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Screening and phenotypic characterization of thermostable amylases producing yeasts and bacteria strains from some Cameroonian soils

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One hundred and nineteen amylases producing strains (29 yeasts and 90 bacteria) were isolated from some Cameroonian soils contaminated by starchy residues and screened for thermostable amylases production. Phenotypic characterization of these amylases producing strains revealed the prominence of ascomycetous yeasts and two kinds of bacteria; the aerobic endospore forming dominated by *Bacillus* spp and aero-anaerobic non spore forming bacteria dominated by lactic acid bacteria of *Lactobacillus spp*. Among yeasts, one designated 04LBA3 produced high title of very high thermostable amylase. It was able to provoke starch hydrolysis halo of 33.7±1.5 mm on starch agar plate, and produced 80 ±0.5 U/ml of amylase in starch broth after 48 h of incubation at 30°C. Concerning amylases producing bacteria, two isolates designated 04BBA15 and 04BBA19 showed very high amylolytic power, the values were 55.0±3.2 mm and 45.3±1.5 mm of starch hydrolysis halo respectively for 04BBA15 and 04BBA19. Amylase production in starch broth were 131.0 and 107.7 U/ml respectively for 04BBA15 and 04BBA19 after 48 h of incubation at 40°C, on the other hand, their crude amylase extract remained 100% of original activity after been heated at 80°C for 30 min. The strain 04LBA3, 04BBA15, 04BBA19 were respectively identified as strain of *Schwanniomyces alluvius, Bacillus amyloliquefaciens* and *Lactobacillus fermentum*. Cluster analysis on the basis of amylolytic activity and thermostability of crude amylase extract showed similarities between strains of same geographic origin, this observation allows us to suggest that amylase activity and thermostabilty can serve as indicators for micro-organisms traceability.

Keywords: Thermostable amylases, amylases producing strains, amylolytic power, phenotypic characterization, microbial traceability.

INTRODUCTION

Thermostable enzymes from micro-organisms have found a number of commercial applications because of their overall inherent stability (Demirijan et al., 2001). The most widely used thermostable enzymes are the amy-lases in the starch industry (Poonan and Dalel 1995; Crab and Mitchinson, 1997; Demirkan et al., 2005). They are among the most important enzymes and are of great significance in present-day biotechnology. Although they can be derived from several sources, such as plants, animal and micro-organisms; enzymes from microbial sources generally meet industrial demands. Starch, which is the substrate of amylase, is the most abundant form of storage polysaccharides in plants and constitute an inexpensive source for production of syrups containing glucose, fructose or maltose which are widely use in food industries. In addition to that, the sugars produced can be fermented to produce bioethanol. The enzymatic liquefaction and saccharification of starch are performed at high temperatures (100-110°C), in this regard thermostable and thermoactive amylolytic enzymes from micro-organisms are of great interest.

Research on source of thermostable enzymes producing micro-organisms proved that, geographic zone where the strains are isolated influence and determine microbial enzyme behaviour (Haki and Rakshit, 2003). A number of

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attempts were made to isolate from exotic ecological zone of the earth some potential thermostable amylases producing micro-organisms and several *Bacilli sp* were found as producers of amylase with significant industrial importance (Antranikian et al., 1987). However, little of these studies described the founding of thermostable amylase producing yeasts. Soils, compost, hot spring and geothermal sites from different area of the earth were found to be conducive biotopes for bacteria which can provide thermo tolerant enzymes (Canganella et al., 1994; Shaw et al., 1995; Young et al., 2001). In Cameroon, cassava farms after harvesting and treatment of tubers, and flour markets are abundant; soils of these sites represent media where natural amylolytic fermentation activities occur (Tatsinkou et al., 2005). Because starch constitutes the main substrate of these media, amylolytic micro- organisms are thus supposed to be naturally present. Intense degradation of starchy residues and alcohol flavour were observed in some of these sites, such flavour is a sign of alcoholic fermentation attributed to yeasts or bacteria. In this regard, bacteria and yeasts of these media may be a potential source of thermostable amylases.

The present study is aimed a screening of thermostable amylases overproducing yeasts and bacteria strains from some Cameroonian starchy soils.

MATERIAL AND METHODS

Sampling of soils

Twenty-eight samples of soils were collected from main geographic zones of Cameroon in four localities: (Ngaoundere, Yaounde, Bafoussam and Mbouda) at the factories where starchy wastes are submitted to natural fermentation. Four kinds of factories were investigated: “garri” factories, corn and cassava mills, cassava plantation after harvesting and treatment of tubers and flour markets. 1 to 5 g of soils were especially collected at the places where degradation of starchy material was remarkable and visible and introduced in polyethylene aseptic bag, age of each factory was recorded, then the samples of soils were brought at the laboratory and analysed in the same week.

Partial characterization of soils

In order to show the relationship between properties of soils samples and frequency of amylolytic biotic factors (bacteria and yeasts), some properties were studied, especially physico-chemical properties (pH, starch and minerals content), and biological properties (total microbial population, amylolytic yeasts, amylolytic bacteria, number of thermostable amylase producing isolates). The soils pH was determined according to method of AFNOR (1994), (Norm NF ISO 10390) . Starch content was determined after crushing according to the method of Keleke et al. (1995). Three minerals were screened; (Calcium, Iron and Magnesium), the choice of these minerals is justified by their ability to act as cofactors of microbial amylases (Forgaty, 1983). Calcium and Magnesium were estimated according to Method of AFNOR (2002) (Norm NF X 31-108), Iron was estimated according to method of Rodier (1978). Numeration of total microbial population was carried out on plate count agar (PCA) containing (gram per liter): 5.0 g tryptone, 2.5 g yeast extract, 1.0 g dextrose, 12.0 g agar n°2. Relative age of soils was considered as the date of the creation of starch activity on the site (age of factory), this was obtained through historic investigation on each site where soils sample were collected.

