Sjournals
Scientific Journals
Scientific Journal of Biological Sciences (2013) 2(12) 244-255
ISSN 2322-1968
doi: 10.14196/sjbs.v2i12.1075

Contents lists available at Sjournals
Scientific Journal of
Biological Sciences
Journal homepage: www.Sjournals.com

Original article

Xylinasic and amylasic activities of some microorganisms in the digestive exudate of termites (Macrotermes Subhyalinus and Macrotermes Bellicosus)

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ARTICLE INFO

Article history:
Received 06 December 2013
Accepted 20 December 2013
Available online 29 December 2013

Keywords:
Enzymes activities
Intestines
Termites macrotermes
Abidjan

ABSTRACT

Termites are terrestrial insects involved in the degradation of complex substances such as cellulose and hemicellulose found into the plant. They thus influence soil properties and contribute to the emission of methane gas. The ability of these Isoptera to digest complexes molecules may associated to the enzymatic activity of intestinal microorganisms. The overall aim of this study was to isolate and make a presumptive identification of cultivable microorganisms in the digestive tract of termites Macrotermes subhyalynus and Macrotermes bellicosus harvested. And search for glycosidase activities produced by the microflora. To achieve this goal 120 insects belonging to two castes (Macrotermes subhyalynus and Macrotermes bellicosus) were collected on termite mounds located at the Felix Houphouet Boigny University of Cocody (Abidjan, Côte d'Ivoire). The intestines of these Isoptera were collected under aseptic conditions and seeded on agar PCA, VRBL, MRS and Sabouraud chloramphenicol. Moreover, the enzymatic activities were investigated in bacteria obtained according to the method of Bernfeld (1955). The results show that in both species of termites, Gram + bacilli had the majority. On nutrient agar, genera Bacillus, Streptococcus and Enterococcus were identified. On MRS agar, the

genera Lactobacillus, Bifidobacterium and Lactococcus have been identified. On agar VRBL, genera Enterobacter, Yersinia, Klebsiella, Citrobacter, Serratia, Escherichia and Yersinia were isolated. Finally, amylasic activities were observed in yeast strains and Lactobacillus. The xylanasic activity was produced by yeast strains of Bacillus and two unidentified strains.

1. Introduction

Termites are abundant terrestrial insects in organic matter and have an important role in the process of degradation of litter in tropical ecosystems (Wood and Sands, 1978). So they are involved in the degradation of complex substances such as cellulose and hemicellulose found in plant (Wood and Sands 1978, Brown, 1998). These insects have been the subject of many studies because of their importance in tropical environments. These studies relate to their role as ecosystem engineers, their influence on soil properties (Anderson et al, 1991. Dangerfield et al, 1998 ; Donovan et al, 2001), their contribute to the emission of methane gas (Eggleton et al., 1999) and finally their role in the degradation of lignocellulose (Hinze et al., 2002).

The digestive tract of most termites contains an abundant and diverse bacterial microflora. According to Brauman et al. (2001), various microorganisms inhabit the gut of the termites with densities from 106 to 107 cells per µl of intestinal volume. The ability of these Isoptera to digest complex molecules is mainly attributed to the action of the intestinal microorganisms (MacKenzie et al, 2007. Braumann and Fall, 2000).

The general objective of this study was to isolate and to make a presumptive identification of cultivable microorganisms in the digestive tract of termites Macrotermes bellicosus and Macrotermes subhyalynus and searching for glycosidase activities produced by the microflora. To achieve this aim we carried out the isolation of bacteria (mesophilic aerobic bacteria, coliforms, lactic acid bacteria and yeast) on agar PCA VRBL, MRS and Sabouraud chloramphenycol; Identification of isolated strains on these plates and Research amylasic, xylanasic and cellulasic activities produced by strains isolated.

2. Materials and methods

2.1. Biological material

The study material consists of soldiers and workers of two species of termites Macrotermes subhyalinus and Macrotermes bellicosus.

2.2. Sampling

After formal identification by an entomologist, 20 per caste and per species insects from three castes (workers, young and old soldiers) of each species of termite was removed without damaging the nest both in Macrotermes subhyalinus than Macrotermes bellicosus. ¶20 insects of each caste represented a sample. Insects are collected on termite located at the Felix Houphouet Boigny University of Cocody (Abidjan, Côte d'Ivoire) and ¶transported to the laboratory in jars lids were perforated.

