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Full Length Research Paper

Simultaneous production of raw starch degrading highly thermostable α-amylase and lactic acid by *Lactobacillus fermentum* 04BBA19

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The widely used thermostable amylases were produced long time ago from *Bacillus* genus. Although, lactic acid bacteria (LAB) fermentation presents several advantages including the reduction of growth of pathogenic microorganisms, no study has yet reported thermostable amylases from lactic acid bacteria. An amylolytic LAB, *Lactobacillus fermentum* (04BBA19) isolated from starchy wastes of a soil sample from the western region of Cameroon was studied for amylase and lactic acid production. The bacterium exhibited maximal amylase and lactic acid production at temperature of 45°C, and within pH range of 4.0 to 6.5. Upon the optimization of various environmental and cultural conditions the yield of amylase and lactic acid reached 732.3±0.4 U/ml and 53.2±0.7 g/L respectively in fermented broth after 48 h of culture. The enzyme was identified as α-amylase, with a very high thermostability revealed by the retention of 100% of original activity after pre-incubation for 30 min at 80°C. The stability was improved significantly with the addition of 0.1% (w/v) $CaCl_2.2H_2O$; the half life of the enzyme in these conditions was 6 h at 80°C. Owing to its aptitude to exhibit a simultaneous production of thermostable amylase and lactic acid, *L. fermentum* (04BBA19) appeared as a potential candidate for the making of high density gruel from starchy material.

Key words: Lactic acid bacteria, thermostable α-amylase, lactic acid, fermentation, high density gruel.

INTRODUCTION

Amylolytic enzymes play important role in the degradation of starch and are produced in bulk from microorganisms and represent about 25 to 33% of the world enzyme market (Asoodeh et al., 2010). The spectrum of amylases application has widened in many fields, such as clinical, medical and analytical chemistry as well as in the textile, food, fermentation, paper, distillery and brewing industries (Saxena et al., 2007; Gangadharan et al., 2008; Ghorbel et al., 2009). Thermostability is one of the main features of many enzymes sold for bulk industrial usage. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling and increased diffusion rate (Saxena et al., 2007). Several thermostable α-amylases have been purified from *Bacillus* sp. and the factors influencing their thermostability have been investigated (Haki and Rakshit, 2003). However, no study has yet dealt with thermostable amylase from lactic acid bacteria (LAB). Amylolytic lactic acid bacteria (ALAB) have repeatedly been isolated from traditional cereal or cassava-based fermented foods (Nguyen et al., 2007), but most of the α-amylases from these LAB presented weak thermostability compared to those of *Bacillus* genus.

The importance of simultaneous production of amylase and lactic acid by LAB is its exploitation in the production of high density gruel with suitable consistency at high dry

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Abbreviations: LAB, Lactic acid bacteria; **ALAB,** amyloytic lactic acid bacteria.

matter content. The method used generally involves hydrothermic treatments, such as drum-drying or extrusion cooking (Mouquet et al., 2003) or enzymatic starch hydrolysis which can be performed after gelatinization. Due to the temperature of gelatinization (80 to 100°C), the amylase needs to be thermostable. The use of thermostable amylases from LAB is advantageous in that 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) (Singh et al., 2006).

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 (Nguyen et al., 2007). This would require a good knowledge of conditions of the production of amylase and lactic acid and their properties. This 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.

MATERIALS AND METHODS

Microorganism

The starch degrading amylolytic lactic acid bacterial strains were isolated from the soil of a flour market in Bafoussam, the capital city of the western region of Cameroon. One of the isolates designated 04BBA19 exhibiting high amylolytic activity was selected and identified using API 50 CH test kit (bioMerieux, France) as *Lactobacillus fermentum* (Tatsinkou et al., 2009) and used for this study.

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 min 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 (8000 x *g*, 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.

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 ($CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $FeCl_3$, 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.

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 to 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.

