ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Department of Biology

Xylanase production by the termite associated fungus,

***Termitomyces* sp. and its role in the termite nest**

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial fulfillment of the Requirement for the Degree of Master of Science in Biology (Applied Microbiology)

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July, 2007

1. INTRODUCTION

About 40 – 60 million years before the advent of human agriculture, three insect lineages: termites, ants and beetles independently evolved the ability to grow fungi for food. Today out of the total > 2,600 species of termites, 330 are known to form an obligate symbiosis with specialized fungi (Ulrich and Nicole, 2002).

1.1. Fungus – cultivating termites

Termites that cultivate a fungus in their nest are known as “fungus – farming” termites (subfamily *Macrotermitinae*). Such termites are distributed through out tropical Africa and Asia, where they are the dominant soil invertebrates (Yoavapa *et al*., 2005).

Colonies of fungus – growing *Termitinae* termites are among the most impressive animal phenomena in the world. As described by Shellman (1997), they can have nest volumes of thousands of liters, may persist for decades and contain millions of sterile helper individuals.

1.2. Fungus garden in termite mound

Termites cultivate the symbiotic fungus on a special structure found in the mound called fungus garden or termite comb. Termite comb is a structure which is made from macerated wood materials gathered by foraging workers that is chewed up and swallowed. When the foragers return to the nest, they evacuate this material very quickly as pseudofeces, passing it on the nest workers which take this material and mold in to the fungus comb (Yoavapa *et al*., 2005).

1.3. *Termitomyces* sp. in termite mound

Vast numbers of fungi are associated with a variety of insects and other arthropods to form symbiosis of various types. According to Richard *et al*. (2000) 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 may be dispersed by their hosts.

The most common types of fungi known to be associated with termites are the Basidiomycetes; *Termitomyces* and Ascomycetes; *Xylaria* (Rogers, 1979).

Figure 1.The mushroom *Termitomyces (source; Tobias, 2000)*

Termitomyces is a paleotropical genus of the oder agaricus. As the name might suggest the species are considered obligate symbionts of termites belonging to the subfamily *Macrotermitinae*. Its mycelium grows on the comb which is made from fecal pellets. As described by Tobias (2000), there are more than 60 *Termitomyces species* described, but reliably only 18 species, collected mainly from West Africa, are reasonably well known, the rest are either synonymies or badly described and difficult to identify. Dawit Abate (1998) also described that these mushrooms are commonly found in Ethiopia as they are in other parts of Africa.

In general, *Termitomyces* have pinkish spores, a cap and stipe at the top of a long 'pseudorrhiza' that arises from the comb and the cap has 'a perforatorium' or 'umbo' that assists the mushroom to penetrate the hard ground (Pegler, 1977). *Termitomyces* produce the mushroom cap of fruit body on the long stalks, when there is rain fall of more than 20 cm/day (Tobias, 2000). As reported by Dawit Abate (1998), the fruiting bodies of these mushrooms have a short season, and are highly perishable.

Termitomyces titanicus (the largest mushroom in the world according to the Guinness book of records) has a cap diameter of up to 1 meter, where as *Termitomyces microcarpus* rarely exceeds 2 cm (Tobias, 2000; Dawit Abate, 1998).

On the termite comb white-spherical yeast-like colony fungal structures are commonly observed. These structures of *Termitomyces* have been described by various authors under different names. Heim (1977) proved these structures exclusively as sporodochia which are found on the combs of *Macroterminae* and represent the anamorph of the genus *Termitomyces*.

Figure 2. Sporodochia (white dots) growing on the termite comb (Turner, 2002).

As reported by Naoya *et a*l. (2005), *Termitomyces* can be isolated by picking the nodules; spherules (sporodochia) formed on the surface of termite comb.

Cultures of *Termitomyces* can also be established from the basidiospores (Dawit Abate, 1998). Botha and Eicker (1991) described the growth characteristics of different species *o*f *Termitomyces* and they identified it as slow grower. Several growing media such as oat meal, sabouraud glucose, malt extract and yeast extract support the growth of most species of *Termitomyces* (Naoya *et al.,* 2005).

1.4. Relationship of *Termitomyces* and *Xylaria* in termite comb

As *Termitomyces*, the genus *Xylaria* is common in fungus comb and it is widely distributed. Even though the saprobic genus, *Xylaria* is associated regularly in combs with *Termitomyces*, the relationship of their association in the comb is not well known. In vitro, *Xylaria* is fast grower than *Termitomyces* (Turner, 2002).

Although *Xylaria* is fast grower, its growth is suppressed in normal fungus combs (Wood and Thomas, 1989). Fungus combs taken out from the mound can become covered with a dense mat and stromata of *Xylaria*, which soon smoothers the *Termitomyces* growth (Turner, 2002).

1.5. Xylan and its structure

The naturally occurring lignocellulosic plant biomasses consist of 20-30% hemicellulose materials which are heterogeneous polysaccharides found in association with cellulose (Kulkarni *et al.,* 1999). These polysaccharides are linear or branched polymers, easily hydrolyzed by mineral acids and give a variety of sugars up on hydrolysis (Amare Gessese, 1998; Suurnakii *et al.,* 1997). The two main hemicelluloses in wood are xylans and glucomannans.

Xylan is the major constituent of hemicelluloses and is the second most abundant renewable resources with a high potential for degradation to useful end

products (Kulkarni, *et al.,* 1999). The β-1, 4-xylans are hetero-polysaccharides with a homo-polymeric back bone chain of 1, 4-linked β-D-xylopyrnose units. The back bone consists of O-acetyl, L-arabino furanosyl, and α-1, 2-linked glucuronic or 4-O-methy glucuronic acid substitutes (Shalom and Shoham, 2003). Xylan is found in large quantities in hard woods than in soft woods (Srinivasan, 1992). The degree of polymerization of hard wood xylans (150-200) is higher than that of soft woods (Kulkarni *et al*., 1999).

Many structural aspects of xylans are unclear because of the difficulties associated with the isolation of xylans from natural raw materials with out significant alternation or loss of the original structure and association with other components (Srinivasan, 1992). Based on the common substitutions found on the back bone, Kulkarni *et al*. (1999) described the chemical structure of xylan as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan.

The three-dimensional structure of xylan molecules has been elegantly described in Kulkarni *et al*. (1999). The xylan back bone shows a three fold left-handed conformation under crystallized conditions and the geometry of the glycosidic linkage is not affected by the side chains. The three – dimensional structure data on xylan, in aqueous environment, is extremely important in understanding the xylan and xylanase interactions.

1.6. Xylanases

Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes. Of all the enzymes involved in the hydrolysis of xylan, attention was mainly focused on the study of xylanases and to some extent *beta*-xylosidases.

Xylanases are glycosidases (O – glycoside hydrolases) which catalyze the endo-hydrolysis of 1, 4 – β- D- xylosidic linkages in xylan. Endo 1, 4- xylanases

randomly cleave the xylan back bone, β- D- xylosidases cleave xylose monomers from the non – reducing end of xylo – oligosaccharides and xylobiose while removal of the side groups is catalysed by α-L-arabinofuranosidase, D-glucuronidases, acetyl xylan esterase, ferulic acid esterases and p-coumairic acid esterases (Collins *et al*., 2006).

