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Research Article

Amylase production by solid-state fermentation of corn (Zea mays) stem using fusarium and mucor

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Abstract: The study aimed at producing amylase from corn stem using Mucor and Fusarium in solid-state fermentation. The fungi used in this study was isolated from the rhizosphere of the corn plant. A solid-state technique was adopted for the fermentation process using spore suspension as inoculum. Various fermentation parameters, such as incubation days, addition of salt supplement, substrate concentration, mycelia suspension and temperature were optimized during fermentation with these organisms. Results show that both organisms produced the highest amount of amylase on day 3 at 28 ± 2°C. Mucor has the highest amylase activity of 12.54 µmol/mg/min when 25g of substrate was used while Fusarium has the highest amylase activity of 13.86 µmol/mg/min when 5g of substrate was used. The optimum temperature for amylase production was observed to be 40°C at pH 8.12 for Mucor, while Fusarium exhibited optimum activity at 20°C and pH 8.34. The study concluded that corn stems are a viable substrate for amylase production using rhizosphere-derived organisms under solid-state fermentation.

Keywords: Rhizosphere, Fermentation, Inoculum, Substrate

1. Introduction

Enzymes are biological substances that, in minute amounts, are capable of altering the rate chemical reactions in biological cells without themselves being altered after the reaction. They are present in the cells of living organisms in varying amounts and locations (Oyeleke & Oduwole, 2009). Of recent, the potential use of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in exploration of extracellular enzymatic activity in several microorganisms (Pabavathy & Nashiyar, 2012). Although enzymes are produced from animal and plant sources, microbial sources have been reported to be the most suitable for commercial applications (Sivakumar et al., 2012). Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan et al., 2003). The first microbial enzyme produced industrially was an amylase from a fungal source (Crueger et al., 1989).

Demand for industrial enzymes has been on the increase in the last few decades. The need for starch converting enzymes in various industries has led to enormous interest in amylase production at a less expensive rate using locally available substrates and organisms (George-Okafor et al., 2013). Amylases are a group of hydrolases that can specifically cleave the O-glycosidic bond in starch and are therefore employed in the starch processing industries for the hydrolysis of starch into simple sugars (Alva et al., 2007). Their action at random locations along the starch chain breaks down long chain carbohydrates, ultimately yielding maltotriose and maltose from amylase, or maltose, glucose and “limit dextrin” from amylopectin through an endo-acting hydrolytic mechanism. This process renders the products available for further saccharification to reducing sugar by amyloglycosidase (Forgarty & Kelly, 1991). The properties and mechanisms of action of amylases depend on the source of the enzyme

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(Lawal et al., 2014). Amylase can be divided into three groups such as: α-amylase (endoamylase), β-amylases (exoamylase), and amyloglucosidase (Sivakumar et al., 2012). Amylolytic enzymes from numerous sources degrade starch, the primary storage polysaccharide in plants (Rao et al., 1998).

Various amylolytic fungi have been used for the production of amylase from starchy materials in submerged agitated culture. However, several researchers have turned their attention to the biodegradation of starch using solid-state fermentation, as this method has been found to be more appropriate system than submerged fermentation for protein enrichment and amylase production from starchy materials (Pandeyet al., 2001). Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale. It is preferred for use in formulations for human or animal consumption, particularly for applications under acidic condition and at around 37oC due to its biocompatibility (Karuki & Imanaka, 1999).

Many attempts have been made to optimize culture conditions and suitable strains of fungi (Abu et al., 2005). The solid substrate acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water, necessary for microbial growth. As the microorganisms in solid-state fermentation (SSF) are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation (Hans et al., 1999). SSF has many advantages over submerged fermentation (SMF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up. Consequently, it widely regarded reported as the most appropriate process for developing countries. A further advantage of SSF is the utilization of cheap and easily available substrates, such as agricultural and food industry by-products (Mostafa & Abd-El-Aty, 2013).

Several agro-wastes are sources of land pollution in developing countries, especially during the harvesting season of such crops. Wastes accumulating from corn stem after harvest is one example, often littering farms and dump sites. This underscores the rationale of the study: to reduce pollution from agro-wastes like corn stems by utilizing them for the production of industrially important enzymes, such as amylase. The importance of this enzyme cannot be overemphasized, as it is widely used not only in the food industry but in breweries, textiles, paper, detergents, among others. However, the high demand for this enzyme has not been met due to the low productivity of the microbes used in its production and their long incubation periods. Therefore, this study was conducted to investigate new organisms capable of producing the enzyme with higher productivity and shorter incubation times.

