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Lethal effects of tungsten and boric acid, and three garlic, basil and caraway essential oils on *Amitermes vilis* (Isoptera: termitidae) and its endosymbiont's cellulolytic activity

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Abstract

Termites are considered as one of the most important and largest groups of insects in terrestrial ecosystems that decompose lignocelluloses. Isolation and characterization of cellulolytic strains from termites will also provide information for understanding the efficient mechanism of lignin degradation in termites. In the present study, some termite specimens were collected from traps in "Khojir" Protected Area in Jajrud district of Pardis County, Tehran Province, Iran, and all were identified as *Amitermes vilis*. Five bacterial isolates in charge of decomposing celluloses were extracted from termite's gut. The isolates ASB1, ASB2, ASB3 and ASB5 were identified as *Bacillus cereus*, *B. circulans*, *B. circulans* and *B. licheniformes*, respectively. A Gram-negative bacterium encoded ASB4 was also characterized but not precisely identified. In order to determine the amount of cellulolytic activities of the strains ASB1 and ASB4, both were grown on Cellulose Congo red agar medium. The clear zones around the mass colonies were measured 5 and 4 mm, respectively. Subsequently, the amount of absorbed glucose was measured by spectrophotometery. The effects of garlic, caraway and basil essential oils along with two mineral compounds, boric acid and tungsten were examined against cellulolytic activities of the five bacterial isolates by congo red assay. All tests were performed with three different concentrations of 10, 50 and 100 ppm in three replicates. The results showed a significant reduction in the cellulolytic activity of all tested bacterial isolates at 100 ppm. The latter was considered a fatal dose for the *Amitermes vilis* termite.

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Introduction

Termites are the most important decomposer insects and mostly found in tropical forest, tropical savanna, and desert ecosystems. The symbiotic gut microbiota of termites plays important roles in lignocelluloses digestion and nitrogen metabolism (Brune and Ohkuma, 2010).

Using synthetic chemicals to control insects including termites cause problems such as contamination of land and water, pest resistance to pesticides, toxic effects of adverse natural enemies, pesticides residual in crops and other natural issues (Rahimzade *et al.,* 2012). Bio control as an applied science could be considered as one of pesticides alternative methods to control termites (Bayon *et al.*, 2000). In the past few years, using compounds extracted from plants as an alternative to chemical pesticides for pest control is taken into consideration. These compounds act as fumigant, contact, and repellent, deterrent to spawning and metabolic disorder in gut microbiota of termites. Moreover, they affect the insect population growth (Valadares *et al.*, 1999).

Termite (order Isoptera) comprises of diverse species, roughly divided into so called higher and lower termites. Lower termites harbor a dense population of prokaryotes and protists (single celled eukaryotes) in their gut. Higher termites comprise only one apical family (Termitidae) but more than three quarters of all termite species. While they also harbor a dense and diverse array of prokaryotes, higher termites lack protists (Ohkuma, 2003).

The genus *Amitermes* is the second largest genus of the Termitinae subfamily after the Microcerotermes (Scheffrahn *et al.*, 2010). *A. vilis* species is categorized among the phylogenetic group of higher termites. The latter, can be found almost anywhere in Iran (Ghayourfar, 1995).

Researches had shown that the cellulolytic endosymbionts existing in their hindguts are mainly responsible for the decomposition of the cellulose. The termites on one hand were considered as beneficial insects since in their ecological cycle, they damage the trees through the decay of organic materials, such as wood and leaves, these in turn, increase soil porosity and improve nutrients involved (Sugimoto *et al.*, 2000, Strassert, 2010).

The symbiotic gut microbiota of termites plays important roles in lignocelluloses digestion and nitrogen metabolism. Termites possess a dual cellulolytic system: in lower termites the cellulases are contributed by both the insect and its gut flagellates, whereas in higher termites, host cellulases and hindgut bacteria participate in fiber digestion. Amino acids are an important substrate for the microbiota (Brune *et al.*, 2010).

Although the bacteria are not believed to contribute considerably to the degradation of cellulose, they play a major role in maintaining the chemical environment of the termite gut. During ATP synthesis in the hydrogenosomes of the flagellates, acetate, H_2, and CO_2 are released. Whereas acetate is resorbed by the termites and is used as the major oxidizable energy source and as an important biosynthetic precursor (Hungate, 1943; Blomquist *et al.*, 1979; Odelson and Breznak, 1983), H_2 and CO_2 are removed by the prokaryotic symbionts.Homoacetogens use H_2 and reduce CO_2 to acetate (Breznak and Switzer, 1986; Kane and Breznak, 199; Brune, 2006).

