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Isolation of a Tannic Acid-Degrading *Streptococcus* sp. From an Anaerobic Shea Cake Digester

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Abstract: An anaerobic digester fed with shea cake rich in tannins and phenolic compounds rich-shea cake and previously inoculated with anaerobic sludge from the pit of a slaughterhouse, enabled six months acclimatization of the bacteria to aromatic compounds. Afterwards, digester waste water samples were subject to successive culture on media with 1 g L⁻¹ tannic acid allowing the isolation of a bacterial strain coded AB. Strain AB was facultatively anaerobic, mesophilic, non-motile, non-sporulating, catalase and oxidase negative bacterium, namely strain AB, was isolated from an anaerobic digester fed with shea cake rich in tannins and phenolic compounds, after inoculation with anaerobic sludge from the pit of a slaughterhouse and enrichment on tannic acid. The coccoid cells occurred in pair, short or long chains and stained Gram-positive. Strain AB fermented a wide range of carbohydrates including glucose, fructose, galactose, raffinose, arabinose, sucrose, maltose, lactose, starch and cellulose. Optimum growth occurred with glucose and tannic acid at 37°C and pH 8. The pH, temperature and salt concentration for growth ranged from 5 to 9, 20 to 45°C and 0 to 15 g L⁻¹, respectively. Strain AB converted tannic acid to gallic acid. These features were similar to those of the *Streptococcus* genus. The determination of tannic acid hydrolysis end products, ability to utilize various organic acids, alcohols and peptides, GC% of the DNA, the sequencing of 16S rRNA gene and DNA-DNA hybridization will permit to confirm this affiliation and to determine the species.

Key words: *Streptococcus* sp., tannic acid hydrolysis, shea cake, anaerobic digester, Burkina Faso

INTRODUCTION

The shea tree (*Butyrospermum paradoxum*) is a specific savanna regions plant of West Africa; the shea walnuts, from of its fruits are used in the production of the shea butter by traditional units and agro-food transformation factories (vegetable oil factory, soap factory, cosmetic...) (Bonkougou, 1987; Maranz *et al.*, 2004). The wastes from the production of shea butter are mainly phenol-rich compounds that cause a major environmental problem in Western Africa and particularly in Burkina Faso.

Phenolic compounds are a group of highly hydroxylated compounds present in extractive fractions of several plant materials. Their most distinguishing characteristic is their reactivity with proteins and related

polyamide polymers (Haslam, 1996). Phenolic compounds in plants include several groups such as simple phenols and phenolic acids (single substituted phenolic ring as catechol or caffeic acid), quinones, flavones, flavonols, flavonoids and coumarins. Tannin is a general descriptive name of polymeric phenolic substances capable of tanning leather or precipitating phospholipids membrane that makes the cell wall impermeable from solution, a property known as astringency. Their molecular weights range from 500 to 3000 g mol⁻¹. Tannins are either hydrolysable or condensed. Hydrolysable tannins are based on gallic acid monomers; condensed tannins, often called proanthocyanidins are based on flavonoid monomers, flavone derivatives or quinone units.

The problem with phenolic compounds in the environment is of great concern in two key regards.

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Firstly, polyphenols notably tannins are bioactive substances; they are vulnerable to polymerization as well as oxidization in air (Champ, 2002). These reactions result in high molecular weight compounds with high capacity to link with protein to form indigestible complexes. In this way, they are discerned like being environmental pollutants by being recalcitrant to biodegradation (He *et al.*, 2007). Secondly, phenolic compounds are toxic to aquatic organisms and microorganisms (Karou *et al.*, 2005). They exert their antibacterial activity through iron deprivation or through non specific interaction with vital proteins such as enzymes (Rohn *et al.*, 2002). Indeed, in the environment, they inhibit microorganisms and enzymes involved in the degradation of biopolymers. A previous study of a continuous anaerobic digester fed with shea cake showed high tannin removal rates and production of organic acids and methane (Ouattara *et al.*, 1992) therefore, the present study was conducted to isolate and to identify a bacterial strain able to degrade phenolic compounds throughout tannic acid hydrolysis.