2. Materials and methods

2.1 Collection of soil sample/corn stem

Soil sample was collected from a farm in Elemere, Moro local government, Kwara state, using sterile McCartney bottles and taken to the laboratory within three hours for immediate analysis, following the method of Lawal et al. (2014). Soil samples around the base of the corn stem were used for microbial isolation. The corn stems were obtained by cutting the stem from the root after uprooting the plants from the soil.

2.2. Isolation of amylolytic fungi

The fungi were isolated from the soil using the pour plate technique. The soil suspension was diluted up to 105. The samples were inoculated on already prepared Potato dextrose agar-chlorampenicol plates. The inoculated plates were incubated at ambient temperature (28 ± 2°C) for 2 days. Colony developments were observed after incubation period (Ratnasriet al., 2014). The desired isolates were sub-cultured repeatedly to obtain pure culture for further studies (Ugoh & Ijigbade, 2013).

2.3. Screening of fungal isolates for amylase production

Primary screening was performed using the starch agar plate method. The isolated fungi were inoculated on the agar plates amended with 2% of starch with 1.5% of agar (Sakthi et al., 2012). The plates were incubated at 28 ± 2°C for 5 days. After flooding with iodine solution, clear zones around the colonies were observed, and plates showing the largest hydrolysis halos were selected for further investigation (Ugoh & Ijigbade, 2013).

2.4. Identification of amylolytic fungal isolates

Isolates obtained were characterized and identified on the basis of their colonial and morphological characteristics, which include macroscopic and microscopic examinations (Soomro et al., 2007). Appropriate references were then made using mycological identification keys and taxonomic description by Harrow (1998).

2.5. Preparation of substrate

The corn stems were chopped into small pieces of uniform sizes, spread on a tray and oven-dried at 70°C for a day, grinded into powdery form with the aid of a Philips blender, sieved with a very fine mesh of about 0.05mm in diameter and stored in an air–tight plastic container for further use (Unakal et al., 2012).

2.6. Preparation of inoculum

A volume of 3 mL sterile distilled water was transferred to a sporulated PDA-chloramphenicol agar slant culture. The spores were then dislodged using the inoculating needle under aseptic conditions and 1mL of the suspension containing about 2x105 spores was used as the inoculum (Kuforiji et al., 2005).

2.7. Production of amylase

The solid-state fermentation (SSF) process was carried out in 250 mL Erlenmeyer flasks containing 10g of processed corn stem, to which 50 mL of distilled water was then added. After sterilization by autoclaving, the flask was cooled and inoculated with 1 mL of fungal spore suspension. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and incubated at 28 ± 2°C (Balkan et al., 2011).

2.8. Enzyme extraction

Crude enzyme from the fermentation process was extracted with 100 mL of sterile distilled water on a rotary shaker for one hour at 120rpm, after which the solution was filtered using 16 folds of cheese cloth (Kalaiarasi & Pavatham, 2015).

2.9. pH determination

The pH of the crude enzyme was measured using a pH meter (Ohaus Starter 2000).

2.10. Amylase assay

Two milliliters of the crude enzyme extracts were introduced into 2 mL of 0.1M phosphate buffer-starch solution (pH 6.8) in a flask. The contents of the flask were incubated at 40°C for 30 minutes. The solution was filtered using No 1 Whatman filter paper. Two milliliter of the filtrate was introduced into a test tube and 2 mL of Dinitrosalicylic acid reagent (D.N.S.A reagent) which contained D.N.S.A (1.0 g); 2M NaOH, (20 mL); Potassium sodium tartarate (30 g); in 100 mL of distilled water was added and boiled for 10 minutes to complete the reactions. The absorbance of the cooled solution was then measured at 540 nm with a Lasany visible spectrophotometer (LI-722). The amylase activity of the culture filtrates was expressed as total reducing sugars released/min/mg amylase activity by I mL of crude enzyme under the assay condition (Oyewale, 2013).

2.11. Optimization of fermentation parameters

2.11.1. Incubation period

Incubation days were varied from 1-5. Crude enzyme was assayed for amylase activity at I day intervals as described under enzyme assay.
Effect of addition of salt supplement: KH2PO4 (0.15g), NH4NO3 (1g), KCl (0.05g), MgSO4 (0.01g) and FeSO4 (0.01g) was suspended in 100 mL of water and used in moistening the substrate before incubation.