Isolation and characterization of microbial strains

Amylolytic micro- organisms were firstly enriched by introduction of 1 g of soil sample in 100 ml Erlenmeyer flasks containing 50 ml of enrichment liquid medium, composed of (gram per litre): 5 g soluble starch, 5 g peptone, 5 g yeast extract 0.5 g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 0.01 g NaCl. Enrichment of thermostable amylases producing bacteria was carried out by heating Erlenmeyer flasks at 90°C for 5 min followed by incubation in an alternative shaker at 37°C and speed of 150 oscillations per second for 24 h. Amylases producing bacteria strains were screened on agar plate, containing (gram per liter): 10 g soluble starch, 5 g peptone, 5 g yeast extract, 0.5 g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 0.01 g NaCl, 15 g agar. Incubation at 37-40°C was carried out for 48 h, after which the plates were stained with lugol solution (Gram iodine solution: 0.1% I_2 and 1% KI). The colonies with the largest halo forming zone were isolated for further investigation.

Amylases producing yeasts were screened by adding chloramphenicol or propionic acid for concentration of 0.1 g/l and 0.01% respectively in above medium. The incubation was carried out at 30°C and 150 oscillations per second in an alternative shaker.

Morphological properties were examined by light microscopy (Olympus microscope BH- 2). Biochemical properties of the isolated bacteria were determined according to the methods described in Bergey’s Manual of Systematic Bacteriology (Claus and Brekeley, 1986) by using the following characteristics; Gram staining, catalase and oxidase activity Voges proskauer (VP) test, nitrate reduction to nitrite, anaerobic growth, gas production from nitrate and glucose, mannose, starch, urea and casein degradation, gelatine liquefaction, acid production from glucose, galactose, lactose, mannose, mannitol, salicin and fructose, citrate utilization, growth with 10% (w/v) NaCl, while biochemical properties of yeasts were studied according to routine method of Kreger VR (1984) using API 20 C Kit (bioMerieux).

The amylolytic power of microbial strain was determined using method of wells by inoculation of 10 µl of each isolate in 4 mm deep micro-wells on the surface of starch agar plate containing (gram per liter): 10 g soluble starch, 5 g peptone, 2.5 g yeast extract. The amylolytic power was defined as the average diameter (mm) of hydrolysis halo provoked by a strain after its inoculation in micro-well on starch agar plate for 48 h incubation at optimum temperature of growth for three assays.

Identification of amylolytic isolates

For bacteria, genus *Bacillus* was identified using identification Key of Reva et al. (2001). Lactic acid bacteria were identified using API 50 CH test Kit (bioMerieux) and APIDENT software version 2.0. The yeasts were identified according to Looder (1970) and Kreger (1984) keys.

Amylases production and assay

Extracellular amylases was produced in submerged fermentation, this production was carried out in 250 ml Ernlenmeyer flasks containing 100 ml of liquid medium for enzyme production. The composition of liquid medium was (gram per liter): 10 g soluble starch, 10 g peptone and 5 g yeast extract. Incubation was at maximum temperature of growth of each strain in alternative shaker at speed of 150 oscillations per second. After 48 h of fermentation at optimum temperature of growth, the culture medium was centrifuged at 8000 g and 4°C for 1 h. The cell free supernatant after centrifuga-

tion was considered as crude enzyme solution and was used for assays.

Enzyme activity was determined by iodine method (Keleke et al., 1995) using soluble starch as substrate. One unit of amylase (U) was defined as the amount of enzyme that was capable to hydrolyse 1 g of soluble starch for 60 min under experimental condition.

For thermostability assay, the crude enzyme extract from each microbial strain was heated at 80°C and pH 6.0 for 30 min and cooled with ice water, then the remaining activity was measured as described previously (Keleke et al., 1995).

Selection of strains

Microbial strains were selected on the basis of three main criteria: the amylolytic power of the strain evaluated on starch agar plate, the thermo stability and the activity of the enzyme.

Statistical analysis

Correlation and principal component analysis were carried out in order to visualize the proximity or the distance between physico-chemical properties of soils and the frequency of thermostable amylases producing strains. This analysis was performed as described by Philippeau (1986). Cluster analysis was also performed using aggregation method of Ward (1963) in order to show similarity or dissimilarity of functional flora of the soils. All these statistical analysis were carried out using two computer's program (Statgraphic plus 5.0 and Statbox 6.4 Software).

RESULTS

Partial characterization of soils

Soils samples were characterized in three ways: relative ages, which are the ages of creation of factories, or plantations where the soils were collected, chemical characteristics (pH, Ca, Mg, Fe) and microbiological characteristics (aerobic mesophile, amylolytic yeasts population, amylolytic bacteria population, amount of thermostable producing isolates). The relative ages were obtained after investigation next to owners of the sites; values were comprised between 5 and 40 years of starch activity on the sites. The oldest factory was found in Bafoussam with relative age of 40 years, this site is a flour market, the youngest factory was found in Ngaoundere with age of 5. Concerning pH, most of soils were near neutral range, while the samples from Mbouda were most acid. For mineral content, only three minerals were screened; Ca, Mg, Fe, the results obtained was comprised between 0.09 and 0.8% (w/w) in our soils samples. For microbiological characteristics, aerobic mesophile were 10^6 to10^9 cfu/g, while amylolytic bacteria and amylolytic yeasts were estimated at (10^2 to 10^4 cfu/g) and (10^2 to 10^3 cfu/g) respectively.