2.3. Isolated microorganisms

2.3.1. Dissection

Twenty termites of the same caste were sterilized in 70% ethanol (w / v) for 5 min, and then rinsed with sterile water. They were then dissected in aseptic conditions (around the flame Bensec) using a stainless steel blade with a sterile forceps and to show up the content of the digestive tract. The homogeneous mixing of the contents of the digestive tracts of five insects caste formed exudate to search for symbiotic microorganisms.

2.3.2. Isolation techniques

The sterile nutritive bubble was inoculated starting from the exsudat and was incubated at 37 C for 24 H.¶ Agar PCA VRBL, MRS and Sabouraud chloramphenicol (laboratory Conia, Spain) were prepared according to the manufacturer's instructions and poured into Petri dishes. They were sowed by streaking from the culture medium and incubated in ovens at 37 ° C and 44 ° C respectively for PCA agar and agar VRBL for 24 h. Sabouraud agar was incubated at 30 ° C for 24 to 48 hours. As for MRS agar incubation is done anaerobically at 37 ° C for 72 h. Anaerobiosis was done in a closed jar where a candle was lit previously.

The isolates were then purified by successive subculture the purity of strains was determined by microscopic observation.

2.4. Identification of bacterial strains isolated

The strain identification was made on the basis of morphological, biochemical and physiological characteristics coupled with the use of taxonomic keys.

2.4.1. Determination of morphological characters

The Gram staining technique was used to determine the morphological characteristics and the type of cell association. It also determined the frequency of occurrence of bacteria according to their shape and position of spores in some sporulated isolates. So to see the prevalence of Gram + bacteria and Gram - in the gut of termites, this frequency was calculated using the following formula:

F = number of colonies of Gram given / total number of colonies

2.4.2. Determination of conventional biochemical characters

Biochemical identification was performed on pure isolates of VRBL agar at 45 ° C, catalase and biochemical characteristics were investigated using the reduced rack Léminor.

2.4.3. Determination of fermentation type

Fermentative types of isolates (CO2 production) were determined in MRS broth for isolates from MRS agar. This medium is left in a range of test tubes (10 ml per tube). Durham tube is disposed in each tube prior to sterilization at 121 ° C for 20 minutes. After inoculation and incubation for 48 hours CO2 production is highlighted by the presence of gas in the Durham tube.

2.5. Conservation techniques isolates

Isolates were stored in two types of media:

- Agar for the conservation of storage at room temperature. Cultures maintained under these conditions are regularly used for laboratory work.
- On MRS broth in an aqueous solution of 30% glycerol (v / v) for storage at - 80 ° C. Two or three colonies were collected on agar 48 hours and mixed with MRS broth submerged in 1 ml of a solution of 30% glycerol. After homogenizing, the tubes are placed at - 80 ° C. This technique is favorable for the preservation of cells for several years in the collections.

2.6. Monitoring bacterial cultures on liquid media for the production of enzymes

Cultures in liquid media were performed at 37 ° C under agitation (180 rpm) to ensure the supply of oxygen to the cells. After incubation, the cultures were collected and centrifuged at 6000 rpm for 30 min at 4 ° C. The collected supernatant is the crude enzyme extract.

2.6.1. Enzyme production

The production of amylase, xylanase and cellulase by different strains were identified on solid media reconstructed in the presence of substrates for these enzymes, according to a method described by Larpent and Larpent-Gourgaud (1985).

2.6.2. Evaluation of growth by optical density of cultures

The optical density of cell suspensions (broth) was measured spectrophotometrically at 600 nm because the absorbance of the bacterial culture is proportional to the cell concentration. This proportionality factor is itself a

function of the size and shape of the cells. An aliquot of the culture medium was used as reference. This approach has allowed knowing the growth zone where the production of the enzyme is high.

2.7. Search enzymatic activities

Polysaccharidasic activities are required in the supernatant at different pH: citrate phosphate buffer (2.6 to 7.0), sodium acetate buffer (pH 3.6 to 5.6), sodium phosphate buffer (pH 5.6 to 8.0), and glycine buffer (pH 8.0 to 10.0).

