Addis Ababa University

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Xylanase and Cellulase production by a termite associated *Xylaria* species

By

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Abbreviations

SSF = Solid-state fermentation

SmF= Submerged fermentation

MEA= Malt extract agar

Sp. = Species

U/g= Unit per gram

pH= Potential of Hydrogen ion

°C= Degree celcius

CMCase= Carboxymethylcellulase

DNS= Dinitrosalycilic acid

NSP= Non starch polysaccharides

I. Abstract

Xyalaria sp. are known to be associated with termites. However, the benefit the termites get from this association is not yet known. Termites collect wood pieces from the surrounding and collect it in the mound. The wood is then converted to soft spongy mass, called comb. The comb is normally invaded with fungal mycelia. It is well known that termites use cellulose as energy source after degradation to glucose with the help of microbial cellulases in the gut. But lignified celluloses can not be digested by cellulases. We hypothesize that the fungus probably helps to delignify cellulose fiber either directly through lignin degradation or through removal of the hemicellulose that cement the lignin to the cellulose fiber. To test this hypothesis we collected termite comb from Zuway and extracted proteins. The extract showed high xylanase activity (24U/g comb) and no detectable cellulase activity. This indicates that the role of the fungus is probably to remove lignin from the cellulose fiber. The fact that there was no detectable cellulase in the comb indicates that the fungus and the termite are not competing for cellulose. The fungus was isolated from the comb in pure culture. It was then grown in culture using submerged fermentation (SmF) and solid-state fermentation (SSF). However, enzyme production in SSF was much higher than in SmF. Maximum enzyme production in SSF using wheat bran was obtained at a substrate to moisture level ratio of 1:0.5 to 1:2. Addition of different sugars to the SSF substrate didn't affect enzyme production, indicating that enzyme production is probably constitutive. The xylanase was optimally active in the pH range of 4 to 6 and at temperature of 40°c. These properties make *Xylaria* xylanase potentially attractive as animal feed supplements.

Key words: *Xylaria, Xylanase, Cellulase*, *Termites*

1. Introduction

1.1. Termite fungus association

Vast numbers of fungi are associated with a variety of insects and other arthropods to form symbioses of various types. The fungi of these associations include necrotrophic (killing and using dead host cells as a nutrient source) and biotrophic (requiring living host cells) parasites, which are dispersed by their hosts. In other interactions insects use fungi directly as food or as sources of enzymes. Symbioses of this type allow the insects to use refractive nutrient resources (Richard, 1995).

1.2. Types of fungi known to be associated with termites and specificity of the association

The most common types of fungi known to be associated with termites are the *basidiomycetes Termitomyces* and *Xylaria* (Rogers, 1979). The genus *Xylaria* (literally meaning dead man's finger) is a fungus with mostly upright, clavet or strap like stromata and probably the largest with in the family *Xylariaceae* comprising more than 500 species of which only about 100 species are identified and known to date (Laessoe 1994; Rogers and Ju, 1994; and Whalley, 1996).

The systematic of insect associated *Xylaria* species is not yet well known. For example, a number of taxa have been equated with *X. nigripes* that undoubtedly are distinct species. A major factor leading to inaccurate identifications has been the failure to observe a significant morphological feature of the ascospores. Boedijn (1959) proposed the new genus *Pseudoxylaria* Boedijn on *Sphaeria* (*Xylaria nigripes*) based in part on the supposition that the ascospores lack a germination site.

Generally, *Xylaria* species are often isolated, but usually cannot be equated with known taxa (Rogers, 2000). There are several reasons for this. First, most *Xylaria's* have not been cultured from ascospores, and thus, their cultural morphology are unknown. Second, many *Xylaria* species in culture are very similar to each other. If such fungi are to be identified to any practical level and made available for comparative purposes, culture should be preserved and gene sequences deposited in GenBank (Rogers, 2000).

Although the specific nature of the relationship is unknown, the association between the *Xylariaceae* and insects, usually termites, is restricted to a few species of *Xylaria* (Rogers, 1979). *Xylaria nigripes* is probably the most common and widespread species and is associated with termite nests. However this termite associated *Xylaria* species would appear to be intricately linked, although the role of the *Xylaria* remains unknown. A critical examination of their taxonomic relationships and ecological activities would certainly prove interesting and worthwhile.

On the other hand, many studies showed that, tropical *Xylariaceous* taxa in the genera *Biscognauxia*, *Hypoxylon* and *Xylaria* were evaluated for their ability to produce wood-decaying enzymes and bring about mass loss and lignin solubilization in angiosperm and gymnosperm wood. All *Xylariaceous* taxa were capable of cellulose and xylan hydrolysis, but few produced enzymes involved in lignin breakdown (Stephen *et al.,* 2003*).* However, there is no report on the enzyme profile of the termite associated *Xylaria* species so far.

1.3. Benefits of the Association

Mutualistic associations between insects and fungi upon which they feed or from which they acquire enzymes for digestion, are often referred to as gardening symbioses (Martin, 1987). Among these, *Xylaria* is one of the fungal groups involved in gardening symbioses. In the fungus gardens of the mounds of the

termite, *Odontotermes redemanni* (Wasmann), only *Xylaria nigripes* (Koltz.) *Sacc.* grows, and the spores of other microorganisms do not proliferate normally (Sannasi, 1968).

1.4. Enzymatic Degradation of Hemicellulose and Cellulose

1.4.1. Hemicelluloses

Hemicelluloses are heterogeneous polysaccharides, which are located between the lignin and cellulose fibres and, depending on wood species, constitute about 20 to 30% of the naturally occurring lignocellulosic plant biomass (Suurnakki *et al.*, 1997; Viikari, 1994; and Viikari *et al.,* 1993).

The two main hemicelluloses in wood are xylans and glucomannans, both of which are present in softwood whereas in hardwood, xylan is the main hemicellulose component (Sjostrom, 1993). In contrast to cellulose, which is crystalline, strong and resistant to hydrolysis, hemicellulose is highly branched and has amorphous structure with little inherent strength. Apart from glucose, it may contain mannose, xylose, arabinose, rhamnose and L-fucose.