Two microbiological concepts were defined for this work in order to explain the frequency of amylolytic and thermostable amylases producing strains in the soils. The first concept was the "Relative Total Amylolytic Power" (RTAP) of a soil sample; which was defined as the sum of amylolytic power of the whole isolates from the soil sample, (each soil has his own RTAP). The second concept was the "Number of Thermostable Amylases Producing Isolates" (NTAPI) from a soil; therefore, each sample of soil is characterized by its own RTAP and NTAPI. The highest value of RTAP and NTAPI were found in sample of soil n°4, which is a soil sample from Bafoussam flour market. The RTAP and NTAPI were 226.9 ±0.1 mm and 3.0 respectively.

Characteristic of soils (relative age, pH, Ca, Fe, Mg, RTAP and NTAPI) were considered as variables and were tested for correlation. Table 1 shows results of Pearson correlation analysis of these variables; calcium and magnesium contents. Correlation coefficients were respectively, 0.522; 0.478; 0.395; 0.499 while NTAPI is only positively and significantly correlated to relative age.

Due to the fact that soils properties were correlated in little groups, principal component analysis was carried out in order to a better visualization of distance and proxi-mity between soils variables (Figure 1). The purpose of the analysis is to obtain small number of linear combina-tion of soils variables (age, starch, pH, Ca, Mg, Fe, RTAP, and NTAPI) which account for most of the varia-bility in the data. In our case four components (F1, F2, F3, and F4) have been extracted, together they account for 84% of variability in original data. For the formation of component F1; the variables NTAPI, Ca, Mg and age account respectively for 20.44, 17.04, 16.84 and 15.90% (Table 2). For the formation of component F2, pH, and Fe account for 31.19 and 19.19 respectively. The contribution of each variable of soils for the principal component (F1, F2, F3 and F4) allow the repartition of variable in four main groups A, B, C, and D (Figure 1).

Amylolytic power of amylases producing yeasts and bacteria strains, activity and thermostability of their crude amylase extract

119 amylolytic isolates were screened from soils samples, each isolate was codified by the soil sample number, its nature (B for bacteria and L for yeast), geographic origin (BA for Bafoussam, NG for Ngaoundere, MB for Mbouda), and isolation order. Those that amylolytic power was upper or equal to 25 mm on starch agar plate after 48 h incubation at optimum temperature of growth were considered as amylases overproducing strains, and were selected for further studies (Table 3). Seven amylases overproducing yeasts strains (04LBA3, 05LBA6, 08LNG8, 14LYA13, 20LBA17, 16LNG15, 26LMB27) were selected while nine amylases overproducing bacteria strains (04BBA15, 04BBA19, 05BBA22, 05BBA23, 14BYA42, 20BBA60, 17BNG51, 23BYA21, 26BMB81) were selected. For amylases overproducing yeasts isolates, the most important amylolytic power was found for strain 04LBA3, this isolate was capable to provoke starch hydrolysis halo of 33.7±1.5 mm after its inoculation in 3 mm micro well on starch agar medium followed by the incubation for 48 h at 30°C (optimum temperature

Table 1. Correlation matrix of physico-chemical and microbiological characteristics of soils.

	Age	Starch	pH	Ca	Mg	Fe	RTAP	NTAPI
Age	1.0							
Starch	0.160 P=0.147	1.0						
pH	0.055 P=0,621	-0.253 P=0.021	1.0					
Ca	0.403 P=0.000	0.498 P=0.000	0.017 P=0.875	1.0				
Mg	0.205 P=0,062	0.403 P=0.000	-0.495 P=0.000	0,387 P=0.000	1.0			
Fe	0.048 P=0.662	-0.130 P=0.241	0.35 P=0.001	0.061 P=0.582	-0.3268 P=0.003	1.0		
RTAP	0.522 P=0,000	0.478 P=0.000	-0.317 P=0.003	0.395 P=0.000	0.499 P=0.000	-0.186 P=0.091	1.0	
NTAPI	0.577 P=0.000	-0.158 P=0.153	0.278 P=0.011	-0.062 P=0.573	-0.118 P=0.287	-0.175 P=0.112	0.202 P=0.067	1.0

