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Substrate-utilization Properties of *Termitomyces* Culture Isolated from Termite Mound in the Great Rift Valley Region of Ethiopia

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Abstract

Termites of the subfamily *Macrotermitinae* are known to live in an obligate symbiosis with *Termitomyces* mushrooms, although the exact benefit of the association is still debating. *Termitomyces* are believed to degrade lignocelluloses into smaller units which then can be used by the fungus-growing *termites*. In this study, extracts of the *termite* comb showed strong xylanase activity (8.27 ± 0.14 unit per g of dried comb) with no cellulase activity. *Termite* comb and wheat bran supported the growth of *Termitomyces* culture in solid state fermentations, in which culture-extracts showed strong xylanase activities (52.25 ± 1.98 and 37.38 ± 1.09 units per g of dried culture, respectively), yet no cellulase activities were detected. Furthermore, we observed that *Termitomyces* cultures were unable to grow on pure cellulose (Avicell). Hence, the isolated *Termitomyces* may be incapable of using cellulose in the studied *termite* nest. As the absence of cellulase activities in the extracts (both comb and culture) and the inability to grow on pure cellulose (Avicell) are unpredicted properties of the fungus, results of this study may add some important data on the ongoing debate for the association between *Macrotermitinae* termites and *Termitomyces* mushrooms.

Keywords: *Termitomyces*, *termite* comb, xylanase, cellulase, Fungus-growing *termites*

1. Introduction

It is well understood that fungi of the genus *Termitomyces* form obligate symbiosis with the sub-family *Macrotermitinae termites* (Nobre et al., 2011; Rouland-Lefe`vre et al. 2006; Korb and Aanen, 2003; Bignell, 2000; Aanen et al., 2009). *Termitomyces* fungi grow on or around *termite* nests, but the exact relationship between them is yet debating. Fungus-growing *termites* feed and grow on lignocellulosic materials; however they have only limited capability to digest it. A number of studies showed that *Termitomyces* fungi help the *termite* partner in the degradation of cellulose to simple sugars (Martin and Martin, 1978, Rouland et al., 1991; Martin, 1987; Grassé and Noirot, 1955). The roles of gut microflora of fungus-growing *termites* have also been reported in cellulose degradation (Liu et al., 2011). Thus, cellulose degradation in the *termite* nest may be performed by both gut microorganisms and *Termitomyces* species, suggests that cellulose degradation may not be the main factor for the obligate association between these organisms. While many studies also reported the role of *Termitomyces* fungi in the degradation of plant lignin to facilitate cellulose degradation by *termites* (Hyodo et al. 2000, 2003; Johjima et al. 2003; Taprab et al. 2005). Collins (1983) and Matsumoto (1976) also proposed the role of *Termitomyces* mycelia as protein sources to the fungus-growing *termites*. On the other hand, the fungal partner may be benefited from the suitable habitat where substrates (plant materials) are collected and supplied by *termites*.

Macrotermitinae termites are commonly distributed throughout tropical Africa and Asia (Aanen et al., 2002). In most parts of Ethiopia where the Great Rift Valley passes through, there are large numbers of mounds constructed by *Macrotermitinae termites*. As expected, in the rainy season, fruiting bodies of *Termitomyces* mushrooms grow on or around the *termite* mounds in the area. As a preliminary survey on the growth properties of *Termitomyces* species, this study employed culture-based isolation and substrate utilization capability of *Termitomyces* fungi from the Great Rift Valley area of Ethiopia.

2. Materials and Methods

2.1. Sampling and comb characteristics

Samples of *termite* combs were collected from 'Kechema', a region located in the Great Rift Valley region of

Ethiopia, which is known for its large number of *termite* mounds and *Termitomyces* mushrooms growing at the rainy season. Sampling was performed at the dry season (winter) when atmospheric temperature raised to 25 °C. The *termite* mound was excavated and fresh *termite* combs were collected aseptically from deep inside the mound.

Three hours after sampling, 10 grams of the combs were grounded to powder, dissolved in 100 ml distilled water and centrifuged at 4000 x g for 15 minutes. The clear supernatants were used to determine pH, enzyme activities (xylanase and cellulase), reducing sugar and total soluble protein contents. Moisture content was determined after drying of the fresh comb to a constant weight. Total nitrogen content of the combs was measured by the Kjeldahl method as described by Sertsu and Bekele (2000). Soluble protein contents were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.3. Isolation and cultivation of Termitomyces culture

White nodules found on the *termite* comb were aseptically picked as previously carried out by Botha and Eicker (1991), transferred to malt extract agar media plates and incubated at 28°C for about 2 weeks. Sub-cultures were then made to purify and pure cultures were stored at 4°C for downstream investigations.