The substrates tested for the presence of polysaccharidasic activities were starch, xylan and carboxymethylcellulose (CMC).

The sugars released in the enzymatic hydrolysis of polysaccharides (soluble starch, carboxymethyl cellulose and xylan) were assayed According to the method Bernfeld (1955) using the DNS.

3. Results and Discussion

After 24 h of incubation, the growth of germs in all broths seeded with Macrotermes bellicosus and Macrotermes subhyalinus exudates resulted in a veil over the middle, increased turbidity and sediment at the bottom of the tubes. Bacterial cultures so obtained were inoculated by streaking the solid media.

a. Characteristics of isolates on solid culture media

Seeding from agar Sabouraud Chloramphenicol the culture broth, used to select the molds and yeasts showed the total absence of mold, however large whitish colonies with regular board has been observed. There was bacterial growth on nutrient agar, VRBL (44 ° C), and MRS agar under anaerobic conditions at 37 ° C and the PCA agar.

b. Characteristics of strains on agar PCA

On agar PCA, circular, irregular bacterial colonies with various colors (yellow, white, red ...) have been highlighted. Table 1 summarizes the characteristics of the strains isolated on the agar. Three presumptive genera, Bacillus, Streptococcus and Enterococcus were identified.

c. Strain characteristics on MRS agar

On MRS agar, colonies are small, whitish, with a regular board. These observations show the presence on this agar of lactic bacteria. Gram staining of isolates after 72 h of culture on MRS agar make sure that they were Gram + bacteria. The homofermentative or heterofermentative character is highlighted by the production of carbon dioxide in MRS broth containing a Durham tube. The results obtained on MRS media are shown in Table 2. The strains were mostly heterofermentative. The genera Lactobacillus, Bifidobacterium and Lactococcus were identified.

d. Strain characteristics on Sabouraud agar

On Sabouraud agar chloramphenycol five strains forming large whitish colonies, rounded and curved board considered as yeasts were isolated. This was only encountered among the workers of Macrotermes subhyalinus.

e. Characterization of strains on agar VRBL

On VRBL agar all colonies had a regular border and were curved, but they had different shapes and colors. These observations demonstrated the presence of different bacterial species. Isolates identified in this study through the Leminor rack come from the gut of Macrotermes bellicosus worker and the worker, the little and the great soldier of Macrotermes subhyalinus on VRBL agar at 44 ° C after 24 hours incubation (Table 3 and 4). Genera Enterobacter, Yersinia, Klebsiella, Citrobacter, Serratia, Escherichia and Yersinia were isolated.

Table 1
isolated bacterial strains agar PCA.

Types of termites	Castes	Shapes and arrangement	Catalase	Gram	Presumptive Genres
Macrotermes subhyalinus	worker	Bacilli, short and small chain isolated	+	+	ND
	Little Soldier	Bacilli and isolated pairs	+	+	ND
		Cocci isolated and chain	+	+	ND
		Coccobacilli pair, chain and cluster	+	+	ND
	Great soldier	Coccobacilli isolated and pair	+	+	ND
		Cocci isolated and pair	+	+	ND
		Isolated bacilli and chain	+	-	ND
Macrotermes bellicosus	worker	Bacilli spore to spore nondeforming Central isolated pair	+	+	Bacillus
	Little Soldier	Spore-forming bacilli in nondeforming central spore, catenary and clusters	+	+	Bacillus
		Bacilli spore deforming plant in clusters, chain	+	+	Bacillus
	Great soldier	Bacilli terminal spore nondeforming catenary	+	+	Bacillus
		Cocci in chain	-	+	Streptococcus (Homofermentative)
		Cocci in short chains	-	+	Enterococcus (Homofermentative)

Cat = catalase (+ catalase production, absence of catalase), ND: not determined PS: little soldier; GS: great soldier, O: worker

Table 2
bacterial isolates sister MRS agar.

