Production of Cellulase and Amylase Enzymes in both Solid and Liquid States by Two Species of Fungi

ABSTRACT

Aims: To isolate fungi capable of simultaneous production of amylase and cellulase enzymes

Study design: The experiment was carried out in aseptic conditions, data were subjected to one way Analysis of Variance (ANOVA), and the means were separated using Least Significance Deference (LSD).

Place and Duration of Study: Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria, between August 2010 and July 2013.

Methodology: Plantain peels and infected bark of a tree were collected for fungi isolation. The isolates were characterized and identified based on colony morphology and microscopic examination. They were later screened for amylase and cellulose activities

Results: Amylolytic and cellulosic fungi were isolated from plantain peels and infected bark of a tree. The isolates were identified as *Rhizopus* and *Fusarium* sp and they were able to produce amylase and cellulase enzymes simultaneously. The enzyme activities were determined on various concentrations of starch and carboxymethyl cellulose (CMC) in liquid medium. At 2 % starch + 0 % CMC, enzymes activities were 127.44 U/ml and 144.59 U/ml glucoamylase and 356.43 U/ml and 263.63 U/ml cellulose for *Rhizopus* and *Fusarium* sp respectively. When the organisms were grown on a solid medium (koji) supplemented with various concentrations of CMC, there was an increase in cellulase activities as CMC increased. Cellulase activity of 1902.02 U/g was recorded by *Rhizopus* sp at 1.5 g CMC supplementation and 1481.18 U/g was from *Fusarium* sp at 1 g CMC supplementation, but highest glucoamylase activities of 322.68 U/g and 302.12 U/g were recorded for *Rhizopus* and *Fusarium* spp respectively at 1 g CMC supplementation. Glucoamylase activities were not significantly affected by CMC supplementation in solid state culture.

Keywords: Cellulase, Amylase, Fungi, Enzyme Production

1. INTRODUCTION

Amylases are enzymes that break down starch or glycogen into simple sugars. They are produced by a variety of living organisms, ranging from microorganisms to plants and humans. Amylases degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes [1], [2]. A great deal of attention is drawn to amylase as a result of its economic and technological importance, isolating an organism suitable for amylase production provided potential new sources of the enzyme [3].

Microorganisms secrete these enzymes to the outside of their cells to carry out extra-cellular digestion. The end products of starch hydrolysis (glucose) are absorbed into their cells. Amylases are of useful applications in the food, textile, detergent and pharmaceutical industries [4]. They are used for starch liquefaction to reduce viscosity, production of maltose, oligosaccharide mixtures, high fructose and maltotetraose syrup. They are also used in textile industry for starch de-sizing [1]. Amylases are used during bread making to break starch in flour into simple sugars. They are also used in clothing and dishwasher detergents to dissolve starches from fabrics and dishes thereby improving cleaning effect [5]. Amylase helps to break down carbohydrates and could be considered as a natural antihistamine. It is often very effective in helping to relieve the symptoms of allergic reactions to insect bites or pollen irritation. Amylase is used to hydrolyse starch into simple sugar for bioethanol production.

Cellulase (EC 3.2.1.4) refers to a class of enzymes that catalyse cellulolysis (hydrolysis of cellulose to β-glucose). Cellulases are produced mainly by fungi, protozoans and symbiotic bacteria in the ruminating chambers of herbivores. However, there are also cellulases produced by a few other types of organisms such as termites [6]. They hydrolyze 1,4-β-glycosidic linkages in cellulose and cereal β-D-glucans [7]. Cellulase hydrolyses cellulose during drying of beans for coffee production, and is widely used in textile industry and laundry detergents. Also in pulp and paper industry, they are used for the production of paper and cellophane as well as biotransformation of waste cellulose to simple sugars. They are used for deinking of fibre surfaces and in improving pulp drainages. In food industry, they are used for production of animal feed and even in pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels. As lytic enzymes, they are of prime importance in protoplast production [8], [9], [10]. Although amylases and cellulases have a number of applications [11], the high cost of production of these enzymes has hindered their industrial application, especially in developing countries. Utilization of plant biomass and agricultural wastes can effectively replace the costly soluble pure substrates with successful solution of garbage disposal problem. As useful as these enzymes are, they are currently being imported into the country but if these enzymes are produced locally and simultaneously from wastes such as those from food processing industries, the high cost of their importation and production can be greatly reduced. Furthermore, this will reduce the problem of environmental pollution caused by organic wastes. Therefore this study was initiated to screen for fungal species capable of simultaneous production of both amylase and cellulase enzymes for efficient utilization of starch and cellulose feedstocks for industrial purposes. The effects of mixed substrates on amylase and cellulase production in both suspended and solid state cultures were also investigated.

2. MATERIALS AND METHODS

2.1 Isolation of Organisms

Plantain peels and infected bark of a tree were collected for fungal isolation. A 1 g sample each was weighed separately and added to 10 ml distilled water, 1 ml of each of the diluents was plated out on Emerson's yeast phosphate soluble starch (YPSs) medium [12]. After growth, colonies were picked and subcultured several times for purity.

