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thermostable amylase from lactic acid bacteria (LAB). The use of thermostable amylases from *Lactobacillus* is of advantage as they are generally non-pathogenic. On the other hand, the major end product of LAB fermentation, lactate, has applications as a preservative, acidulant and flavouring agent in the food industry, because of the tartness provided by lactate and also because lactate is generally regarded as safe (GRAS) [6].

Thus a thermostable amylase producing lactic acid bacterium would be a potential candidate for food industries and especially for the making of high density gruel from starchy raw material as corn or wheat [7]. This would require a good knowledge of the conditions required to optimally produce amylase and lactic acid of good quality. The present study deals with the co-production of thermostable α-amylase and lactic acid from a LAB, *Lactobacillus fermentum* 04BBA19, isolated from a starchy waste of a soil sample from the western region of Cameroon.

2. Background and significance

2.1. Amylolytic lactic acid bacteria

Amylolytic lactic acid bacteria (ALAB) have been reported from different tropical starchy fermented foods, made especially from roots as cassava and sweet potato or grains as maize sorghum and rice. Strains of *Lactobacillus plantarum* have been isolated from African cassava-based fermented products [8], *L. plantarum* A6 (LMG 18053) have been isolated from retted cassava in the Congo [9] and *Lactobacillus manihotivorans* OND32 have been isolated from cassava sour starch fermentations in Colombia [10]. Amylolytic strains of *Lactobacillus fermentum* were isolated for the first time from Benin maize sourdough (ogi and mawè) by Agati et al. [11]. Sanni et al. [12] described amylolytic strains of *L. plantarum* and *L. fermentum* strains in various Nigerian traditional amylaceous fermented foods. ALAB are generally screened in fermented amylaceous foods. Owing to their relatively high starch content, starchy biomass appears as an important eco-niche for the screening and isolation of ALAB, which can be industrially applied to convert starch into mono- and disaccharides for lactic acid fermentation. The composition of the microbiota and in particular the occurrence of ALAB is determined by the way the raw material is processed [13]. Most ALAB isolated belong to the *Lactobacillus* genus, however few studies reported the existence of amylolytic activity in some strains of *Bifidobacterium* isolated from the human large intestinal tract [14, 15]. The distribution of amylolytic microorganisms in the human large intestinal tract has been investigated in various individuals of different ages using anaerobic cultures techniques. So far, twenty one amylolytic bifidobacteria have been isolated from adult faeces and tested for rice fermentation [16].

Owing to the ability of their α-amylases to partially hydrolyze raw starch, ALAB can ferment different types of amylaceous raw material, such as corn [17], potato [18], or cassava [19] and different starchy substrates [20, 21, 8]. Amylolytic LAB utilize starchy biomass and convert it into lactic acid in a single step fermentation. ALAB are mainly used in food fermentation, they are involved in cereal based fermented foods such as European sour rye bread, Asian salt bread, sour porridges, dumplings and non-alcoholic beverage production.

Few of them are used for production of lactic acid in single step fermentation of starch [1]. The common method to produce lactic acid from starchy biomass involves the pretreatment for gelatinisation and hydrolysis (liquefaction and saccharification). The liquefaction of the starch is carried out at high temperatures of 90–130 °C for 15 min followed by enzymatic saccharification to glucose and subsequent conversion of glucose to lactic acid by fermentation [22, 1]. This two-step process involving consecutive enzymatic hydrolysis and fermentation makes it economically unattractive. The bioconversion of carbohydrate materials to lactic acid can be made much more effective by coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived glucose into a single step. This has been successfully employed for lactic acid production from raw starch materials with many representative bacteria including *Lactobacillus* and *Lactococcus* species [23, 20, 24, 21].

Because at industrial scale, the use of glucose addition is an expensive alternative, there is interest in the use of a cheaper source of carbon, such as starch, the most abundantly available raw material on earth next to cellulose. This, in combination with amylolytic lactic acid bacteria may help to decrease the cost of the overall fermentation process. Amylolytic lactic acid bacteria can convert the starch directly into lactic acid. Development of production strains which ferment starch to lactic acid in a single step is necessary to make the process economical. Very few bacteria have been reported so far for direct fermentation of starch to lactic acid [1, 25, 26] Approximately 3.5 billion tonnes of agricultural residues are produced per annum in the world [27]. The use of a specific carbohydrate feedstock depends on its price, availability, and purity. Although agro-industrial residues are rich in carbohydrates, their utilization is limited [27]. Different food/agro-industrial products or residues form the cheaper alternatives to refined sugars as substrates for lactic acid production. Sucrose-containing materials such as molasses are commonly exploited raw materials for lactic acid production. Starch produced from various plant products is a potentially interesting raw material based on cost and availability. Laboratory-scale fermentations have been reported for lactic acid production from starch by *Lactobacillus amylophilus* GV6 [20], *L. amylophilus* B4437 [28], *Lactobacillus amylovorus* [29, 23, *Lactococcus lactis* combined with *Aspergillus awamorii* [30] and *Rhizopus arrhizus* [31]. *L. amylophilus* NRRL B4437 [32], *L. amylovorus* [17] and *L. amylophilus* GV6 are exceptions that have been described to actively ferment starch to lactic acid and this may lead to alternative process of industrial lactic acid production [23, 20]. To make the process cost effective in terms of substrate, various groups have worked on acid/enzyme hydrolysis of starchy substrates followed by *Lactobacillus* fermentation or simultaneous saccharification and fermentation by co-culture/mixed culture fermentations. It has been reported that starch is used as substrate in two steps [1].

2.2. Thermostable amylases

Amylases 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, animals and microorganisms; enzymes from microbial sources generally meet industrial

demands. The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistries, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries. Thermostability is one of the main features of many enzymes sold for bulk industrial usage. Thermostable α-amylases are of interest because of their potential industrial applications. They have extensive commercial applications in starch liquefaction, brewing, sizing in textile industries, paper and detergent manufacturing processes. [33,34, 35]. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination and cost of external cooling, a better solubility of substrates, a lower viscosity allowing accelerated mixing and pumping [36]. Several thermostable α-amylase have been purified from *Bacillus sp.* and the factors influencing their thermostability have been investigated, but the thermostability of amylases from lactic acid bacteria have attracted very few scientific attention. *Lactobacillus amylovorus*, *Lactobacillus plantarum*, *Lactobacillus manihotivorans*, and *Lactobacillus fermentum* are some of the lactic acid bacteria exhibiting amylolytic activity which have been studied [37, 10, 38, 5, 39, 40]. However, most of α-amylase from these bacteria presented weak thermostability compared to those of genus *Bacillus*. Owing to the important acidification of fermenting medium by most lactic acid bacteria, the production of thermostable amylase by a lactic acid bacterium under submerged or solid-state fermentation can help to reduce the risk of contamination caused by undesirable micro-organisms during the process [41, 42]. Another advantage is the non-pathogen character of the genus *Lactobacillus* that allows their utilization in food fermentation processes.

