nm (Miller, 1959). One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar from carboxymethyl cellulose per ml per min under the assay conditions. A calibration curve was established with glucose from which reducing sugars were calculated.

Determination of amylase activity

Amylase activity was determined according to Demoraes et al. (1999) using 0.5 ml of 0.5 % gelatinized soluble starch, buffered with 0.2 ml of 0.05 M citrate buffer (pH 4.8). The reaction mixture contained 0.2 ml citrate buffer, 0.5 ml of substrate solution (gelatinized soluble starch) and 0.3 ml of the crude enzyme. The reaction was carried out at 40° C for 30 min. The reaction was stopped by addition of 1 ml of 3, 5 dinitrosalicylic acid reagents (DNSA) and boiled for 10 minutes followed by addition of 3 ml of distilled water. Thereafter, the absorbance was taken at 540 nm (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar (glucose equivalent) from soluble starch per ml per min under the assay conditions.

RESULTS AND DISCUSSION

Simultaneous production of cellulase and amylase from a mixture of carboxymethyl cellulose and soluble starch

Table 1 shows that *Aspergillus fumigatus* IB-A1 was able to simultaneously produce cellulase and amylase from a mixture of carboxymethyl cellulose and soluble starch. In a medium containing 1 % soluble starch, the maximum cellulase and amylase activities were 1.143± 0.081 U/ml/min and 2.144± 0.240 U/ml/min respectively. When cultivated with 1 % carboxymethyl cellulose, the maximum cellulase and amylase activities were 2.913± 0.030 U/ml/min and 1.558± 0.172 U/ml/min respectively. In other words, using starch as the carbon source led to higher amylase activity while production of cellulase was favoured by using CMC as the carbon source. However, when cultivated in a medium containing 0.5 % soluble starch and 0.5 % carboxymethyl cellulose, the maximum cellulase and amylase activities were 2.746± 0.170 U/ml/min and 2.177± 0.321 U/ml/min respectively. As shown in Table 1, it is noted that the total enzyme activity was higher when a mixture of soluble starch and CMC was used as the carbon source. When *Aspergillus fumigatus* IB-A1 was cultivated in the medium in which a mixture of soluble starch and carboxymethyl cellulose was replaced with either soluble starch only or carboxymethyl cellulose only, the cells grew well on the loofa sponge as revealed by physical observation. However, the growth was better in a medium containing

cellulose than in the medium containing soluble starch. The results are in agreement with those reported by Khokhar et al. (2011) on the amylase and cellulase production by *Aspergillus* and *Penicillium* species. Several species of fungi have been reported to be able to produce both amylase and cellulases in both submerged and solid-state cultures (Ogbonna et al. 2018) and the relative proportions of the enzymes depend on the species and the substrate used.

Optimum carboxymethyl cellulose to soluble starch ratio for enzyme production

The optimum ratio of carboxymethyl cellulose to soluble starch for enzyme production was determined by varying their ratio in the medium. Cultivation of *Aspergillus fumigatus* IB-A1 in a medium containing 0.7% carboxymethyl cellulose and 0.3 % soluble starch gave the highest cellulase activity of 2.907± 0.32 U/ml/min while the medium containing 0.4% cellulose and 0.6 % starch gave the highest amylase activity of 2.442± 0.21 U/ml/min (Fig 1). It is noted that the cellulase activities increased with increase in CMC ratio up to 70% but the activity remained high even when the medium contained 0.9% CMC and 0.1% starch. On the other hand, amylase activity increased with increase in the concentration of soluble starch up to 0.6% and remained high even in a medium containing 0.9% starch and 0.1% CMC. Thus, the optimum ratio of soluble starch and CMC would depend on the relative concentrations of cellulose and starch in the substrate to be hydrolyzed. When about the same cellulase and amylase activities are desired, the ratio of CMC to soluble starch in the medium should be maintained between 4:6 and 5:5.

Comparison of enzymes production from cassava peel in submerged and in alternating air phase – liquid phase cultures

In a submerged culture, cellulase and amylase production by *Aspergillus fumigatus* IB-A1 were 6.122± 0.320 U/ml/min and 4.342± 0.210 U/ml/min respectively. However, in the alternating air phase - liquid phase culture, the cellulase and amylase activities increased to 8.106± 0.620 U/ml/min and 5.206± 1.24 U/ml/min respectively (Figure 2). This shows that alternating air phase-liquid phase cultivation led to a significant increase in the cellulase and amylase activities over the values obtained in submerged culture. Morphological observation showed that higher cell growth was achieved in

alternating air phase- liquid phase culture than submerged culture. This may have contributed to the higher enzymes production in the alternating air phase- liquid phase than that of submerged. It is noteworthy that regardless of the culture

system used, the activities of the two enzymes were higher when cassava peel was used as the carbon source when compared with the use of either soluble starch, CMC or a mixture of soluble starch and CMC.