Termite species	Caste	Shape and arrangement	Catalase	Gram	Fermentation type	Presumptive Genera
Macrotermes subhyalinus	worker	Isolated bacilli chain	-	+	heterofermentative	Lactobacillus
	Little Soldier	Bacilli isolated, pair and chain	-	+	heterofermentative	Lactobacillus
		Coccobacilli, pair	-	+	heterofermentative	Bifidobacterium
	Great soldier	Hull chain	-	+	homofermentary	Lactococcus
		Bacilli, pair, chain	-	+	heterofermentative	Lactobacillus
Macrotermes bellicosus	worker	Coccobacilli, pairs and clusters	-	+	heterofermentative	Bifidobacterium
		Bacilli, pair, chain	-	+	heterofermentative	Lactobacillus
	Little Soldier	Bacilli isolated, pair, chain	-	+	heterofermentative	Lactobacillus
		Coccobacilli pair clusters	-	+	heterofermentative	Bifidobacterium
	Great soldier	Bacilli isolated chain, pair and isolated	-	+	heterofermentative	Lactobacillus

Table 3
Morphological and biochemical characteristics of bacterial strains isolated on VRBL (worker Macrotermes bellicossus).

	Numbers of strains						
Settings	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Gram	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-
Morphology of isolates	Bacilli	Bacilli	Bacilli	Coccobacilli	Bacilli	Bacilli	Bacilli
Mobility	-	-	-	-	+	+	-
Catalase	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+
TDA	-	-	+	-	-	-	-
Citrate	+	+	+	+	+	+	-
Indole	-	-	-	-	-	-	-
Urea	-	-	+	+	-	-	+
LDA	-	-	+	-	-	-	-
LDC	+	-	-	-	-	+	-
- H 2 S	-	-	-	-	-	-	-
- Gas	+	+	+	+	+	+	+
- Glucose	+	+	+	+	+	+	+
- Lactose	+	+	+	+	-	+	+
- Mannitol	+	+	+	+	+	+	+
Presumptive Genres	Enterobacter	Enterobacter	Yersinia	Klebsiella	Citrobacter	Serratia	Yersinia

- = Absent, + = presence.

Table 4
morphological and biochemical characters isolated on VRBL (large and small soldiers Macrotermes subhyalinus) bacterial strains.

	Numbers of strains								
	Isolates (little soldier Macrotermes subhyalinus)						Isolates (great soldierMacrotermes subhyalinus)		
Settings	1	2	3	4	5	6	1	2	3
Gram	-	-	-	-	-	-	-	-	-
oxidase	-	-	-	-	-	-	-	-	-
Morphology of isolates	Bacilli	Bacilli	Cocobacilles	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Mobility	-	-	-	+	-	+	-	-	+
Catalase	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+	+	+
TDA	-	-	-	-	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-
Urea	+	+	+	-	-	-	+	+	+
LDA	-	-	-	-	-	-	-	-	-
LDC		-	+	+	-	-	-	+	-
- H 2 S	-	-	-	-	-	-	-	-	-
- Gas	+	+	+	+	+	+	+	+	+
- Glucose	+	+	+	+	+	+	+	+	+
- Lactose	+	+	+	+	+	-	+	+	+
- Mannitol	+	+	+	+	+	+	+	+	+
Presumptive Genres	Yersinia	Klebsiella	Klebsiella	Serratia	Klebsiella	Enterobacter	Yersinia	Klebsiella	ND

- = Absent, + = presence , ND: Not Determined.

f. acterial cultures for producing enzymes

Enzyme activities produced

Bacteria were developed quite well in minimal medium where they produced amylasic and xylanasic activities. Yeast strains (LOS) and Lactobacillus (LBPs) produce amylasic activities. The xylanasic activity was produced by the following starins : LOS BPB (Bacillus), SPS (unidentified strains) and SGS (unidentified strains) (Fig. 1).