2.3. Lactic acid

Lactic acid a water soluble and highly hygroscopic aliphatic acid is present in humans, animals and microorganisms. It is the first biotechnologically produced multi-functional versatile organic acid having wide range of applications [1], namely as a preservative in many food products. It can be produce by LAB trough fermentation or synthetically from lactonitrile [43]. It is non-volatile, odorless organic acid and is classified as GRAS (Generally Recognized As Safe) for use as a general purpose food additive. The lactic acid consumption market is dominated by the food and beverage sector since 1982 [1]. More than 50% of lactic acid produced is used as emulsifying agent in bakery products [44]. It is used as acidulant/flavoring/pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants. Lactic acid or its salts are used in the disinfection and packaging of carcasses, particularly those of poultry and fish, where the addition of aqueous solutions during processing increased shelf life and reduced microbial spoilage. The esters of calcium and sodium salts of lactate with longer chain fatty acids have been used as very good dough conditioners and emulsifiers in bakery products. The water retaining capacity of lactic acid makes it suitable for use as moisturizer in cosmetic formulations. Ethyl lactate is the active ingredient in many anti-acne preparations. The natural occurrence of lactic acid in human

body makes it very useful as an active ingredient in cosmetics [45]. Lactic acid has long been used in pharmaceutical formulations, mainly in topical ointments, lotions, and parenteral solutions. It also finds applications in the preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses and controlled drug delivery systems [45]. Because of ever-increasing amount of plastic wastes worldwide, considerable research and development efforts have been devoted towards making a single-use, biodegradable substitute of conventional thermoplastics. Biodegradable polymers are classified as a family of polymers that will degrade completely – either into the corresponding monomers or into products, which are otherwise part of nature – through metabolic action of living organisms. The demand for lactic acid has been increasing considerably, owing to the promising applications of its polymer, the polylactic acid (PLA), as an environment-friendly alternative to plastics derived from petrochemicals. PLA has received considerable attention as the precursor for the synthesis of biodegradable plastic [46]. The lactic acid polymers have potentially large markets, as they many advantage like biodegradability, thermo plasticity, high strength etc., have potentially large markets. The substitution of existing synthetic polymers by biodegradable ones would also significantly alleviate waste disposal problems. As the physical properties of PLA depend on the isomeric composition of lactic acid, the production of optically pure lactic acid is essential for polymerization. L-Polylactic acid has a melting point of 175–178 °C and slow degradation time. L-Polylactide is a semicrystalline polymer exhibiting high tensile strength and low elongation with high modulus suitable for medical products in orthopedic fixation (pins, rods, ligaments etc.), cardiovascular applications (stents, grafts etc.), dental applications, intestinal applications, and sutures [45].

3. Materials and methods

3.1. Samples

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 frequently submitted to natural fermentation. Four kinds of factories were investigated: "gari" factories, corn and cassava mills, cassava plantation after harvesting and treatment of tubers and flour markets. At the site where degradation of starchy material was remarkable and visible, one to five grams of soils were collected and transferred to polyethylene aseptic bag, the factory age recorded; the samples were finally transported to the laboratory and analyzed in the same week.

3.2. Screening of thermostable amylases and lactic acid producing bacteria

The starch degrading amylolytic lactic acid bacterial strains were isolated from different samples of soil. 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 37-40°C and speed of 150 oscillations per minute 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$_2$O, 0.01g FeSO$_4$.7H$_2$O, 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 pre-selected and tested for Gram staining and catalase activity.

Preliminary tests were carried out to determine the heat stability of the amylase of each isolate as we described previously [47, 48]. The gas production from glucose, growth at different temperature (10, 40, 45 °C) as well as the ability to grow in different concentration of NaCl was determined as described by Schillinger and Lucke [49] and Dykes et al [50]. The isolates which were Gram positive and catalase negative, non-motile and producing heat stable amylase and lactic acid were finally selected and identified using API 50 CH test kit (bioMerieux, France). The APILAB PLUS database identification was used to interpret the results.

3.3. Microbial growth, amylase and lactic acid production

In order to study microbial growth, amylase and lactic acid production, the microorganism was propagated at 40°C for 70 h in 50 ml of a basal medium containing: soluble starch, 1% (w/v); yeast extract, 0.5 % (w/v) placed in 100 ml Erlenmeyer flask with shaking at 150 oscillations per minute in an alternative shaker (Kotterman, Germany). The initial pH of the medium was adjusted to 6.5 using 0.1 M HCl. After removal of cells by centrifugation (8000xg, 30 min, 4°C) in centrifugator (Heraeus, Germany), the supernatant was considered as the crude enzyme solution and was also used for lactic acid evaluation.

3.4. Optimisation of raw starch degrading thermostable amylase and lactic acid production

The amylase and lactic acid production was optimized by studying the effect of cultural and environmental variables (carbohydrate and nitrogen sources, metal salts and surfactants) individually and simultaneously. The effect of carbohydrate sources was studied by replacing soluble starch in basal medium with different sugars, gelatinized and raw natural crude starch sources (glucose, fructose, maltose, amylose, amylopectine, cassava, corn, rice tapioca, and sorghum flours at final concentration of 1% (w/v)). Nitrogen sources were tested by replacing yeast extract with various nitrogen sources (peptone, tryptone, beef extract, soyabean meal, ammonium sulphate, and urea at final concentration of 1.5% (w/v)). The effect of metal salts was studied by adding individually various metal salts (CaCl$_2$.2H$_2$O, MgSO$_4$.7H$_2$O, FeSO$_4$.7H$_2$O, FeCl$_3$, NaCl at concentration of 0.1% (w/v)). Similarly the effect of surfactants was studied by supplementing the culture medium with Tween 80 and Tween 40 at concentration of 1.5% (v/v).

All media containing gelatinized starch sources were autoclaved at 121 °C for 20 min, while for the media containing raw starch flour, starch powder was sterilized by washing in ethanol and added to sterile nutrient broth.

3.5. Partial enzyme purification

The culture supernatant was supplemented with solid ammonium sulphate to 65% (w/v) final concentration, with mechanical stirring at 4°C. The suspension was retained for 1 h at 4 °C, and centrifuged at 8000 g for 30 min at the same temperature. The resultant supernatant was brought to 70 % w/v ammonium sulphate saturation at 4°C. 50-70% (w/v) ammonium sulphate precipitate was recovered, dissolved in 0.1 M phosphate buffer and dialysed using Spectra/PorR, VWR 2003 dialysis membrane overnight against the same buffer at 4°C and used as partial purified enzyme solution.

3.6. Effect of temperature and pH on activity and stability

The optimal temperature for amylase activity was determined by assaying activity between 30 and 100°C for 30 min in 50 mM phosphate buffer. Measurement of optimum pH for amylase activity was carried out under the assay conditions for pH range of 3.0-10.0, using 50mM of three buffer solutions: Tris-HCl (pH 3.0), Na_2HPO_4-Citrate (pH 4.0 – 6.0), and Glycine-NaOH (pH 7.0-10.0).

The temperature stability was determined by incubating the partial purified enzyme solution in water bath for temperature range of 30-100°C for 30, 60, 90, 120, 180 min and then cooled with tap water. The remaining α-amylase activity was measured and expressed as the percentage of the activity of untreated control taken as 100%. The first order inactivation rate constants, k_i were calculated from the equation: $\ln A = \ln A_0 - k_i t$, where A_0 is the initial value of amylase activity and A the value of amylase activity after a time t (min).

For the determination of pH stability, the enzyme was incubated in a water bath at 60 °C at varying pH value for 30 min. The residual activity was detected under the same conditions and expressed as the percentage of the activity of untreated control taken as 100%.

3.7. Effect of metal salts and chelating agent

The effect of metal salts and EDTA on amylase activity was determined by adding 0.05 to 0.1% (w/v) of metal salts ($CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, NaCl, $FeCl_3$, $CuSO_4.5H_2O$) and EDTA to the standard assay. The effect of metal salts and chelating agent on amylase activity were evaluated by pre-incubating the enzyme in the presence of effectors for 30 min at 60°C. The remaining amylase activity was determined and expressed as the percentage of the activity of untreated control taken as 100%.