Table 1 Evaluation of the ability of *Aspergillus fumigatus* IB-A1 to produce and secrete cellulase and amylase from a mixture of carboxymethyl cellulose and soluble

Culture conditions	Cellulase activity (U/ml/min)	Amylase activity (U/ml/min)	Total activity (U/ml/min)
Cultivated with soluble starch (1%) only	1.143± 0.081	2.144± 0.240	3.287± 0.321
Cultivated with carboxymethyl cellulose (CMC) 1% only	2.913± 0.030	1.558± 0.172	4.471± 0.202
Cultivated with soluble starch (0.5%) and CMC (0.5%)	2.746± 0.170	2.177± 0.321	4.923± 0.491

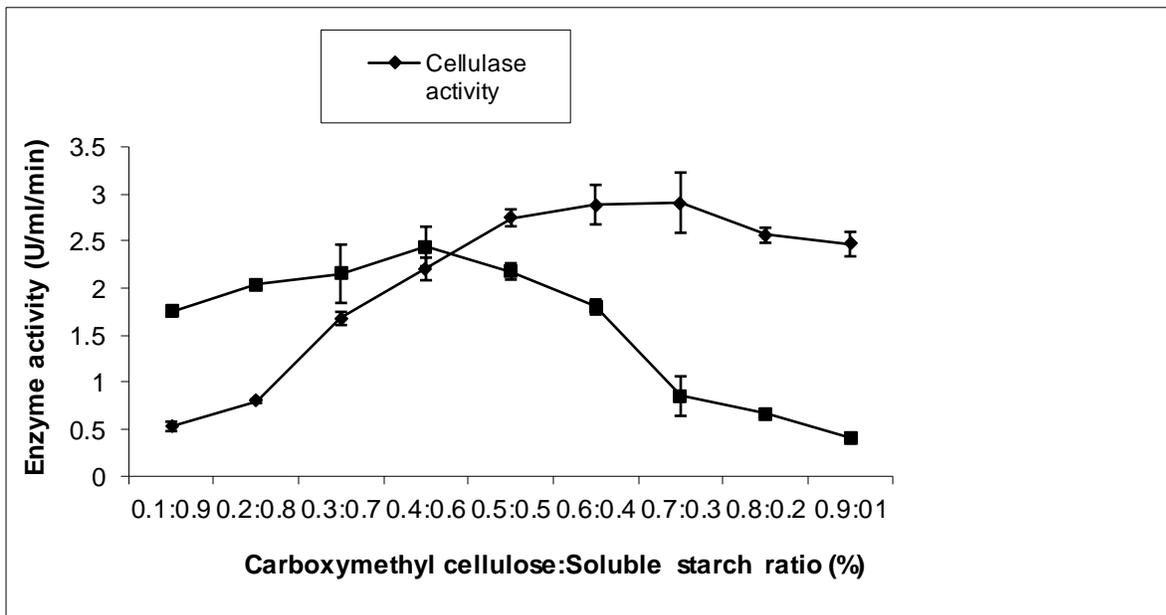


Figure1. Effects of the ratio of carboxymethyl cellulose to soluble starch on enzyme production by *Aspergillus fumigatus* IB-A1

The effects of the ratio of the air phase to liquid phase on enzyme production are shown Figure 3. Exposing the immobilized cells to 3 hours in air phase and 21 hours in submerged culture broth resulted in the maximum cellulase and amylase activities of 8.55 ± 0.32 U/ml/min and 5.285 ± 0.34 U/ml/min respectively. Regardless of the ratio of the air phase to the liquid phase investigated, the cellulase activity remained higher than that of amylase activity. However, cellulase appeared to be more sensitive to the ratio of the air phase to the submerged phase (Figure 3). In this system,

the function of the air phase is mainly for aeration of the immobilized cells (exposure to air) while the liquid phase is to supply the soluble nutrients and to extract the produced enzymes into the culture broth. A major advantage of the alternating air phase – liquid phase culture is that it reduces the problem of oxygen limitation, which is a major problem in submerged cultures. It combines the advantages of submerged cultures and those of the solid-state cultures (Roble et al., 2020; Ugwu et al., 2021).