1.4.2. Hemicellulose-degrading enzymes (Hemicellulases)

Due to the complex structure of hemicelluloses, several different enzymes are needed for their enzymatic degradation or modification. The two main glycosyl hydrolases depolymerising the hemicellulose backbone are endo-1,4- ß -D xylanase and endo-1, 4- ß -D mannanase (Suurnakki *et al.,* 1997). Since xylan is a complex component of the hemicelluloses in wood, its complete hydrolysis requires the action of a complete enzyme system, which is usually composed of ß-xylanase, ß -xylosidase, and debranching enzymes such as alpha-L-arabinofuranosidase, alpha-glucuronidase, acetylxylan esterase, and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone. All these enzymes act cooperatively to convert xylan to its constituents

(Sunna and Antranikian, 1997). Xylanases attack randomly the backbone of xylan to produce both substituted and non-substituted shorter chain oligomers, xylobiose and xylose (Hudson, 1992).

1.4.3. Celluloses

Cellulose is the most abundant biopolymer on earth. It is the main structural component of plant cell walls, constituting up to 50% of the mass in trees. Cellulose is a linear polymer composed of D-glucose residues joined by ß-1,4-glucosidic bonds. The cellulose molecule forms a straight, almost fully extended chain, where glucose residues are rotated 180° relative to each other along the main axis, which means that the repetitive unit is the glucose dimer, cellobiose, rather than glucose (Anu, 2006).

1.4.4. Cellulose degrading enzymes (Cellulases)

Cellulases are O-glycosyl hydrolases (GHs) that hydrolyse ß-1,4-glucosidic bonds in cellulose. Functionally, cellulases have traditionally been classified into two distinct classes: cellobiohydrolase (1,4- ß-D-glucan cellobiohydrolase, EC 3.2.1.91) which release cellobiose from the nonreducing ends of the cellulose chain; and endoglucanase (1,4- ß –D glucan glucanohydrolase, EC 3.2.1.4), which cut cellulose chains at random positions in less crystalline regions, creating new chain ends. Extreme endoglucanases, often called CM-cellulases (carboxymethyl-cellulases) have little activity towards crystalline cellulose, but hydrolyse readily CMC, acid-swollen cellulose and even barley ß -glucan in a random fashion, resulting in a rapid fall in the degree of polymerisation (Kleman-Leyer *et al.*, 1994).

1.5. Industrial application of xylanases

1.5.1. Xylanase as animal feed supplements

Enzymes are proving to be extremely important as feed supplements, especially for monogastric animals. The enzymes that are found important for this application are xylanases, proteases, phytases, and amylases (Amare Gessesse, 1998). Of this xylanases are the most important.

The use of xylanase in poultry feeds has predominantly been related to the hydrolysis of fibre or nonstarch polysaccharide (NSP) fractions in cereal grains. These NSPs cannot be digested by the endogenous enzymes of poultry and can have antinutritive effects. The two main NSPs in cereals are ß-glucan in barley and oats and pentosans in wheat, triticale, and rye (Friesen *et al.*, 1992). Numerous researchers have demonstrated that the soluble-NSP fraction, not the total NSP fraction, is responsible for antinutritive responses (Classen and Bedford, 1991). These NSPs can bind to large amounts of water, and as a result, the viscosity of fluids in the digestive tract is increased. The increased viscosity causes problems in the small intestine because it reduces the substrate–enzyme interaction, which reduces nutrient availability (particularly fat) (Friesen *et al.*, 1992) and results in increased amounts of sticky droppings.

Inclusion of xylanase in poultry diet stimulates growth rates by improving digestibility, which also improves the quality of the animal litter (Bedford, 1996; Beg, *et al.*, 2001). For example, chicken feed based on wheat, rye, and many other grains is incompletely digested without added enzymes. These grains tend to be too viscous in the chicken's intestine for complete digestion. Xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta. It also changes hemicellulose to sugars so that nutrients formerly trapped within the cell walls are released. The chickens get sufficient energy from less feed. The barn is cleaner because the feed is more

thoroughly digested so the chicken waste is drier and less sticky. In addition, chicken eggs are cleaner because the excrement in the laying area is drier. The evidence to date suggests that this technology has a future, particularly for poultry (Classen and Bedford, 1991; Campbell and Bedford 1992).

Different studies showed that supplementation of xylan rich feed with commercially available xylanases do not completely eliminate the stickness of the litter (Bedford and Morgan, 1996), which may show that no complete depolimerization takes place. This indicates the need to search for new xylanases having better efficiency under the condition of the animal gut.

1.5.2. Lignocellulose bioconversion

Cellulases, together with hemicellulases, are among the most important group of enzymes that are employed in the processing of ligno-cellulosic materials for the production of, fuel, and chemical feedstocks (Bhat, 2000; Saha, 2003).

1.5.3. Other Applications of Cellulase and Xylanases

Cellulase and hemicellulases find interesting application in textile, detergent and pulp and paper industries (Campbell and Bedford, 1992; Jain, 1995; and Kulkarni *et al.*, 1999; Wong *et al.*, 1988). Today, these enzymes account for approximately 20% of the world enzyme market. Currently, most commercially available cellulase and xylanase are derived from *Trichoderma* and *Aspergillus*.

1.6. Methods of Cultivation

1.6.1. Submerged and solid-state fermentation

The production of enzyme and other commercially important products by filamentous fungi in submerged fermentation have long been established. However, in recent years studies on solid-state fermentation (SSF) have increased

significantly. SSF is defined as a culture in which a microorganism grows on a moist insoluble solid material in the absence or near absence of free water. The choice of the kind of fermentation depends on the physiological adaptation of the organism, cost and availability of the substrates. While in submerged fermentation (SmF), the microorganism is exposed to hydrodynamic forces, in SSF growth is restricted to the surface of the solid matrix. Solid-state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Tengerdy, 1998). However, both SSF and submerged fermentation have several advantages and drawbacks.

1.6.2. Advantages and drawbacks of solid state and submerged fermentation processes

The use of SSF for enzyme production offers several advantages. Some of the advantages include high productivity, extended stability of products and low production costs, water limitation of the system so that a higher product concentration and volumetric productivity can be attained. With increasing progress and application of rational methods in engineering, SSF will achieve higher levels in standardisation and reproducibility in the future. This can make SSF the preferred technique for special fields of application such as the production of enzymes and food (Holker and Lenz, 2005). However, scale up of SSF processes is the main drawback for large industrial application. On the other hand, submerged fermentation (SmF) offers several advantages due to the very well known engineering aspects such as fermentation modeling, bioreactor design and process control. It has been considered that there would not be biochemical differences between an enzyme produced by the same fungal strain in either SSF or SmF. However, it has some drawbacks. These are the product from SmF is diluted, the cost is high, and it requires sophisticated machinery and therefore expensive for developing countries.