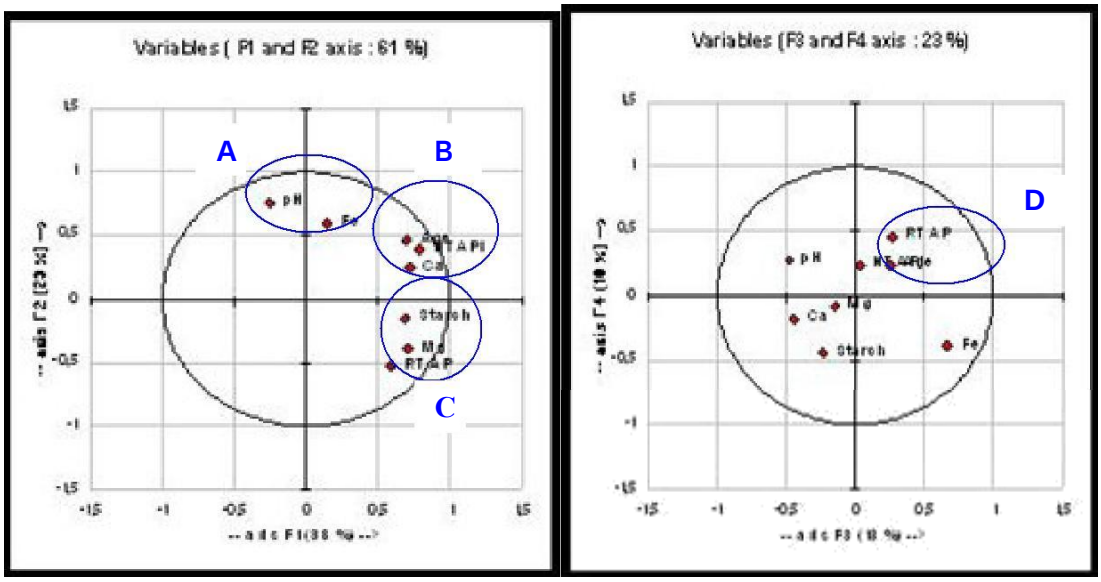

Figure 1. Principal component analysis of soils variables (age, pH, Ca, Mg, Fe, RTAP, and NTAPI) . The components F1, F2, F3, and F4 contribute respectively for 38, 23, 13 and 10% for the variability of soils data. The proximity between variables allows their repartition in four mains groups A, B, C, D.

of growth). The amylase production and its thermostability were also the most important among yeasts with the respective values of 80.0±0.5 U/ml and 85% of original activity preserved after heat treatment at 80°C for 30 min.

Concerning amylases overproducing bacteria, two isolates (04BBA15 and 04BBA19) showed very high amylolytic power, the values were 55.0 ± 3.2 and 45.3 ± 1.5 mm respectively for 04BBA15 and 04BBA19 after incubation at 40°C for 48 h o n starch agar plate. The amylase production reached 131.0 and 107.7 U/ml respectively for 04BBA15 and 04BBA19. On the other hand, both crude amylase extracts remained 100% of original activity after been heated at 80°C for 30 min.

Morphological and biochemical characterization of amylases overproducing isolates

Yeast isolates were identified on the basis of their morphological, physiological and biochemical properties according to the Lodder (1970) and Kreger (1984) Keys. Important characteristics are shown in Table 4, the column

Table 2. Contribution of variables for the principal component formation.

	F1	F2	F3	F4
Age	15,90	11,93	5,88	7,35
Starch	15,48	1,17	4,98	25,00
pH	2,22	31,79	21,27	9,88
Ca	17,04	3,34	18,04	4,48
Mg	16,84	8,47	1,97	0,88
Fe	0,65	19,19	41,04	18,78
RTAP	11,43	15,66	6,68	26,23
NTAPI	20,44	8,44	0,13	7,39

1 and 2 presents shape and cell size respectively. Ovoid shape (O) was prominent among yeasts isolates while rectangular (R) was minor. The cell size varied from 2.0 to 7.5 µm in diameter. Column 3 presents the asexual reproduction mod, unilateral and multilateral bundings cells (B) were observed after 18 h of culture at 30°C for strains 04LBA3, 08LNG8, 14LYA3, 16LNG15, 20LBA17, 26LMB27; while binary fission (BF) was observed only for strains 05LBA6 after the same time of culture. Two kinds of filaments were observed (column 4); true mycelium (M) were observed for strain 08LNG8, while pseudo-mycelium were observed for strains 04LBA3, 14LYA13, 20LBA17, 26LMB27. None mycelium was observed for strains 05LBA6 and 16LNG15. The column 5 and 6 presents the spore formation and the amount after cultivation on sporulation medium of MacClary et al (1959). The behaviour of yeasts after cultivation on sporulation medium was essential for their discrimination in two main groups: *Ascomycetes* groups that were able to give spores on the sporulation medium: this group was represented by strains 04LBA3, 20LBA17, 14LYA13, 26LMB27; their vegetative cells were trans-formed directly into asci after 4 days of incubation at 30°C in sporulation medium. The asci contained one to four spheroid ascospores. The second main group found in the soils was unable to release spore after one week of incubation at the same temperature, this group was classified according to yeast taxonomy as *Deuteromycetes,* and represented by strains 05LBA6, 08LNG8, and 16LNG15. Physiological and biochemical profile included nitrate reduction, fermentation of sugars (Glucose, Galactose, Saccharose, Maltose, Trehalose, Lactose, Cellobiose, Melibiose, Raffinose, Xylose, Starch), ethanol production and growth at 37°C are summarized within column 7 to 20 of Table 4. Strain 04LBA3 for example has negative reaction (-) to nitrate reduction test while fermentation of above sugars were all positive (+). According to their morphological character and biochemical profile, the strains 04LBA3, 05LBA6, 08LNG8, 14YA13, 16LNG15, 20LBA17, 26LMB27 were respectively identified as strains of *Schwanniomyces alluvius, Tricosporon pullulans, Endomycopsis filbulgera, Saccharomyces cereviasiae, Cryptococcus albidus, Schizosaccharomyces pombe, Saccharomyces diastaticus.*