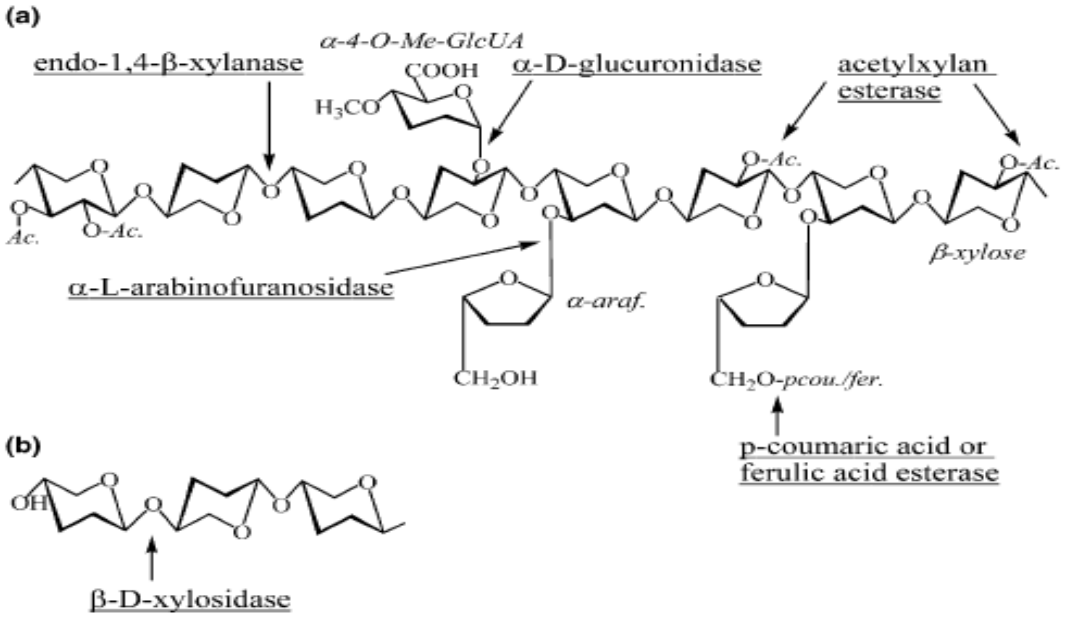

Figure 3. A). Structure of xylan and the sites of its attack by xylanolytic enzymes. B) Hydrolysis of xylo-oligosaccharides by *beta*-xylosidases. Adapted from (Collins *et al.,* 2006).

The main products of the reaction catalyzed by endoxylanase are xylo-oligosaccharides of different chain length which thus produced are acted up on by *β*-xylosidase to produce xylose (Amare Gessese, 1998).

In addition to the production of a variety of xylanolytic enzymes, many microorganisms produce multiple xylanases. These may have diverse physiochemical properties, structures, specific activities and yields, as well as over lapping but dissimilar specificities, there-by increasing the efficiency and extent of hydrolysis, but also the diversity and complexity of the enzymes. This multiplicity may be the result of genetic redundancy, or post – translational processing (Collins *et al*., 2006).

1.6.1. Xylanase producing microorganisms

Microorganisms are rich sources of xylanase enzymes, which are produced by diverse genera and species of bacteria, actinomycetes and fungi. Although xylanases are produced by fungi, yeast and bacteria, filamentous fungi are preferred for commercial production as the levels of the enzyme produced by fungal cultures are higher than those obtained from yeast or bacteria (Pandey *et al*., 1994). In many microorganisms xylanase activity has generally been found in association with cellulases, *β*-glucosidases or other enzymes (Collins *et al.,* 2006).

1.6.2. Factors affecting xylanase production

The yield of xylanases in a fermentation process is governed by a few key factors in addition to the standard parameters. When xylanase fermentation is carried out on complex heterogeneous substrates, various factors have a combined effect on the level of xylanase expression. They include substrate accessibility, rate and amount of release of the xylo-oligosaccharides and their chemical nature and quantity of xylose released, which acts as the carbon source and as an inhibitor of xylanase synthesis in most of the cases (Kulkarni *et al*., 1999). pH, temperature and agitation also affects xylanase yield (Kulkarni *et al*., 1999; Collins *et al*., 2006).

1.6.3 Biotechnological Potentials of xylanases

Xylanases have high industrial potential and used in various processes. The different areas of application of xylanases are summarized as follows.

1.6.3.1. Animal feed supplement:- Although cereals like wheat, barley, triticale, rye and oats are commonly used as animal feed, they contain relatively high proportion of anti-nutritive carbohydrates like *beta*-glucon, pentosans and arabinoxylan which are collectively known as non-starch polysaccharides (NSPs) (Friesen *et al*., 1992). The content of NSPs in the diet is inversely related to the apparent metabolizable energy (AME) of wheat (Annison and Choct, 1991).

A lot of feed additives are being currently used and new concepts are continuously developed. Enzymes as additives to animal feed have had a great impact on the livestock industry. As reported by Amare Gessese (1998), xylanases, proteases, phytases and amylases are found important in such industry.

The use of xylanase in poultry feeds has predominantly been related to the hydrolysis of fiber or NSP fractions in cereal grains. These NSPs can not be digested by the endogenous enzymes of poultry and can have anti-nutritive effects (Friesen *et al*., 1992). The intestinal viscosity caused by water soluble NSPs dramatically reduces the nutritive value of the feed (Annisoon and Choct, 1991). Feed additive that contain xylanase help to such anti-nutritive factor or NSPs thus liberating the nutrients for easier digestion, absorption and utilization (Zhang *et al.,* 1996). Endo – *beta*- D-xylanase (xylanase) is added to feeds to catalyze depolymerization of this polysaccharide. McCleary (2004) demonstrated that endo-cleavage by xylanase of just one bond per thousand in the arabinoxylan back bone can significantly remove viscosity properties.

Xylanase also changes the hemicellulose to sugars so that nutrients formerly trapped with in the cell walls are released. This means the chickens get sufficient energy from less food (Zhang *et al*., 1996).

Xylanase could also improve the quality of the environment by reducing the out put of excreta and pollutants, such as phosphate and nitrogen, including ammonia (Petterson and Aman, 1989). In addition, chickens eggs become

cleaner because the excrement in the laying area is drier (Classen and Bedford, 1991).

Although xylanases have proven to be highly beneficial, the use of xylanase in animal fed is still in its infancy. Most of the problems should be solved before their full potential is realized. As explained in Amare Gessese (1998) supplementation of xylan- rich feed with xylanase doesn't completely eliminate the stickiness of the litter, which may show that no complete depolymerization takes place. Therefore searching xylanases having better efficiency under the condition of the animal gut should be of an interest.

1.6.3.2. In baking industry:- Xylanases were introduced to the bakery industry in the 1970s and they are currently used frequently in combination with amylases, lipases and various oxido-reductases (Elliot, 1996). Xylanase enhances the dough and bread quality leading to improved dough flexibility, machinability and stability and a larger loaf (10%) volume as well as an improved crumb structure(Collins *et al.,* 2006). Flour generally consists of approximately 80% starch and 12% proteins with arabinoxylan content varying from 2- 3% in wheat flour and this small amount is an extremely important functional ingredient as it can bind almost 10 times its own weight with water, accounting for almost 30% of the water binding capacity of wheat flour (Elliot, 1996). The exact mechanism of the functionality of xylanases in bread making is not yet fully elucidated, but currently believed that the redistribution of water from the arabinoxylan in the flour to the starch and gluten phases is important (Collins *et al.,* 2006).

1.6.3.3. In beer making:- Xylanase has the ability to break the hemicelluloses down in to sugars. This indicates extraction of more fermentable sugars from barley for making beer. It also helps processing the spent barley for animal feed and in addition, added xylanase can reduce the viscosity of the brewing liquid improving its filterability (Mohagheghi, 1986).