In this way, they contribute substantially to the nutrition of the termites. Methanogens use CO_2 or the methyl group of acetate as an electron acceptor (Odelson and Breznak, 1983; Breznak and Brune, 1994). When CO_2 is used, H_2 acts as an electron donor. Thus, methanogenesis represents a further important hydrogen sink in the termite gut. Oxygen, which diffuses into the hindgut, is consumed by facultatively or even obligately aerobic bacteria in the gut periphery (Brune *et al.*, 1995). The oxygen sink resulting from this activity is a prerequisite for the survival of the obligately anaerobic flagellates.

The mechanism of action of essential oils depends on their chemical composition, and their antimicrobial activity is not attributable to a unique mechanism but is instead a cascade of reactions involving the entire bacterial cell (Burt, 2004). So essential oils and mineral compounds can cause disturbances in the bacterial enzyme activities and their energy production in lower concentrations (Tiwari, *et al.*, 2009).

In this research, isolation and characterization of cellulolytic bacteria from termites and the impact of some natural and chemical compounds on their enzyme activites were evaluated. The aim of study is a significant reduction in the cellulolytic activity of endosymbionts of termite's gut with effects of garlic, caraway and basil essential oils along with two mineral compounds, boric acid and tungsten. The latter decrease function of the termite's symbiont system in the digestion of lignocelluloses is the hydrolysis of cellulose and hemicelluloses and mortality rate of *A. Vilis* Termite. This might be one of the first reports on cellulolytic bacteria of the *A. Vilis* Termite.

Materials and methods

Sampling Site

The termites were collected from the traps placed near ash trees within the "Khojir" Protected Area in Jajrud District of Pardis County, near Tehran. 50 traps were made and placed between termite infested trees with 5 meters distance between them.

The traps consisted of five thin wood slices (25 × 6 × 0.5 cm) that were buried in ground stations by a PVC pipe (20 cm in diameter) with a lid to protect them from rain and direct sunlight. They had a great potential for attracting and harboring thousands of the termites inside the crevices of the slices. Trapped termites were transferred to the laboratory. All traps were contained a plastic boxes, with cardboard inside. Distilled water was sprayed everyday on the inner walls of the container in order to keep the relative humidity above 80%.

All tested termites (Fig. 1 & 2) were identified as the *Amitemes vilis* species by Iranian Research Institute of Plant Protection (IRIPP).

Fig. 2. A sampling site showing highest mean number of *Amitermes vilis*.

Fig. 1. A worker termite identified as *Amitermes vilis*.

Essential oils providing

The three plants that were used for hydrodistillation were garlic (*Allium sativum*), caraway (*Carum carvi*) and basil (*Ocimum basilicum*). A water based *clevenger* apparatus was used to extract these essential oils from the plants.

Bacteria Isolation and Culture

The whole body of termite workers were submerged in 70% ethanol with forceps and then gently swirled for approximately 10 seconds to remove any surface contaminants. The specimens were taken out from the ethanol solution and allowed to dry out for about 20 seconds. Sterile fine-tipped forceps were used to

hold the worker's abdomen and the tip of the abdomen was grabbed by another pair of forceps to gently pull the gut upward or downward in a 45 degree angle. If the gut is pulled at a straight angle and with too much force it is likely to break apart.10 gut samples suspensions were cultured in a Trager U medium, nutrient agar, potato dextrose agar and M1 media. Each medium was supplemented with 1% Carboxy methylcellulose (CMC) as cellulose source. (Husseneder *et al.,* 2010). The Trager U medium was used for isolating obligated anaerobic bacteria (Trager, 1934). Whereas nutrient agar and potato dextrose agar were used for screening bacteria and fungi, respectively. M1 agar was also used because of its tendency to support the growth of cellulolytic bacteria (Upadhyaya *et al.* 2012).

Isolation and Screening of Cellulose-Degrading Bacteria

Confirmation for the isolation of cellulose-degrading ability of bacterial isolates was performed by streaking the bacteria on the cellulose Congo- Red agar medium with the following composition (g/l): KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, congo-red 0.2 g, and gelatin 2 g; in distilled water at pH 6.8– 7.2. The congo-red was used to isolate all bacteria with any degree of cellulolytic activities. Colonies showing discoloration of congo-red were considered as positive cellulose-degrading bacterial colonies (Lu *et al.,* 2004). Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony (Gupta *et al.,* 2012).

Identification of Bacterial Isolates

Bacterial isolates were identified by certain morphological and biochemical tests such as gram staining test by Lay method (1994), motility test (Cappuccino and Sherman, 1992), fluorescent pigmentation on King's B medium (Murray *et al.*, 2003), carbohydrate fermentation test and indole test (Lay, 1994), methyl red test (Cappuccino and Sherman, 1992), Voges Proskauer test (Lay, 1994), citrate and oxidase test (Ijong, 2003), catalase test (Lay, 1994), oxidative/fermentation glucose test (Leboffe *et al.,* 2008), levan production (Sangiliyandi *et al.,* 1999), spore staining test (André *et al.*, 2013), Growth on 6.5% NaCl (Acharya, 2014), nitrate reduction test (Skerman, 1967) and arabinose fermentation Test (Dickey, 1979).