3.8. Analytical methods

Cell growth was evaluated by reading the absorbance of culture medium at 600 nm using a Secoman spectrophotometer and numeration of total colony forming unit by 10-fold serial dilution of fermented broth and pour plating on MRS-starch agar (De Man Rogosa and Sharpe medium in which glucose has been replaced by soluble starch (Prolabo-Merck Eurolab, France)). In order to evaluate the capacity of microorganism to acidify the culture

medium, the pH of the fermented broth was measured using an electronic pH meter (Mettler Seven S20, Japan)

The amylolytic power of Lactic acid bacteria was determined using the method of wells by inoculation of 10 μl of microbial strain in 4 mm depth micro-wells on the surface of MRS-starch agar plate. The starch hydrolysis halo was revealed after 48 h of incubation using iodine solution. The amylolytic power was defined as the average diameter (mm) of hydrolysis halo provoked by a strain after its inoculation in micro-well on MRS-starch agar plate for 48 h incubation at optimum temperature of growth for three assays

The activity of amylase both in crude and purified extracts was assayed by iodine method. In a typical run, 5 ml of 1% soluble starch solution and 2 ml of 0.1M phosphate buffer (pH 6.0) were mixed and maintained at a desired temperature for 10 min, then 0.5 ml of appropriately diluted enzyme solution was added. After 30 min the enzyme reaction was stopped by rapidly adding 1ml of 1M HCl into the reaction mixture. For the determination of residual starch, 1 ml of the reaction mixture was added to 2.4 ml of diluted iodine solution and its optical density was read at 620 nm using a spectrophotometer (Secoman). One unit of amylase activity (U) was defined as the amount of enzyme able to hydrolyse 1 g of soluble starch during 60 min under the experimental condition. The lactic acid was determined according to Kimberley and Taylor [51]. The nature of amylase (endo-acting or exo-acting) was determined according to Ceralpha method (Megazyme) which uses a blocked maltoheptaoside as substratre [57].

The affinity of the enzyme preparation from selected LAB toward raw cassava starch was studied by incubating 0.2 g of raw cassava flour with 1ml of the enzyme solution at 60 °C for 15 min. After centrifugation, the α-amylase activity of the supernatant was measured and the adsorption percentage was calculated as follows: $Adsorption\,(\%) = \dfrac{A-B}{A} \times 100$, A is the original α-amylase activity and B is the α-amylase activity in the supernatant after adsorption on raw potato starch granules.

For the determination of raw starch digestibility, raw cassava was used and the reaction mixture containing 100 U of α-amylase preparation from the selected LAB and 100 mg of raw cassava starch in a final volume of 10ml dispensed in 100ml Erlenmeyer flasks were incubated in alternative water bath shaker at 60°C and 150 oscillations per min. After a time interval of 6 h, the reducing sugars liberated in the reaction mixtures were determined by dinitrosalicyclic acid method [58].

Light microscopy was used for the examination of the effect of enzyme on raw starch granules using Olympus microscope BH-2.

4. Results and discussion

4.1. Biochemical properties of amylolytic LAB isolated

From the 28 samples of soil collected from different localities of Cameroon, 90 amylolytic isolates were screened but only 9 isolates (04BBA15, 04BBA19, 05BBA22, 05BBA23,

14BYA42, 20BBA60, 17BNG51, 23BYA21, 26BMB81) presented very high amylolytic power (≥15mm) and were qualified as amylase overproducing isolates. The amylolytic power was defined as the average diameter (mm) of starch hydrolysis halo (Fig.1.) provoked by a strain after its inoculation in micro-well on MRS-starch agar plate for 48 h incubation at optimum temperature of growth for three assays. The amylolytic power is an expression of the capacity of an isolate to degrade starch during the culture. Among the amylase overproducing isolates, two (04BBA19, 26BMB81) were aero-anaerobic non spore forming, gram positive and catalase negative bacteria; this characteristic is proper to lactic acid bacteria. Microscopic observation showed rod cells. Biochemical characteristics of these isolates were carried out using API 50 CH kit bioMerieux system, the results are summarized in Table 1, the isolates were tested for their possibility to ferment 50 carbohydrates, and this fermentation profile was use for their numerical identification. According to their biochemical profile, 04BBA19 and 26BMB81 were respectively identified as *Lactobacillus fermentum* and *Lactobacillus plantarum.* The strain 04BBA19 (*Lactobacillus fermentum*) presented a very high amylolytic power, as it was able to cause a starch hydrolysis halo of 45 ±1.5mm on MRS-starch agar plate after 48 h of incubation at 40 °C; consequently it was selected for further studies. The preliminary test of thermostability carried out on its crude extract amylase showed that it produce a very high thermostable enzyme and it was selected for an application on simultaneous production of thermostable amylase and lactic acid from starchy material.

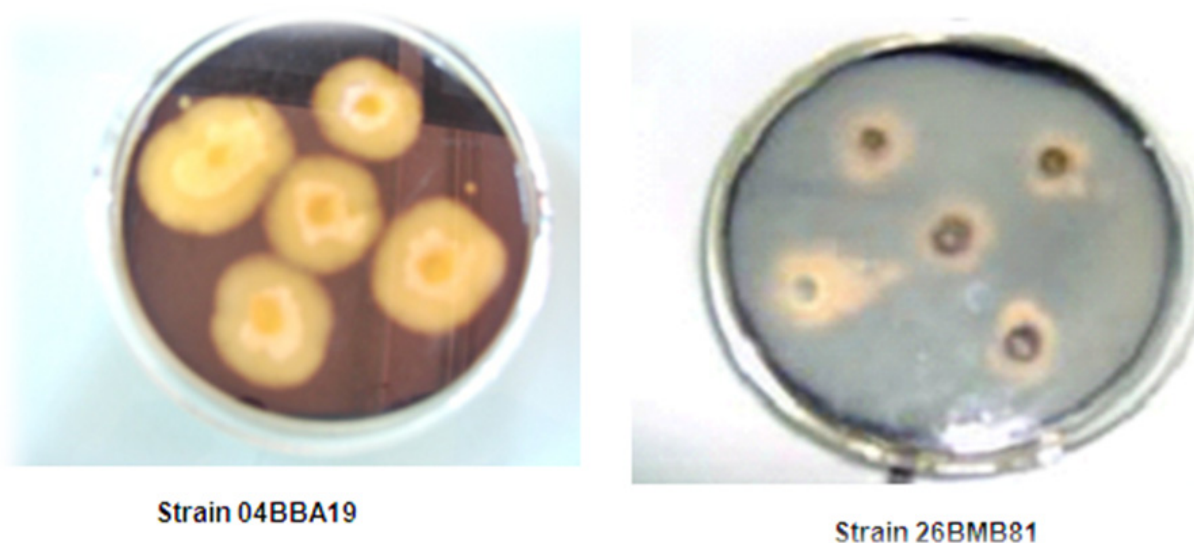
Strain 04BBA19

Strain 26BMB81

Figure 1. Plate assays for detection of amylase activity of lactic acid bacteria (04BBA19, 26BMB81) on MRS-starch agar plate medium. The diameter of hydrolysis halo was revealed by flooding the plates with Iodine solution (0.1% I_2+1% KI) after 48 h of culture at 40°C

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 temperature of growth	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	α-Methyl-D-mannoside	α-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-gh-Gluconate	5-Keto-Gluconate	Identification species (API 50CHL)
04BBA19	-	-	nc	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	*Lactobacillus fermentum*
26BMB81	+	+	+	+	+	+	+	+	+	+	nc	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	*Lactobacillus plantarum*