2.2 Identification of Fungal Isolates

The isolates were characterized and identified based on colony morphology and microscopic examination. Among the characteristics used were colonial characteristics such as surface appearance and colour of the colonies. Microscopic examination revealed the type of hyphae i.e. septate or aseptate, and the

vegetative mycelia. Slide culture method was used to identify the isolates to the generic level and appropriate references were then made. The isolates were stored in starch agar slants until required.

2.3 Screening for Amylase and Cellulase Production

The isolates were grown in broth containing 1 % (w/v) starch and 0.5 % CMC as carbon sources. They were incubated at room temperature for 72 h, after which enzyme assay was carried out. The cellulase and glucoamylase activities were measured.

2.4 Effect of Mixed Substrates Concentrations on Enzyme Production

The isolates were inoculated into media containing varying concentrations of both starch and carboxymethyl cellulose in the following proportions: 1.0 % (w/v) starch and 0 % (w/v) CMC; 0.8 % (w/v) starch and 0.1 % (w/v) CMC; 0.6 % (w/v) starch and 0.2 % (w/v) CMC; 0.4 % (w/v) starch and 0.3 % (w/v) CMC; 0.2 % (w/v) starch and 0.4 % (w/v) CMC and 0 % (w/v) starch and 0.5 % (w/v) CMC. The broths were incubated at room temperature for 3 days, after which the enzyme activities were assayed.

The isolates were further inoculated into another set of media containing varying concentrations of both starch and CMC in the following proportions: 2 % (w/v) starch and 0% (w/v) CMC; 1.5 % (w/v) starch and 0.5 % (w/v) CMC; 1 % (w/v) starch and 1 % (w/v) CMC; 0.5 % (w/v) starch and 1.5 % (w/v) CMC; and 0 % (w/v) starch and 1.5 % (w/v) CMC. They were incubated at room temperature for 3 days; after incubation enzyme activities were assayed.

2.5 Solid State Cultivation

2.5.1. Preparation of Koji

Three test tubes containing 10 ml distilled water, 20 g rice bran and a piece of white cloth were sterilized in an autoclave at 121 OC for 15 min. On cooling, the sterile water from each of the tubes was emptied into 3 slants containing a 72 h old culture of the isolate. The spores were dislodged with a sterile wire loop. Cooked rice (100 g) was carefully weighed into the sterile white cloth; 20 g rice bran was added and thoroughly mixed together. It was inoculated with 30 ml of fungi spore suspension harvested from the 3 slant cultures. It was thoroughly mixed together to ensure even distribution of the spores and the white cloth was tied and incubated for 72 h at room temperature. Every 24 h during the incubation period, the cloth was untied and the contents mixed thoroughly, retied and incubated again. After the incubation, 5 g of the koji was suspended in 10 ml of distilled water. It was thoroughly mixed and the supernatant was used for enzyme assay.

2.5.2. Effect of CMC supplementation on enzyme production in solid state culture

Rice (400 g) was cooked and divided into four portions containing 100 g each in 4 pieces of sterile white cloth. Then 0.5 g Carboxymethyl cellulose (CMC), 1 g CMC, 1.5 g CMC or 2 g CMC was added to each of the four portions of 100 g of cooked rice. Each was thoroughly mixed together and inoculated with already prepared spore suspension of the isolate. It was mixed thoroughly to ensure even distribution of the spores within the rice grains. The cloths were tied and incubated for 72 h at room temperature, but every 24 h during the incubation period, the cloth was untied and the contents mixed thoroughly, retied and incubated. After the incubation, 5 g of the koji was suspended in 10 ml of distilled water. It was thoroughly mixed and the supernatant was used for enzyme assay.

2.6. Analytical Methods

2.6.1. Glucoamylase Assay

The glucoamylase assay was determined by measuring the amount of reducing sugar released from starch solution. The reaction mixture contained 0.5 ml of 1 % (w/v) soluble starch, 0.2 ml of 0.1M sodium acetate buffer (pH 5.6), and 0.3 ml crude enzyme solution. The mixture was incubated at 50 OC in a water bath for 30 minutes. The reaction was terminated by adding 1 ml of 3, 5-dinitrosalicylic acid (DNSA) and boiled in a boiling water bath for 10 minutes. Four millilitres of distilled water was added after cooling and absorbance taken at 540 nm using a spectrophotometer. Control tubes were set up which contained the reaction mixture but lacked the crude enzyme solution [13], [14].

One unit of glucoamylase activity was defined as the amount of enzyme which released 1 µg glucose equivalent from starch per ml per minute under the assay condition.

2.6.2. Cellulase Assay

The method used involved estimating the amount of reducing sugar produced by the activity of the enzyme on buffered 0.5 % CMC. The reaction mixture containing 0.5 ml of supernatant (the crude enzyme) and 0.5 ml of 0.5 % CMC in 0.05M sodium citrate buffer (pH 4.8) was incubated at 50 OC in a water bath for 30 minutes. The reaction was terminated by adding 3 ml DNSA and then boiled for 10 minutes in a boiling water bath. The control tubes contained the reaction mixture but lacked the crude enzyme solution. Absorbance was taken at 630 nm using a spectrophotometer [13], [15].