Detection of D-Glucose in Cellulolytic Enzyme Solutions

The bacterial suspensions were prepared by transferring single colony from the CMC agar plates to 100mL of CMC liquid medium. The initial pH was adjusted to 7.0. The incubated Erlenmeyer flasks were incubated at 28°C for two days on a shaker at 200 rpm. 2mL from each culture was seeded into 200mL of CMC liquid medium in 500mL flasks. The flasks were further incubated on a shaker at 150 rpm for 7 days at 28°C. Culture samples were taken every 24 hours during incubation period, and their cell-free supernatants (CFSs) were obtained by centrifugation (10,000×g, 5min) and analyzed for cellulolytic activities. Meanwhile, the reduction of D-glucose was estimated spectrophotometrically by adding glucose to 4 different concentrations of 5, 10, 15 and 20 ppm. The results were made into a curve as a standard benchmark to compare it to the amount of glucose that the bacteria produced in the same culture. Cell growth was monitored by measuring the optical density at 276 nm and 574 nm by using spectrophotometry (Jurick *et al.* 2012; Yu-Kyoung Kim *et al.*, 2012, Saptarini *et al.*, 2014).

Analyzing the effects of mineral compounds (boric acid and tungsten) and essential oils (garlic, caraway and basil) on bacteria cellulolytic activity Assay

Three concentrations of 10, 50 and 100ppm from each essence were made in the NA+1% CMC medium (Jayashree *et al.*, 2007). The bacterial isolates were spot inoculated on the medium surface, each with three replicates. After 48 hours, surfaces of the treated cultures were flooded

with 0.1% congo-red reagent and left for 20 minutes. Then the plates were washed with 1M NaCl. The mean of clear zones around the colonies were determined (Gautam *et al.*, 2012, Husseneder *et al.*, 2010, Ghose, 1987).

Results

Screening and Selection of Cellulolytic Bacteria

Among different bacterial isolates, 5 strains with maximum cellulolytic activity on the congo red assay were selected (Fig., 3). The diameters of the clear zones associated with each bacterium are shown in the table 1.

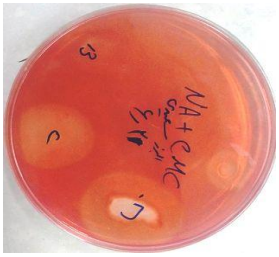

Fig. 3. Zone of clearance on cellulose congo red agar plates for Isolate ASB4 After 48 hours incubation.

Table 1. The five bacterial strains with maximum cellulolytic activities on congo red agar medium.

Bacteria Isolates	Maximum clearing zone (mm)
ASB1	5
ASB2	2.5
ASB3	3.8
ASB4	4
ASB5	2

Identification of Isolated Bacteria

The bacteria were identified based on certain morphological and biochemical tests (Table 2). Four Gram positive isolates were identified as *Bacillus cereus*, *B. circulans* and *B.licheniformers*. A Gram-negative bacterium encoded ASB4 was not identified by these tests, without exact assignment (Table 2). The exact identification would require higher molecular methods which involves characterization using 16s-rRNA techniques.

Table 2. Key tests were used to identify the bacterial isolates within in *Amitermes vilis* hindgut.

Tests / Isolate	ASB1	ASB2	ASB3	ASB4	ASB5
Gram Staining	+	+	+	-	+
Shape	Rod	Rod	Rod	Rod	Rod
Motility	V	+	-	+	+
Fluorescent on King's B medium	-	-	-	-	-
Levan production	-	-	-	-	-
Spore	+	+	+	-	+
O/F	+	+	+	+	+
NaCl 6.5%	-	+	+	+	+
carbohydrate fermentation	+	+	+	+	+
Oxidase	+	+	+	-	+
Catalase	+	-	-	+	-
VP	-	+	-	-	-
Methyl red	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Citrate	-	-	-	-	+
Arabinose	-	-	-	-	+
Species	*Bacillus cereus*	*B. circulance*	*B. circulance*	Unknown	*B. licheniformes*

Production of Cellulolytic Enzymes

Two bacterial isolates encoded ASB1 and ASB4 exerting the largest clear zones around their mass colonies were selected. Both bacterial isolates were grown in CMC liquid medium for cellulolytic excitation. Then the optical density (OD) wavelengths were measured at 276 nm and 574 nm. Comparing with standard benchmark of D-glucose,

the absorption rate of glucose for both isolates were 0.0016 and 0.0450 nm at OD 276 and 0.1205 and 0.0665 nm at OD 574, respectively (Fig. 4).