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 to 10.0, using 50 mM of three buffer solutions: Tris-HCl (pH 3.0), Na_2HPO_4-Citrate (pH 4.0 to 6.0), and Glycine-NaOH (pH 7.0 to 10.0). The temperature stability was determined by incubating the partial purified enzyme solution in water bath for temperature range of 30 to 100°C for 30, 60, 90, 120, 150, 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%.

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%.

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 agar, in which glucose have been replaced by soluble starch. 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 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 0.1 M 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 1 ml of 1 M 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) is 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 (1996). The nature of amylase (endo-acting or exo-acting) was determined according to Ceralpha method (Megazyme) which uses a blocked maltoheptaoside as substratre (Talamond et al., 2002). The affinity of the enzyme preparation from *L. fermentum* (04BBA19) toward raw cassava starch was studied by incubating 0.2 g of raw cassava flour with 1 ml 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:

$$Adsorption\ (\%) = \frac{A - B}{A} \times 100$$

Where, *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 *L. fermentum* (04BBA19) and 100 mg of raw cassava starch in a final volume of 10 ml dispensed in 100 ml 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 (Miller, 1959). Light microscopy was used for the examination of the effect of enzyme on raw starch granules using Olympus microscope BH-2.

RESULTS AND DISCUSSION

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) (Figure 1) 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±0.5 g/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. (2005), Liu and Xu (2008) 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 (Singh et al., 2006) or to the rise of protease levels (Hiller et al., 1996). The acidification was also expressed by the decrease of initial pH of culture broth (Figure 1). The initial pH of culture broth declined significantly and reached a value of 3.0 around 50 h of incubation and then remained constant. The study of cell growth and amylase production as a function of temperature (Figure 2a) 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 (Burhan et al., 2003; Goyal et al., 2005). These results are contrary to the findings of Chandra et al. (1980) 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 (1975) 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 (Figure 2b). Maximum amylase and lactic acid production was achieved for pH range of 4.0 to 6.5. These results could be explained by the fact that pH generally act by inducing morphological change in microorganism which facilitates enzyme production (Gupta et al., 2003).

Optimisation of amylase and lactic acid production

Amylase production is known to be induced by a variety of carbohydrate, nitrogen compounds and minerals (Muralikrishna and Nirmala, 2005; Gangadharan et al., 2008). 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