One unit of cellulase was defined as the amount of enzyme which released 1 µg of glucose from cellulose per ml per min under the assay conditions.

2.6.3. Statistical Analysis

All the experiments were done in triplicates and the results expressed as mean±standard deviation. The data were subjected to one way Analysis of Variance (ANOVA), and the means were separated using Least Significance Deference (LSD).

3. RESULTS AND DISCUSSION

3.1. Identification of Isolates

The two isolates with appreciable glucoamylase activities were identified. Based on their cultural, morphological as well as microscopic characteristics, ABIBM was identified as *Fusarium* sp while AFPW was identified as *Rhizopus* sp (Table 1).

Table1: Microscopic characteristics and identification of the isolates.

Isolates	Morphological characteristics	Microscopic characteristics	Suggested Species

AFPW	Fast growing fungus quickly filing the plate with a dense white cottony aerial mycelium.	Mycelium aseptate, with many hyphal branches connecting groups of unbranched sporangiophores.	*Rhizopus* sp
ABIBM	Fast growing fungus white, cottony or woolly.	Septate mycelium, macroconidia sickle-shaped, curved at the pointed ends, many-celled; microconidia oval elongated and curved.	*Fusarium* sp

SOURCE: [16], [17], [18].

3.2. Production of Amylase and Cellulase Enzymes by the Isolates in Suspended Cultures

The isolates were screened for simultaneous production of glucoamylase and cellulase enzymes. This was done by growing the isolates in a broth containing 1% starch and 0.5% CMC. Glucoamylase and cellulase activities (118.48 U and 238.15 U) from *Fusarium* sp were higher than the activities (9.14 U and 102.34 U) produced by *Rhizopus* sp (Figure 1).

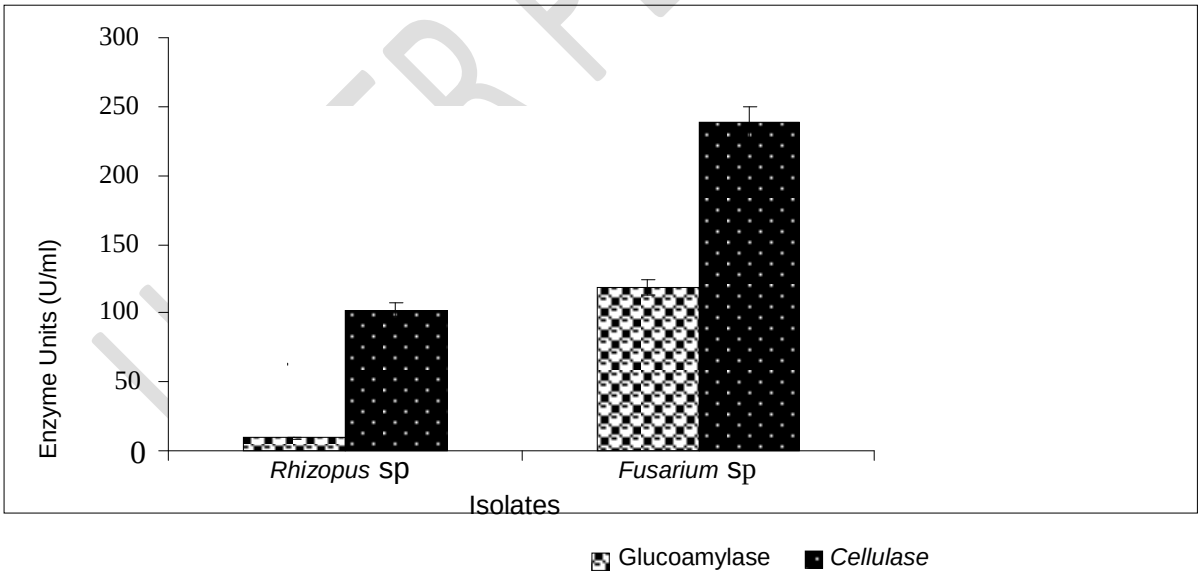


Figure 1: Screening of the isolates for the production of both glucoamylase and cellulase enzymes in suspended culture

The two strains of fungi (*Fusarium* and *Rhizopus* sp) were able to produce glucoamylase and cellulase enzymes simultaneously but the cellulase activities were higher than those of glucoamylase.

3.3. Effect of Ratios of Starch to Carboxymethyl Cellulose (CMC) on Enzymes Production in Liquid Medium

The effect of ratios of starch to CMC on glucoamylase and cellulase production by *Rhizopus* sp in suspended culture was investigated. Both the glucoamylase and cellulase activities were highest in a medium containing 1 % starch and 0 % CMC. The enzyme activities decreased with a decrease in starch concentration and almost no glucoamylase activity was detected in the culture broth containing 0.5 % CMC + 0 % starch while cellulase activity was very low. On the whole, cellulase activities were higher than glucoamylase activities at all the ratios tested (Figure 2A). However, when the starch concentration was increased to 2 % (Figure 2B), there was an increase in enzyme activities. Cellulase activities were still higher than glucoamylase activities and the activities of both enzymes decreased with a decrease in starch concentration. The activities in the medium containing 1.5 % CMC and 0 % starch were very negligible.