2.11.2. Effect of substrate concentration

Various masses of the substrate were utilized for fermentation, with 5, 15, 20and 25g used for enzyme production.

2.11.3. Effect of mycelia suspension as inoculum

Dry mycelia mass was obtained as described by Abdullah (2005). One mg/mL from the dried mycelia suspension was used to inoculate 10g of substrate before incubation.

2.11.4. Effect of temperature

The optimal temperature for amylase activity was determined by incubating the substrate enzyme at20, 30 and 40oC; preparing the substrate as described earlier.

3. Results

3.1. Isolation, screening and identification of amylolytic fungi

Eight fungi with amylase potentialities were isolated and labeled isolate 1, 2, 3, 4, 5, 6, 7 and 8. Among the eight isolates screened, two had the highest potentiality, giving the largest zone of hydrolysis (Table 1). After screening method, the two organisms that had the highest zone of hydrolysis were identified.

Isolate 1 had colonies which were initially white and cottony but later turned grey with age as sporangia were produced on potato dextrose agar plate. Hyphae were thick and non-septate with large diameter, the sporangiospores were erect and sometimes branched

and/or unbranched, which bear single sporangium containing large number of spherical spores with round columella. There was however absence of rhizoids and it was later identified as Mucor sp.

Table 1: Screening for Amylase Production

Isolates	Zone of hydrolysis (mm)
Isolate 1	28
Isolate 2	80
Isolate 3	15
Isolate 4	20
Isolate 5	5
Isolate 6	15
Isolate 7	10
Isolate 8	20

Isolate 2 had colonies that were white and fluffy but later turned black with age as spores were produced on potato dextrose agar plate. The microconidia was spindle shaped, mycelium was septate bearing crescent conidia on the conidiophores and it was later identified as Fusariumsp.

3.2. Fermentation for amylase production

The result obtained for fermentation with Mucor sp and Fusarium sp at 28± 2oC for five incubation days revealed that the organisms had highest amylase activity on day 3 (9.99 µmol/mg/min at pH 7.7 for Mucor sp and 11.22 µmol/mg/min at pH 8.3 for Fusarium sp and lowest on day 1 (2.64 µmol/mg/min at pH 7.5 for Mucor sp µmol/mg/min and 1.98 µmol/mg/min at pH 7.9 for Fusarium sp) [Figure 1].

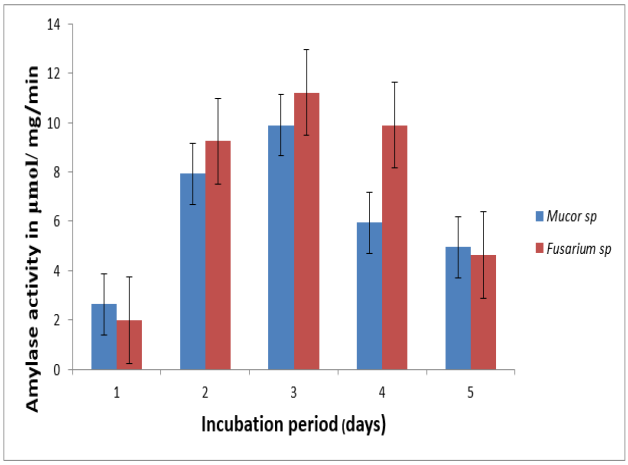

Fig 1: Effect of incubation days on amylase activity

3.3. Effect of optimization of fermentation parameters on amylase activity

3.3.1. Effect of salt supplement

Addition of salt supplement resulted in a reduction in amylase activity in both organisms. However, the peak of activity was also recorded on day 3 for both organisms (7.92 µmol/mg/min at pH 8.05 for Mucor sp and 9.99 µmol/mg/min at pH 7.97 for Fusarium sp) and the lowest enzyme activity was on day 1 for Mucor sp (3.96 µmol/mg/min at pH 7.38) and Fusarium sp(1.98 µmol/mg/min at pH 7.05) [Figure 2].