1.6.3. Substrates used for the production of enzymes in SSF systems

Agro-industrial residues are generally considered the best substrates for SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce different enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, wheat straw, saw dust, etc. The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus require screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate.

1.6.4. Factors affecting enzyme production in solid-state fermentation systems

The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism; pre-treatment of the substrate; particle size (inter-particle space and available surface area) of the substrate; water content and water activity of the substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter; period of cultivation; maintenance of uniformity in the environment of SSF system (Pandey, 1992; 1994).

1.7. Production of hemicellulases and cellulases by termite associated *Xylaria*

The majority of microorganisms growing on plant residues in nature usually produce both cellulolytic and xylanolytic enzymes due to the close association of cellulose and xylan in plant cell walls. However, a number of microorganisms are

only able to degrade xylan (Biely, 1993). For example, *T. lanuginosus* has been shown in numerous studies to produce extremely high levels of xylanase but do not produce cellulose- degrading enzymes (Singh *et al.*, 2000; Gomes *et al.*, 1993; and Purkarthofer *et al.*, 1993). To date, there is no report whether termite associated *Xylaria* species produces lignocellulose degrading enzymes or not. The termites collect small pieces of wood from the surroundings and transport it to the mound. The collected wood pieces are decomposed and become very soft forming what is called comb. The comb is invaded by *Xylaria* mycelia. However, the benefit the termite gets from this association is not yet known. However it is well known that termite use cellulose as energy source (Wilson 1971; Breznak 1984; Wood, 1988; Wood and Thomas 1989). The cellulose is converted to glucose with the help of bacterial cellulases in the termite gut. But lignified cellulose is hardly digested by bacterial cellulases. To render it more digestible the cellulose fiber must be freed from the lignin sheeth. Lignin is cemented to the cellulose fiber with the help of hemicelluloses. Therefore, one possibility is that the fungus helps to remove lignin coat from the cellulose fiber either by selectively removing the lignin or degrading the hemicellulose component. The sugar derived from the hydrolysis of hemicelluloses may serve as energy source by the fungus, probably for the termite too. However, if the fungus hydrolyzes cellulose as energy source, it will compete with the termite for energy soure. Therefore, there is a need to know whether termite associated *Xylaria* produce hemicellulase and cellulase activity and the level of production of each enzyme.

2. Objectives

Main objectives of this study were:

* To isolate termite associated *Xylaria* species from termite mound.

*To investigate whether termite associated *Xylaria* produce cellulase and

Xylanase or not.

*To characterize the enzyme produced and evaluate their potential biotechnological application.

*To get better understanding of the ecological significance of the association between termites and *Xylaria.*

3. Materials and Methods

3.1. Isolation of the fungus. After the collection of combs from the termite mounds from Zuway area it was brought to Mycology laboratory in Addis Ababa University and humidified with distilled water. After about 4-5 days vegetative growth was observed. From this vegetative part, portion was cut aseptically and transferred to Malt extract agar plate. Mycelia grown on agar plate were serially transferred to another agar plate until the pure culture was obtained. The fungus was maintained on malt extract agar (MEA) slants at 4°C.

3.2. Extraction and determination of enzyme activity from termite comb

Fresh comb was dug out from a termite mound and transported to the laboratory in Addis Ababa. The moisture level measured in situ was 50%. Ten gram of the

comb was mixed with 50ml distilled water and vigorously shaken and centrifuged. The residue resuspended again with distilled water and re-extracted until no enzyme activity was detected. The activity of both xylanase and cellulase was tested following the standard assay condition out lined below in section 3.8.

3.3. Cultivation of the organism

To check whether *Xylaria* species produce xylanase and cellulase activities, Innoculum was made from 4-5 days old MEA culture. Two agar blocks were used as innoculum and grown using liquid medium on shaker flask and SSF at 28°C. The solid-state fermentation experiments were carried out in 250ml Erlenmyer flasks containing 10-gram wheat bran as substrate. Distilled water was added in such away that the final moisture be 50%.

3.4. Enzyme production through solid-state fermentation using different substrates

Wheat bran was purchased from the local market; other substrates like comb, Teff straw, wheat straw, baggase, and Acacia were collected locally and were chopped into pieces before used as substrate. Substrates were sterilized at 121°C for 30 minutes. All enzymes are collected totally from each substrate and assayed following the standard assay procedure out lined in section 3.8 for activity. Extraction was carried out as follows:

Wet weight was determined by using a balance. The content of the flask was totally withdrawn in blue-capped test tubes; distilled water was added and vigorously shaken followed by centrifugation. Extraction and assaying was repeated until no activity is detected in the filtrate from the supernatant (Amare Gessesse and Gashaw Mamo).

3.5. Time course of enzymatic production

Set of fermentation flasks with 50% moisture level was incubated at 28°C. One flask was taken every other day for extraction and determination of xylanase and cellulase activities.

3.6. Effect of solid to moisture ratio on enzyme production. The effect of moisture level on xylanase and cellulase production was tested by varying the wheat bran to moisture ratio within the range of 1:0.5 to 1:4 with a premise that one gram of wheat bran is equivalent to one milliliter of water as adopted from (Amare Gessesse and Gashaw Mamo, 1999). The culture was incubated at 28°C. After 10 days of incubation the culture was extracted and enzyme activity assayed following standard assay procedure.

3.7. Effect of additives on xylanase and cellulase production

Wheat bran was supplemented with different sugar sources and the effect of these additives on xylanase and cellulase production was evaluated. The additives include lactose, sucrose, yeast extracts, glucose, xylose, tryptones, at 5% w/w. After 10 days growth at 28°C, the culture was harvested, enzymes extracted as above and the level of xylanase and cellulase activities measured following standard assay procedure.

3.8. Analytical methods

3.8.1. Xylanase Activity Assay

Xylanase activity was measured using birch wood xylan as substrate. The reaction mixture in a total volume of 1ml contained 1% xylan, 50mM citrate phosphate buffer and 0.1ml appropriately diluted enzyme. Reaction mixture was incubated for 10min at 40°C. After 10 minutes, the reaction was stopped by adding 2ml DNS, and boiled for 5 minutes. Absorbance of the resulting colored

compound was measured spectrophotometrically at 540nm (Miller, 1956 as cited in Amare Gessesse and Gashaw Mamo, 1999). Xylanase activity was expressed in international unit (U) where one unit is defined as the amount of enzyme which released one micromole of xylose per minute under the assay condition.