Concerning bacteria, two kinds of amylases over producing bacteria were found in our samples of soils: the aerobic endospore forming bacteria dominated by genus *Bacillus*, and aero-anaerobic non-spore forming bacteria dominated by lactic acid bacteria. Morphological observation and biochemical tests performed on aerobic endospores forming strains are shown in Table 5. Rod Gram positive cell that are 0.1 to 1.0 in diameter were observed (Column 1 to 3). Sporulating cells from strains 05BBA23, 17BBNG21, 20BBA60, 23BYA21 contained a subterminal (T) spore while sporulating cell from 04BBA15, 05BBA22, 14BYA42 contained a central (C) spore. Catalase and oxidase tests (column 9 and 10) were positive for all strains of this group whereas urease test (column 11) was positive only for strains 04BBA15, 05BBA22, 14BAYA42 and negative for strains 05BBA23, 17BNG21, 20BBA60, 23BYA21. Aesculin hydrolysis test was positive for strains 04BBA15, 05BBA23, 14BYA42, and 23BYA21 and negative for strains 05BBA22, 17BNG21, and 20BBA60. All aerobic endospore forming bacteria isolated from our soils samples were negative for casein hydrolysis excepted strain 23BYA21; however they were all positive for hyppurate and starch hydrolysis. None resistance was observed for antibiotics as chloramphenicol, polymycin, streptomycin while strains 04BBA15, 05BB22, and 14BYA42 were resistant to naxidilic acid. All strains of this group was also able to provoke acid fermentation of cellobiose, they were also tested for their ability to acid fermentation of others carbohydrates (Fructose, Galactose, Lactose, Mannose, Raffinose, Salicin, Xylose). Citrate and succinate utilization was positive for strains 04BBA15, 05BBA22, 05BBA23. Almost strains of this group were able to growth at 50°C, none was capable to growth in the presence of NaCl (10%). Nitrate reduction was positive for strains 04BBA15, 05BBA22, 05BBA23, 17BNG21 and negative for strains 14BYA42, 20BBA60, and 23BYA21. Voges Prokauer reaction (acetoin production) was positive for strains 05BBA22, 05BBA23, 14BYA42 and negative for 04BBA15.

According to Gordon et al (1976) and Reva et al (2001) keys the aerobic endospore forming bacteria 04BBA15, 05BBA22, 05BBA23, 14BYA42, 17BN21, 20BBA60, 23BYA21, isolated in our soils samples were respectively identified as strains of *B. amyloliquefaciens, B. subtilis, B. lentus, B. amylolyticus, B. brevis, B. subtilis, B. megaterium*

Among amylase overproducing bacteria isolated from our sample of soils, strains 04BBA19 and 26BMB81 were aero- anaerobic non spore forming bacteria, on the other hand catalase test was negative for both isolates, this characteristic is proper to lactic acid bacteria. Biochemical characteristics of these isolates were carried out using API 50 CH kit bioMerieux system, the result are summarized in Table 6, the isolates were tested for their possibility to ferment 50 carbohydrates, and this fermen-

Table 3. Amylolytic power of amylases overproducing yeasts and bacteria strains, amylase activity and thermostability of their crude amylase extract.

Sites	Localities	Yeasts				Bacteria			
		Strains cod	Amylolytic power (mm)	Amylase activity (U/ml)	Thermostability (%)	Strains cod	Amylolytic power (mm)	Amylase activity (U/ml)	Thermostability (%)
Market flour	Bafoussam	04LBA3	33,7±1,5^{a*}	80,0±0,5^{a}	85 ,0±0,5^{a}	04BBA15	55,0±3,2^{a}	131,0±1,3^{a}	100±1,5^{a}
	Bafoussam	05LBA6	26,0±1,2^{b}	61,7±1,5^{c}	36,2±1,8^{d}	04BBA19	45,3±1,5^{b}	107,7±0,7^{b}	100±1,2^{a}
Mills	Ngaoundere	08LNG8	26,5±0,7^{b}	62,0±0,9^{c}	20,2±2,3^{f}	05BBA22	26,1±0,7$_{d}$	60,0±0,7$_{e}$	37,2±1,1$_{d}$
	Yaounde	14LYA13	25,1±1,4^{b}	59,1±3,1^{c}	25,3±1,5^{e}	05BBA23	25,1±0,3	63,0±1,2	47,0±2,5
	Bafoussam	20LBA17	26,2±0,5^{b}	62,4±1,5^{c}	57,4±0,7^{b}	-	-	-	-
Cassava plantation	Ngaoundere	16LNG15	28,2±2,3^{a}	67,0±0,5^{b}	30,5±0,7^{b}	14BYA42	26,0±1,2^{b}	59,0±0,4^{f}	63,0±1,0^{c}
	Yaounde	-	-	-	-	20BBA60	28,3±0,5^{cd}	67,1±0,3^{b}	63,1±0,3^{b}
Gari production workshop	Mbouda	26LMB27	28,5±2,4^{a}	57,2±0,5^{d}	53,1±1,2^{c}	17BNG51	31,2±1,2^{c}	74,0±0,5^{c}	28,0±1,5^{f}
						23BYA21	26,0±0,7^{d}	63,1±1,2^{e}	65,0±0,3^{c}
						26BMB81	29,3±0,5^{cd}	71,3±2,5^{c}	29,1± 2,1^{f}

*Means with different superscripts within columns are significantly different (P<0.05).

Table 4. Morphological and biochemical characteristics of amylases overproducing yeasts isolated from soils.