In order to determine growth and enzyme production potential of *Termitomyces* using different carbon sources, solid state fermentation media were prepared. To 10 g of (either wheat bran, *termite* comb, wheat straw, bean straw, teff (*Eragrostis tef*) straw, saw dust or Avicell) in 250 ml Erlenmeyer flasks, 15 ml stock mineral salt solution (KH2PO4 0.05g, MgSO4.7H2O 0.02g, NH4NO3 0.1g, CaCl2.2H2O 0.01g and 1ml of 1% FeCl3) were added and autoclaved. The cooled solid media were then inoculated with pure cultures of *Termitomyces* agar blocks. After 10 days of incubation at 28°C, cultures were extracted using100 ml sterile distilled water. Pure supernatants were used as crude enzyme sources to assay xylanase and cellulase activities. The effect of moisture level on growth and enzyme productions were also studied upon varying the percentage of water in the medium from 36% to 72%.

2.4. Enzyme assays

Xylanase and cellulase activities were determined by measuring the amount of reducing sugar released from the monosaccharaides following the standard dinitrosalicylic acid (DNS) method (Miller, 1959). One unit (U) of enzymes activity was defined as the amount of enzyme that releases 1µmol reducing sugar per minute.

3. Results and Discussion

Macrotermitinae termites are known to form obligate symbiosis with the genus *Termitomyces*; however, the benefit of the association is not clearly understood. A number of studies indicated that *Termitomyces* can play a significant role in the degradation of cellulose to smaller units that can be used by the fungus-growing *termites* (Martin and Martin, 1978; Martin, 1987; Rouland-Lefèvre et al., 1991), suggesting the role of *Termitomyces* in the cellulose utilization by fungus-growing *termites*. The fungal partner is likely benefited through obtaining feed materials that are collected by the *termites*. In this study, we carried out culture-based investigations of *Termitomyces* culture isolated from a *termite* mound in the Great Rift Valley region of Ethiopia.

The pale creamed *termite* combs (Figure 1b) collected had low moisture content (52.7 ± 2.55% w/w) and were acidic (pH, 4.5 ± 0.05) (Table 1), which probably help the fungus to reduce bacterial competition, as most bacteria require high water activity and a pH of around neutrality. Total nitrogen and soluble protein content of the combs were 1.5 ± 0.08% and 29.5 ± 0.51 mg g-1, respectively. Although *termite* combs are mainly composed of lignocellulosic materials and have poor level of proteins, the detection of such level of soluble proteins is mostly associated with the presence of large number of *Termitomyces* nodules. This suggests the potential of *termite* combs as source of protein for the fungus-growing *termites*.

Comb extracts showed strong xylanase activities (8.27 ± 0.14 U g^{-1}), but no cellulase activities were detected (Table 1). While *termite* combs are composed of mainly cellulose, the absence of cellulase activities in the extracts may put some doubts on the potential of these fungi to utilize cellulose. In contrast, a significant amount of reducing sugar (404.8 ± 2.51 µg g-1 of comb) which most likely derived from lignocellulosic comb materials was detected. Nevertheless, the presence of high xylanase activities and the absence of cellulase indicate that the amount of reducing sugars detected in the comb extract might be associated with the action of *Termitomyces* xylanase and other hydrolytic enzymes of microbial sources.

Pure cultures of *Termitomyces* fungi were obtained after successive sub-cultures of the first culture obtained from nodules on fresh combs (Figure 1c). On malt extract agar media, *Termitomyces* grew very slow (about 4.5 cm in 2

weeks), but were greatly improved upon supplementation with comb extract (about 6 cm in 2 weeks). This is likely an indication that the fungal partner might get some more nutrients from the comb. Wheat bran, *termite* comb, Avicell, saw dust and the possible substrates of the fungus-growing termites at the sampling area (wheat straw, bean straw, sugarcane bagasse, and teff straw) were used as growth substrates to test the growth and enzyme production of capability the isolated *Termitomyces* cultures. As expected, *termite* comb strongly supported growth and xylanse production (Table 2), which further indicated the strong dependence of *Termitomyces* on the comb. As wheat bran supported the growth of these *Termitomyces* cultures better than the remaining substrates (Table 2), it was selected for further laboratory investigation of the culture. While many studies reported the capability of *Termitomyces* species to produce cellulase (Martin and Martin, 1978; Martin 1987; Rouland-Lefèvre et al., 1991), we did not detect cellulase activities in the extracts of all substrates used. This was further supported by providing pure cellulose (Avicell) to the culture where no growth was observed (Table 2).

Using wheat bran as a growth substrate, optimum moisture content of the solid state growth media and the course of xylanase production were assessed (Figure 3a and b, respectively). Maximum growth and xylanse activities (42.2 ± 0.5 U g-1) were observed at 58% moisture level (at the 10th day of incubation), which is near to the in situ moisture level of the comb (52.7 ± 2.55%).

Overall, the absence of cellulase activities in the extracts of the fresh *termite* combs collected and solid state growth cultures along with the inability of the fungus to grow pure cellulose (Avicell) may raise some doubts on the general conclusions for *Termitomyces* fungi to degrade celluloses. While further detail molecular-based investigations of the organism are ongoing, this study may add some important further information to the current understanding of the ecology of fungus-growing *termites* and *Termitomyces*.