3.8.2. Cellulase Activity Assay

CMCase assay was investigated for CMC-saccharifying activity by incubating 0.1ml of enzyme solution with 0.45ml of CMC (Carboxymethylcellulose) (1%) and 0.45ml of citrate phosphate buffer (100mM, pH 5.0) for 10 minutes at 40°C. Released sugar was estimated by dinitrosalicylic acid (DNS) reagents spectrophotometrically at 540nm. Cellulase activity was expressed in international unit (U) where one unit is defined as the amount of enzyme, which released one micromole of glucose per minute under the assay condition.

3.8.3. Optimum temperature, pH, thermostability and pH stability for xylanase and cellulase activity

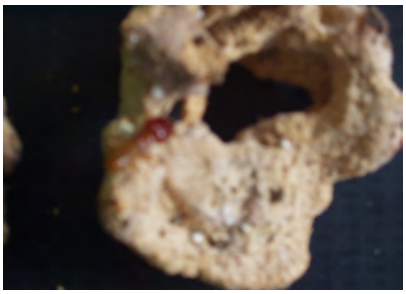
For the estimation of optimum temperature, pH, temperature and pH stability, activity was determined by carrying out the above standard assay at several temperature or pH value. To determine optimum temperature for activity, the reaction mixture was incubated for 10 minutes in the temperatures range of 30°C and 80°C. Optimum pH for activity was determined by assaying activity at different pH values (3 to 10). The buffers used were Citrate-phosphate buffer (pH 3.0 to 7.0) Tris buffer (pH 7.5 to 9.0) and Glycine-NaOH buffer (pH 10). For temperature stability, each enzyme was incubated in the standard buffer (pH 5) for 30 minutes at different temperature and the residual activities were measured following standard assay conditions. For the determination of pH stability each enzyme was mixed with different buffers of varying pH and incubated at 40°C for an hour. Residual activities were assayed following the standard assay condition.

4. Results

4.1. Isolation of the termite associated *Xylaria* species

The fungus was identified as *Xylaria,* because the pure isolate possesses all the morphological features that any *Xylaria* species should possesses. For example when it was grown on SSF in the lab, the vegetative part protrude as white, thin finger like structure (Fig. 1). As time of incubation keep on increasing, the white color was changed to black, which is some how the indicative of any *Xylaria* species in the field.

Mound of termite around Zuway



Comb

Vegetative growth

Xylaria on the plate

Figure 1. Pictorial representation of the mound of termite, comb of termites, vegetative growth and the fungus on Malt extract agar plate.

4.2. Determination of xylanase and cellulase activities from the comb obtained from the natural environment

In the natural environment the fungus produced high level of xylanase activity. However, no cellulase activity was detected (Table 1).

Table 1. Level of xylanase and cellulase activity measured from the natural comb taken from termite mound. Values are expressed in units per gram of the comb

Parameters	Amount
pH	4.6
Moisture level	50%
Xylanase	24U/g fresh weight of comb

Cellulase	Not detectable

4.3. Quantitative determination of enzyme activity

The termite associated *Xylaria* species produced xylanase activity both in liquid culture and solid-state fermentation. However, the level of enzyme production in SSF was much higher than that produced using liquid culture. Therefore, enzyme extracted from SSF was used as enzyme source for further characterization of the enzymes.

4.4. Enzyme production under SSF using different substrates

The termite associated *Xylaria* sp. was tested under SSF using different substrates. Of the six different substrates tested, only two (wheat bran and termite comb) supported significant enzyme production (Table 2). No enzyme production was detected when baggasse and teff straw were used as substrates. In the other two substrates (acacia and wheat straw), only traces of enzyme activity was detected. In this work, we did not supply any nutrients or saline solution to the growth medium, and only distilled water was used to humidify the solid substrates.

Table 2. Effect of different substrate on Xylanase and CMCase production from termite associated *Xylaria* sp. After 10 days of incubation the clear filtrate was used for assays of enzyme activities. Fermentation was carried out in duplicate Nutrient sources. Amount of enzymes produced are expressed as u/g of the moldy substrate.

Substrates	Cellulase production (U/g moldy substrates)	Xylanase production (U/g of moldy substrates)
Wheat bran	2.52	32.55

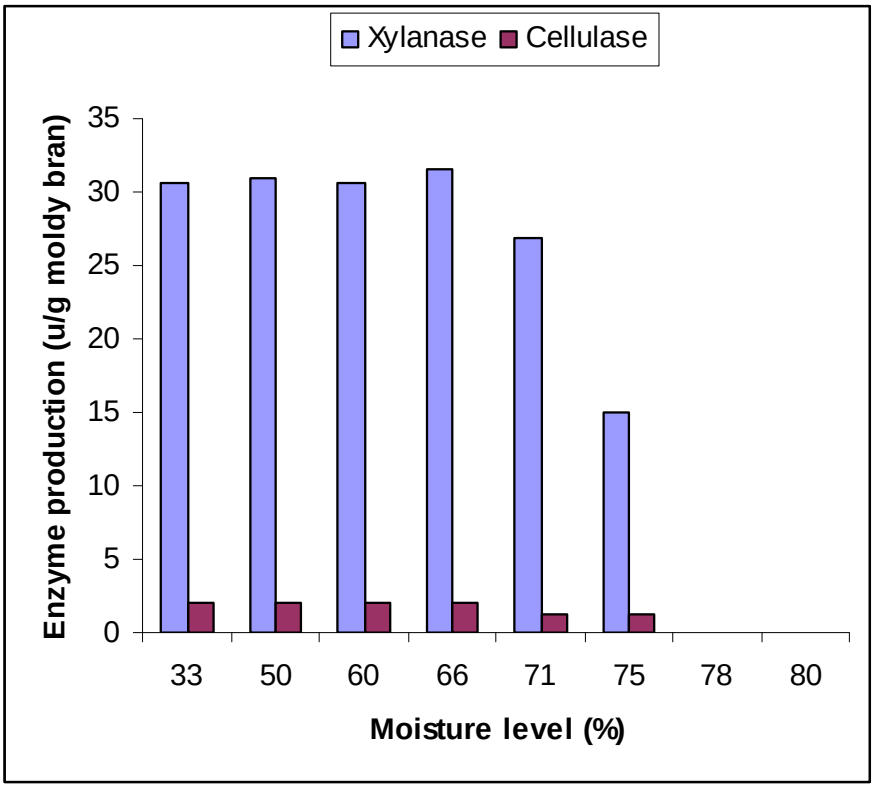
Comb	2.96	42.66
Teff straw	ND	ND
Wheat straw	0.19	0.29
Baggase	ND	ND
Acacia	0.20	0.24

NB: ND= Not Detectable

4.5. Effect of solid to moisture ratio on production of enzyme from *Xylaria* species

The moisture level had marked influence on the level of xylanase and cellulase production. Better growth and enzyme production was observed between a wheat bran to moisture ratio of 1:0.5 to 1:2 (Fig. 2). Above this growth and enzyme production decreased significantly. No enzyme production was observed at a substrate to moisture ratio of 1:3.5 and above.