1.6.3.4. Treating plant wastes: - Annually a large amount of xylan containing waste is released in the form of industrial, agricultural and municipal wastes. Treating plant waste by xylanase dissolves the xylan and reduces the amount of organic wastes that should be disposed in to the land fill. As reported by Amare Gessese (1998), this treatment might not be efficient in treating the plant waste as it is composed of complexed polysaccharides. Therefore the use of other plant polymer degrading enzymes together with xylanase might involve in efficient hydrolysis of lignocellulosic wastes.

1.6.3.5. Other applications of xylanase: - Xylanases also have potential applications in many industrial processes. Xylanases were found to be important in the process of Kraft pulp bleaching for substantial reduction in the level of chlorine bleach used in subsequent bleaching operations, with concomitant reduction in the level of chlorinated organic compounds (Amare Gessese, 1998; Vikari *et al., 1994)*. In the production of fuel alcohol, xylanase decreases the viscosity of the mash and prevents fouling problems in distilating equipment (Elliott, 1996). Xylanases, along with cellulase and pectinase also aids in the maceration (chewing up) of fruit and vegetable materials which decreases the viscosity of the juice or woin. Xylanases could also be used to improve retting of flax fibers. Retting is the decomposition of the outer stem of the flax plant, necessary before the fibers are processed in to linen (Mohaghehi, 1986). Others also described xylanase as it improves the performance of detergents that are especially effective in cleaning grass stains.

1.7. Solid state fermentation for the production of industrial enzymes

Solid state fermentation (SSF) is defined as any fermentation process performed on a non – soluble material that acts both as physical support and source of nutrients in absences of free flowing liquid (Pandey 1992). 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 as the enzyme source (Pandey *et al.*, 1994).

The hyphal mode of fungal growth and their good tolerance for low water activity (aw) and high osmotic pressure conditions make fungi efficient and competitive in natural micro-flora for bioconversion of solid substrates (Pandey *et al.*, 1994). The selection of a suitable strain for the required purpose depends up on a number of factors, in particular up on the nature of the substrate and environmental conditions (Nigam and Singh, 1994). Therefore the selection of a particular strain remains a tedious task, especially when commercially competent enzyme yields are to be achieved.

Agro-industrial residues are generally considered the best substrates for the SSF processes. Some of the substrates that are commonly used for SSF include; sugar cane bagasse, wheat bran, rice bran, maize bran, wheat straw, saw dust, corn flour, wheat flour and pre-treated willow (Pandey *et al*.,1994). The selection of substrate for enzyme production in a SSF processes depends up on several factors mainly related with cost and availability of the substrates, and thus may involve screening of several agro-industrial residues (Doelle *et al.,1997*). Pandey *et al*. (1994) described wheat bran as the key and most commonly used substrate.

The use of SSF for enzyme production offers several advantages over submerged fermentation (SmF). Some of the advantages include high productivity; low Production costs, low risk of contamination due to the inability of

many organisms at low water activity and it doesn't required complex machinery, equipment and control systems (Kheng and Omar, 2005; Maucrice, 1998). SSF seems to have theoretical advantage over SmF. Nevertheless, SSF has several limitations. The most significant problem of SSF is the high heterogeneity, which makes difficult to focus one category of hydrolytic processes, and lead to poor trials of modeling (Maucrice,1998) , labour intensive , lack of uniformity in the substrate, limitation on the choice of the type of the SSF (Pandey,1992).

In general, 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 surface area) of the substrate.

1.8. Benefits of termite-fungus association

Termites feed and grow on wood which contains lignified cellulose (Sands, 1977). However, they have only limited ability to digest lignified cellulose (Wood and Thomas 1989; Wood, 1988). Cellulose is consumed and digested with the help of cellulolytic gut microflora of the termite (Breznak, 1982). However, cellulases produced by those gut microflora are not capable of digesting the native cellulose because in its native form cellulose microfibriles are embedded in a sheath of lignin and hemicelluloses making the cellulose inaccessible for cellulases (Amare Gessesse, 1998). Lignin is cemented to cellulose fibers with the help of hemicelluloses. Thus to make the cellulose digestible the termite gut microflora need to remove either the lignin and/or the hemicellulose fraction. However, in the termite gut no lignin degrading microorganism and very few xylanolytic microorganisms have been reported (Ajit *et al*., 1994). This indicates that probably the termite ingest a delignified cellulose. Termites form an obligate association with *Termitomyces* and some *Xylaria species.* To date the benefit the termite get from the associated fungi is not clearly known.

One hypothesis we had was that these fungi help to remove the lignin sheath from cellulose by removing the hemicellulose component of the lignified celluloses which thus enable the termite to consume delignified cellulose.

Lignocelluloses which termites feed on are very poor in protein (Naoya *et al.,* 2005) and yet they survive with it. Thus another hypothesis we had was that the fungal mycelia could serve as a protein source.

1.9. Objectives of the study

1) To study whether *Termitomyces* is associated with termites in the Rift Valley area of Ethiopia.

2) To examine the role of the associated fungal species in the termite nest

3) To grow *Termitomyces,* characterize its enzyme and evaluate its potential for industrial application

2. Materials and Methods

2.1. Chemical and physical characteristics of termite comb

Ten g of fresh comb was grounded to a powder, dissolved in 100 ml distilled water and centrifuged. pH and enzyme activity was measured from the supernatant. Enzyme activities measured include; xylanase, cellulase, protease and pectinase.

To measure the moisture content, the weight of the comb after complete drying and getting a constant weight in 105^0C oven was subtracted from its previous fresh weight. The reduced weight indirectly indicates the amount of moisture level in the comb.

Total nitrogen amount of the comb was measured following the Modified Kjeldhal method described by Sahlemedhin Sertsu and Taye Bekele (2000).

Soluble protein content was measured following the method of Lowry *et al.* (1951) with BSA (bovine serum albumin) used as standard. The concentration of reducing sugar from the comb was measured by a colorimetric assay (Miller, 1959) using xylose as standard.

2.2. Isolation of *Termitomyces*

Samples of termite comb were taken from a termite mound around Nazareth in rift valley area of Ethiopia using sterile glass bottles. Isolation of the organism was performed following the method used by Botha and Eicker (1991). *Termitomyces* sporodochia (white dots) (Fig. 4) obtained on the termite comb were aseptically transferred to MEA plates, and incubated at 28^0C for15 days. Sub-cultures were made on the same medium and the organism was maintained at 4^0C on MEA slants for further investigation.

2.3. Growth of *Termitomyces* in liquid medium

Termitomyces was grown in liquid medium containing (g/l) (KH_2PO_4 0.3 g, $MgSO_4.7H_2O$ 0.12 g, 1% $FeCl_3$ 6.6ml, $CaCl_2.2H_2O$ 0.066 g, NH_4NO_3 0.5 g), and carbon source (0.5 g). The carbon sources used were CMC and Birch wood xylan. The culture was inoculated with *Termitomyces* and incubated at room temperature on rotary shaking. After 23 days of incubation a sample was removed, centrifuged and the clear supernatant was used as enzyme source.

2.4 Solid state fermentation

2.4.1 Growth of *Termitomyces* on solid substrates

To 10 g of wheat bran in 250 ml Erlenmeyer flask, 15 ml stock mineral salt solution (KH_2PO_4 0.05g, $MgSO_4.7H_2O$ 0.02g,NH_4NO_3 0.1g, $CaCl_2.2H_2O$ 0.01g and 1ml of 1% $FeCl_3$) was added and sterilized by autoclaving and inoculated with agar blocks of *Termitomyces*. After 10 days of incubation at 28^0C, the culture was extracted by adding 100 ml distilled water, filtering and centrifuging. The filtrate was used to assay enzyme activity.