Test number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Strains cod	Cellmorphology	Cell size(µm)	Division	Filament Sporulation		Spores number	Nitrate	Glucose	Galactose	Saccharose	Maltose	Trehalose	Lactose	Cellobiose	Melibiose	Raffinose	Xylose	Starch	Ethanol	Growth at 37°C	Identification species
04LBA3	O	7,5	B	P	+	4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	*Schwanniomyces alluvius*
05LBA6	O	3,5	BF	-	-		+	+	+	+	+	+	+	+	+	+	-	+	+	+	*Trichosporon pullullans*
08LNG8	O	4,3	B	M	+		+	+	+	+	+	+	-	+	+	-	-	+	+	+	*Endomycopsis filbulgera*
14LYA13	O	5,3	B	P	+	2	-	+	+	+	+	+	-	+	+	+	-	+	+	+	*Saccharomyces cerevisaiae*
16LNG15	O	4,0	B	-	+		-	+	+	+	+	+	-	-	+	+	-	+	+	+	*Cryptococcus albidus* -
20LBA17	R	3,5	B	P	+		+	+	+	+	+	+	+	+	-	+	-	+	+		*Schizosaccharomyces pombe*
26LMBA27	R	2,0	B	P	+	4	+	+	+	+	+	+	+	+	+	+	-	+	+	+	*Saccharomyces diastaticus*

+; positive reaction, -; negative reaction, O; ovoid, R, rectangular, B, bundings, BF; binary fission, M; mycelium and P; pseudomycelium.

Table 5. Morphological and biochemical characteristics of amylases overproducing *Bacillus* isolated from soils.

Tests number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	15	17	18	19	20	21	22	23	24	25	26	27	28	29	
Strains cod	**Cell morphology**	**Size (µm)**	**Gram**					**Starch hydrolysis**	**Catalase**	**Oxydase**	**Urease**	Chroramphenicolresistance				**Cellobiose acid**	**Fructose acid**	**Galactose acid**	**Lactose acid**	**Mannose acid**	**Raffinose acid**	**Salicin acid**	**xylose acid**	**Citrate utilization**	Succinateutilization	**Growth at 50°C**	Growth in10%NaCl	**Anaérobiose**	**Nitrate reduction**	**Voges Proskauer reaction**	**Identification species**
04BBA15	R	0,5	+	+C	+	-	+	+	+	+	+	-	+	-	-	+	-	-	-	+	+	-	+	+	+	+	-	-	+	-	*Bacillus amyloliquefaciens*
05BBA22	R	1,0	+	+C	-	-	+	+	+	+	+	-	+	-	-	+	+	-	-	-	+	-	+	+	+	+	-	-	+	+	*Bacillus subtilis*
05BBA23	R	0,7	+	+T	+	-	+	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+	-	+	+	+	-		+	+	*Bacillus lentus*
14BYA42	R	0,8	+	+C	+	-	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+		-	-	-	+	-	-	-	-	*Bacillus amylolyticus*
17BNG21	R	0,3	+	+T	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	+	-	-	+	-	*Bacillus brevis*
20BBA60	R	0,1	+	+T	-	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-	-	*Bacillus subtilis*
23BYA21	R	0,2	+	+T	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	*Bacillus megaterium*

R, rod; +=positive reaction, -=negative reaction, C= central, T= terminal.

tation profile was use for their numerical identification using APIDENT software 2.0. According to their biochemical profile, 04BBA19 and 26BMB81 were respectively identified as strain of *Lactobacillus fermentum* and *Lactobacillus plantarum.*

DISCUSSION

The soil is a vital medium that physico-chemical composition varies with the ecological zone on the earth (Min et al., 1999). The presence of different micro-organisms and their survival in the soil can be justified by composition of soil. Organic matter in the soil plays the role of substrate for fermentation; this reason explains the high amount (10^6 to 10^9cfu/g) of aerobicmesophile microflora in our samples of soils. Soil appears therefore as an important biotope for searching and the exploration of industrial micro-organisms. The occurrence of amylolytic yeasts and amylolytic bacteria in our samples of soils is priorily linked to the presence of starch residues. Generally, in the nature, amylolytic micro-organisms are bacteria or mould however(few species of yeasts were described as starchy degrading micro-organisms (Laluce et al., 1988; Verna et al., 2003, Shigechi et al., 2003), the same observation was notified in our samples.

Each sample of soil was characterized by two concepts already defined above, its RTAP and NTAPI. RTAPI indicates the virtual amylolytic activity in a soil because it is the amylolytic activity of the whole amylolytic bacteria and yeasts that are supposed to be present in a soil sample while NTAPI indicates the frequency of thermostable amylase producing strains in a sample of soil. Correlation analysis of soils data allows us to think that the aptitude of a strain to degrade starchy material is probably linked to starch content in its original medium e.g. in the soil. Several authors have reported that starch is amylase inducer for many bacterial strains (Burhan et al., 2003, Gomes et al. 2003; Muralikrishna and Nirmala, 2005). This imply that the search of industrial microbial enzyme producing strains must be oriented by the nature of substrate degrade in soil. In this study, the age of factory where sample of soils was col-lected appears as an important variable affecting the frequency of thermostable amylases produc -ing strains; the oldest factories have given the best thermostable amylases producers. Laluce et al. (1988) obtained similar results concerning ethanol producers. On the other hand, principal components analysis gives a better visualization of proximity and distance be-

Table 6. biochemical characteristics of amylases overproducing *Lactobacillus* isolated from soils.