A



B

Figure2. Effects of moisture content on enzyme production of the termite associated *Xylaria*: Pictorial representation (A), Graphic representation (B).

4.6. Time course on enzyme production

Maximum xylanase production was observed under SSF after 10 days of incubation at 28°c while cellulase production was more or less the same from the 6th to 12th day (Fig.3).

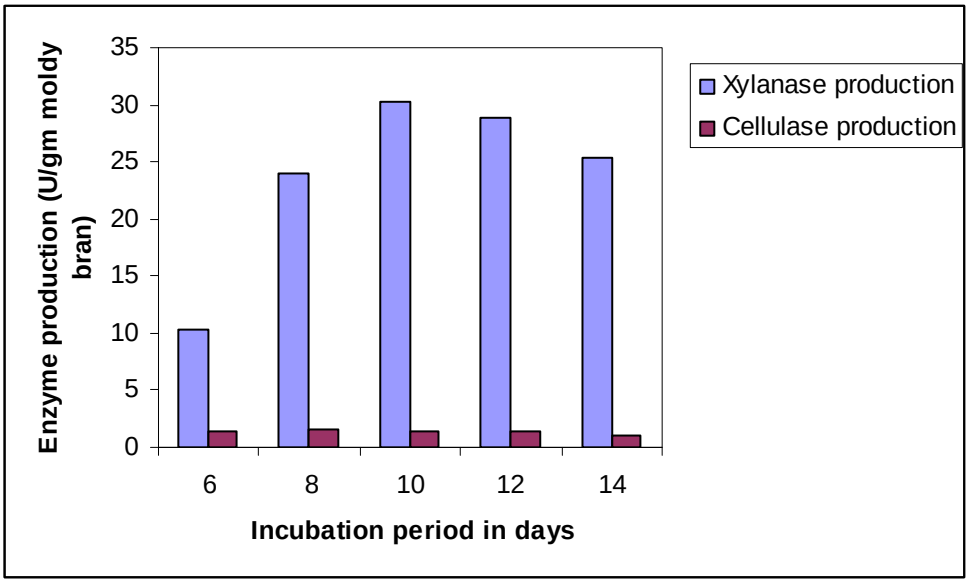


Figure.3. Time course on xylanase and CMCase production in SSF from the termite associated *Xylaria* species.

4.7. Effects of different Additives on Xylanase and CMCase production by *Xylaria* spp.

Addition of different sugars to Wheat bran didn't affect xylanase and cellulase production by *Xylaria* species. Addition of yeast extract and tryptone (5%w/w) slightly increased xylanase production but didn't have any effect on cellulase production (Table 3).

Table 3.Effects of different Additives on Xylanase and CMCase production from *Xylaria*

Additives	Xylanase production(U/g moldy bran)	Cellulase production (U/g moldy bran)

None	32.2	2.5
Xylose	32.6	3.1
Glucose	31.3	2.8
Sucrose	29.5	2.6
Lactose	31.2	2.2
Yeastextracts	39.3	2.5
Tryptone	34.9	2.4

4.8. Properties of the enzymes

4.8.1. pH profile of *Xylaria* cellulase and xylanase

The pH optima of both xylanase and cellulase were determined in three different buffers (Tris-HCl buffer, pH 7.5-9, Citrate-phosphate buffer, pH 3-7 and Glycine-NaOH buffer, pH10). *Xylaria* xylanase showed optimum activity at pH 5.0 and exhibited 95% of its initial activity at pH 5.6. Above pH 6, activity fell sharply (Fig.4).

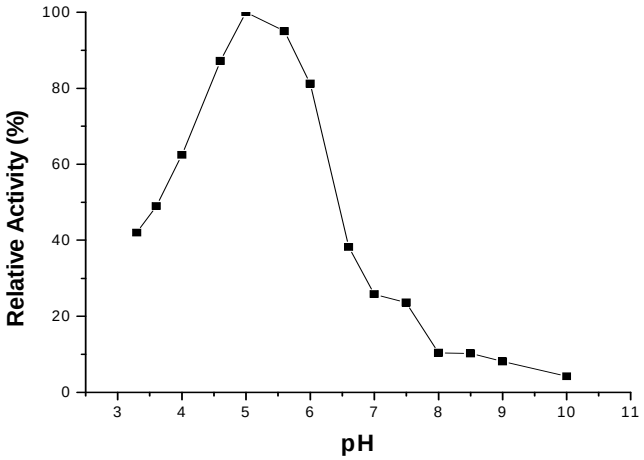


Figure 4. pH profile of *Xylaria* sp. xylanase. Activity was assayed at different pH values and at 40°c. Values given are averages of two experiments.

The optimum pH for the CMCase activity was 4.6. At pH 7.0, 71% of the maximum cellulase activity was retained. From pH 4.6 to 6 the enzyme retained more than 80% of its maximum. However, above pH 6, activity fell slowly (Fig. 5).