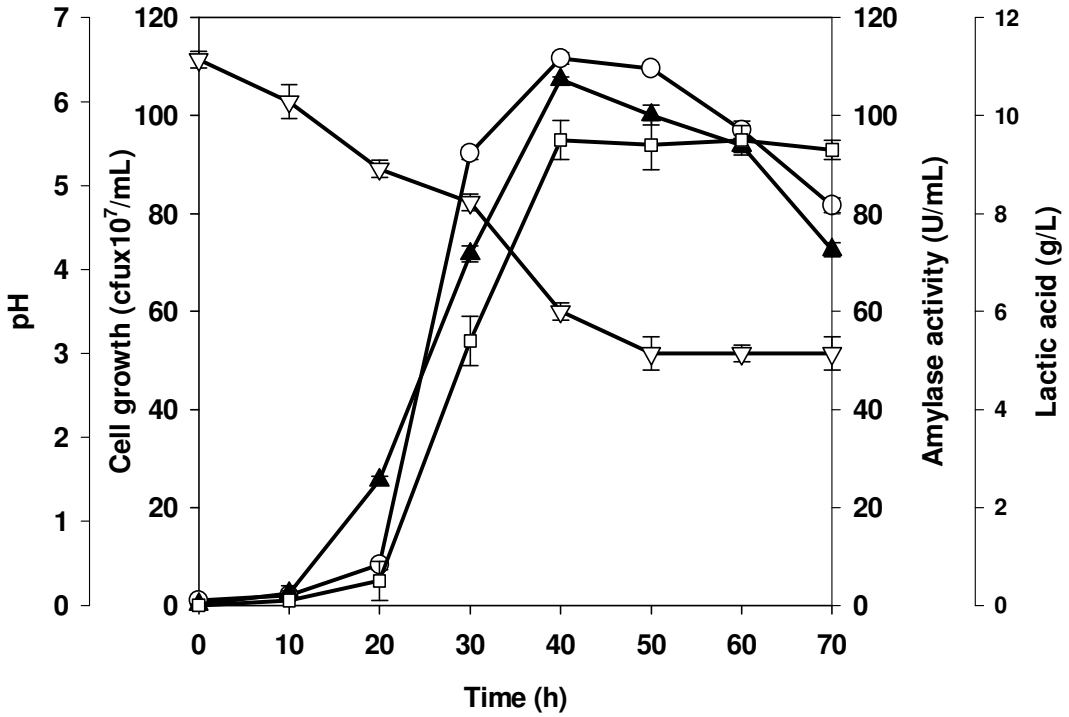

Figure 1. Time course of growth (○), pH (▽), α-amylase (▲) and lactic acid (□) production by *L. fermentum* (04BBA19) in 1% (w/v) soluble starch medium at 40°C, pH 6.0. The data shown are averages of triplicates assays within 10% of the mean value.

combination of carbohydrates, nitrogen and minerals (Goyal et al., 2005; Sodhi et al., 2005). From the use of different carbohydrate sources in this 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±0.5 U/ml after 48 h 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. (2004), Saxena et al. (2007) 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 Teodoro and Martin (2000), 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. (1996) 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 the hydrolysis of α-1, 4 linkages than those of α-1, 6. The amylase production increased with the soluble starch concentration (Figure 3), reaching a maximum (180.5 ± 0.3 U/ml) at the concentration range of 8 to 16% (w/v). These optima starch concentrations for amylase production by *L. fermentum* (04BBA19) are higher than those observed for amylase production in *Bacillus* sp. PN5 reported by Saxena et al. (2007). 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. 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 1).

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 (Gao et al., 2008). Among nitrogen sources used in this study, soya bean meal and yeast extract showed significant effect on α-amylase and lactic