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

Table 1. Biochemical characteristics of amylases overproducing *Lactobacillus* isolated from soils

4.2. Amylase and lactic acid production

In the presence of starch as carbon source at 40°C, *L. fermentum* 04BBA19 strain grew, exhibited amylolytic activity and produced lactic acid in the culture medium. The amylase production pattern in *L. fermentum* 04BBA19 (Fig.2) indicates that the induction of amylase took place during the lag phase (after 10 h of incubation) in the presence of starch. The level of amylase production increased significantly during the exponential phase of growth. Lactic acid production became visible around 15 h after incubation and also increased considerably during the exponential phase of growth. Cell growth, amylase and lactic acid production reached maxima values at the same time (40 h of fermentation). The values of those maxima were 1.1×10^9 cfu/ml, 107.3±0.5 U/ml, 8.7±0.5g/l for cell growth, amylase activity, and lactic acid production respectively. Such coincidence shows that amylase production by *L. fermentum* 04BBA19 was tightly linked to cell growth. These results are in agreement with the report of Goyal et al. [53], Liu and Xu [54] on the relationship between pattern of cell growth and amylase production. The decline of cell growth and amylase production after the peak occurred around 50 h of incubation and could be attributed to the rise of lactic acid concentration in fermented broth [6] or to the rise of protease levels [55]. The acidification was also expressed by the decrease of initial pH of culture broth (Fig. 2). The initial pH of culture broth declined significantly and reached a value of 3.0 around 50 h of incubation and then remained constant.
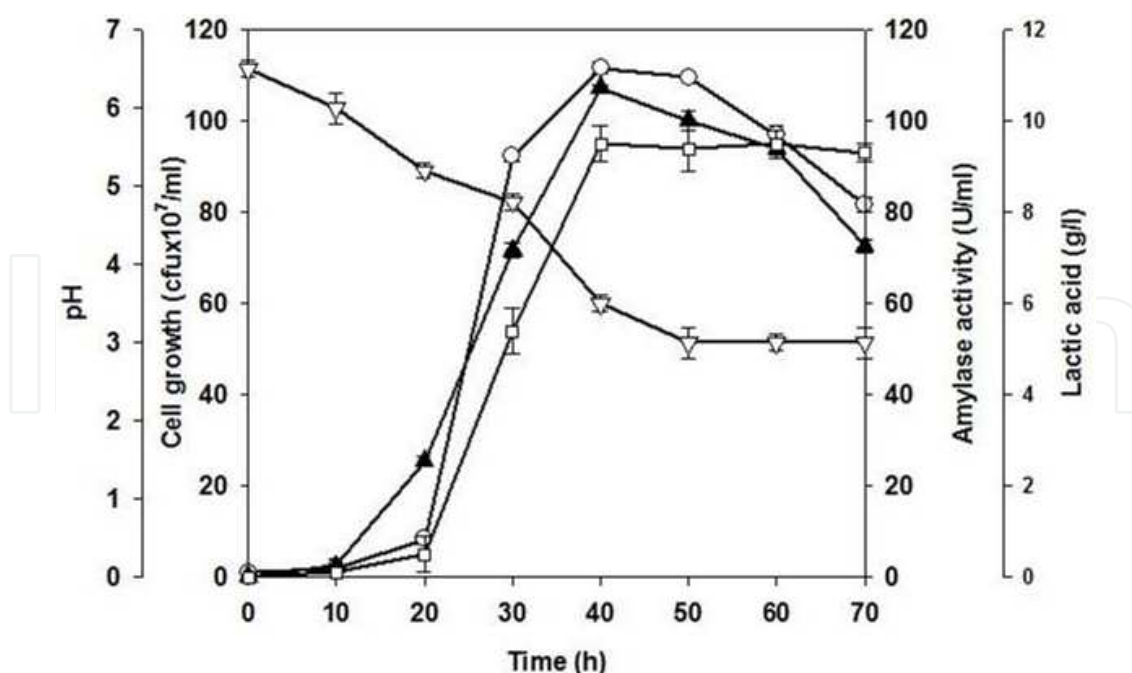
Figure 2. Time course of growth (○), pH (▽), α-amylase (▲) and lactic acid (□) production by *L. fermentum* 04BBA19 in 1% (w/v) soluble starch medium at 40°C, pH 6.0. The data shown are averages of triplicates assays within 10% of the mean value.

The study of cell growth and amylase production as a function of temperature (Fig. 3a) showed that *L. fermentum* 04BBA19 exhibited maximal growth and amylase activity at 45°C, confirming thus the strong relationships between cell growth and amylase production. On the other hand the maximum value of lactic acid was produced at the same temperature. Many other investigators reported that maximum amylase production occurred at the optimum growth temperature [56, 53]. These results are contrary to the findings of Chandra et al.[57] who studied the growth and amylase production of *Bacillus licheniformis* CUM 305. They have observed that this microorganism grew very well at 30°C, but did not produce α-amylase at that temperature. In addition, Saito and Yamamoto [58] found α-amylase production at 50°C and cell growth at a temperature lower than 45°C for another strain of *B. licheniformis*.

The amylase and lactic acid production by *L. fermentum* 04BBA19 was influenced significantly by initial pH of culture broth (Fig. 3b). Maximum amylase and lactic acid production was achieved for pH range of 4.0-6.5. These results could be explained by the fact that pH generally act by inducing morphological change in microorganism which facilitate enzyme production [59].