MATERIALS AND METHODS

Source of isolation: The sample for microorganism isolation was a mud sample collected from January 21, 2007 to March 16, 2008, from the purification station of Ouagadougou slaughterhouse. This station is an anaerobic lagoon functioning in strict anaerobe. Samples were collected in sterile bottles at 1.5 m depth and immediately transported to the laboratory at ambient temperature. These samples were used to inoculate an anaerobic digester containing shea cake which was subsequently incubated for 6 months at 35°C for microorganisms' acclimatization prior to isolation.

Culture media: The anaerobic technique of Hungate (1969) modified by Macy *et al.* (1972), was used for microorganisms cultivation. The basal medium contained (L⁻¹): 0.4 g NH₄Cl, 0.5 g KH₂PO₄, 1 g NaCl, 0.33 g MgCl₂.6H₂O, 0.05 g CaCl₂.2H₂O, 0.25 g cysteine-HCl, 2 g yeast extract (Difco), 1 mL trace-element mineral solution (Widdel and Pfennig, 1982) and 1 mg resazurin. The pH was adjusted to 7 with 1 M KOH solution. The medium was then boiled under a stream of argon and cooled to room temperature. Five milliliter aliquots were dispensed into Hungate tubes, degassed under argon and subsequently sterilized by autoclaving at 121°C for 15 min. Prior to inoculation 0.05 mL of 10% (w/v) NaHCO₃ and 0.05 mL of 5% (w/v) Na₂S.9H₂O were injected from sterile stock solutions; afterwards, substrates were injected from concentrated anaerobic sterile stock solutions to obtain the desired final concentration.

Enrichment: For enrichment, 0.5 mL liquid sample from the anaerobic digester was inoculated into 5 mL basal medium containing 1 g L⁻¹ tannic acid and then incubated at 37°C. The enrichment culture was subcultured every 72 h during three weeks under the same conditions prior to isolation.

Isolation: From the last enrichment culture, a sample (0.5 mL) was serially diluted tenfold to inoculate tubes containing basal medium with 1 g L⁻¹ tannic acid and 3.2% agar (roll tubes). Well isolated colonies that developed in roll tubes were picked and serially diluted in fresh media. This procedure was repeated until only one type of colony was observed. The purity was then checked on cells grown on basal medium supplemented with 10 mM glucose and 0.2% Biotripcase (Difco) in both aerobic and anaerobic conditions. The purification was confirmed by light microscopy.

Morphology and sporulation test: Microorganism morphology was assayed by optic microscopy before and after coloration (Gram coloration, flagella coloration, spore coloration). For testing heat resistance, cells grown in basal medium containing glucose were exposed to 80, 90 and 100°C for 10 min. The cultures were cooled quickly to ambient temperature and inoculated into fresh glucose-containing medium and growth was recorded after 24 h incubation at 37°C. Conditions for sporulation that were tested included growth in the presence of glucose or tannic acid and without added carbon sources.

Growth parameters: For all experiments basal medium containing 0.2% yeast extract and 10 mM glucose was used. The experiment was performed in triplicate. The pH of the pre-reduced anaerobic medium was adjusted with 0.1 M KOH or 0.1 M HCl to obtain a range of initial pH between 3.0 and 10.0. The temperature range for growth was set between 20 and 50°C. Different amounts of NaCl were weighed directly in Hungate tubes prior to dispensing 5 mL medium to obtain the desired NaCl concentrations (0-30 g L⁻¹). The growth was monitored by measuring the Optical Density (OD) at 580 nm and the average maximal growth rate (μ_{max}) was determined for each temperature, pH and NaCl concentration as described by Ouattara *et al.* (1992).

Electron acceptors: Sulfate and the thiosulfate (20 mM each) were tested as electron acceptors in basal medium containing 10 mM glucose, from pre-sterilized and concentrated stock solutions.

Substrate utilization: All experiments were performed in triplicate on three successive cultures with an inoculum

subcultured at least once under the same experimental conditions. The substrates tested were injected into Hungate tubes containing 5 mL pre-sterilized basal medium, from pre-sterilized and concentrated stock solutions. The following substrates were used: 20 mM carbohydrates (arabinose, fructose, galactose, glucose, sucrose, lactose, maltose, xylose, raffinose, starch and cellulose) and 5 mM tannic acid. Concentrated stock solution were prepared, neutralized if necessary, set anaerobic by gassing with argon and sterilized by filtration (0.2 µm pore size Millipore filter). Tannic acid was tested with yeast extract (0.2%) as carbon source. An increase in OD₅₈₀ in tubes containing added substrates, compared with control tubes without substrate, was considered to be positive growth.