Test number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Strains cod	**Heterofermentative**	**Gaz production**	**Optimum temperatureofgrowth**	**Growth at 10°C**	**Dextran**	**Ammonia from Arginine**	**Nitrate reduction**	**Glycerol**	**Erythritol**	**D-arabinose**	**L-arabinose**	**Ribose**	**D-xylose**	**L-xylose**	**Adonitol**	**ß methyl-D-Xyloside**	**Galactose**	**Glucose**	**Fructose**	**Mannose**	**Sorbose**	**Rhamnose**	**Dulcitol**	**Inositol**	**Mannitol**	**Sorbitol**	**-Methyl-D-mannoside**	**-Methyl-D-glucoside**	**N-Acetyl-Glucosamine**
04BBA19	+	+	45°C	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	nc	-	-	-	-	-
26BMB81	-	+	40°C	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	+

Test number	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	
Strains cod	**Amygdalin**	**Arbutin**	**Aesculin Salicin**		**Cellobiose**	**Maltose**	**Lactose**	**Melibiose**	**Sucrose**	**Trehalose**	**Inulin**	**Melezitose**	**Raffinose**	**Starch**	**Glycogen**	**Xylitol**	**ß Gentiobiose**	**D-turanose**	**D-lyxose**	**D-tagatose**	**D-fucose**	**L-fucose**	**D-arabitol**	**L-arabitol**	**Gluconate**	**2-Keto-Gluconate**	**5-Keto Gluconate**	**Identification species (API 50CHL)**
04BBA15	-	-	nc	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	*Lactobacillus fermentum*
26BMB81	+	+	+	+	+	+	+	+	+	+	nc	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	*Lactobacillus plantarum*

+, positive reaction; -negative reaction, nc, non conclusive.

tween soils variables. Four mains groups the group A shows that the variability of pH can be attributed to the concen-tration of iron in the soils, this can be explained by the hydroxylation process of iron allowing the increase of pH. This process occurs in the nature in the presence of ions Fe^{2+} (Alloway, 1995). Group B confirms that the frequency of thermo-stable amylases producing strains is linked to the age of factory where soils samples were collected. Group C testifies the importance of starch and Mg for the expression of amylolytic activity, while group D proves the importance of duration of starch on a site for the induction of amylolytic activity to a microbial strain.

Phenotypic characterization of the amylolytic yeasts in the present study showed species of yeasts with similar biochemical profile to those