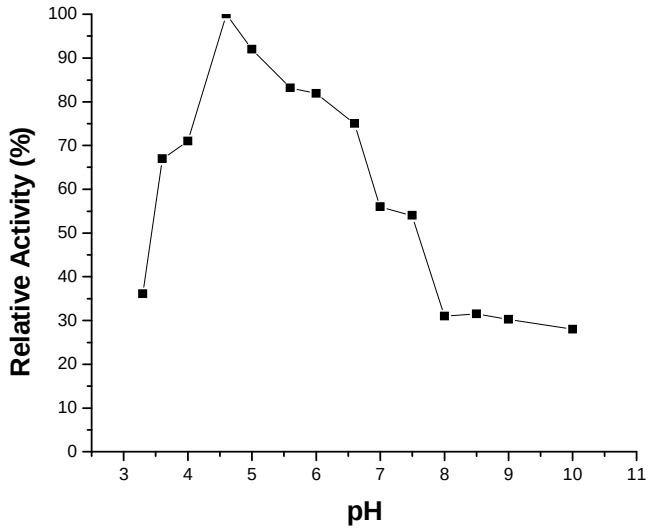


Figure 5. pH profile of *Xylaria* sp. cellulase. Activity was assayed at different pH values and at 40°c. Values given are averages of two experiments

pH stability of *Xylaria* xylanase and cellulase were determined by incubating the enzyme at 40°C for one hour in different pH values. *Xylaria* species xylanase was almost 100% stable in the narrow range of pH, 4.0 to 6.0. Above pH 6, stability of the enzyme linearly falls until it was 0 at pH 10 (Fig. 6).

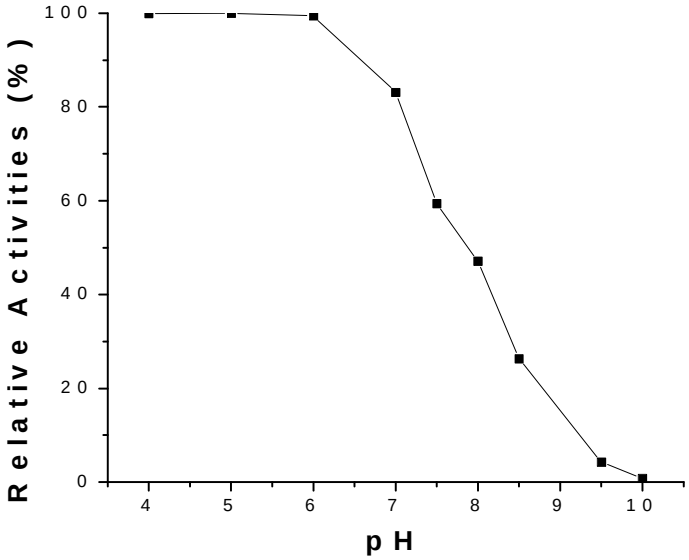


Fig.6 pH stability of *Xylaria* sp. xylanase. The enzyme was incubated for an hour at the indicated pH at 40°C. Residual activity was then measured at 40°C under the standard assay conditions. Values given are averages of two experiments

Similarly *Xylaria* species CMCase retained almost 100% of its original activity in the pH range of 4.0 to 6.0. Over 75% of its original activity was retained at pH 7.0. Above pH 7, activity fell sharply (Fig. 7).

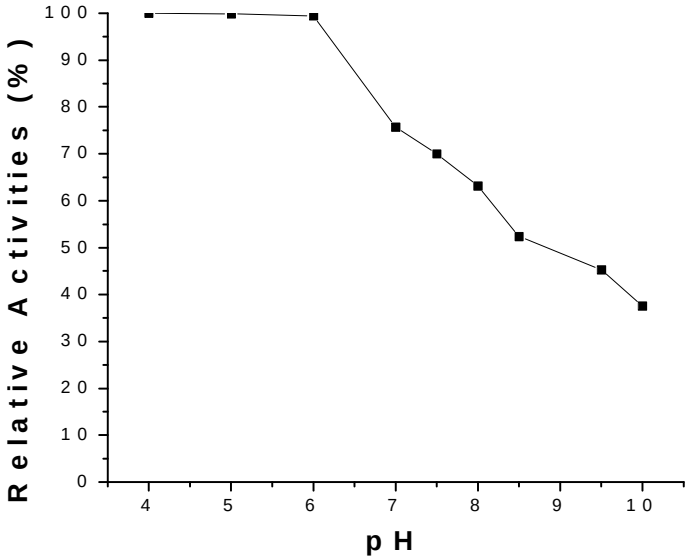


Fig.7. pH stability of *Xylaria* sp. cellulase. The enzyme was incubated for an hour at the indicated pH at 40°C. Residual activity was then measured at 40°C following the standard assay conditions. Values given are averages of two experiments.

4.8.2. Effect of temperature on the activity of Xylaria species xylanase and cellulase

Figure 8 shows temperature profile of *Xylaria* species xylanase assayed at different temperature and pH 5. Maximum xylanase activity was obtained between 50°C-55°C. About 80% of the maximum activity was retained at 60°C. Above 60°C the activity was fallen sharply.

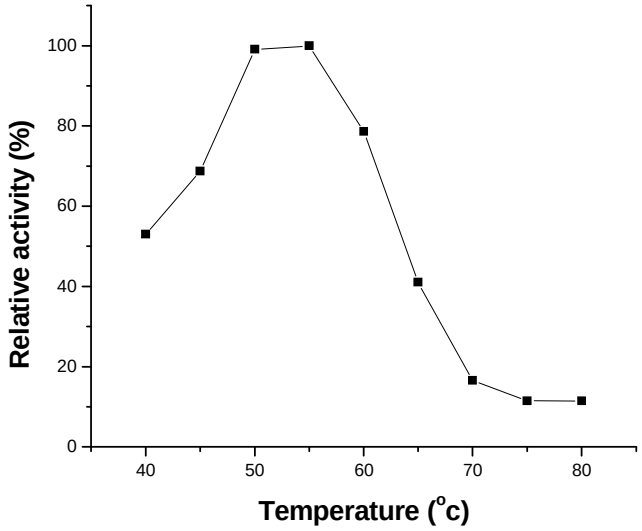


Fig 8.Temperature profile of *Xylaria* sp. Xylanase. Activity was measured at different temperatures in 50 mM citrate-phosphate buffer pH 5. Values given are the averages of two experiments.

On the other hand, the optimum temperature of the CMCase was 60°C. Between 60°C and 70°C, the activity fell sharply. Up to 80°C, the enzyme retained more than 40% of its maximum activity (Fig.9).