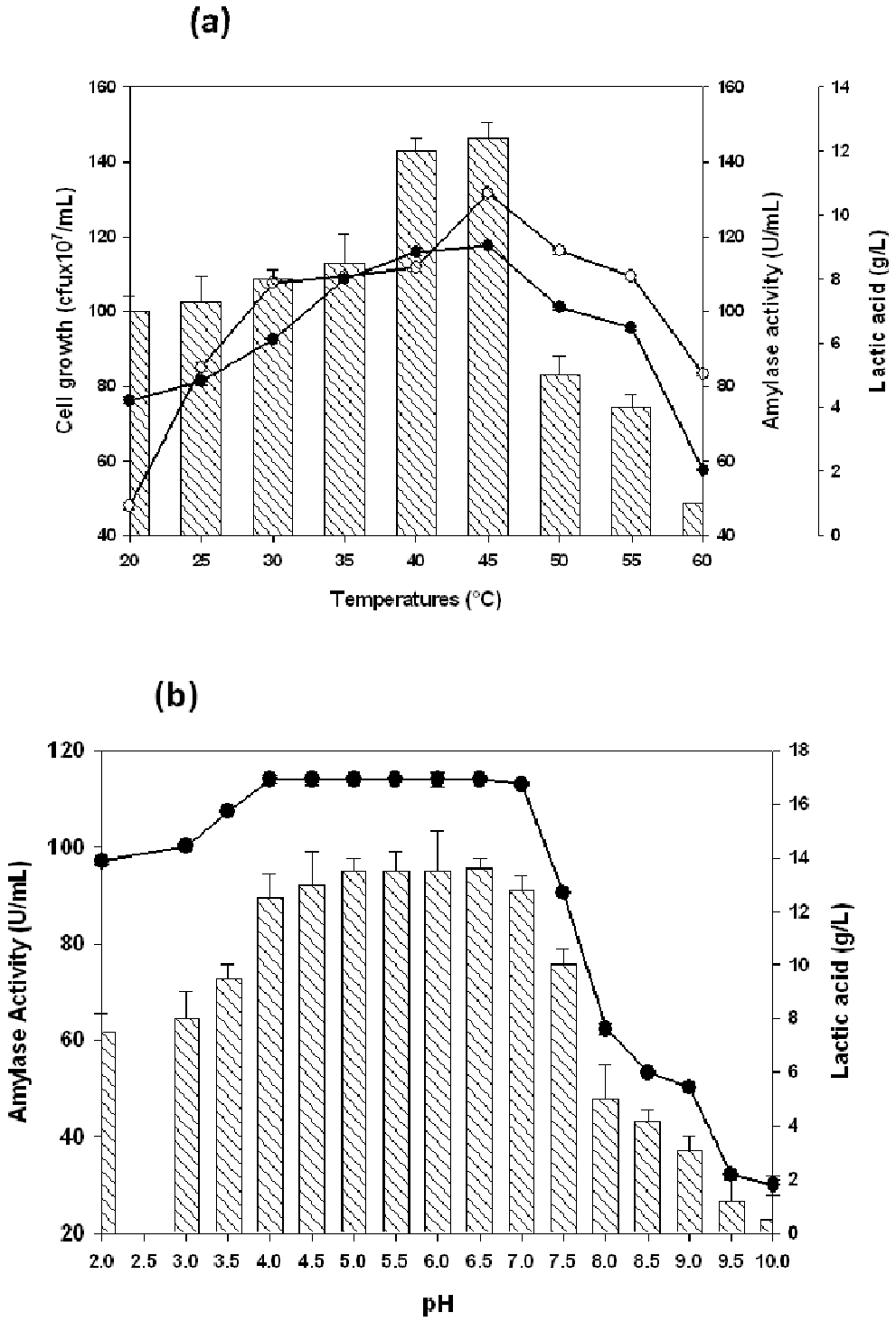

Figure 2. (a) Effect of temperature on microbial growth (○), α-amylase (●) and lactic acid (▨) production. (b) Effect of initial pH of culture broth on α-amylase (●) and lactic acid (▨) production. The data shown are averages of triplicate assays within 10% of the mean value.

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. (2005) 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,

Table 1. 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.

Parameter	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)		
$CaCl_2$. $2H_2O$	412.1±0.6^{a}	33.2±0.1^{a}
$MgSO_4$. $7H_2O$	315.1±0.4^{b}	31.2±0.5^{a}
FeSO4. $7H_2O$	237.3±0.7^{c}	20.2±0.4^{b}
NaCl	315.2±0.9^{b}	22.1±0.6^{b}
$CuSO_4.5H_2O$	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±0.7c
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}

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 ($p<0.05$).