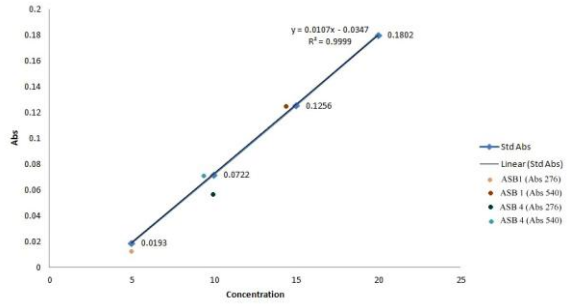

Fig. 4. The absorption rate of glucose by two bacteria (ASB1 and ASB4) grown in the CMC liquid medium were measured as 0.0016 and 0.0450 nm at OD_{276} and 0.1205 and 0.0665 nm at OD_{574}, respectively. Standard benchmark of D-glucose was used as control.

The effects of mineral compounds (*boric acid and tungsten*) *and essential oils* (*garlic, caraway and basil*) *on cellulolytic activity of bacteria within termite's guts*

According to the results derived from the ANOVA test, the caraway essential oil had the most and the garlic essential oil had the least deterrence effect on the cellulolytic activity of the bacterial isolates extracted from termite's guts. In the case of garlic, this effect was measured 8.1 mm and 2.9 mm on both strains of ASB1 and ASB4, respectively. Interestingly, at lower concentrations of garlic essential oil we observed an enhance effect of the cellulolytic activity in ASB4. Whereas the sensitivity, of the ASB4 to basil EO compared to the ASB1 was distinguishable. This was the same with caraway EO, the impact of caraway EO strain on ASB4 being the most effective and the ASB1.

The effects of mineral compounds in cellulolytic activities of both strains were determined. In the case of Boric acid, has shown the most susceptibility to boric acid rather than ASB4.

The effect of tungsten compound on ASB1 and ASB4 was 2.53 mm and 4.56 mm the least and most, respectively (Fig.s 5).

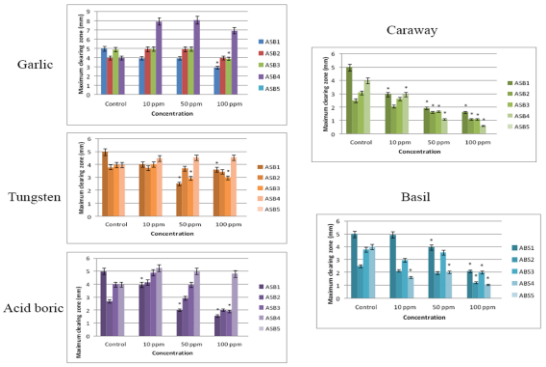

Fig. 5. Effects of garlic, caraway, basil, tungsten and boric acid on cellulolytic activity of endosymbionts in the digestive system of *Amitermes vilis*. Values are mean of 3 replicates; Correlation is significant at the 100 ppm, based on LSD $\alpha \leq 0.05$.

Discussion

In this study, several types of bacteria with distinct characteristics were isolated from the *Amitermes vilis* termite's gut. These were further characterized mainly by morphological, physiological and biochemical tests. Four species of bacilli were identified as *Bacillus cereus*, *B. circulans* and *B. licheniformers* and a Gram-negative bacterium, encoded as ASB 4, was remained unidentified. Although, these methods were enough to distinguish the isolates, but on accurately identification needs PCR-based analyses.

Regarding the enzymatic activities of the bacterial isolates, we found two efficient cellulolytic bacteria isolates (ASB 1 and ASB 4) which could produce relatively close amounts of glucose in 5, 10 and 15 ppm concentrations compared to standard glucose solution. Also their enzymatic activity assays were determined, we found the strain ASB 1 showed a good enzyme activity in both 10 ppm and 15 ppm concentrations, whereas the strain ASB 4 was active at 5 ppm concentration.

Since at the 100 ppm concentration, a significant reduction in cellulolytic activity of the bacteria could be seen; therefore a conclusion can be drawn that the mentioned essential oils and mineral compounds can disrupt the balance of *Amitermes vilis*'s digestive system through synergistic or antagonistic effects in 100ppm concentration.

The results from the effects of two mineral compounds (boric acid and tungsten) and three essential oils (garlic, caraway and basil) on cellulolytic activity of the bacterial isolates were significant and were coincided with results obtained by Burt (2004), Nazzaro *et al*. (2013), Sun OG *et al*. (2007) and Katsuda *et al*. (2004). We also found a synergist effect of garlic essential oil on the ASB4 bacterial isolate at low concentrations. These particular effects of some plant essential oils on cellulolytic activities of bacteria are well documented.

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