To test enzyme production using other subtrates wheat bran was replaced by 10 g of termite comb, teff straw, wheat straw, sugar cane bagasse, saw dust, and bean straw. After 10 days of incubation at 28^0C the SSF was extracted until no enzyme activity was detected in the filtrate and enzyme activity was detected using the standard procedure.

2.4.2. Time course of enzyme production

To determine the time for maximum enzyme production, *Termitomces* was grown in SSF and samples were taken at 2 days interval, and the extracted enzyme was measured following the standard procedure.

2.4.3. Moisture content of the solid media

The effect of moisture level on enzyme production was studied by varying the percentage of water in the medium from 36% to 72%. All the liquid added in to the flask and moisture content of the wheat bran (4%) was taken in to consideration in calculating the percentage of water (w/w) in the medium. After 10 days of incubation the enzyme was extracted and assayed following the standard assay procedure.

2.4.4. Effect of additives for enzyme production

Wheat bran was supplemented with different carbohydrates as carbon source at a concentration of 5% (w/w) and the effect of these additives on the level of xylanase production was evaluated. The additives include xylose, glucose, yeast extract, lactose, tryptone and termite comb extract. The enzyme was extracted on the optimum time of growth and its activity was measured following the standard assay procedure.

2.5. Enrichment of wheat bran for feed application

Termitomyces species was grown on wheat bran with 58% moisture level at 28^0C for 10 days. The content of the flask was then air dried and powdered. The protein content, xylanase and protease activity of the dried mouldy bran powder was analyzed following the standard procedure.

2.6. Enzyme assays

Xylanase activity was determined by measuring the amount of reducing sugar released from xylan following the dinitrosalicylic acid (DNS) method (Miller, 1959).The reaction contained 1% birch wood xylan in 0.2 M acetate buffer pH 5.0 and 100 µl enzyme preparation. After 10 minute incubation at 50^0C, the reaction was stopped by adding 2 ml DNS reagent followed by boiling for 5 minutes. Absorbance was measured on spectrophotometer at 540 nm against a reagent

blank. One unit (U) is defined as the amount of enzyme that releases1µ mol of reducing sugar equivalent to xylose perminute. The amount of xylanase in SSF was expressed as U/g.

To determine cellulase activity 0.5% CMC dissolved in acetate buffer pH 5 was used as substrate. One unit (U) of cellulase activity was defined as the amount of enzyme that releases 1µ mol reducing sugar equivalent to glucose per minute.

Pectinase activity was determined by using 0.5% poilygalactouronic acid dissolved in acetate buffer pH 5 as substrate. One unit (U) of pectinase activity was defined as the amount of enzyme that releases 1µmol reducing sugar released from polygalactouronic acid.

Protease activity was assayed according to the method described by Amare Gessesse and Berhanu Gashe (1997) with some modification. To 450 µl of 1% casein, 50 µl of enzyme source was added and incubated for 30 minutes at 50 ^{0}C. The reaction was stopped by adding equal volume of 10% trichloroacetic acid (TCA). After incubation for 20 minutes at room temperature, the reaction mixture was centrifuged at 10,000 rpm for 5 minutes, then 500 µl of the supernatant was taken and 2.5 ml of 0.5 M Na_2CO_3 was added. Finally, 500µl of 1:10 diluted 1N folin ciocalteaus's phenol reagent was mixed and absorbance at 660 nm was measured. One unit of protease activity (U) is defined as the amount of enzyme which resulted in the release of 1 µg amino acid equivalent to tyrosine per minute.

2.7. Characterization of *Termitomyces* xylanase

The optimal pH for *Termitomyces* xylanase was determined by assaying enzyme activity at various pH values, Buffers used were sodium acetate (pH 3.6 – 5.5), maleate (pH 5.2 – 6.8) and tris-HCl (pH 7.5 – 9) buffers. To test pH stability the enzyme was diluted in different buffers and incubated at room temperature for one hour. The residual enzyme activity was measured under the standard assay conditions.

The optimal temperature of *Termitomyces* xylanase was determined by assaying the enzyme activity at various temperatures and a pH 5.0 under the standard conditions. Thermal stability was investigated by incubating the enzyme from 40^0C – 70^0C for 30 minutes. The residual activity of the enzyme was determined following the standard procedure. The enzyme was also incubated at different temperature values and withdrawn at time intervals to measure the residual activity following the standard enzyme assay conditions.

To determine the effect of metal ions, the enzyme was pre-incubated with different metal ions for 30 minutes at room temperature, and then the enzyme activity was measured under the standard assay conditions. The final concentration of ions in the reaction mixture was 5 mM. The salt solutions used were NaCl, KCl, $MnCl_2$, $ZnCl_2$, $FeCl_3$, $CuCl_2$ and $CaCl_2$.

3. Results

Figure 4. Pictorial representation of mound of termites

3.1. Properties of the termite comb

The comb excavated from termite mound around Nazareth had pale creamed color. It has a pH of 4.5 and a moisture content of 52.7%. The amount of total nitrogen and soluble protein were 1.56 % and 29.5 mg/g of the comb. Also 405 µg/g of comb of reducing sugar was detected. In the comb a significant amount (8.27U/g) of xylanase was detected but no cellulase, protease and pectinase were detected.

Table 1. Physical and chemical properties of the termite comb. Values given are at least the averages of two experiments.

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Properties Tested	Result description
➢ Enzyme activities	
• Xylanase	8.27U/g
• Cellulase	No activity
• Protease	No activity
• Pectinase	No activity
➢ Total soluble protein	29.5mg /g of comb (dry weight basis)
➢ Total crude protein	9.8%
➢ Total nitrogen	1.56 % of the comb
➢ Total reducing sugar	405 µg / g of comb (dry weight basis)
➢ pH	4.5
➢ Moisture content	52.7%
➢ Color of the comb	Pale creamed

Figure 5. Pictorial representation of comb of termites

3.2. Isolation of *Termitomyces* and its cultural characterstics

In this study, a pure culture of *Termitomyces* was isolated by picking the nodules (sporodochia) from the termite comb. On MEA media plates, *Termitomyces* has grown 4.2 cm in 15 days of incubation which is slower than another termite associated *Xylaria* (Table 2*)*. However, up on addition of comb extract on MEA it has grown to 6 cm on the same time of incubation. *Termitomyces* and *Xylaria* were grown on the same plate and *Xylaria* overgrown on the plate.

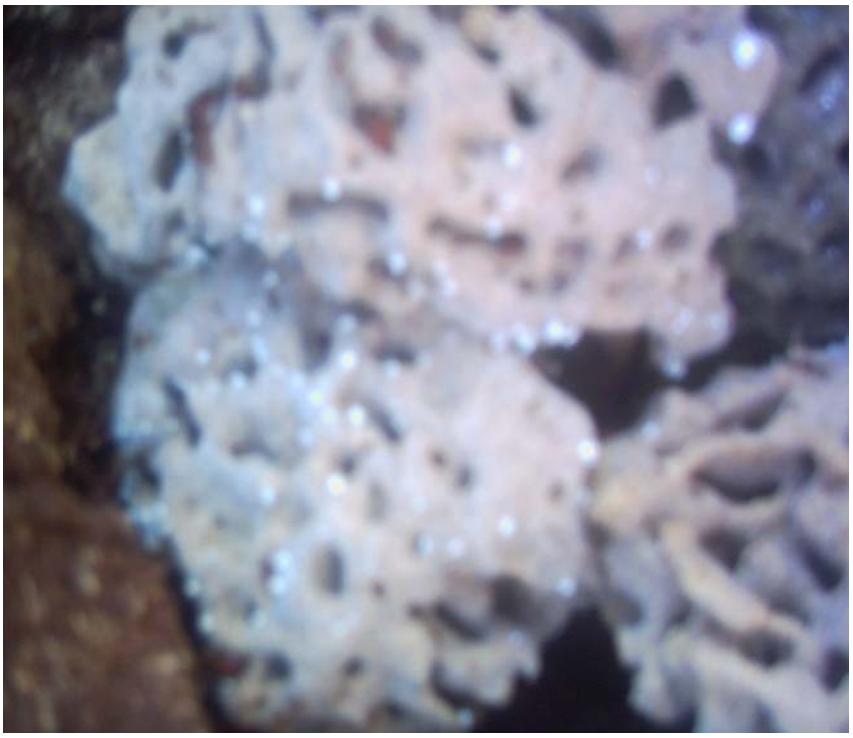

Figure 6. Pictorial representation of sporodochia on the termite comb

Table 2. Growth characteristics of *Termitomyces* on plates. Values given are at least the averages of two experiments.