Analytical techniques: Bacterial growth was monitored by measuring OD₅₈₀ from anaerobic Hungate tubes inserted into the cuvette holder of a spectrophotometer (Shimadzu CS-930). Gallic acid production was measured and expressed as OD₂₆₃ (Dalsager, 1984; Mondal *et al.*, 2001).

RESULTS

Enrichment and isolation: To isolate different tannic acid-degrading microorganisms, an enrichment culture method was used. This enrichment culture was designed to select strain able to grow on tannic acid as carbon source. Cultures developed in medium containing 1 g L⁻¹ tannic acid within 3 weeks incubation at 37°C as shown by growth and organic acid production. After several transfers in the liquid medium, these bacteria were then screened for their ability to degrade tannic acid. Several isolates were obtained by using the roll-tube method. One of these isolates, designated strain AB, was selected for further characterization.

Morphology: Strain AB was isolated from a shea cake digester containing aromatic compounds, after enrichment on tannic acid. Cells of strain AB were non-motile cocci that occurred in pairs, short or long chains and stained Gram-positive (Fig. 1). Flagella were not revealed after coloration. Spores were not observed and cells did not survive 10 min heat treatment at 80°C, indicating an absence of heat-resistant cells.

Growth, physiology and metabolic properties: Strain AB was a mesophilic, facultatively anaerobic, chemo-organotrophic bacterium. The optimum growth temperature for the strain was 37°C; growth was observed between 20 and 40°C; no growth occurred at 50°C. The optimum pH for growth was pH 8 and the pH range for



Fig. 1: Phase-contrast micrographs of tannic acid degrading bacterium (Strain AB)

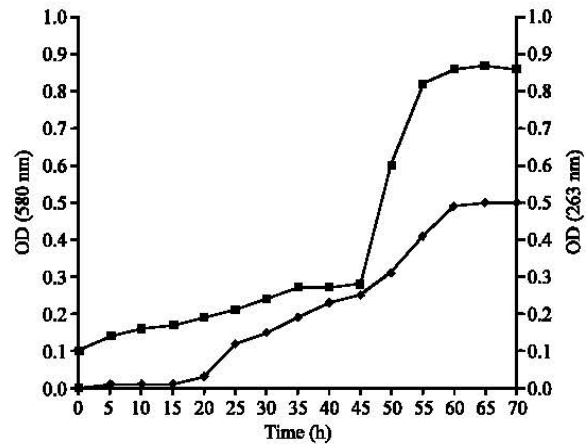


Fig. 2: Transformation of 5 mM tannic acid by strain AB. Growth is indicated by an increase in optical density (■) and the transformation of tannic acid to gallic acid (◆)

growth was pH 5-9. The optimum NaCl concentration for growth was 5 g L⁻¹ and the range for growth was 0-20 g L⁻¹.

The strain AB fermented a wide range of carbohydrates including arabinose, fructose, galactose, glucose, sucrose, lactose, maltose, xylose and raffinose. Cellulose and starch were slightly fermented. Fermentation was accompanied by pyruvic acid production. Strain AB hydrolyzed tannic acid at concentration of 5 mM to gallic acid. Yeast extract stimulated growth, but was not required. The growth rate of strain AB was much lower than that on glucose after 24 h. During growth on tannic acid (5 mM) the intermediate compound (gallic acid) was produced after a lag of two days (Fig. 2). A concentration in tannic acid superior to 25 g L⁻¹ inhibits the growth of the strain. Sulfate and thiosulfate could not be used as electron

acceptors but stimulate the growth of the strain. The strain AB produced neither oxidase nor catalase.

DISCUSSION

Phenotypically, strain AB is Gram-positive, facultatively anaerobic, cocci, non-sporulating, non-motile occurring singly, in pairs or long chains. The strain grows at mesophilic temperatures and ferments a wide range of carbohydrate. Acid is produced during fermentation of carbohydrate. Strain is a chemo-organotrophic bacterium. These phenotypic characteristics can be used to establish a connection between isolate AB and the genus *Streptococcus*.