4.3. Optimisation of amylase and lactic acid production

Amylase production is known to be induced by a variety of carbohydrate, nitrogen compounds and minerals [60, 61]. In order to achieve high enzyme yield, efforts are made to develop a suitable medium for proper growth and maximum secretion of enzyme, using an adequate combination of carbohydrates, nitrogen and minerals [53, 62].
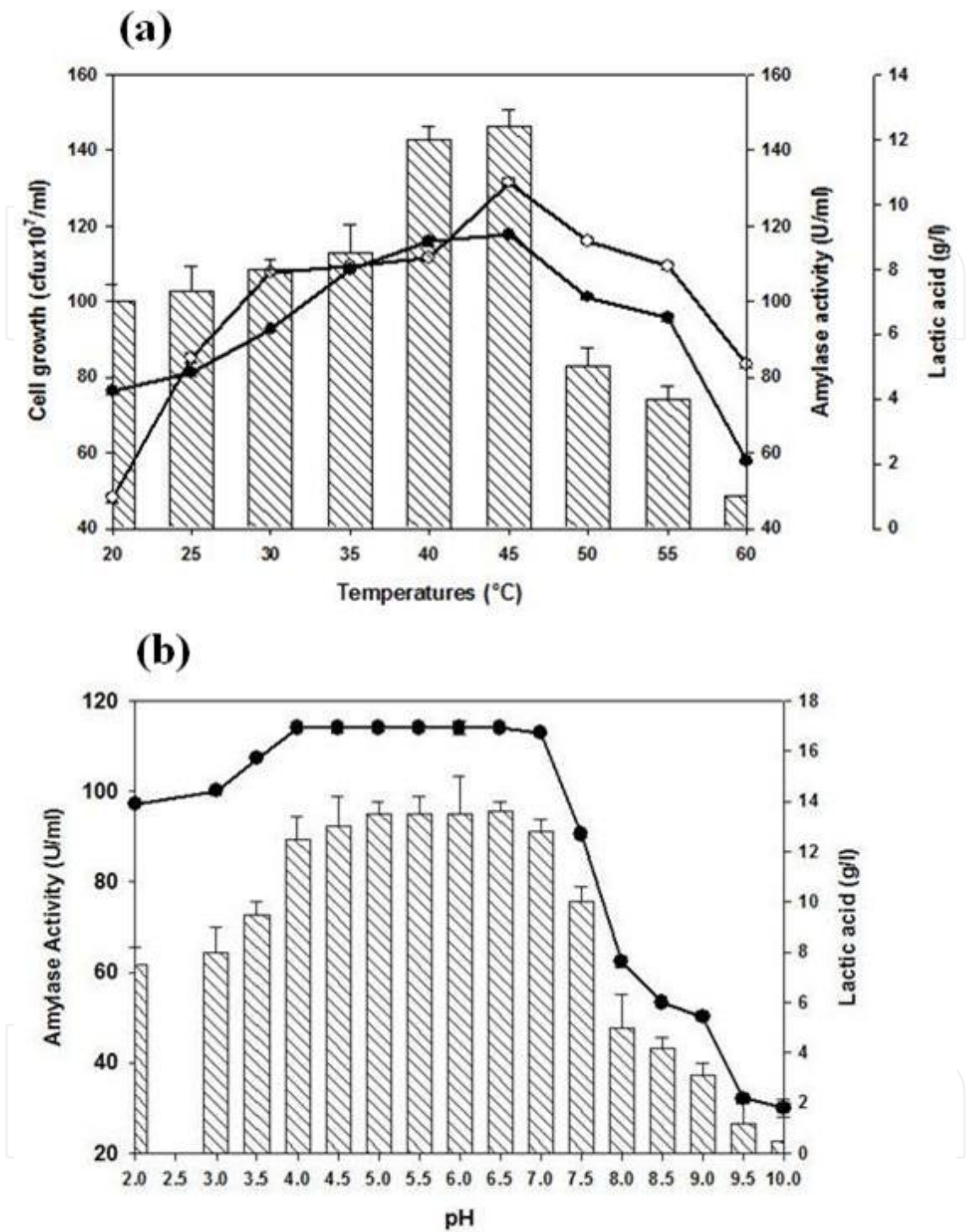
Figure 3. (a) Effect of temperature on microbial growth (○), α-amylase (●) and lactic acid (▨) production. (b) Effect of initial pH of culture broth on α-amylase (●) and lactic acid (▨) production. The data shown are averages of triplicate assays within 10% of the mean value.

From the use of different carbohydrate sources in the present study, soluble starch proved to be the best inducer of amylase production (Table 1). In the presence of soluble starch at concentration of 1% (w/v), the enzyme yield reached 107.0±1.2 U/ml after 48 hours of fermentation, while in the presence of raw cassava starch at the same concentration, the

enzyme yield was 67.1±0.5 U/ml. These results are in agreement with the reports of Cherry et al. [63], Saxena et al. [4] who reported maximum amylase production when starch was used as carbohydrate source. In the presence of glucose and fructose, amylase production was almost nil; and that was a proof that glucose and fructose repressed amylase synthesis by *L. fermentum* 04BBA19. This observation is in agreement with the reports of Theodoro and Martin [64] showing that synthesis of carbohydrate degrading enzymes in some microbial species leads to catabolic repression by substrate such as glucose and fructose. Similar results were observed by Halsetine et al. [65] for the production of amylase by the hyperthemophilic archeon *Sulfolobus solfataricus*. According to them, glucose prevented α-amylase gene expression and not only secretion of performed enzyme. Since amylase yield is higher with amylose (92.3 U/ml) as carbohydrate source than with amylopectin (50.1 U/ml), the *L. fermentum* 04BBA19 amylase is more efficient for hydrolysis of alpha-1,4 linkages than those of alpha-1,6. The amylase production increased with the soluble starch concentration (Fig. 4), reaching a maximum (180.5 ± 0.3 U/ml) at the concentration range of 8-16 % (w/v). These optimum starch concentrations for amylase production by *L. fermentum* 04BBA19 are higher than that observed for amylase production in *Bacillus* sp. PN5 reported by Saxena et al. [4]. This microorganism presented an optimum soluble starch concentration of 0.6% (w/v) for amylase production. The lactic acid production also increased with the soluble starch concentration, the optimum starch concentration for lactic acid production was achieved at the same range of concentration for amylase production.
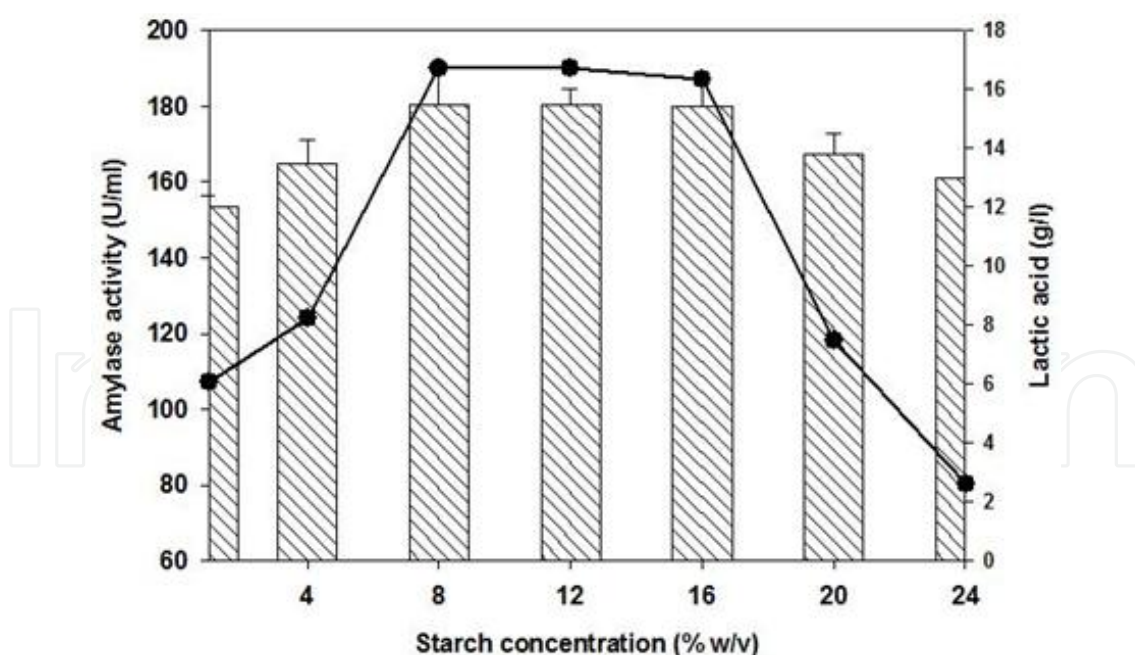

Figure 4. Effect of starch concentration on α-amylase (●) and lactic acid production (▨) by *L. fermentum* 04BBA19. The data shown are averages of triplicate assays with SD within 10% of mean value

Among the various gelatinized starchy sources tested, corn and sorghum flour were found to be the most suitable for α-amylase and lactic acid production by *L. fermentum* 04BBA19

while for the raw starchy sources tested, potato starch was most suitable (Table 2). On the other hand the level of lactic acid was more important when corn and sorghum flours were used. The good production of α-amylase and lactic acid when these starchy flours are used is based on their composition; they also contain proteins and vitamins which are required by lactic acid bacteria for their growth, enzymes and acids production [66].