Properties tested	Result description	Time of Incubation(days)
Growth diameter on plates; ➢ *Termitomyces* ➢ *Xylaria*	4.2 cm 9 cm	15 10
Growth diameter of *Termitomyces* on plates that contain comb extract	6 cm	10
Growing *Termitomyces* and *Xylaria* on the same plates	*Xylaria* has grown over *Termitomyces*	10

3.3. Xylanase and cellulase production by *Termitomyces* sp. using SSF and SmF

Solid and liquid substrate fermentation media were used for xylanase and cellulase production. In SSF, *Termitomyces* has shown growth on wheat bran and gave maximum amount of enzyme (42.2U/g) after 10 days of incubation (Table, 3). However, no growth was detected on Avicell. In SmF *Termitomyces* produced very little amount of xylanase (0.00694U/ml) on xylan after 23 days of incubation on shaker flask at room temperature but no cellulase production was detected up on incubation on CMC.

Table 3. Amount of xylanase produced on solid and liquid state fermentation. Values given are at least the averages of two experiments.

Fermentation type	**Substrate**	**Growth**	**Enzyme examined**	**Amount of enzyme**	**Time of Incubation (days)**
Solid	Wheat bran	Yes	Xylanase	42.2 U/g	10
			Cellulase	-----	
	Avicell	No	Cellulase	-----	10-20
			----------	------	
Liquid	Xylan	Yes	Xylanase	0.00694U/ml	23
			Cellulase	-------	
	CMC	No	Cellulase	-----	23
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3.4. Enzyme production using different substrates

Different agricultural residues (wheat bran, teff straw, wheat straw, sugar cane bagasse, saw dust, molasses and bean straw) and termite comb were used as substrate for the growth of *Termitomyces.* Maximum xylanase activity was observed on termite comb (52.24U/g) followed by wheat bran (37.37U/g). Bean straw and wheat straw supported the growth of *Termitomyces* better than teff straw (Table 4). Almost no enzyme production was detected on saw dust.

Table 4. Growing of *Termitomyces* on different solid substrates on the production of xylanase. Values given are at least the averages of two experiments.

Growing Substrates	**Amount of enzyme(U/g)**
Termite comb	52.24
Wheat bran	37.37
Bean straw	13.54
Wheat straw	11.19
Sugar cane bagasse	1.07
Teff straw	0.010
Saw dust	NS

Key: NS-not significant

3.5 Time course of enzyme production

Under SSF using wheat bran, *Termitomyces* showed maximum xylanase production (42.2U/g) after 10 days of incubation at 28^0C (Fig. 5). After the 10^{th}day enzyme production falls rapidly, declining by 86.4% after the 16^{th} day.

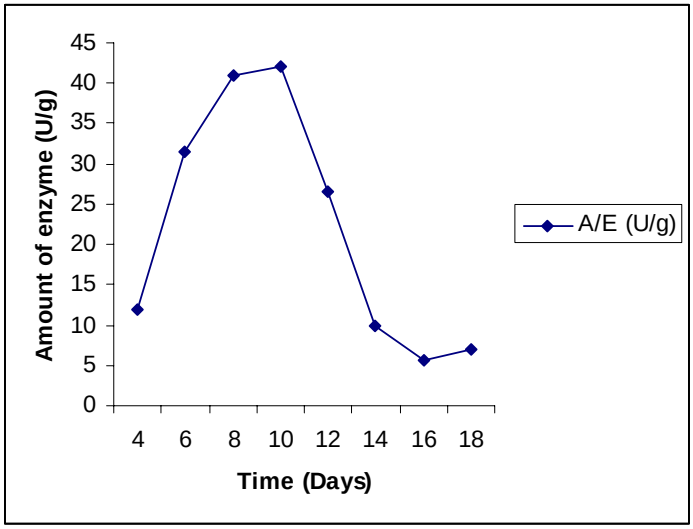

Figure 7 Time course of xylanase production on SSF by *Termitomyces* sp. using wheat bran as carbon source. Values given are at least the averages of two experiments.

3.6. The effect of moisture content of the solid substrates

Enzyme production was affected by the moisture content of the substrate. Maximum amount of xylanase (42.2U/g) was observed around 58% moisture level. Above this level enzyme production and growth decreased significantly. At moisture content of 75% and above no significant growth and enzyme production was detected (Fig. 6).

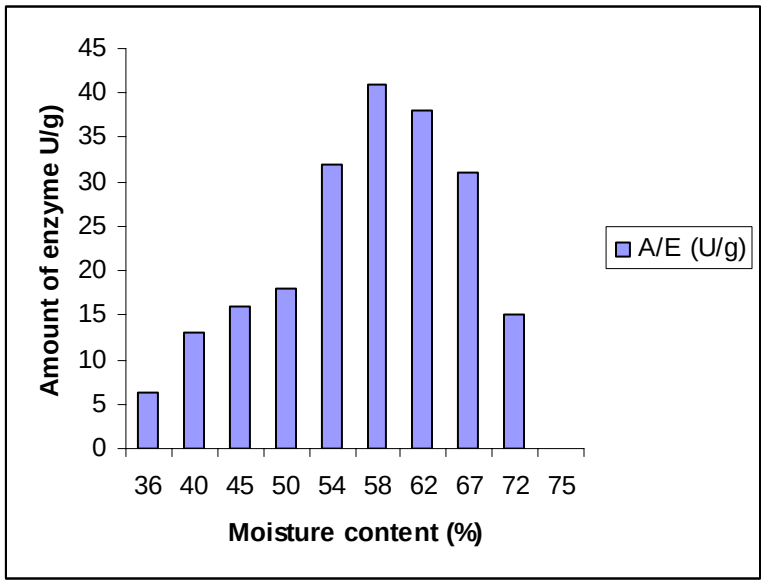

Figure 8.The effect of moisture level on the production of *Termitomyces* sp. Values given are at least the averages of two experiments.

3.7. The effect of additives on enzyme production

Xylanase production was repressed up on addition of different sugars. At a concentration of 5% (w/w) xylose, lactose, sucrose and glucose showed significant repression of xylanase production. On the other hand, addition of yeast extract and comb extract increased xyanase production by 22% and 27.2% respectively (Table 5), while tryptone didn't show a significant effect.

Table 5.The effect of different additives on xylanase production by *Termitomyces* species grown using solid state fermentation. Values given are at least the averages of two experiments.

Additives	Amount of Xylanase (U/g)
None	40.9
Xylose	21.7
Glucose	19.7
Sucrose	25.8
Lactose	11.2
Yeast extract	49.8
Tryptone	32.4
Comb extract	52

3.8. Enzyme activity of the dried powdered mycelium of *Termitomyces sp.*

Termitomyces grew under SSF using wheat bran for 10 days and the content of the flask was allowed to dry to a constant weight at room temperature and grounded in to a powder. The dried powdered mycelium along with the remaining wheat bran has shown 28.3U/g of xylanase and 47U/g protease (Table 6).