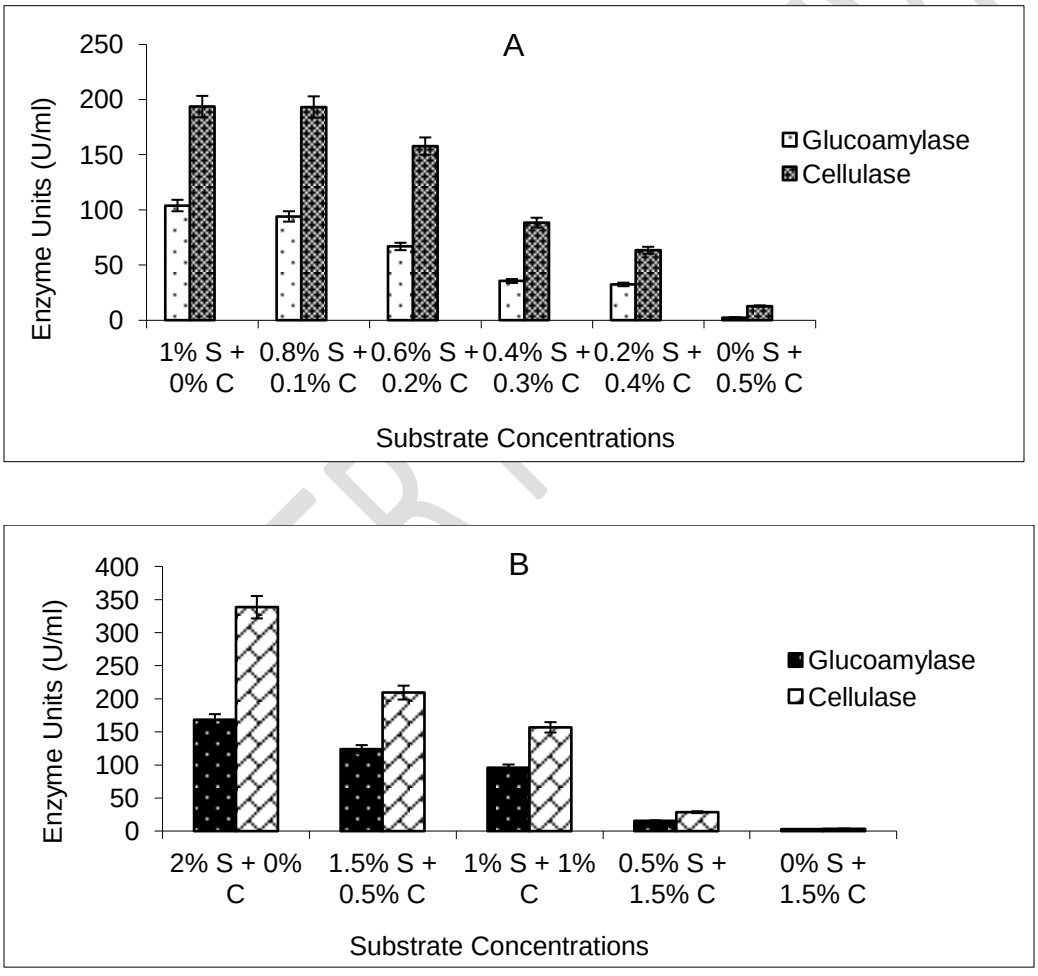
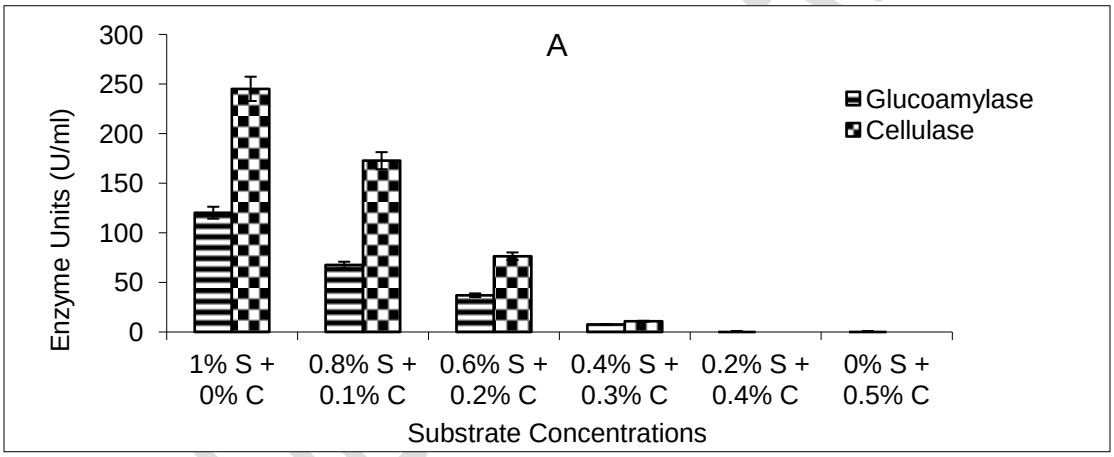


Figure 2: Effect of the ratios of starch to cellulose on enzyme production by *Rhizopus* sp.