It demonstrated that some bacteria were able to developed physiological faculties because of their permanent contact with tannins in the culture medium (Smith *et al.*, 2005). Indeed, several bacteria degrading aromatic compounds were isolated either by inoculums enrichment in medium containing these compounds (Long *et al.*, 2009) either from alimentary tract of animals feeding on tannin-rich food (Sasaki *et al.*, 2005).

Thus, *Streptococcus* sp. is recovered often in herbivorous alimentary tract. A *Streptococcus* sp. capable of degrading tannic acid-protein complexes has been isolated from the cecum of koalas (Osawa, 1990); *Streptococcus caprinus* was isolated from the ruminal contents of feral goats browsing tannin-riche *Acacia* species (Brooker *et al.*, 1994). In the same way Nelson *et al.* (1995) isolated *Streptococcus* strain; they observed chain formation when the concentration of tannic acid became higher.

Chamkha *et al.* (2002) isolated a strain of *Streptococcus gallolyticus* from the anaerobic digester fed with shea cake, after enrichment on tannic acid. The digester has previously been inoculated with anaerobic sludge from the pit of a slaughterhouse. Growth of *S. gallolyticus* was inhibited by tannin concentration greater than 17 g L⁻¹. Strain AB, isolated in similar conditions, is inhibited by tannic acid concentration greater than 25 g L⁻¹ and formed long chain than *Streptococcus gallolyticus*. Nelson *et al.* (1995), after phase-contrast microscopic examination, for the same strain degrading tannin, showed an increase in chain formation as the concentration of tannic acid in the medium was increased.

Strain AB differs from other bacterium of *Streptococcus* genus ever isolate by his capacity to form very longue chains and to stand high concentration of tannic acid (25 g L⁻¹). Tannic acid degradation made in much reduced time (less 72 h). Cellulose and starch fermentation by this strain is also a particularity.

Table 1: Comparison of characteristics of stain AB and *Streptococcus gallolyticus*

Characteristics	Strain AB	<i>Streptococcus gallolyticus</i> *
Morphology	Cocci in pairs or long chains	Cocci in pairs or short chains
Size	ND	< 2 µm
Gram reaction	+	+
Spores	-	-
Mobility	-	-
Optimum temperature (°C)	37	40
pH optimum	8	7
NaCl (g L ⁻¹)	0-20	0-40
Tannic acid tolerance (g L ⁻¹)	25	17
Yeast extract requirement	-	-
Growth on other organic compounds		
Carbohydrate ¹		
Tannic acid	+	+
Gallic acid	ND	+
Procatechic acid	ND	+
p-coumaric acid	ND	+
Ferilic acid	ND	+

*Chamkha *et al.* (2002), Carbohydrate¹: Carbohydrate fermented by two strains (fructose, galactose, glucose, lactose, maltose and raffinose). ND: Non determined

The metabolism of aromatic compounds by the type strain *Streptococcus gallolyticus* CIP 36 11T, *S. gallolyticus* CIP 107089, *S. gallolyticus* CIP 107090, *S. gallolyticus* CIP 107091 is established. These strains hydrolyzed tannic acid to gallic acid and decarboxylated it to pyrogallol (Chamkha *et al.*, 2002).

Based on this alone, strain AB can be regarded as similar to *Streptococcus gallolyticus* species. In addition there are numerous phenotypic resemblances that also set these two strains together (Table 1). Based on the evidence presented there, it is proposed that strain AB be designated a species of *Streptococcus* genus.

CONCLUSION

The results of our experiments clearly show that the inoculum acclimatized on the shea cake digester can be used for the degradation of phenolic compounds. Successive enrichment methods allowed the selection of a stable bacterium able to biodegrade tannic acid. The morphological, biochemical and physiological knowledge of the strain AB showed its capacity to hydrolyze tannic acid that allowed us to associate it to *Streptococcus* genus. The measurement of the end products during the metabolism of tannic acid and other aromatic compounds by strain AB, the determination of the GC% of DNA the 16S rRNA sequencing and DNA-DNA hybridization as well will permit to confirm this affiliation and to determine the species.

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