Table 6 Amount of xylanase and protease from the dried powdered mycelium of *Termitomyces* species. Values given are at least the averages of two experiments.

Enzymes	Amount of enzyme (U/g)
Xylanase	28.3
Protease	47

3.9. Properties of *Termitomyces xylanase*

3.9.1. pH profile of *Termitomyces* xylanase

Termitomyces xylanase showed maximum activity at pH 5.5 and exhibited more than 85% of its activity in the pH range of 4.5-6.5 (Fig. 7).

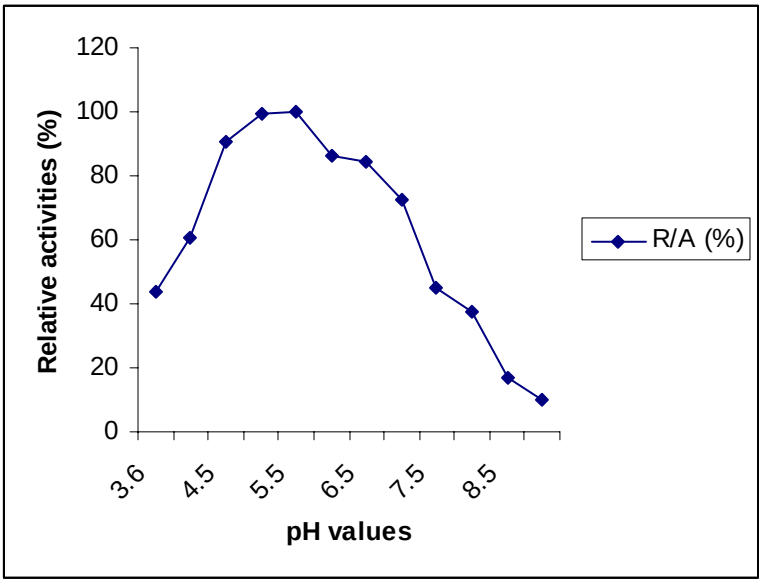

Figure 9. pH profile of *Termitomyces* species Xylanase at 50 ^{0}C. Buffers used were: Sodium acetate buffer (pH 3.6-5.5), Maleate buffer (pH 5.5-6.8), Tris-HCl buffer (pH 7.5-9.0). Values given are at least the averages of two experiments.

3.9.2. pH stability of *Termitomyces* xylanase

Termitomyces xylanase was stable in a broad pH range with maximum stability in the pH range of 4.5 - 6.5 (Fig. 8). However, the enzyme decreased its activity at pH values above 7.5.

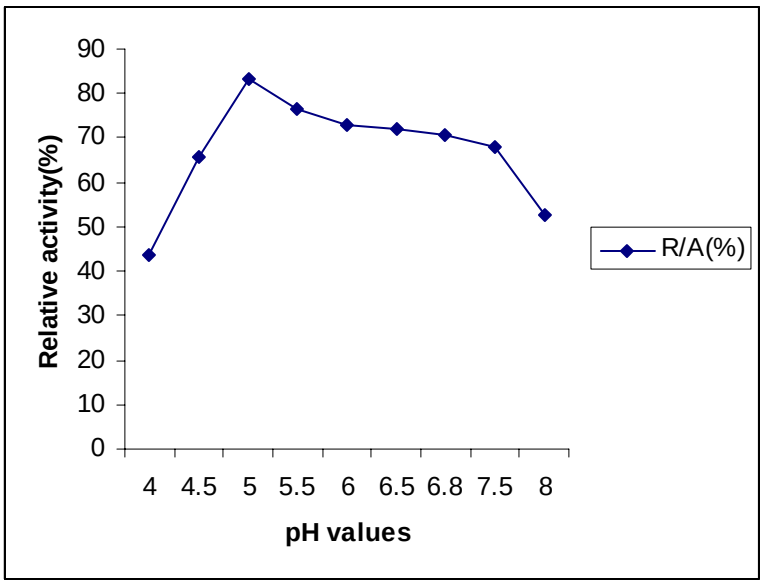

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Figure 10 .The effect of pH on the stability of *Termitomyces* xylanase. Buffers used were Sodium acetate (pH 4-5.5), Maleate (pH 5.5-6.8) and Tris-HCl (pH 7.5-9.0).Values given are at least the averages of two experiments.

3.9.3. Temperature profile of *Termitomyces* xylanase

The temperature profile of *Termitomyces* xylanase was studied at pH 5 using 1% birch wood xylan as substrate. Maximum activity was observed at 55-60^{0}C (Fig. 9).

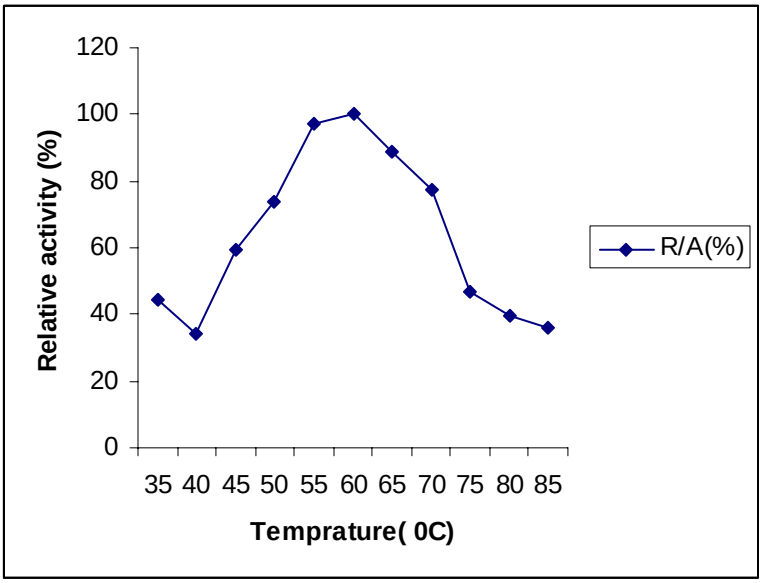
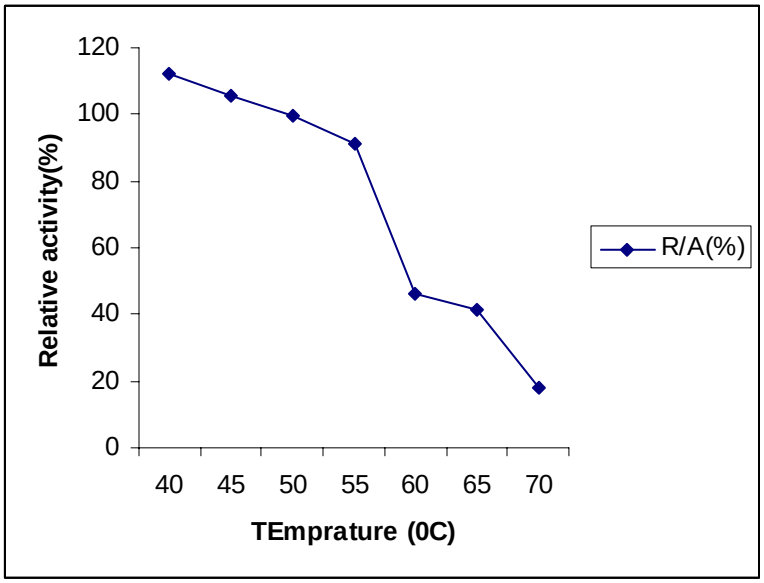