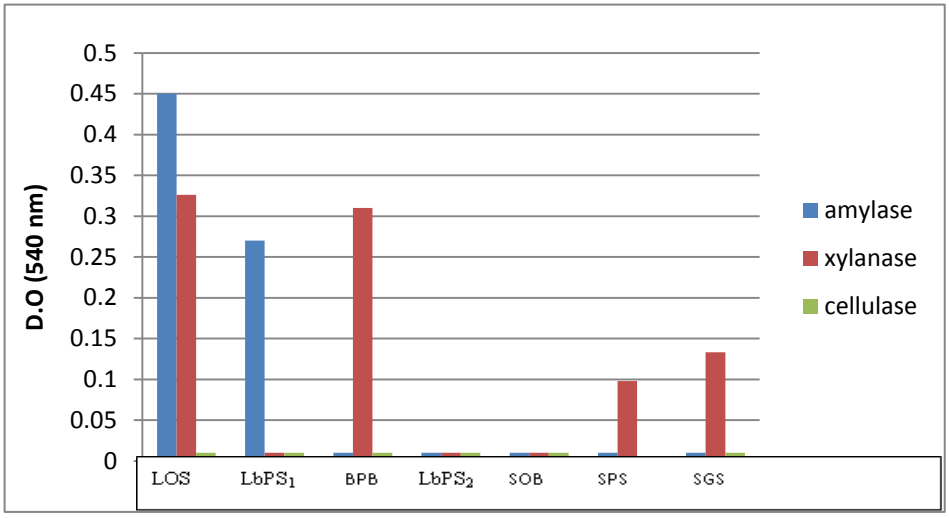

Fig. 1. Production of enzymatic activities of the studied isolates.

LOS: strains isolated from yeast Worker
LBPs: Lactobacillus strains isolated from Little Soldier Macrotermes subhyalinus
BPB: Bacillus strains isolated from Little Soldier Macrotermes bellicosus
SOB: Worker isolated from unidentified strains Macrotermes bellicosus
SPS: isolated little soldier unidentified strains Macrotermes subhyalinus
SGS unidentified strains isolated from Grand Soldier Macrotermes subhyalinus

Determination of pH activity optimum

Three strains (LOS LBPs and BPB) from MRS agar and PCA were selected and grown in liquid media for the production of hydrolases namely amylasic and xylanasic activities. The incubation was done at 37 ° C for 60 min.

- Study of the pH optimum of the xylanase activity of BPB strain at 37 ° C

The optimum pH of the xylanase activity produced by this strain is 4.0 in citrate phosphate buffer (Fig. 2).

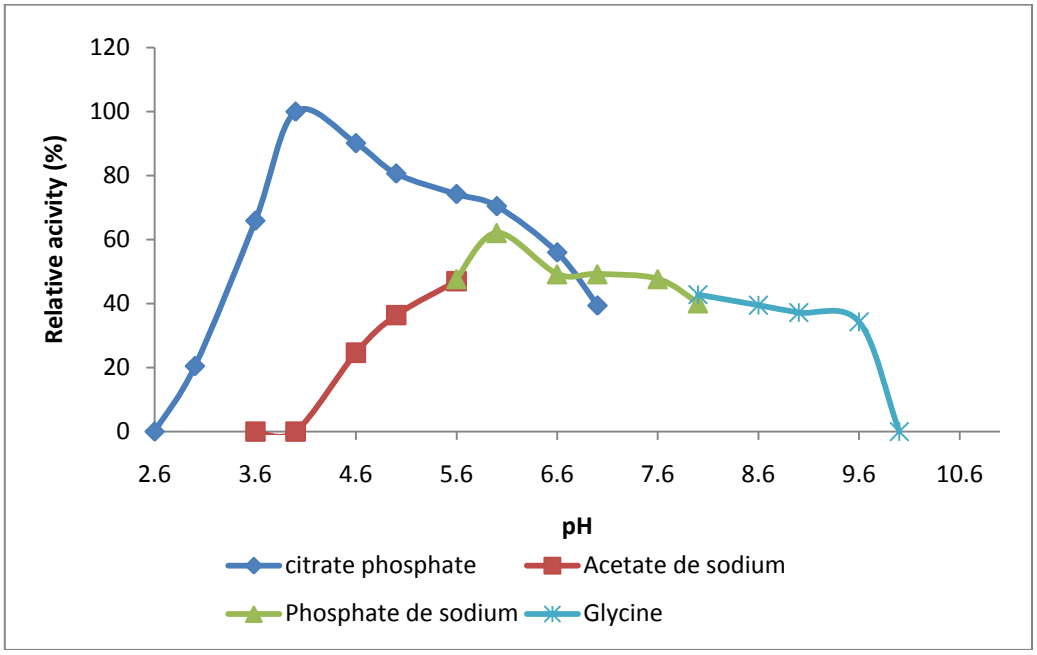

Fig. 2. pH optimum of the xylanasic activity of the isolate BPB.