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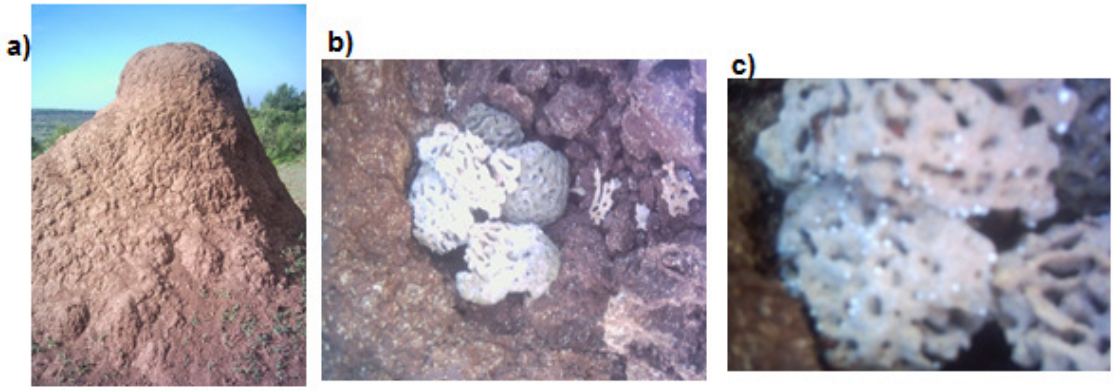

Figure 1. Pictures showing (a) the mound, (b) excavated termite comb and (c) the white nodules picked for isolation of Termitomyces culture.

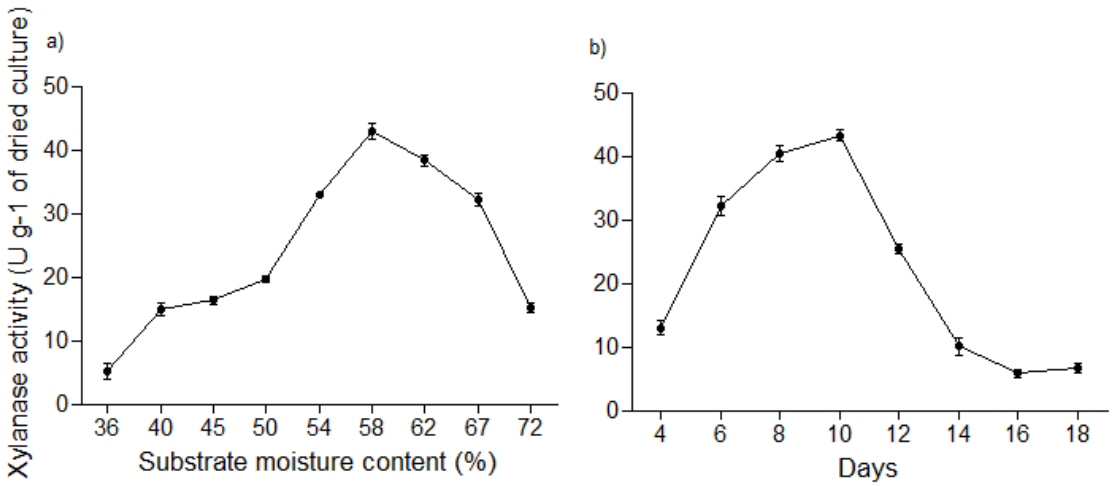

Figure 3. a) The effect of moisture level on the growth and enzyme production by *Termitomyces* culture using wheat bran as sole carbon source, b) time course of xylanase production on solid state fermentation *Termitomyces* culture using wheat bran as carbon source. Error bars indicates standard deviation of three experiments.

Table 1. Table 1. Properties of the *termite* comb excavated from a *termite* mound in Rift Valley region of Ethiopia.

Properties	Mean ± SD
Xylanase activity (U g^{-1})	8.27 ± 0.14
Cellulase activity (U g^{-1})	ND
Total soluble protein (mg g^{-1} of dried comb)	29.5 ± 0.51
TN (% of dried comb)	1.5 ± 0.08
Total reducing sugar (µg g^{-1} of dried comb)	404.8 ± 2.51
pH	4.5 ± 0.05
Moisture content (%)	52.7 ± 2.55

ND: not detectable

Table 2.Growth nature and enzyme production (Units per gram of the dried culture) of the *Termitomyces* culture in different agricultural residues.

Substrates	**Growth**	**Mean ± SD (U g^{-1})**
Termite comb	+++	52.25 ± 1.98
Wheat bran	++	37.38 ± 1.09
Bean straw	+	13.58 ± 0.89
Wheat straw	+	11.18 ± 0.06
Sugar cane bagasse	+	1.11 ± 0.04
Teff straw	Poor	0.10 ± 0.01
Avicell	no	_
Saw dust	no	_

Key: '+' represents relative growth potential on malt extract media in a specific time limit.

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