The ratios were varied between 1 %S : 0 %C to 0 %S : 0.5 %C (A) or between 2 %S : 0 %C to 0 %S : 1.5 %C (B). S denotes starch while C denotes carboxymethylcellulose.

The effect of ratios of starch to CMC on glucoamylase and cellulase production by *Fusarium* sp in suspended culture is shown in Figure 3A. Both the glucoamylase and cellulase activities were highest in a medium containing 1 % starch without CMC. The enzyme activities decreased with a decrease in starch concentration and almost no enzyme activity was detected in the culture broth containing 0.5 % CMC without starch. On the whole, cellulase activities were higher than glucoamylase activities at all the ratios tested. When the starch concentration was increased to 2 % (Figure 3B), there was increase in enzyme activities, especially that of glucoamylase. Cellulase activities were still higher than glucoamylase activities and the activities of both enzymes decreased with a decrease in starch concentration. The activities in the medium containing 1.5 % CMC and 0 % starch were negligibly small.



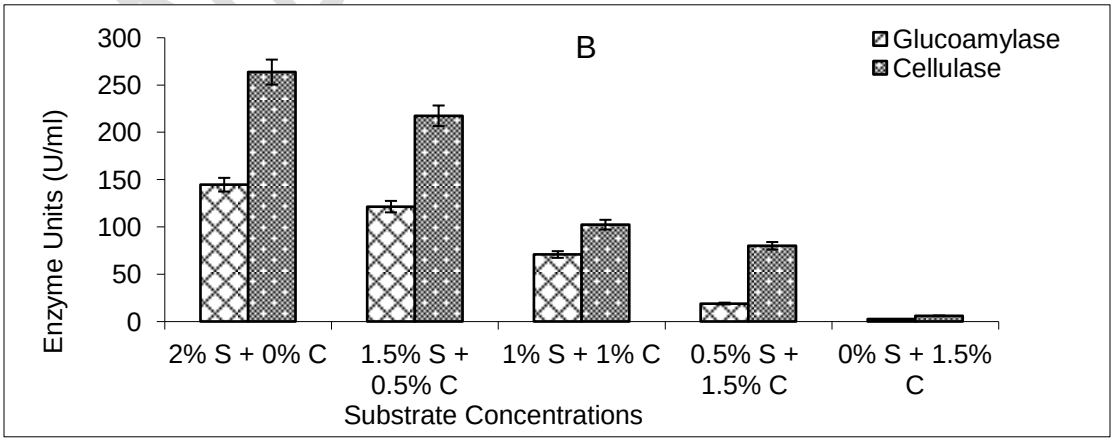


Fig 3: Effect of ratios of starch to cellulose on enzyme production by *Fusarium* sp.

The ratios were varied between 1 %S : 0 %C to 0 %S : 0.5 %C (A) or between 2 %S : 0 %C to 0 %S : 1.5 %C (B). S denotes starch while C denotes carboxymethylcellulose.

The composition of media plays an important role in the production of enzymes. Growth and enzyme production of any organism are greatly influenced by both environmental conditions as well as the nutrients available in the growth medium [19],[4]. Substrate concentration of 2 % starch and 0 % CMC gave the highest activity in a liquid medium. In suspended culture, both glucoamylase and cellulase activities decreased as the concentration of starch decreased and CMC concentration increased. Sohail *et al*. [20] reported that amylase enzyme production decreased as the concentration of starch increased. Rajoka [21] and Devi and Shankar [22] reported low cellulase activity in medium containing soluble carbon sources like CMC.

The result obtained in this study is higher than that obtained by Adebiyi *et al*., [23] and Fadahunsi and Garuba [24] who reported amylolytic activity of 45.33 U/ml for *Rhizopus* sp amylase grown on Sorghum bicolor starch and 30.1 U/ml for *Aspergillus favus* implicated in the bio-deterioration of starch-based fermented foods respectively. Also Tuysuz *et al*.,[25] reported amylase activity of 64.9 U/ml from a thermophilic bacterium *Anoxybacillus rupiensis* T2. Akinyosoye *et al.* [26] has reported *Rhizopus stolonifer* as capable of producing amylases.

3.4. Enzyme Production by the Isolates in Solid Culture

Rhizopus and *Fusarium* spp were grown in solid medium by inoculating them into cooked rice grains (koji). Glucoamylase and cellulase activities of *Rhizopus* sp in solid state culture (371.52 U, 1086.36 U) were higher than those of *Fusarium* sp (368.16 U, 708.7 U) (Figure 4). Enzyme activities in solid state cultures were significantly higher when compared with enzyme activities in suspended cultures.