Study of optimum pH of the xylanasic activity of the LOS strain at 37 ° C

The optimum pH of the xylanasic activity produced by this strain is 6.0 in sodium phosphate buffer (Fig. 3).

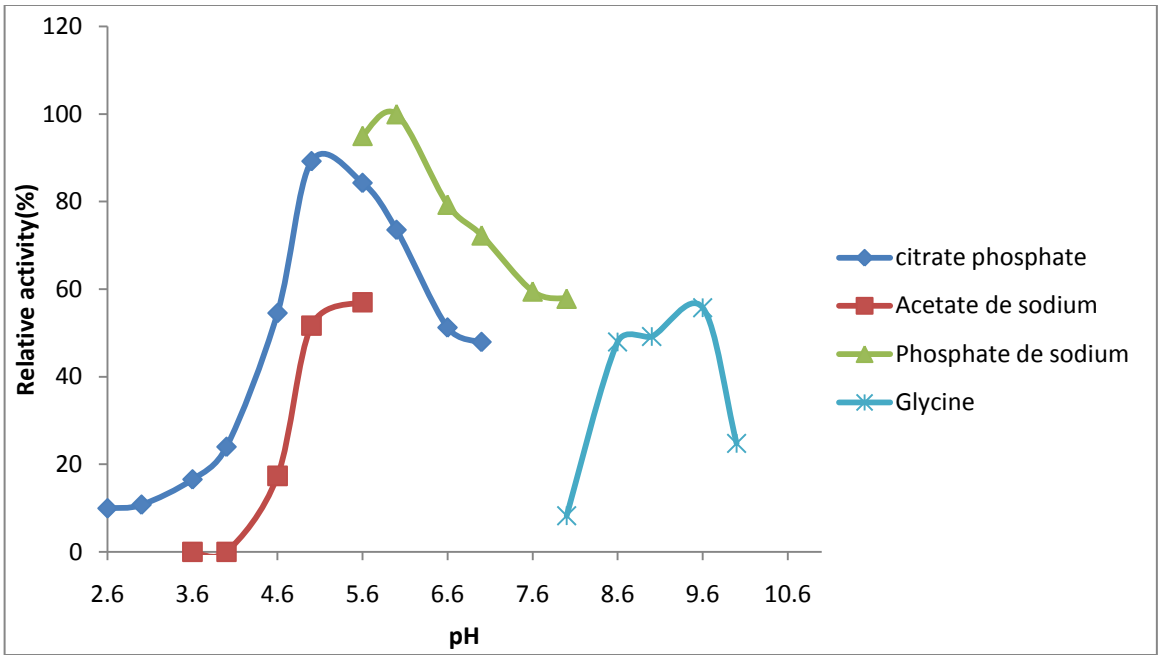

Fig. 3. pH optimum of the xylanasic activity of the isolate LOS.