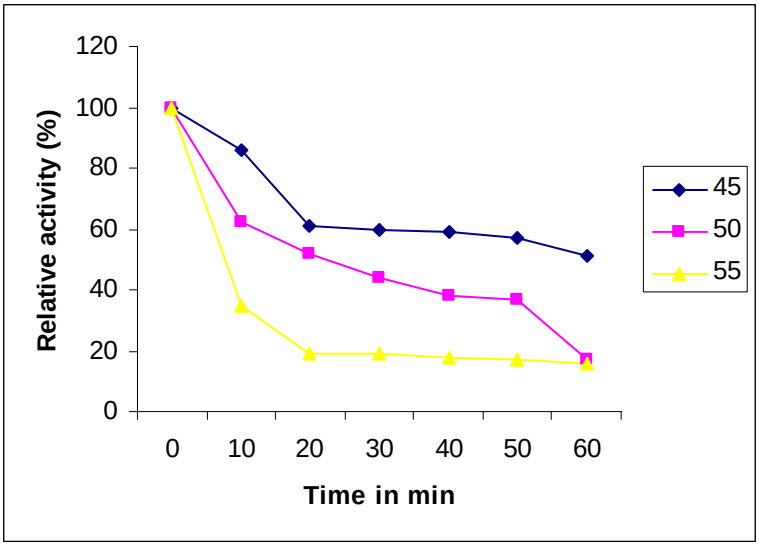
Figure 11.Temperature profile of *Termitomyces* xylanase; assayed at different temperature values and pH 5. Values given are at least the averages of two experiments.

3.9.4. Temperature stability of *Termitomyces* xylanase

The effect of temperate on the enzyme stability was determined by maintaining the enzyme at various temperatures ranging from 40^0C - 70^0C for 30 minutes in 0.1 M sodium acetate buffer pH 5. The enzyme retained more than 90% of its original activity between 40-55^0C (Fig. 10A). At 60^0C and above the enzyme retained less than 50% of its original activity. The stability of the enzyme was also determined by incubating the enzyme for one hour at 45^0C, 50^0C and 55^0C at pH 5. The enzyme has showed good stability up to 30 minutes at 50^0C and up to 40 minutes at 45^0C (Fig. 10B).



A)



B)

Figure 12.The effect of temperature on the stability of xylanase. Effect of temperature on the stability was tested by incubating the enzyme at different temperature values for 30 minutes in sodium acetate buffer pH 5 (Figure 10 A).The enzyme was also incubated at 45, 50 and 55^{0}C for 1 hr with 10 minutes interval at pH 5 (figure 10 B).

3.9.5 Effect of metal ions on *Termitomyces* xylanase

Data on the effects of mono, di, and trivalent cations on enzyme activity is presented on (Table, 7). Of all the metal ions tested only Zn^{2+} brought about an increase in xylanase activity while CU^{2+}, Mn^{2+} and Fe^{3+} significantly inhibited xylanase activity. Other metal ions did not have significant effect on the activity.

Table 7. Effect of metal ions on the activity of the crude *Termitomyces* sp. Xylanase Values given are at least the averages of two experiments.

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Metal ion added (Final concentration 5mM)	**Xylanolytic Activity (%of control)**
None	100
Na^{+}	107.7
K^{+}	109.4
Mn^{2+}	49.3
Zn^{2+}	131
Cu^{2+}	58.7
Fe^{3+}	26.2
Ca^{2+}	93.9

4. Discussion

Of all the intriguing activities and properties of termites, none seems as widely recognized or as often quoted as their ability to utilize wood as a food source. Many species prefer wood that is partially decayed by the associated fungi (Ajit *et al.,* 1994). Termites especially the *subfamily Macrotermitinae* is known to form symbiosis with *Termitomyces* and some *Xylaria* species. However, the benefit the termite and the fungus get from this association is not clearly understood.

The gut of trmites has cellulolytic prokaryotes which are believed to play important role in the hydrolysis of cellulose to glucose (Breznak, 1982). However, cellulose in its native form is not easily digestible. This is because in the cell walls of plants, cellulose microfibriles are found embedded in an aqueous gel of xylan which again is enclosed by a layer of lignin (Amare Gessesse, 1998).

Turner (2002) suggested that fungi in termite nest are capable of degrading cellulose and use it as carbon and energy source. However, if these fungi are able to degrade cellulose, the fungus and the termite may compete for the energy source.

When we start this study we had a hypothesis that the fungus in the termite garden may help to remove the lignin sheath from the cellulose microfibriles through selective degradation of the hemicellulose which cement the lignin to the cellulose. To test this hypothesis *Termitomyces* and the other termite associated fungi *Xylaria,* were isolated from the termite comb, and tested for their ability to utilize different wood components.

The termite comb (fungus garden) contained high xylanase (hemicellulase) and no cellulase activity, indicating that fungi in the termite garden are not capable of degrading cellulose.

To further test the ability of termite associated fungi to produce different plant cell wall degrading enzymes under laboratory condition, both *Termitomyces* and *Xylaria* were grown under SSF using wheat bran as substrate. Wheat bran contains a significant amount of cellulose (Chotborsk, *et al.,* 2004). Both organisms grew very well and produced very high amount of xylanase activity, but no cellulase activity was detected. This could indicate that both organisms are unable to utilize cellulose. Furthermore, when both organisms were grown using pure cellulose (Avicell) as substrate in SSF and using CMC in SmF, no growth was observed, further confirming the inability of both organisms to utilize cellulose as a carbon and energy source.

Therefore, high hemicellulase production and no cellulase production by the two fungi indicate that, there is no competition for cellulose between the termite and the fugal species. The fungi rather help to free the cellulose from lignin by degrading the hemicellulose that cements it to the cellulose. The lignin free cellulose is then consumed by the termite and further degraded with the help of cellulases in the termite gut produced by the gut microflora. In the absence of these associated fungi the *Macrotermitinae* termite may not be able to feed on lignocellulosic biomasses.

Another benefit the termite gets from this association is probably protein supplement. Lignocellulosic biomass is well known to have very low protein content around 1.5% (Browning, 1967) and yet termites survives feeding on such protein deficient diet. However, in this study analysis of total protein content in the termite comb using the Kjeldhal method gave a crude protein amount of 9.8% (around 6 folds higher than lignocelluloses) and a soluble protein content of 29.5 mg/g of comb.

The fungus may probably get its nitrogen from inorganic nitrogen sources in the soil convert it to organic nitrogen. When termites consume the mycelial rich comb, they are not only taking the energy source in the form of pure cellulose but

also get sufficient nitrogen. The nitrogen excreted by termites is likely be used by the fungus thus keeping a closed nitrogen cycle in the termite mound.

The termite comb had an acidic pH and low moisture content (52.7%). This probably helps to keep away bacterial competition, as most bacteria require high water activity and a pH of around neutrality and above. On the other hand the condition in the termite comb is an ideal substrate for the growth of many fungi. However, to date only few fungi are known to be associated with termites. What prevent other fungi, such as *Trichoderma* from growing in the comb remains unknown.

From all termite comb samples we excavated, we were able to isolate both *Termitomyces* and *Xylaria*. Examining the surface of the comb under the natural conditions, *Termtomyces* seems to dominate. But when grown in culture, *Xylaria* showed a much faster growth rate than *Termitomyces*. When the two organisms were grown on one agar plate, *Xylaria* over grow *Termitomyces* very easily. If *Xylaria* is such a fast growing organism and if both organisms are found together, what makes *Termitomyces* dominant in termite mound remain unknown. One possibility is that the termite some how produce factors that favor *Termitomyces* and suppress *Xylaria*.