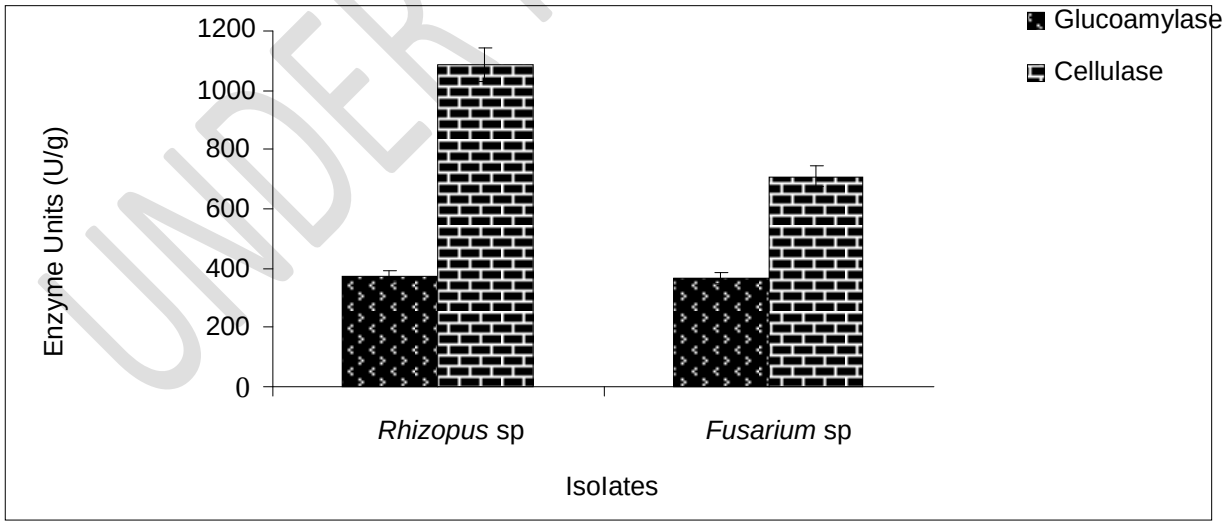


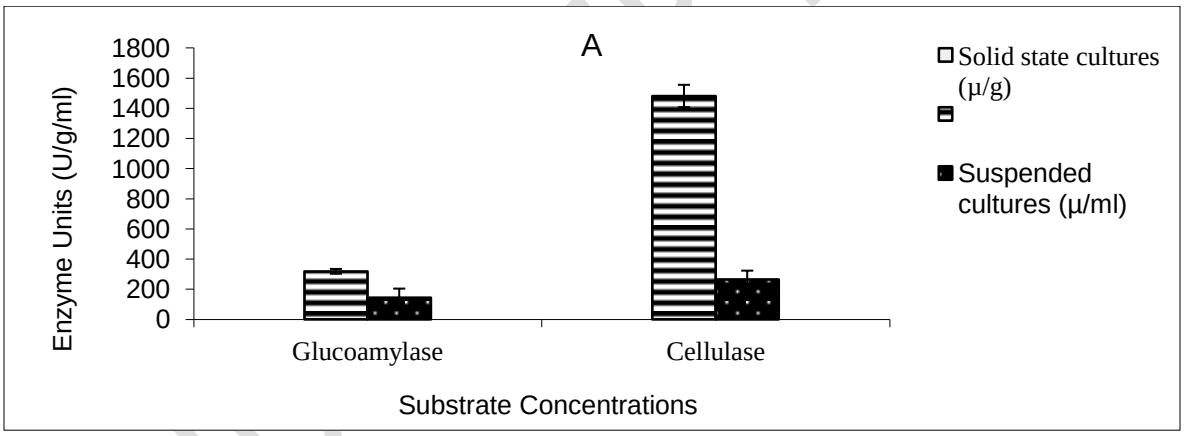
Fig 4: Enzyme production by the isolates in solid state culture

In solid state cultures, supplementation of CMC to starch resulted in increase in cellulase activity (up to 1.5 %) but had no significant effect on glucoamylase. This compares favourably with the reports given by

Narasimha et al. [27], that among various soluble organic sources and lignocelluloses tested, 1g carboxymethyl cellulose and sawdust supported maximum production of cellulase by *A. niger*. Furthermore *A. flavus* gave the highest cellulase activity on sawdust [28]. *A. niger* isolated from the soil produced highest cellulase activity at 1% CMC concentrations, higher CMC concentrations resulted in a decline of cellulase production [29].

3.5. Comparison of Enzyme Production in Solid State and Suspended Cultures

As shown in Figures 5A and 5B, enzymes production by both *Fusarium* and *Rhizopus* spp were higher in solid state culture than in suspended culture. Furthermore, for the two strains of microorganisms, cellulase activities were significantly higher than glucoamylase activities. It is also important to note that *Rhizopus* sp (Figure 5A) produced higher glucoamylase and cellulase enzymes than *Fusarium* sp (Figure 5B).