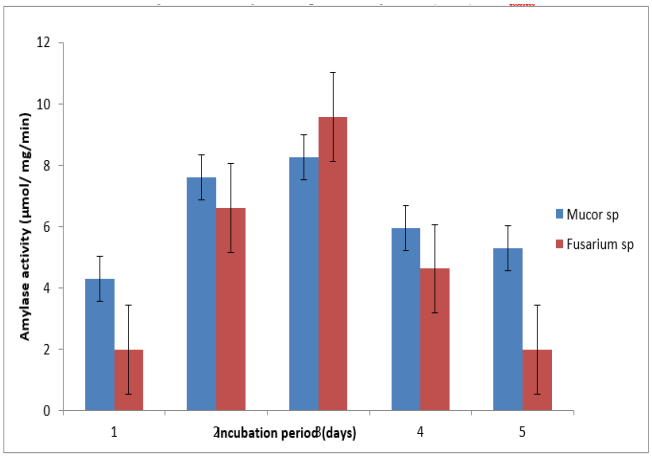

Fig 2: Effect of addition of salt supplement on amylase activity

3.3.2. Effect of substrate concentration on amylase activity

For Mucor sp, the peak of enzyme activity in the substrates varied (25, 20, 15, 10 and 5g) was (12.54, 12.54, 11.22, 9.9 and 11.55 µmol/mg/min at pH 8.08, 8.12, 8.3, 7.7 and 8.3 respectively) on day 3 and the lowest on day 1 for the 25, 20, 15, 10 and 5g (6.6, 3.96, 1.98, 2.64, 4.29 µmol/mg/min at pH 8.10, 8.28, 8.03, 7.51 and 7.94 respectively) substrate. For Fusarium sp the peak of enzyme activity was also on day 3 for the 25, 20, 15, 10 and 5g substrate (13.2, 12.54, 13.86, 11.22 and 13.86 µmol/mg/min at pH 7.82, 8.10, 8.12, 8.3 and 8.22 respectively) and lowest on day 1(2.31, 3.63, 2.64, 2.64 and 2.31 µmol/mg/min at pH 7.23, 7.16, 7.96, 7.94 and 7.05 respectively) [Figures 3 and 4].

3.3.3 Effect of using mycelia suspension as inoculum on amylase activity

The highest amylase activity was recorded on day 3 for both organisms when mycelia suspension was used instead of spore suspension (10.56 µmol/mg/min at pH

7.52 for Mucor sp and 12.21 µmol/mg/min at pH 8.04 for Fusarium spand the least activity was on day 1 (1.98 µmol/mg/min at pH 7 or Mucor sp and 2.6 µmol/mg/min at pH 7.36Fusarium sp respectively) [Figure 5].

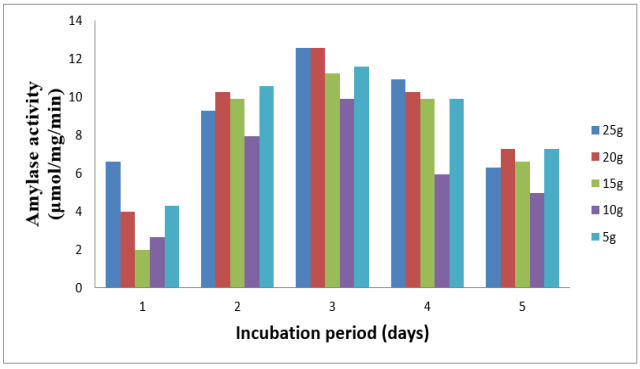

Fig 3: Effect of substrate concentration on amylase activity during fermentation with Mucor sp

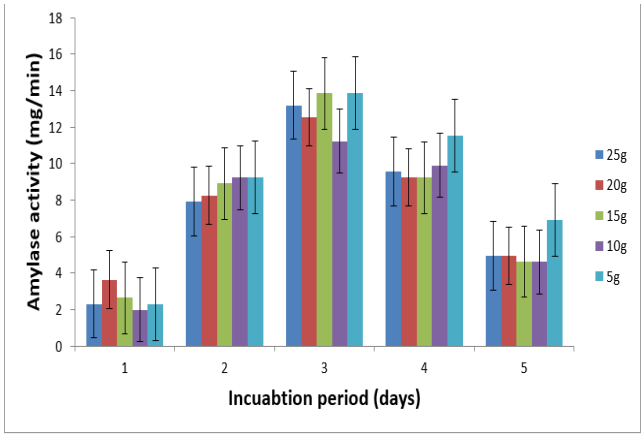

Fig 4: Effect of substrate concentration on amylase activity during fermentation with Fusarium sp