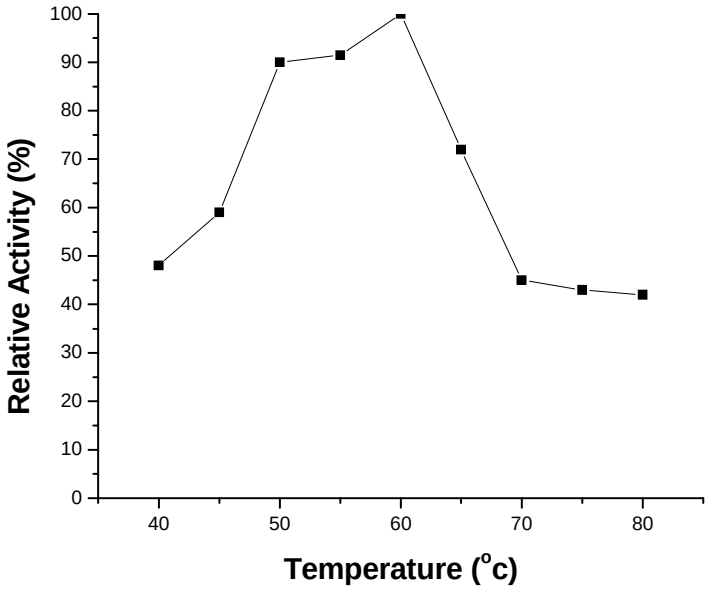


Fig 9. Temperature profile of *Xylaria* sp. cellulase. The enzyme was incubated at different temperatures in 50 mM citrate-phosphate buffer (pH 5). Activity was then measured following the standard assay conditions. Values given are averages of two experiments.

4.8.2.1. Effects of temperature on the stability of cellulase at pH 4 and 5

Thermostability was studied by incubating the enzyme at different temperatures (40°C-75°C) for 30 min at pH 4 and 5. At both pH values the enzyme retained more than 70% of its original activity at 55°C and below. Above 55°C enzyme stability was falling sharply. Above 50°C, the enzyme showed better stability at pH 4 than pH 5 (Fig. 10).

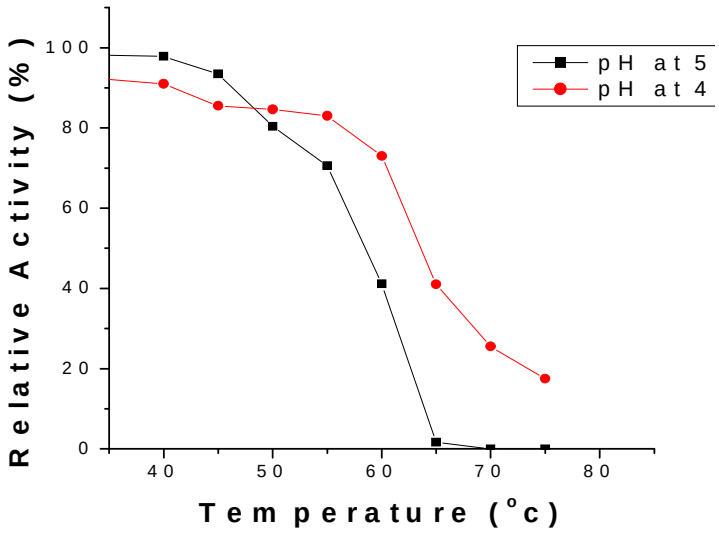


Figure 10. Temperature stability of *Xylaria* sp. cellulase. The enzyme was incubated for 30 min at different temperatures in 50 mM citrate-phosphate buffer (pH 4 and 5). Residual activity was then measured at 40°C following standard assay conditions. Values given are averages of two experiments.

4.8.2.2. Effects of temperature on the stability of xylanase at pH 4 and 5

Thermostability of *Xylaria* species xylanase was studied by incubating the enzyme at different temperatures (40°C-75°C) for 30 min at pH 4 and 5. At both pH values the enzyme retained more than 50% of its original activity at 50°C and below. Above 50°C enzyme stability was falling sharply (Fig. 11).

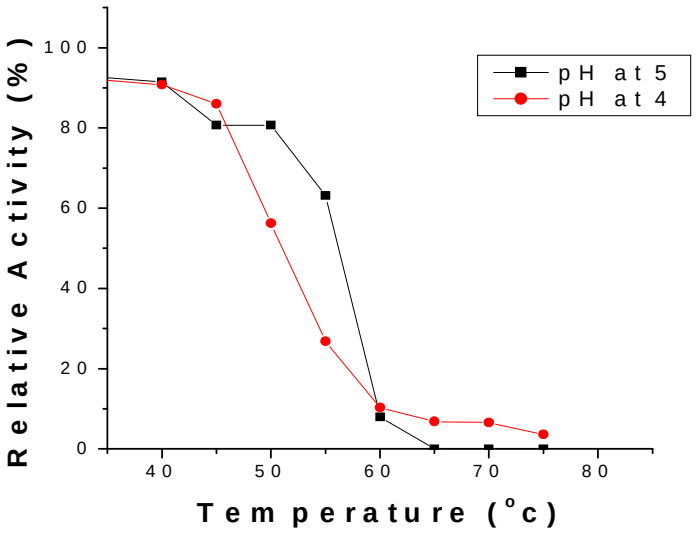


Figure 11. Temperature stability of *Xylaria* sp. xylanase. The enzyme was incubated for 30 min at different temperatures in 50 mM Citrate-Phosphate buffer (pH 4 and 5). Residual activity was then measured at 40°C under the standard assay conditions. Values given are averages of two experiments.

5. Discussion

Xylaria species is known to be associated with termites. However, the benefit the termite gets from this association is not known. Termites collect wood pieces from the surrounding area and accumulate it in the mound. It is known that termites feed on the cellulose because termites harbor cellulose degrading bacteria in their gut. But lignified cellulose is hardly digestible. Therefore we suggested that the fungus might play a role in removing the lignin sheath either through selective degradation of lignin or through degradation of xylan, which bind the lignin with the cellulose fiber. To test this we recovered comb from termite mound and extracted the protein. When the extract was assayed for activity a high level of xylanase activity was found while no cellulase activity was detected (Table1). This probably indicates that the fungus plays a role in removing the lignin from cellulose fiber by degrading the hemicellulose fraction that cements the lignin to the cellulose fiber. The delignifed cellulose fiber will be taken up by the termite and easily digested by bacterial cellulases in the gut. The fact that there is no detectable cellulase activity shows that the fungus is not competing with the termite for cellulose. But earlier there was a hypothesis by (Batra and Batra, 1979), proposing that *Xylaria* is fast growing, because of rapid uptake of cellulose digestate and thus suggest competition between the fungus and the termite.

The moisture content of the comb immediately after it was recovered from the termite mound was 50%. When the comb was moistened and kept in the lab, vegetative part emerged after 4-5 days from which the fungus was isolated and identified as *Xylaria* species. The fungus was then grown in liquid culture and solid-state fermentation using wheat bran as substrate. Compared to SmF, the fungus produced more enzymes under SSF. This probably indicates that SSF provides the fungus with an environment closer to its natural habitat as reported by Roberto da *et al.,* (2005).