Among nitrogen sources used in the present study, soya bean meal and yeast extract showed significant effect on α-amylase and lactic acid production. Soya bean meal, rich in protein is a potential nutrient for lactic acid fermentation. Similar results were obtained by several authors. Goyal et al. [53] reported that soybean meal presented a positive effect and was the best nitrogen source for raw starch digesting thermostable α-amylase production by the *Bacillus* sp I-3 strain. The yeast extract was also reported to be a potential nutrient for lactic acid fermentation, since it contains vitamins, amino acids [66]. Though all nitrogen sources are positively influencing enzyme production by *L. fermentum* 04BBA19, an inverse behaviour has been observed with other bacterial strains, for instance, Tanyildizi et al. [67] reported zero effect of yeast extract on amylase production by *Bacillus* sp.

All metal salts tested in this study increased amylase and lactic acid production by *L. fermentum* 04BBA19, except $CuSO_4.5H_2O$ that acted as inhibitor. The inhibition of amylase production by $CuSO4.5H2O$ was also reported by Wu et al. [68] for the *Bacillus* sp CRP strain. Copper ion acted as poisonous compound for this strain and consequently inhibited amylase synthesis. The effect of $CaCl_2.2H_2O$ was the most important, and was in agreement with the observation of Gangadharan et al. [61] who described the rise of amylase production by *B. amyloliquefaciens* when $CaCl_2.2H_2O$ was supplemented to the culture medium. The supplementation of metal ions has been reported to provide good growth and also influence higher enzyme production. Most α-amylases are metalloenzymes and in most cases, Ca^{2+} ions are required for maintaining the spatial conformation of the enzyme, thus play an important role in enzyme stability [61].

From the surfactants tested in this study, Tween-80 appeared to be the best surfactant sources for amylase production by *L. fermentum* 04BBA19. Similar results were obtained by Reddy et al. [69]. These authors reported that the supplementation of culture medium with Tween-80 resulted in a marked increase in the yields of thermostable β-amylase and pullullanase by *C. thermosulfurogenes* SV2, and that the stimulation of enzyme production was greater when the surfactants were added after 18 h of incubation of culture. Beside stimulation, the surfactants caused and increased secretion of the enzymes into extracellular fluid [59].

From various environmental factors tested for α-amylase and lactic acid production by *L. fermentum* 04BBA19, it has been observed that all factors that increase amylase synthesis also positively affect lactic acid production. The optimization of the basal medium by supplementation of all carbohydrate, nitrogen, mineral and surfactant sources (excepted $CuSO_4.5H_2O_4$) in culture medium resulted to a significant improvement of enzyme and lactic acid yield. In the optimized medium, amylase activity and lactic acid content reached 732.4±0.4 U/ml and 53.2±0.4 g/l respectively.

Parameters	Enzyme yield (U/ml)	Lactic acid(g/l)
Carbohydrate sources (1% w/v)		
Glucose	0.1±0.0[d*]	14.3±0.5[a]
Fructose	0.2±0.0[d]	12.1±0.5[b]
Maltose	0.1±0.0[d]	12.8±0.4[b]
Amylose	92.3±0.1[b]	12.2±0.1[b]
Amylopectin	50.1±0.5[c]	10.3±0.5[c]
Soluble starch	107.3±0.5[a]	8.7±0.5[d]
Nitrogen sources (1.5% w/v)		
Yeast Extract	107.3±0.5[b]	8.7±0.5[b]
Beef extract	92.4±0.5[c]	7.3±0.2[b]
Peptone	88.3±1.7[d]	7.1±0.3[b]
Tryptone	75.3±0.5[e]	6.5±0.5[b]
Soya bean meal	397.3±0.4[a]	29.2±0.4[a]
Ammonium sulphate	95.4±1.5[c]	7.2.±0.8[b]
Urea	76.3±0.3[e]	5.3±0.6[c]
Minerals (0.1% w/v)		
CaCl$_2$. 2H$_2$O	412.1±0.6[a]	33.2±0.1[a]
MgSO$_4$. 7H$_2$O	315.1±0.4[b]	31.2±0.5[a]
FeSO4. 7H$_2$O	237.3±0.7[c]	20.2±0.4[b]
NaCl	315.2±0.9[b]	22.1±0.6[b]
CuSO$_4$.5H$_2$O	12.2±0.6[e]	3.2±0.3[d]
Surfactants (1.5% w/v)		
Tween-40	209.5±0.1[b]	27.3±0.4[b]
Tween-80	215.1±0.3[a]	35.2±0.3[a]
Gelatinized starchy sources (1 %w/v)		
Corn flour	303.5±0.2[a]	36.3±0.6[a]
Cassava flour	182.3±0.4[c]	24.2±0.8[d]
Sorghum flour	305.8±0.7[a]	35.2±0.1[a]
Rice flour	187.3±0.8[c]	30.1±0.5[b]
Tapioca flour	237.4±0.6[b]	27.2±0.7[c]
Raw starchy sources		
Cassava starch	67.1±0.5[c]	21.3±0.4[a]
Potato starch	87.2±0.5[a]	23.4±0.1[a]
Cocoyam starch	78.6±0.2[b]	22.7±0.4[a]
Media		
Basal medium	107.5±0.3	8.7±0.5[b]
Optimized medium	732.3±0.4	53.2±0.4[a]

Table 2. Effect of different parameters on α-amylase and lactic acid production by *L. fermentum* 04BBA19 in submerged state fermentation at 45 °C and initial pH 6.5.

The basal medium contained soluble starch, 1% (w/v); yeast extract, 0.5 % (w/v); while the optimized medium contained all parameters without $CuSO_4.5H_2O$. The data shown are averages of triplicate assays with SD within 10% of mean value. For each group of parameters (Carbohydrate, Nitrogen, Mineral, Starchy sources, media), means with different superscripts within columns are significantly different ($p<0.05$).

4.4. Enzyme properties

The amylase produced by *L. fermentum* 04BBA19 showed high affinity toward cassava raw starch granules with 80% adsorption and brought about 79% hydrolysis of 1% (w/v) suspension of raw cassava starch. On the other hand, the enzyme was able to hydrolyze blocked p-nitro phenyl methyl heptaoside, releasing a yellow compound (p-nitro phenol) with maximum absorption at 530 nm. This result was a proof that amylase from *L. fermentum* 04BBA19 is an endo acting amylase (α-amylase), since the blocked p-nitro phenyl methyl heptaoside is known to be hydrolysed only by endo-acting amylases [38].

The enzyme exhibited maximum activity at 60-70°C and maintained 100% of its initial activity at 80°C for 30 min of heat treatment (Fig 5-a). When the enzyme was treated for the same time (30 min.) at 90°C and 100°C, the remaining activities were 90 and 87% respectively. These results showed the thermophilic character and very high thermostability of α-amylase from *L. fermentum* 04BBA19. In general, most of lactic acid bacteria do not produce amylases. However, this property have been observed in some genera of lactic acid bacteria, especially in *L. plantarum* and *L. amylovorus* [37], *L. manihotivorans* [70, 13], *L. fermentum* OGI E1 [38]. But amylases produced by these strains are not thermostable. Traditionally high thermostable and thermophiles amylases are found in *Bacillus* and *Thermococcus* genera as: *B. amyloliquefaciens* [71]; *B. licheniformis* [72]; *B. stearothermophilus* [73]; *B. subtilis* and *T. aggreganes* [74], *T. profundus* [75], *Bacillus* sp PN5 [4], *B. cohnii* US147 [35], *Chromohalobacter* sp. TVSP 101 [76].