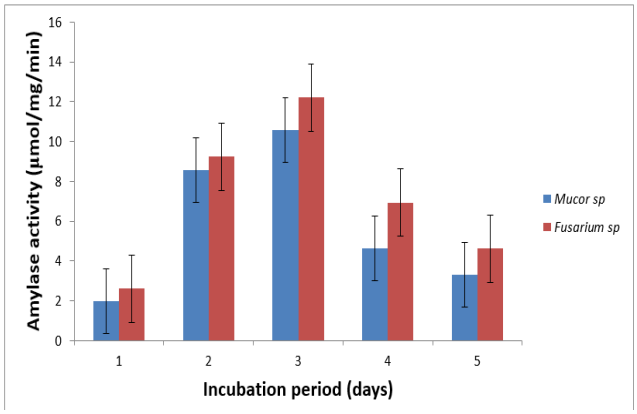

Fig 5: Effect of fermentation with mycelia suspension on amylase activity

3.3.4 Effect of temperature on amylase activity

Both organisms produced up to day 4 under a temperature of 20 and 40^{o}C while the highest enzyme activity was recorded on day 3 at 30^{o}C. Highest enzyme activity was on day 4 (11.88 µmol/mg/min at pH 8.18 for Mucor sp and 12.54 µmol/mg/min at pH 8.54 Fusarium sp respectively) and the lowest enzyme activity was on day 1 (1.98 µmol/mg/min at pH 7.07 for Mucor sp and 2.29 µmol/mg/min at pH 7.04 for Fusarium sp, respectively) at 20^{o}C.

The peak of enzyme activity was on day 3 for both organisms at 30^{o}C (9.99 µmol/mg/min at pH 7.7 for Mucor sp and 11.22 µmol/mg/min at pH 8.3 for Fusarium sp, respectively), while the lowest enzyme activity was on day 1 (2.64 µmol/mg/min at pH 7.5 for Mucor sp and 1.98 µmol/mg/min at pH 7.94 for Fusarium sp, respectively).

At 40^{o}C, the highest amylase activity was also on day 4 (12.87 µmol/mg/min at pH 8.17for Mucor spand 12.21 µmol/mg/min at pH 8.54 for Fusarium sp, respectively) and the lowest activity on day 1 (2.64 µmol/mg/min at pH 7.09 for Mucor sp and 3.96 µmol/mg/min at pH 7.06 for Fusarium sp respectively) [Figures 6 and 7].

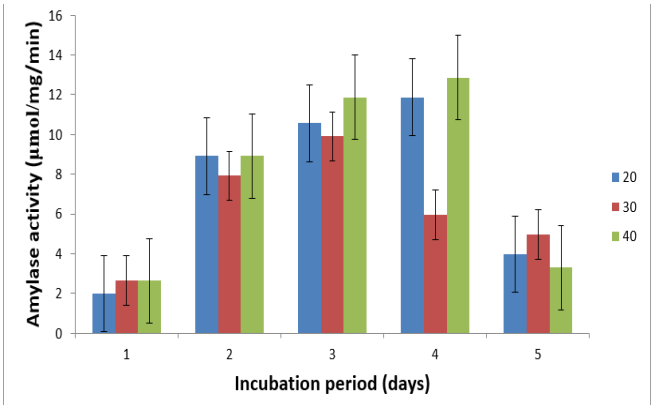

Fig 6: Effect of temperature on amylase activity during fermentation with Mucor sp.

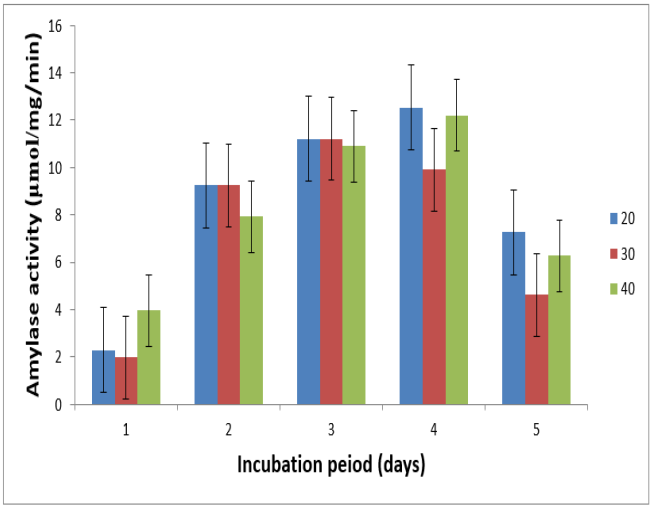

Fig 7: Effect of temperature on amylase activity during fermentation with Fusarium sp.

4. Discussion

As the soil samples houses a vast array of organisms, different species of fungi with amylase potentialities were isolated from it. Among the eight isolates, two gave the highest zone of hydrolysis after screening and these two organisms were later identified as Mucor sp and Fusarium sp (Kawo & Abdulmumin, 2009).