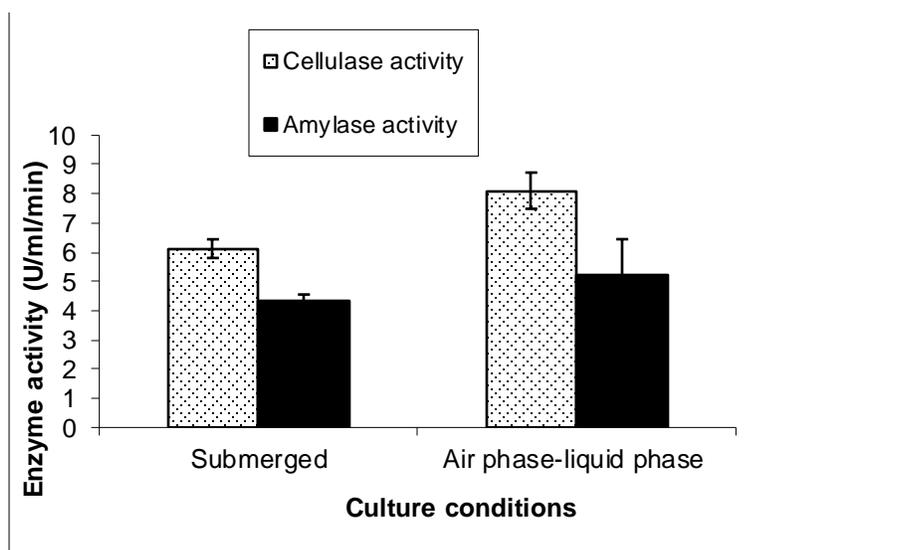


Figure2. Comparison of submerged and alternating air phase-liquid culture in cassava peel medium. The ratio of air phase to liquid phase was 6h:18h.

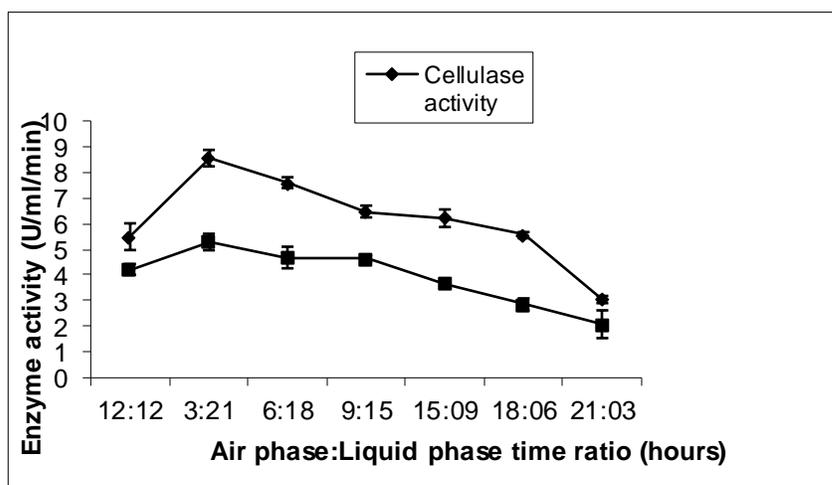


Figure 3. Effects of the ratio of air phase to liquid phase on enzyme production by *Aspergillus fumigatus* IB-A1, using cassava peel as the carbon source.

Many previous workers have reported higher enzyme production by immobilizing cells in loofa sponge. Samia et al (2013) reported higher specific β -glucosidase activity by cell immobilized on loofa sponge (*Luffa cylindrica*) than free cell. Also, Velichkova et al (2014) reported that immobilization of *Aspergillus awamori* K-1 and *Trichoderma viride* SL-45 on loofa sponge led to higher cellulase activity when compared with that of the suspended cells. However, the present study is the first report on simultaneous production of amylase and cellulase in an alternating air phase-liquid phase culture. It is noted that both the cellulase and amylase activities were higher in this culture system when compared with the conventional suspended submerged cultures.

CONCLUSION

Aspergillus fumigatus IB-A1 is capable of simultaneous production of cellulase and amylase in media containing either soluble starch, CMC, a mixture of the two or cassava peel as the carbon sources. The activities of the two enzymes were higher when cassava peel was used as the carbon source. Production of the two enzymes were further enhanced by alternatingly exposing the cells to air phase and liquid phase.

Conflict of interest

There is no conflict of interest

Acknowledgement

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AUTHOR CONTRIBUTIONS

EIB conducted the experiments and drafted the manuscript. YM designed and supervised the work and reviewed the manuscript. OJC conceptualized the project, supervised the work and reviewed the manuscript. All authors read and approved the final draft of the manuscript.

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