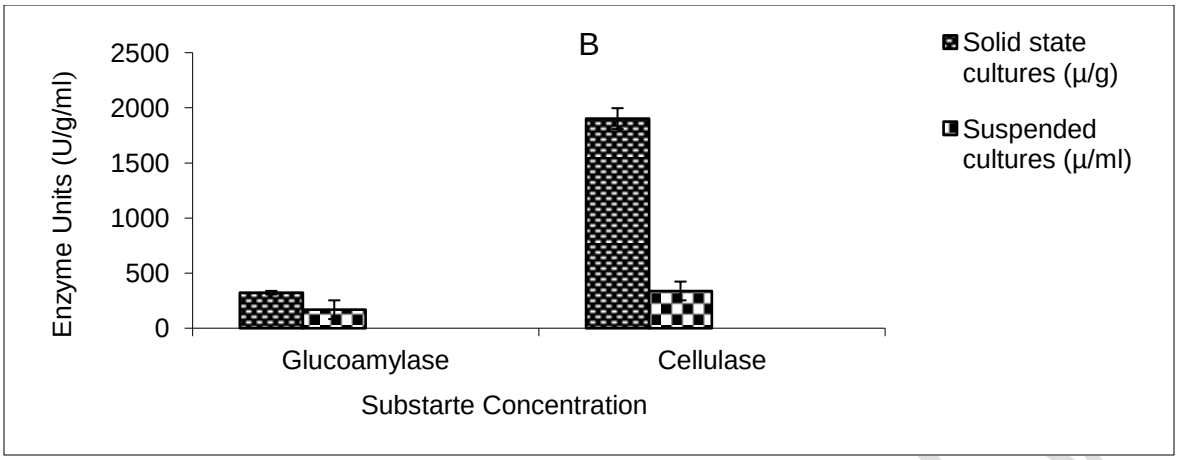


Fig 5: Comparison of enzyme production by *Fusarium* sp (A) and *Rhizopus* sp (B) in solid state and suspended cultures

The result of this study compares favourably with Kahil and Hassan [30] who reported an activity of 96.5 U and 86.1 U for cellulase (as filter paperase activity) from *A. niger* and *T. koningii* grown on agro-waste under solid-state fermentation condition. Mukherjee *et al.* [31] reported an exoglucanase activity of 50 U/ml from potato peel inoculated with *R. oyzae*. This is higher than the report from other works. *Trichoderma reesei*, in comparison, produced 0.8 U/ml while *F.oxysporium* gave 1.92 U/ml [32]. Cellulase activity reported for *Aspergillus niger, A. terrreus* and *Rhizopus stolonifer* were 0.12 ± 0.002, 0.1 ± 0.003 and 0.46 ± 0.03 respectively in solid state fermentation of cassava waste [33].

Simultaneous production of cellulase and glucoamylase enzymes is not common. These enzymes are of utmost important in industries, the enzymes are expensive and as they have to be imported, they contribute to the high cost of production. This study has shown that *Fusarium* and *Rhizopus* sp are able to produce amylase and cellulase enzymes simultaneously in appreciable concentrations. These organisms can then be harnessed for the production of these enzymes for industrial use.

REFERENCES

1. Prasanna, AV. Amylases and their Applications. African Journal of Biotechnology. 2005:4(13): 1525 – 1529.
2. Arun, S,ManthiriKani, S, Jegadeesh, G, Ravikumar, M. Submerged fermentation of amylase enzyme by *Aspergillus flavus* using Cocos nucifera meal. Kathmandu University Journal of Science, Engineering and Technology. 2010:6(11): 75 - 87.
3. Aullybux, A, Puchooa, D. α-amylase production on low-cost substrates by *Naxibacter* sp isolated from Mauritian soils. Br. Microbiology Research Journal. 2013:3:478-491.
4. Asrat, B, Girma, A. Isolation, production and characterization of amylase enzyme using the isolate *Aspergillus niger* FAB-211. International Journal of Biotechnology and Molecular Biology Resaerch. 2018:9(2): 7 - 14.
5. Wikipedia. Amylase. Retrieved 11/02/2014 from http://en.wikipedia.org/wiki/Amylase. 2009
6. Brune, A, Ohkuma, M. Role of the termite gut macrobiota in symbiotic digestion. In: David Edward Bignell (ed.), Biology of Termites: A Modern Synthesis. Springer, New York. ch. 16; 2010.
7. Wikipedia. Cellulase Retrieved 11/02/2014 from http://en.wikipedia.org/wiki/Cellulase. 2011b
8. Bhat, MK. Cellulases and related enzymes in biotechnology. Biotechnology Advances. 2000:18: 355 - 383.