Fermentation with these organisms (Mucor sp and Fusarium sp) at room temperature resulted in a peak in amylase activity on day 3. This can be explained from the fact that most fungi require a minimum of 2 days to attain their log phase, as such at day 3 the organisms are at their full capacity, having attained their log phase and are able to secrete more enzymes that will break down the substrate to yield appropriate substances to foster their growth. On the other hand, the least amylase activity was on day 1, the explanation for this could be that the organisms while in their lag phase are yet to secrete sufficient enzymes. This result is in line with the work of Ratnasri et al. (2014).

The pH changes of 7.5 to 8.79 were observed in the culture media throughout the incubation period. The increase in pH of the medium may also be as a result of an increase in the amounts of enzymes and other metabolites released into the medium. This is contrary to the work of Oladapo (2013), who reported a change in pH of between 5.5 and 7.6.

The study shows that supplementing the substrate with various salt suspensions resulted in a decrease in enzyme activity in Mucor sp and Fusarium sp. However, when the substrate was utilized without salt supplementation, enzyme activity increased. The highest amylase activity was observed at day 3, which was lower when the substrate was utilized without salt supplementation. One possible explanation is that these salts may have hindered the organisms' ability to produce the enzymes needed to break down the complex carbohydrate moieties in the substrate, resulting in a reduced amylase activity. The least activity which was on day 1 which was also low compared to what was recorded in the absence of salt supplementation. This is in agreement with the work of Ferreira et al. (2015).

When the amylase activity of both organisms was examined under different substrate concentration, Mucor sp has the highest activity when 20 and 25g of substrate was used at day 3 of fermentation. This could be because increasing the amounts of substrates prompt the organism to produce more enzymes to meet its growth requirement, using the substrate as energy source. In other words, to support growth the organism produces additional enzymes that aid in substrate decomposition and the release of essential growth factors. From this, it can be deduced that increasing the substrates concentration leads to higher amylase production, whereas, using smaller amounts of substrates (15, 10 and 5g) resulted in reduced enzyme activity on the same day. However, an increase in enzyme activity was also recorded with Fusarium sp when using 5g of substrate. This may indicate that a smaller substrate mass reduces stress during colonization, allowing the limited metabolites produced to enhance enzyme activity. In other words, enzyme production in this organism appears to be higher with a small substrate amount compared to using of 25, 20, 15 and 10g of substrate. This is in agreement with the work of Oladapo (2013).

The use of mycelia suspension as inoculums increased enzyme activity for Fusarium sp when compared to spore suspension on day 3 of fermentation. This may be attributed to the time required for spores to develop into mycelia, whereas, the mycelia are already active on the substrate, thereby increasing enzyme activity. A similar trend was observed in Mucor sp., consistent with the findings of Abdullah (2005).

On day 4, at 20^{o}C, Mucor sp produced more enzyme than at 30^{o}C.The highest enzyme activity was recorded at 40^{o}Cwhile at 30^{o}C the peak of enzyme activity was on day 3 contrary to what was observed at a temperature of 20 and 40^{o}C.It may be deduced from this that enzyme activity by this organism can occur over a wide range of temperatures. This is similar to the work of Mohapatra (1998). However, Fusarium sp produced more enzyme under a temperature of 20^{o}C than at 30 and 40^{o}C, suggesting that Fusarium sp produces higher enzyme levels at lower temperatures. This is in line with the work of Lefuji et al. (1996).

5. Conclusion and recommendation

In conclusion, corn stem is a viable substrate for amylase production as both organisms were able to utilize it. Fusarium sp and Mucor sp produced amylase under alkaline conditions and best at pH between 7.7 and 8.5. Both organisms produced highest amylase on day 3 of fermentation under 28 ± 2^{o}C, after which activity declined. In contrast, at temperature between 20 and 40^{o}C, both organisms continued to produce enzyme up to up to day 4, with a reduction in activity observed on day 5. Furthermore, while Mucor sp is likely to produce enzyme at high temperature, Fusarium sp should produce better at low temperature. If Fusarium sp is used in subsequent studies, parameters should be optimized at 20^{o}C, so as to increase productivity. More organisms should be isolated from the rhizosphere of

the corn plant; screened in order to obtain more isolates with better potential for amylase production. Higher amount of substrate should be used when fermenting with Mucor sp, especially at 40°C.

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