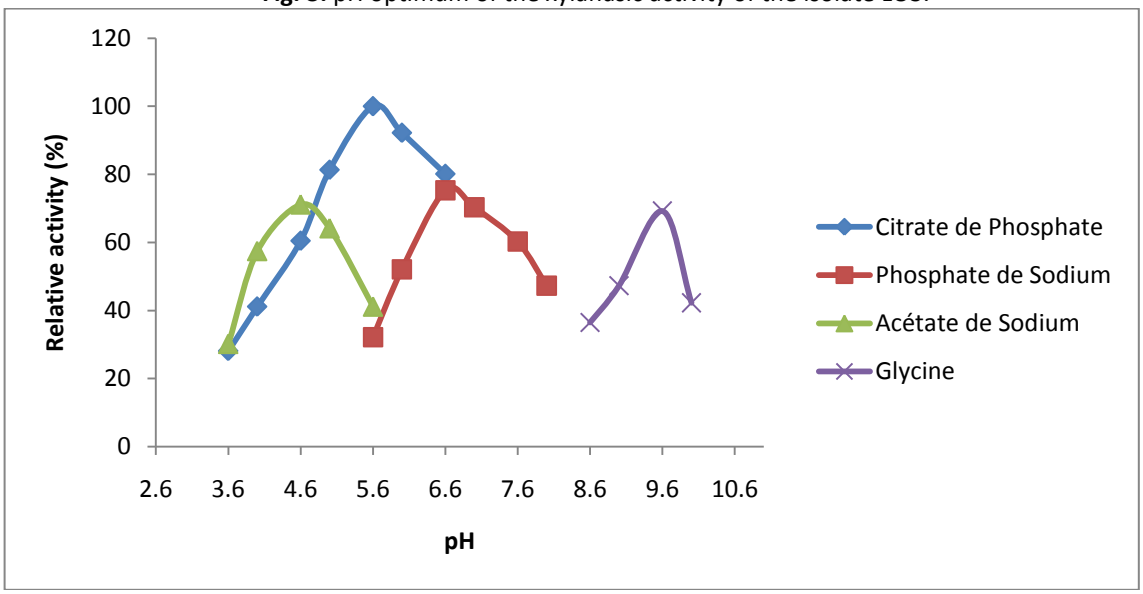

Fig. 4 . pH optimum of amylase activity of the isolate LBPs.

Strains produced an amylase activity, the pH optimum is 5.0 in sodium acetate buffer (Fig.4).

The amylase activity produced by the LOS strain is optimal at pH 5.6 in citrate phosphate (Fig. 5).

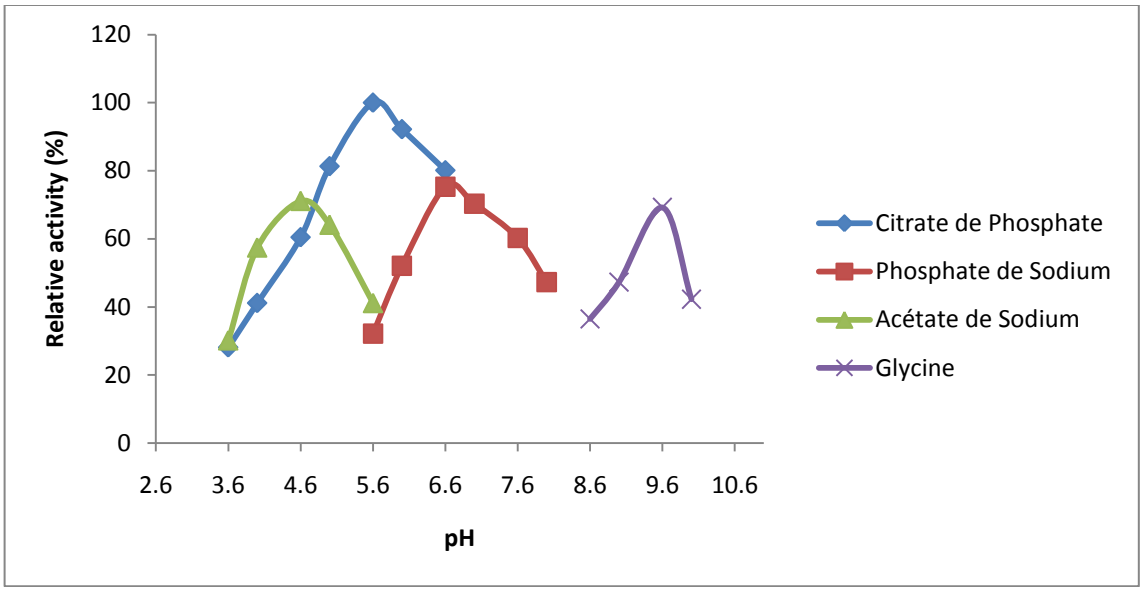

Fig. 5. pH optimum of amylasic activity of the LOS isolate.

There is a strong presence of microorganisms in the exudate of the intestine of both termite species considered here. Indeed, microbiological analyzes have revealed a diversity of microorganisms composed mostly of Gram+. The diversity of such microflora (bacteria, fungi, protozoa etc..) has been reported since 1958 and that is related to the evolution of these insects (Grasse and Noirot, 1958). Brauman et al. (2001) showed that various microorganisms are hosted in the intestinal tract of all termites, with a density of 106-107 cells / µl of intestinal volume (Bignell et al, 1983. Brauman et al, 2001).