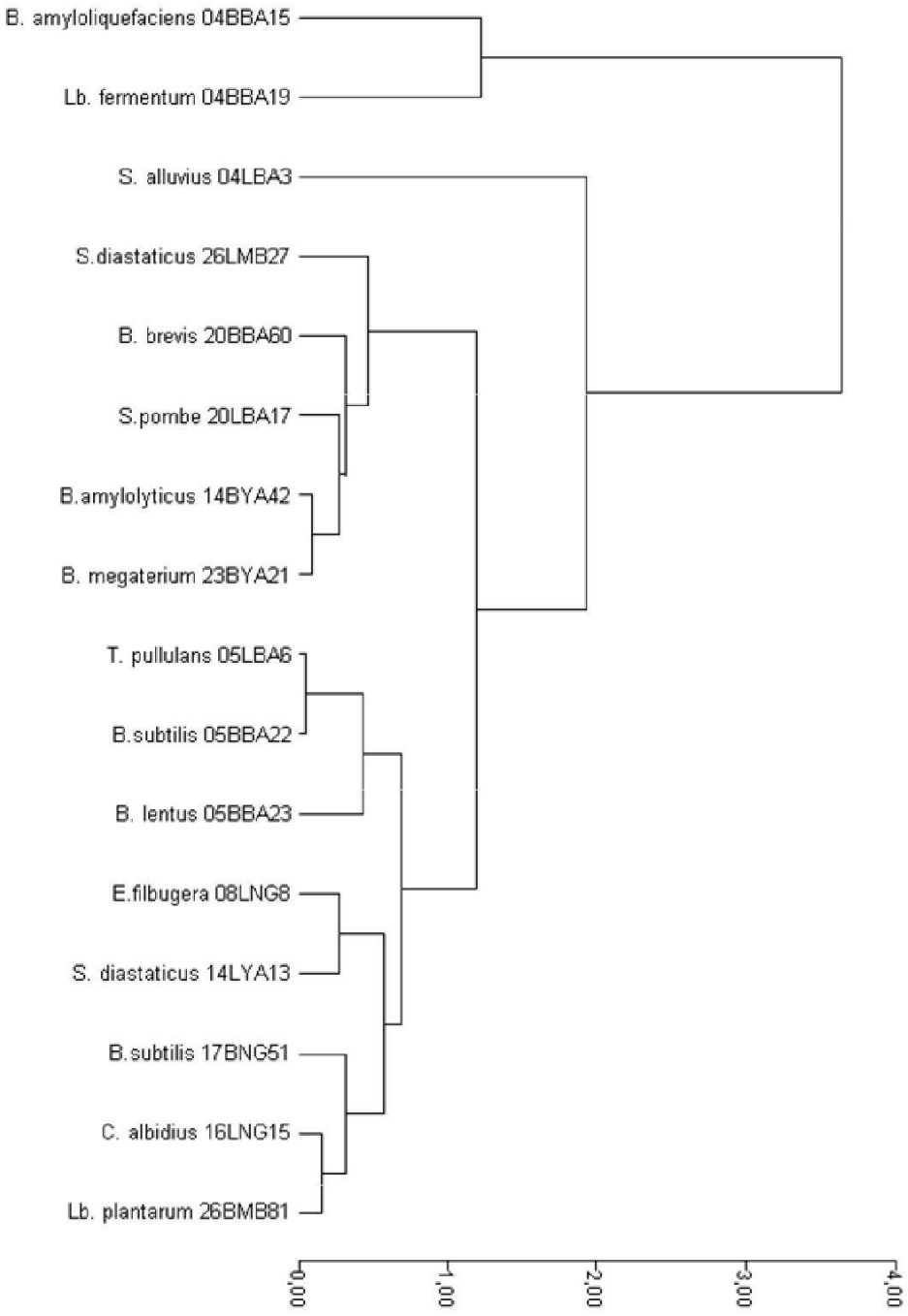

Figure 2. Dendrogram showing the classification of strains of amylases overproducing bacteria and yeasts on the basis of their amylase profile (activity and thermostability).

found by several authors; e.g. *C. antartica, C. tsukubaensi, S. alluvius*, *S. Castelli, S. occidentalis, T. pullulans, Lipomyces kononenkoe, Lipomyces starkeyi, Saccharomycopsis, S. cerevisiae*, *var. diastaticus, S. filbuligera, Pichia panomala, Brettanomyces naardenensis* (Sills and Stewart 1982; Tubb and Hammond, 1986; Steyn, 1995, Rey and Nanda, 1996). However, the particularity of this work is the founding of an amylolytic yeast (strain 04LBA3) presenting a particular character due to its high amylolytic activity and heat stability of its crude amylase extract. The capacity to degrade starch is not widespread among yeasts, but yeasts that are capable of degrading starch have been investigated as promising micro-organisms for the conversion of starchy materials to "single-cell" protein or ethanol (Verna et al., 2003). Many of these yeasts are of industrial significance

in biomass-conversion biotechnology either as a source of the hydrolytic enzymes or as a source of the appropriate gene for transfer to others organisms.

The thermostability of yeast 04LBA3 amylase was similar to those of bacteria as *Bacillus lentus* (El-Aassar et al., 1992), *Bacillus coagulans* (Fatma and Refai, 1991) and *Bacillus caldolyticus* (Heinein and Heinein, 1972). This yeast was isolated in the sample of soil with bacteria strains 04BBA15 and 04BBA19, which release very high thermostable amylase. The behaviour of yeast strain 04LBA3, particularly, the thermostabilty of its amylase, brings out some hypothetic considerations, taking into account the natural medium where the yeast strain have been isolated. Bacterial fermentation is the main phenomenon taking place in starchy soils of flour mills. The bacteria considered are usually thermo-resistant. Cohabitation of bacteria and yeast in such medium may have induced gene transfer from bacteria to yeast, justifying the unusual thermal behaviour of the yeast strain 04LBA3 amylase, comparable to amylase from bacteria. However, to our knowledge, such genetic transformation has not been reported between bacteria and yeasts, generally this kind of phenomenon is observed between a virus and bacteria on the same medium (Hofer, 1985; Deak et al., 1986; Cocconcelli et al., 1986) . On the other hand, gene mutation could be another reason explaining this particular behaviour of yeast strain 04LBA3.

Phenotypic characterization of amylase overproducing bacteria proved and confirmed that hydrolytic enzymes from bacteria are generally more thermostable than those from fungus (yeasts or moulds). Most of these bacteria belong to the genus *Bacillus*. Although the strain 04BBA15 is mesophile its amylase was very thermostable and can be compared to the heat stability of some thermophile or hyper-thermophile bacteria as *Thermococcus profoundus* (Kwak et al., 1998), *Thermus sp* (Shaw et al., 1995) and *Rodothermus marinus* (Gomes et al., 2003) isolated from thermal ecosystems. Similar result was obtained by Ramesh and Lonsane (1989), these authors' isolated *Bacillus licheniformis* M27 a mesophile producing a very thermostable amylase.

Lactic acid bacteria were found among amylase producing bacteria but only one (strain 04BBA19) identified as *L. fermentum* was able to release very high thermostable amylase, that is normal because this strain presented thermophile character, its optimum temperature of growth is 45°C. Thermophile micro-organisms generally produce very high thermostable enzymes (Haki and Rakshit, 2003). Morlon-Guyot et al. (2002) reported the presence of amylolytic activity for some lactic acid bacteria, *L. plantarum* , and *Lactobacillus mannihotivorans* isolated from cassava fermentation, but their amylase are not thermostable.

According to the cluster analysis on the basis of amylase profile (activity and thermostabilty) (Figure 2) the similarities between amylases producing strains were mainly observed for the strains coming from the same geographic origin, this suggests that enzymes activities as amylase activity and heat stability can be used as indicators for micro-organisms traceability. Among isolates obtained for this work, amylases for bacteria strains (*B. amyloliquefaciens* 04BBA15, *Lb fermentum* 04BBA9) and yeast strain *S. alluvius* 04LBA3 are the best for degradation of starch at high temperature. These strains are from Bafoussam flour market origin, the particular character of this yeast isolate brings out a hypothetic consideration, the cohabitation of bacteria and yeasts imply a synergic relationship between both kinds of micro-organisms, this synergy may affect positively amylolytic activity of yeast and the properties of its amylase. The above hypothesis suggests then scientific interest in investigating amylolytic bacteria yeasts are in mixed culture.

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