9. Suurnakki, A, Niku-Paavola, MI, Buchert, J, Viikari, L. Enzymes in pulp and paper processing. In: Aehle, W. (ed.), Enzymes in industry. Wiley-VCH.Weinhelm, Germany. 2004.
10. Penttila, M, Limon, C, Nevalainen, H. Molecular biology of *Trichoderma* and biotechnology applications. In: Arora D (ed). Handbook of Fungal Biotechnology. Marcel Dekker Inc, New York 2004.
11. Karmakar M, Ray RR. Current trends in research and application of microbial cellulases. Research in Microbiology-Journal. 2011:6: 41 - 53.
12. Olutiola, PO, Famurewa, O, Sonntag, HG. An Introduction to General Microbiology (A Practical Approach). Hygiene - Institut Der Universitat Heidelberg, Germany, 1991.
13. Miller GL. Use of Dinitrosalycylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959:31: 426 - 428.
14. DeMoraes, LMP, Filho, SA, Ulhoa, CJ. Purification and some properties of an α-amylase glucoamylase fusion protein from *Saccharomyces cerevisiae*. World Journal of Microbiology and Biotechnology. 1999:15: 561 - 564.
15. Pecĭulyte, D. Isolation of cellulolytic fungi from waste paper gradual recycling materials. Ekelogija 2007:53(4): 11 - 18.
16. Pitt I. John (ed.). A Laboratory Guide to Common *Penicillium* species. Presented at a workshop held at University of Georgia, Athens, Georgia, USA. ISBN 064303949X: 064303949X 1985.
17. Beneke, ES, Rogers, AL. Medical Mycology Manual (2nd ed). Burges Publishing Coy, Minneapolis, Minnesota; 1971.
18. Klich, MA, Pitt, JI. A Laboratory Guide to the Common *Aspergillus* Species and Their Teleomorphs. Commonwealth Scientific and Industrial Research Organization (CSIRO), Division of Food Processing. Pp 58. 1988.
19. Singh, R, Kapoor, V, Kumar, V. Influence of carbon and nitrogen sources on the a-amylase production by a newly isolated *Thermophilic Streptomyces* sp. MSC702 (MTCC 10772). Asian J. Biotechnol. 2011:3: 540 - 553.
20. Sohail, M, Ahmad, A, Shahzad, S, Khan, SA. A survey of amylolytic bacteria and fungi from native environmental samples. Pakistan Journal of Botany. 2005:37 (1): 155 - 161.
21. Rajoka, MI. Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena*. Electronic Journal of Biotechnology. 2004:7(3): 259 - 266.
22. Devi, RN, Shankar, S. Bioconversion of cellulose into fermentable sugars by *Saccharomyces cerevisiae* cells for the production of ethanol using cellulolytic fungi isolated from soil. The Internet Journal of Microbiology. 2009:7 (2): 1 - 7.
23. Adebiyi, AO, Adebiyi, AP, Olaniyi EO. Nutritional composition of Sorghum bicolor starch hydrolysed with amylase from *Rhizopus* sp. African Journal of Biotechnology. 2005: 4(10): 1089 - 1094.
24. Fadahunsi F, Garuba EO. Amylase production by *Aspergillus flavus* associated with the Bio-deterioration of starch-based fermented foods. New York Science Journal, 2012: 5(1): 13 - 18. (ISSN: 1554- 0200). http://www.sciencepub.net/newyork
25. Tuysuy, E, Gonul-Baltaci, N, Omeroglu, MA, Adiguzel, A, Taskin, M, Ozkan H. Co-production of amylase and protease by locally isolated Thermophilic bacterium *Anoxybacillus rupiensis* T2 in sterile and non-sterile media using waste potato peels as substrate. Waste and Biomass Volarization 2020:11: 6793 - 6802.
26. Akinyosoye, FA, Adeniran, HA, Oboh, G. Effects of cultural changes on amylolytic activities of *Rhizopus stolonifer* isolated from decomposing agro-industrial wastes. Nigerian Journal of Biochemistry and Molecular Biology. 2004:19 (1): 41 - 46.
27. Narasimha, G, Sridevi, A, Vishwanath, B, Chandra, S, Rajasekhar, R. Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. African Journal of Biotechnology. 2005:5(5): 472 - 476.
28. Ojumu, TV, Solomon, OB, Betiku, EI, Layokun, KS, Amigun, B. Cellulase production by *Aspergillus flavus* linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. African Journal of Biotechnology. 2003:2 (6): 150 - 152.

29. Ja'afaru, IM, Fagade, EO. Optimization studies on cellulose enzyme production by an isolated strain of *Aspergillus niger* YL128. African Journal of Microbiology Research. 2010:4 (24): 2635 - 2639.
30. Kahil, T, Hassan, HM. Economic co-production of cellulase and α-amylase by fungi grown on agro-industrial wastes using solid-state fermentation conditions. Middle East Journal of Applied Sciences. 2015:5 (1): 184-195.
31. Mukherjee, S, Karmakar M, Ray RR.. Production of extracellular exoglucanase by *Rhizopus oryzae* from submerged fermentation of agro waste. Recent Research in Science and Technology. 2011:3(3): 69-75.
32. Ramanathan, G, Banupriya, S, Abirami, D. Production and optimization of cellulase from *Fusarium oxysporium* by submerged fermentation. Journal of Scientific and Industrial Research. 2010:69: 454 - 459.
33. Pothiraj, C, Balaji, P, Eyini, M. Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. African Journal of Biotechnology. 2006:5 (20): 1882 - 1885.