The microbiological analysis of the exudate from the intestine of the three castes (small and great soldiers and workers) of two termites species (Macrotermes bellicosus and Macrotermes subhyalimus) shows that these insects harbor a variety of bacteria in their intestinal tract , some of which are difficult to cultivate. However we could isolate from these termites some kinds of fermentative microorganisms such as Lactobacillus, Bifidobacterium, Enterococcus and Streptococcus as well as in the worker termites among soldiers. Analysis of fermentation types showed that the majority of bacteria grown on MRS agar are hetero, suggesting that these microorganisms are capable of converting glucose to lactate, acetate or ethanol and CO2. Indeed, termites can effectively hydrolyze cellulose with their intestinal microflora (Breznak and Brown, 1994). Thus, these microorganisms may be important for operation in biotechnology. This is for example the production of bioethanol from lignocellulosic residues abundantly present in our ecosystems (Mackenzie et al., 2007). Yeasts has been isolated only in the worker Macrotermes subhyalinus. Bacillus was isolated only from the tract of the little soldier in Macrotermes bellicosus.

In addition to these fermentative microorganisms, we could also isolate genera of the Enterobacteriaceae family, among which may be mentioned of Citrobacter, Enterobacter, Escherichia, Klebsiella, Serratia, Shigella and Yersinia. Microorganisms from the Enterobacteriaceae family were observed in Macrotermes bellicosus (worker cast) intestinal tract.

This study shows that the bacterial population is not varying only from one species of termite to another but also from one caste to another within the same species. The microbial diversity was discussed by Brown (1998), who showed the presence of microorganisms in the intestinal tract of termites including several fungi as well as bacteria. The composition of the bacterial population can be explained in part by the diet of termites (Brauman et al., 2001). In fact Anderson et al. (1991) showed that the nature of the intestinal microflora is depend on bacterial species but also on the of the nest implementation site. In another study, Brauman et al., 2001 confirmed that microbial diversity was related to the presence of a dense population of bacteria and less dense population of archaea in Macrotermes subhyalinus and Macrotermes bellicosus.

One explanation for this is the variety of habitat for termites which differs depending on the species. This microflora is much more varied than speculations so far have taken place on culturable microorganisms. Indeed, recent studies by Warnecke et al. (2007) showed that much of the termite symbiotic microorganisms cannot be

isolated or are fastidious for the in vitro culture techniques (Ohkuma and Kudo, 1996; Schmitt-Wagner et al, 2003. Bradley et al, 2004. Hongoh et al, 2005.Hongoh et al, 2006).

Both termite species studied are higher termites. The literature reveals that in addition to their symbiosis with bacteria, these termites develop an original symbiosis with the fungus Termitomyces that grows in nests (Anklin-Mühlemann et al., 1995). Rouland et al. (1988) and Anklin-Mühlemann et al. (1995) showed that the fungi grower termite in this symbiosis cannot digest cellulose compounds and wood in the absence of the fungus.

The amylasic activity was the induced more by the addition of starch in the culture medium. So the best amylasic activities were produced by yeasts strains of and lactic acid bacteria (Lactobacillus). The yeast strains of Lactobacillus and produce amylases whose optimum pH activity are respectively 5.6 in citrate phosphate buffer and acetate buffer in 5.0 phosphate at 37 ° C. Similarly Kostinek et al. (2007) and Rasooli et al. (2008) showed the production of amylase by Bacillus licheniformis during the fermentation of cassava. Bacillus and yeast produced xylanasiques activities with pH respective of 4.0 in citrate phosphate buffer and 6.0 in sodium phosphate buffer at 37 ° C. Indeed Symbiotic microorganisms' termite family Nasutitermitidae (Brennan et al., 2004) also produce xylanasic activities. Similarly xylanase from symbiotic fungus Termytomices sp. has even been purified (Faulet et al., 2005).

4. Conclusion

This study permitted to isolate and identify several types of microorganisms in the digestive tract of Macrotermes bellicosus and Macrotermes subhyalinus termites. It also illustrated glycosidase activities produced by this microflora. It was noticed that these insects harbor significant microbial diversity dominated by Gram-positive bacteria that are represented in all castes of the two termite species, while in Macrotermes bellicosus only the workers home Gram- bacteria, they are present in all Macrotermes subhyalinus castes . Yeast, Bacillus and lactic acid bacteria isolated from the two termites species have shown good potential for the amylase and xylanase enzyme production.

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