Fig. 6 shows the thermostability pattern of α-amylase from *L. fermentum* 04BBA19 at 80°C, 90°C and 100°C when the time of heat treatment is beyond 30 min. Table 3 presents the thermal inactivation rate constant (k_i) and half-life (T) at these temperatures. The half-life of this enzyme is higher than that of α-amylase from *B. licheniformis*: 120 min at 70°C [76]. The thermal stability was considerably improved by addition of 0.1% (w/v) $CaCl_2.2H_2O$. Goyal et al. [53] obtained a half-life value of 3.5 h at 80°C with α-amylase from *Bacillus* sp.I-3 in the presence of 0.1 % (w/v) calcium chloride, while under the same conditions; α-amylase from *L. fermentum* 04BBA19 displayed a half-life of 6.1 h.

Due to its high thermostability, α-amylase from *L. fermentum* 04BBA19 could be highly competitive in industrial bioconversion reactions, as compared to α-amylase from *Bacillus*. In addition, this competitiveness is enhanced by the fact that lactobacilli, due to their non-pathogen character, are easily used in food industry [6].

The *L. fermentum* 04BBA19 α-amylase is active and stable in pH range of 4.0 – 7.0 (Fig. 5-b), which is the pH range of many foods. In this respect, this amylase could be used in starch hydrolysis, brewing and baking.
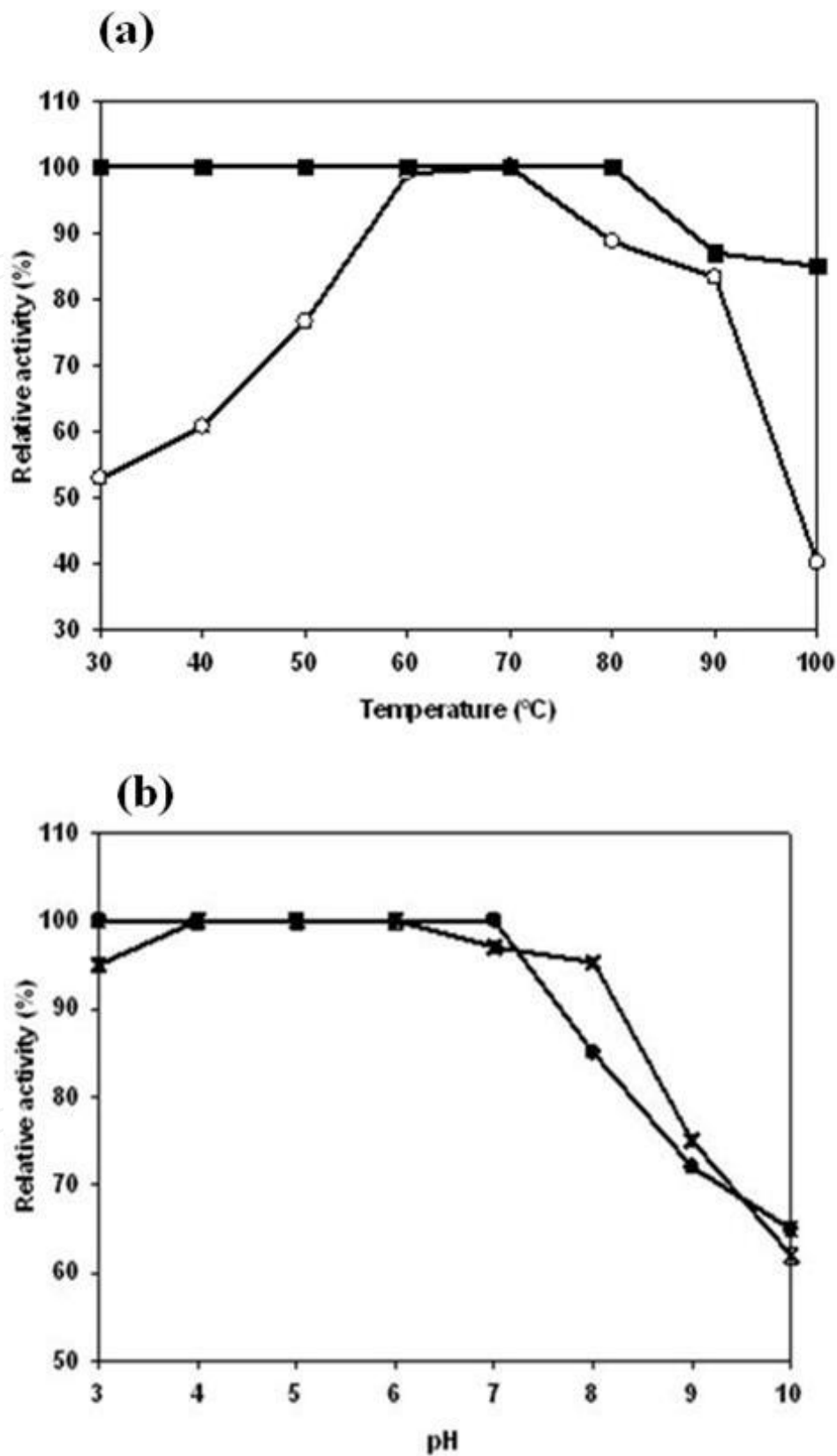
Figure 5. (a) Effect of temperature on activity (○) and stability (■) of α-amylase from *L. fermentum* 04BBA19. (b) Effect of pH on activity (✕) and stability (■) of α-amylase from *L. fermentum* 04BBA19. The data shown are averages of triplicate assays within 10% of the mean value.

The metal salts generally act on activity of enzyme through their ions. The enzyme activity was highly improved by Ca^{2+}, while Fe^{2+}, Fe^{3+}, Na^+ and Mg^{2+} had less significant effect. On the contrary Cu^{2+} and EDTA acted as inhibitors (Fig. 7). The behaviour of the enzyme towards metal ions, particularly calcium, indicates its metalloenzyme nature, which is confirmed by the action of EDTA.
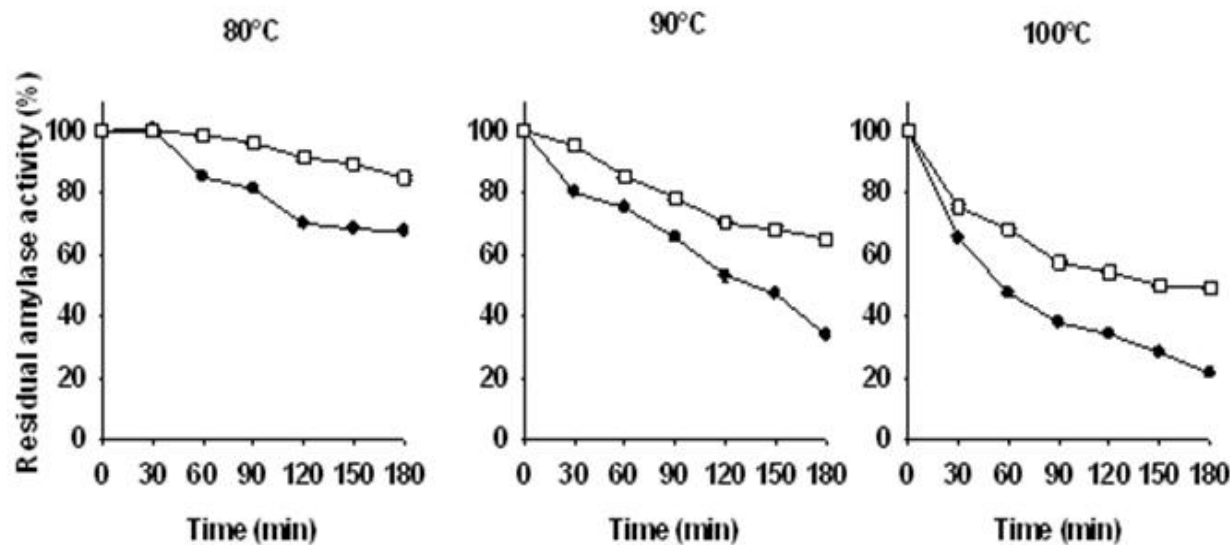

Figure 6. Thermostability pattern of α-amylase from *L. fermentum* 04BBA19, at 80, 90, 100°C without $CaCl_2.2H_2O$ (●) and with 0.1% (w/v) $CaCl_2.2H_2O$ (□). The enzyme was pre-incubated at optimum pH, for 30, 60, 90, 120 and 180 min at temperatures (80, 90 and 100°C). The remaining activity was determined incubating the enzyme at optimum temperature, 60°C for 30 min. The data shown are averages of triplicate assays with SD within 10% of mean value.