Termitimyces xylanase was optimally active in the pH range of 4.5-6.5 and a temperature of 55-60^{0}C. Xylanses which are active in the acidic pH range (pH 4.8-6) are considered to be suitable for application as animal feed supplement (McCleary, 2004; Tony *et al*., 2005). This enables the enzyme to adapt to the gut condition of the animal. Different researchers showed that xylanases have a huge potential of xylanase as animal feed supplement, especially for monogastric animals. However, cost of enzyme is a limiting factor for wider application of xylanase in the animal feed industry. In most industrial enzyme production processes up to 30-40% of the enzyme production cost is accounted for by the growth substrate (Kulkarni *et al.,* 1999). Thus finding cheap ways of enzyme

production is expected to substantially reduce production cost and enable wider application of xylanases as animal feed supplement. Amare Gessese and Gashaw Mamo (1999) described wheat bran to contain a good balance of nutrients to support growth of many microorganisms. The fact that *Termitomyces* grow in cheap substrate as wheat bran and produce high xylanase activity may allow cheap production of xylanase.

Optimum xylanae production was obtained at 58% moisture content which is close to what is found in termite comb. Above 65% moisture content xylanase production was decreasing. This probably is due to reduction in the porosity of wheat bran with increasing moisture content (Raimbault and Alazard, 1980).

Addition of yeast extract to the wheat bran leads to a slight increase in xylanase production while addition of xylose, glucose, sucrose and lactose repressed the xylanase production, which might be due to the catabolite repression of the xylanase production. This probably indicate that yeast extract provide some growth factors for the fungus. However, when wheat bran was supplemented with comb extract, growth and enzyme production was significantly improved indicating that the fungus gets some growth factors from the comb extract, probably produced by the termite. Understanding the nature of such growth promoting factors could help to enhance enzyme production by this fungus under SSF for animal feed application.

Growth of *Termitomyces* on SSF using cheap substrates at low moisture level, in addition to offering a cheap method of enzyme production could offer a possibility of protein enrichment of lignocellulose biomass. The protein content of *Termitomyces* mycelium is very high. Botha and Eicker (1992) described the presence of different essential amino acids like isoleusine, leusine, lysine, threonine, tryptophan and valine from *Termitomyces* sp. Therefore, this might make the mouldy wheat bran ideal for animal feed supplement.

Besides the production of *xylanase, Termitomyces* produced a significant amount of proteases using wheat bran under SSF. Although proteases are used to break down proteins in to amino acids inside the animals gut system and improve overall body development, it may also affect the stability of the xylanase during extraction and storage. Therefore the use of appropriate protease inhibitors during extraction or selection of protease deficient mutants may help to solve the effect of proteases.

Another potential application of *Termitomyces* xylanase would be biopulping which is a solid-substrate fermentation (SSF) process where lignocellulosic materials are treated with fungi prior to pulping in order to reduce energy during mechanical pulping or to reduce chemical consumption during chemical pulping processes. The fact that no cellulase is produced along with xylanase makes it ideal for biopulping as cellulase decreases the strength of the fibers. The beneficial effects of this cellulase free xylanase treatment can be described to the reduction of the binding capacity of fibers and improved fibrillation (Jacobs *et al.,* 1998).

Termitomyces species xylanase was sensitive to Mn^{2+}, Fe^{3+} and Cu^{2+}, which might suggest the necessity of eliminating these metal ions (salt solutions) from the oligosaccharide, polysaccharide or glycoprotein substrate when it needs to be degraded before its hydrolysis by the enzyme. A similar result has been reported to Cu^{2+} and Fe^{3+} for xylanases from *Micrococcus* sp. AR- 135 (Amare Gessese, 1998), *Staphylococcus sp*. SG – 13 (Gupta *et al*., 2002). The stimulation of *Termitomyces* xylanase activity in the presence of Zn^{2+} might help the digestion of macromolecules that are not readily attacked by the xylanase from other organisms under these conditions.

5. Conclusion

- *Termitomyces* and and some *Xylaria sp*. are Identified to be associated with termites in rift valley of Ethiopia

- Termite associated fungi are unable to utilize cellulose but produced high hemicellulase activity.

- High hemicellulase activity could benefit the termite in removing the lignin from the cellulose fibers allowing consumption and easily digestion, which otherwise is difficult to digest.

- The relationship between termites and *Termitomyces* could be mutualism rather than competitive as both benefits from the association.

- The results obtained from this work strongly indicate that the SSF system using wheat bran as substrate is an economical method for the production of xylanase at extremely low operational cost.

- *Termitomyces* produced highest amount of xylanase around 58% moisture levels which is near to its natural environment (53%).

- The properties of *Termitomyces* xylanase are suitable for animal feed supplement.

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Acknowledgements

First of all I wish to express my deepest and warmest thanks to my advisor Dr. Amare Gessesse for his continuous and unreserved guidance and giving me constructive feedback in the study. I am also grateful to Dr. Dawit Abate for his assistance in the identification of the fungus and providing me very crucial ideas and materials.

I also acknowledge Addis Ababa University Department of Biology for the financial support for this research work.

I would like to express my gratitude towards W/t Zenebech Aytenew for her assistance in many ways and the whole staff at Laboratory of Mycology and Applied Microbiology for having a good time.

My deepest thanks then goes to my brother Fikadu Zeleke for his financial assistance and shouldering the responsibility of our family. Fik! Without you it was really impossible and even could not be started.

I would like to give my special thank to Woinshet Gizaw for her continuous support financially and morally. This work was completed because of you. Really, it is very much and beyond my expectation to express all. Thanks woin!

Finally, I would like to express my deepest love to my family, especially my mother for her deep enthusiasm and helping me in all aspects.

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Abbreviations

AME	Apparent metabolizable energy
AW	Water activity
BSA	Bovine serum albuin
CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic acid
MEA	Malt extract agar
SmF	Submerged fermentation
NSP	Non-starchy polysaccharides
SSF	Solid state fermentation
TCA	Trichloroacetic acid

Abstract

A xylanase producing symbiotic fungus *Termitomyces sp.* was isolated from the termite mound around Nazareth found in Rift Valley area of Ethiopia. The culture was prepared from sporodochia found on the termite comb. *Termitomyces sp.* was slow grower (4.2cm in 2 weeks) than another termite associated fungal *sp*. *Xylaria* (9 cm/10 days). 405µg reducing sugar, 8.27U xylanase, 1.56% total nitrogen and 29.4mg soluble protein were analyzed in one gram of comb. pH and moisture content of the comb were 4.5 and 52.7% respectively. *Termitomyces sp*. produced xylanase more (42.2U/g) in SSF using wheat bran as carbon source than SmF (0.007U/ml) on xylan medium. The fungus produced xylanase on wheat bran, termite comb, wheat straw, bean straw and sugar cane bagasse using SSF. At 58% moisture level and 10 days of incubation, the highest level of xylanase produced on termite comb (52.24U/g) followed by wheat bran (37.37). No growth was observed on Avicell on SSF and CMC in SmF. Comb extract and yeast extract enhances enzyme production by 27.2% and 22% respectively, but xylose, glucose, sucrose, and lactose strongly repressed the enzyme production. The xylanase exhibited maximum activity at 55-60^{0}C and at pH 5.5, but it retained more than 85% of its activity in the pH range 4.5-6.5. The enzyme was stable up to 55^{0}C for 30 minutes incubation. *Termitomyces* xylanase was stimulated by Zn^{2+}. However, it was inhibited by Mn^{2+}, Fe^{3+}, and Cu^{2+}. Those properties make this enzyme potentially attractive for industrial applications like animal feed supplement and biopulping of wood.

Key words: *Termitomyces* sp., Termite Comb, Xylan, Xylanase, Termites