Both xylanase and cellulase from *Xylaria* spp. show higher activity in the acidic pH range. Xylanases that are active at a temperature around 40°C and pH around 4.8 have good potential application as animal feed supplements (Tony *et al.,* 2005). Enzymes with such characters can easily adapt to the condition existing in digestive tract. Therefore, the xylanase from *Xylaria* sp. possess properties which is ideal for animal feed supplement. Moreover, the fact that the organism produce high level of xylanase activity using wheat bran in SSF further increase its potential application. This is because the whole SSF culture can be dried, grounded, and blended with other feed. Thus, in addition of supplying xylanase, the fungal mycelia could serve as a protein source. But there is a need to study the nutritional value and safety of such formulation.

Optimum temperature of xylanase extracted from the termite-associated *Xylaria* species was 55°C. This is similar to xylanases from *T. reesei* and *Penicillium* species but lower than temperature optima of the xylanase of *T. terrestris* and *S. celluphilum* (Roberto da *et al.*, 2005).

The result of the CMCase show that the enzyme has 97.8% of its maximum activity at 40°C and 93.5% of its maximum activity at 45°C treatment for 30 minutes and started to undergo significant denaturation from this temperature on wards.

Of the six substrates tested only the termite comb and wheat bran supported good growth and enzyme production under solid state fermentation. In nature termites collect acacia from which it forms the comb. But in the lab it didn't support growth and enzyme production. As no supplement was included, Acacia may not supply all nutrients required for growth. This probably indicates that in nature termite may supply required nutrients, for example nitrogen for the fungus in the form of excrements. That is probably why comb support the best growth and enzyme production. The probable reason why wheat bran supported good growth and enzyme production under SSF could be that it contains all the necessary

nutrients to the requirements of the fungus. Many studies also showed that wheat bran contains a good balance of nutrients to support growth of many organisms (Amare Gessesse and Gashaw Mamo, 1999).

Comparing the level of production of the two enzymes, the level of xylanse production is about 13 to 14 fold higher than cellulase. The fact that the fungus produces high level of xylanase activity but very low level of cellulase activity under the laboratory condition and the fact that no cellulase activity was extracted from the termite comb (Table 1) has ecological significance. The fungus produce an extracellular enzyme especially hemicellulases (xylanase) which has the capacity to digest or degrade the xylan part of the wood pieces collected by termites and frees the cellulose from lignin to be taken up by the termite. On the other hand, no cellulase production under natural condition and little cellulase production under lab condition by the fungus suggest that there is no competition between the fungus and the termite for cellulose. Therefore, this could mean that *Xylaria* species preferentially produce high xylanase activity and hence is more favored fungal symbiont than other microorganisms in the mound to live with the termite. Production of little or no cellulase compared to xylanase is not unique to *Xylaria* species. Other fungi, such as *T. lanuginosus* are known to produce xylanase activity but not cellulase activity (Biely, 1993; Singh *et al.*, 2000; Gomes *et al.*, 1993; and Purkarthofer *et al.*, 1993).

The moisture level of the comb, measured immediately after recovery from the mound, was 50 %(or a substrate to moisture ratio of 1:1). In the lab maximum growth and enzyme production under SSF was obtained at a substrate to moisture ratio of 1:0.5 to 1:2.

It seems that the slight wet of the medium increases substrate porosity and facilitates utilization of the medium by the organism. If the substrate is too moistened, the substrate porosity decreases which prevents oxygen penetration. Cell growth and oxygen consumption rate increased in conjunction with an

increase in moisture content (Kim *et al.,* 1985). However, the enhancement of enzyme production occurred up to a certain moisture level then leveled off.

The ability of the fungus to grow and produce enzyme activity in the substrate to moisture ratio of 1:0.5 to 1:2 may have important implication for the association between the fungus and the termite. The termite mound is very resistant to flooding or from attack by rain. However, during the dry season the moisture level in the mound may fall down. But, the fungus grows and produces the required enzymes at a substrate to moisture ratio of as low as 1:0:5 (33% moisture). During the rainy season, moisture level may rise, but *Xylaria* optimally grows and produces enzymes up to substrate to moisture ratio of 1:2 (66% moisture level). This probably offers flexibility for the fungus depending on the season. Therefore, normal life could continue in the mound.

Furthermore, the fact that the fungus produces enzyme even at low level of moisture may be advantageous in decreasing the risk of contamination or competition by bacteria or other group of microorganisms in the soil which are known to require high level of water activity. This may be one reason why this fungus is dominating in the termite mounds. Reports in the literature also show that many *Xylariceous* species in the tropics do posses a kind of adaptation to conserve water (Whalley, 1996).

Maximum xylanase production by *Xylaria* was reached on the 10th day, extended almost until the 12th day, and then, started to decline. However, some fungi like *A. ochraceus* when grown in SSF require up to 14-day fermentation for maximum xylanase production (Biswas, *et al*., 1988; 1990). Therefore, from application point of view, reduction in fermentation time will reduce the production cost of the enzyme.

6. Conclusions

Termite associated *Xylaria* species was isolated from termite mound. Protein extract from termite comb showed high xylanase activity and no detectable cellulase activity. This probably indicates that the fungus plays a role in removing the hemicellulose fraction from wood. In its native form cellulose fibers are found sheathed with lignin. Lignified cellulose is not digestible by cellulase in the termite gut. Because the lignin is cemented to the cellulose fiber with hemicelluloses, removal of hemicelluloses frees the cellulose fiber from lignin. The delignified cellulose could then be taken up by the termite and easily digested by the gut microflora.

The fact that no detectable cellulase activity from the comb may indicate that the fungus and the termite are not competing for cellulose.

In the laboratory the fungus produce high xylanase activity and low cellulase activity using solid-state fermentation.

The xylanase was optimally active in the acidic pH range (4 to 6) and temperature of 40°C. These properties make it ideal as animal feed supplement. The organism grows and produces high enzyme activity using SSF with wheat bran as substrate and at a moisture level of as low as 33%. This could offer the possibility to easily dry the SSF culture and blend it with animal feed. While the enzyme helps to degrade antinutritive components in the feed, the fungal mycelium may serve as a protein source. However, further work is needed to evaluate this possibility as well as the nutritive value and safety of such formulations.

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