	Temperatures					
	80 °C		90°C		100°C	
$CaCl_2.2H_2O$ (% w/v)	k_i ($10^{-3}.min^{-1}$)	T (min)	k_i ($10^{-3}.min^{-1}$)	T (min)	k_i ($10^{-3}.min^{-1}$)	T (min)
0	3.4	204.0	5.6	123.8	7.9	87.7
0.1	1.9	364.8	2.6	266.6	3.8	182.4

Table 3. Inactivation rate constant (k_i) and half-live (T) of amylase from *L. fermentum* 04BBA19 at 80, 90 and 100°C in the absence and the presence of 0.1% (w/v) $CaCl_2.2H_2O$.

The main ALAB that have been isolated for the past decade are summarized in Table 4. No study has dealt with the thermostability of their amylases, except the case reported by Aguilar et al. (2000) concerning the properties of the extracellular amylase produced by *L. manihotivorans* LMG 18010ᵀ. This strain produced an amylase with a moderate themostability exhibiting maximum activity at 55°C.

The strain *L. fermentum* 04BBA19 appears as the first ALAB producing highly thermostable amylase. The potential industrial application of this strain could be the bioconversion of inexpensive raw material as starch into lactic acid in single step process. On the other hand

this strain and its amylase are potential candidates for food industries (making of high density gruels, baking, brewing) and for the production of biodegradable plastic from starchy raw material.
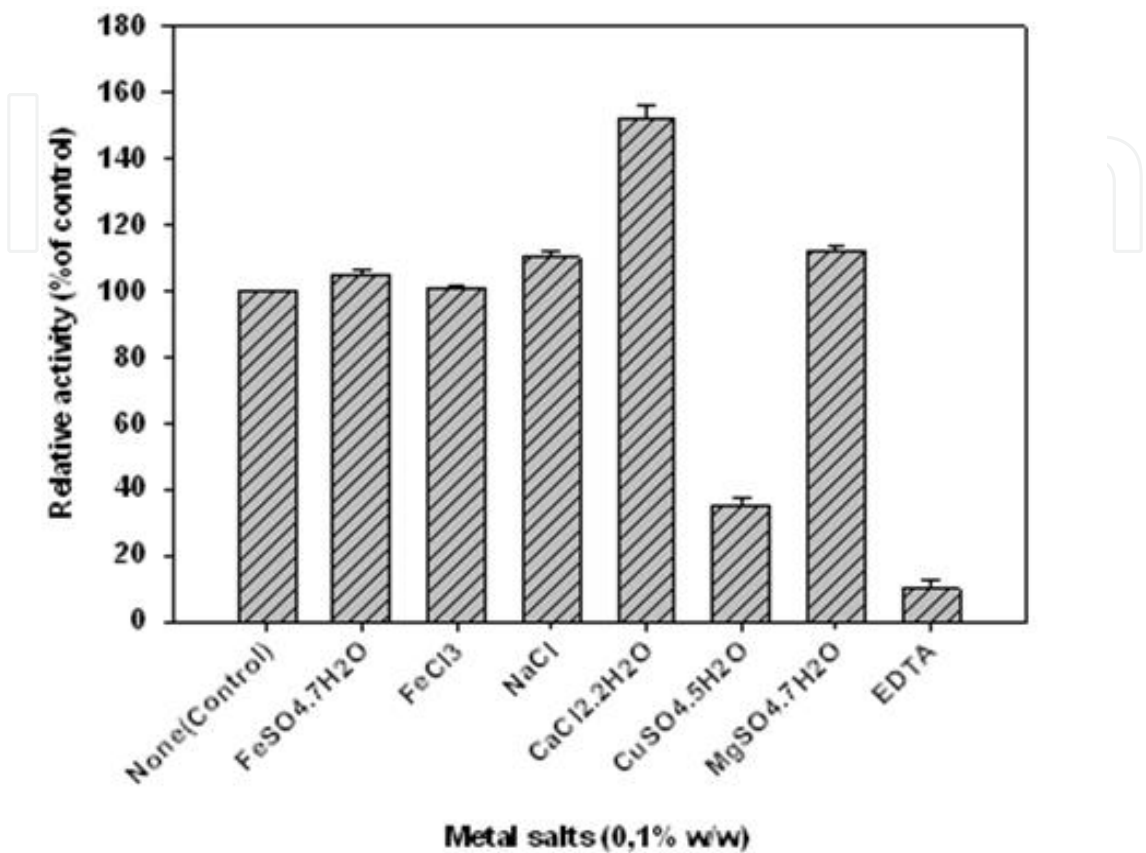

Figure 7. Effect of metal salts and EDTA on the activity of α-amylase from *L. fermentum* 04BBA19. The data shown are averages of triplicate assays with SD within 10% of mean value.

Bacteria	Strains	References
L. fermentum	04BBA19	[47, 48]
L. manihotivorans	LMG18010T	[69]
L. fermentum	Ogi E1	[11]
L. fermentum	MW2	[11]
L. fermentum	K9	[12]
L. acidophilus	L9	[78]
L. amylovorus	ATCC33622	[23]
L. amylovorus	B-4542	[29]
L. amylovorus		[17]
L. manihotivorans	OND32T	[10, 13]
L. manihotivorans		[13]
L. manihotivorans	LMG 18011	[79]
L. acidophilus		[78]

L. plantarum	A6	[80, 9]
L. plantarum	LMG18053	[9]
L. plantarum	NCIM 2084	[81]
Streptococus. bovis	148	[82]
Lactobacillus sp	LEM 220,	[85]
Lactobacillus sp	LEM 207	[85]
Leuconostoc sp		[86]
Leuconostoc	St3-28	[80]
S. macedonicus		[87]
L. amylolyticus		[88]
L. amylophilus	JCIM 1125	[84]
L. amylophilus	B 4437	[28, 32]
L. amylophilus	GV6	[20]
Bifidobacterium adolescentis	Int57	[15]
B. adolescentis	ZS8	[16]

Table 4. The main amylolytic lactic bacteria strains isolated during the past two decade

5. Conclusion

L. fermentum 04BBA19 which is a soil isolate produced very high thermostable α-amylase. This is the first study dealing with high thermostable amylase from a lactic acid bacterium. According to its properties, this enzyme is a good candidate for starch hydrolysis at high temperature. An economical process could be attained through the use of this enzyme at the liquefaction stage at high temperatures.

On the other hand the fact that thermostable amylase and lactic acid production can be combined in single fermentation step would not only provide a way to make gruels with high energy density, but also improve its safety, since lactic acid bacteria fermentation is an efficient way to inhibit food-borne pathogens.

Owing to the importance of this finding, further studies will focus on the development of an accurate method for preparing high energy density complementary food using local starchy sources and the *L. fermentum* 04BBA19 strain.

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