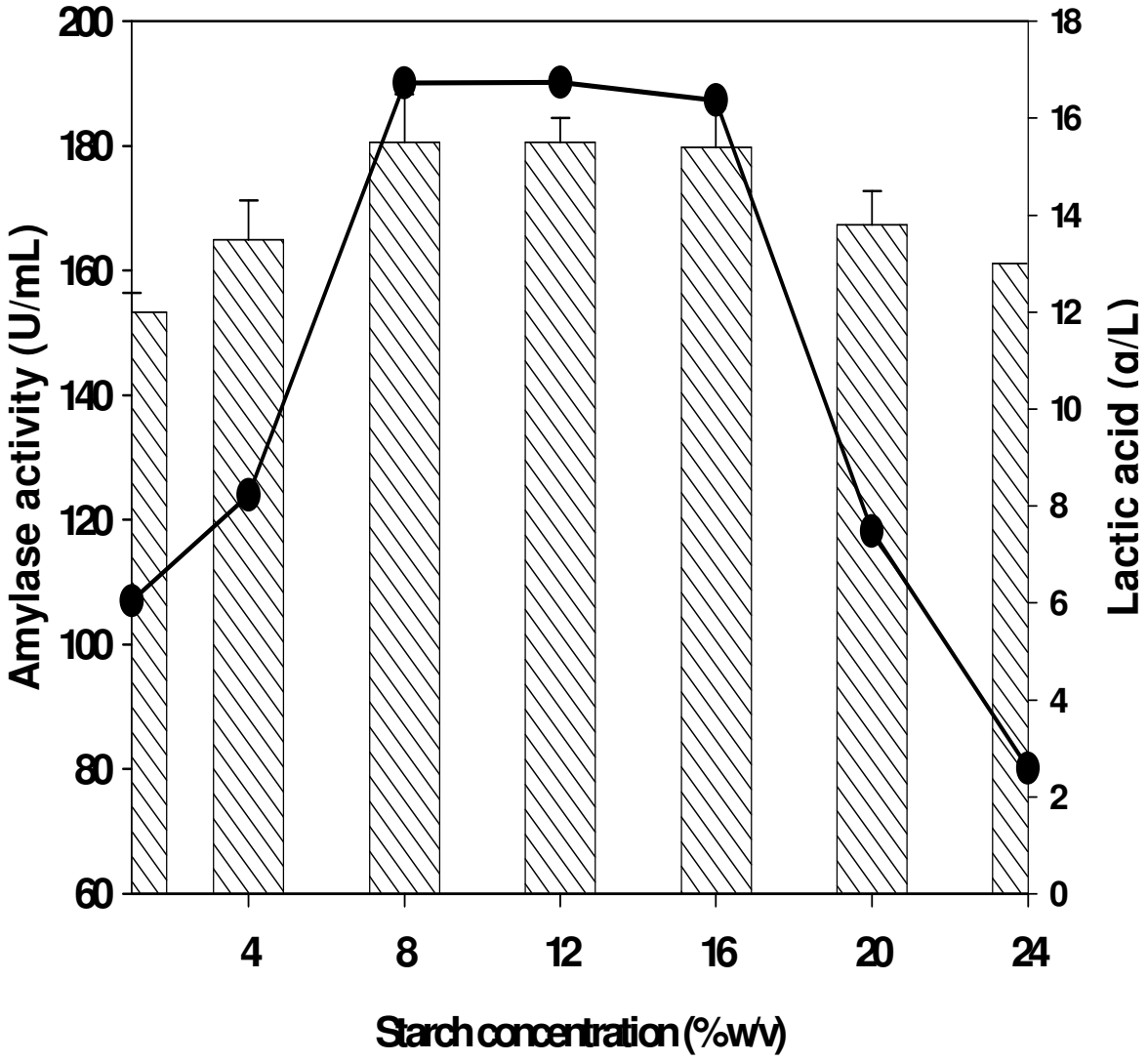

Figure 3. Effect of starch concentration on α-amylase (●) and lactic acid production (▨) by *L. fermentum* (04BBA19). The data shown are averages of triplicate assays with SD within 10% of mean value.

since it contains vitamins, amino acids (Gao et al., 2008). enzyme production by *L. fermentum* (04BBA19), an inverse behaviour has been observed with other bacterial strains, for instance, Tanyildizi et al. (2005) 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 $CuSO4.5H_2O$ was also reported by Wu et al. (1999) 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. (2008) who described the rise of amylase production by *Bacillus 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 (Gangadharan et al., 2008). 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. (1999). 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 (Gupta et al., 2003). 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 732.3±0.4 U/ml and 53.2±0.4 g/L respectively.

Enzyme properties

The amylase produced by *L. fermentum* (04BBA19) showed high affinity towards 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-nitrophenol) 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 (Talamond et al., 2002). The enzyme exhibited maximum activity at 60 to 70°C and maintained 100% of its initial activity at 80°C for 30 min of heat treatment (Figure 4a). When the enzyme was treated for the same time (30 min) at 90 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 lactic acid bacteria do not produce amylases. However, this property have been observed in some genera of lactic acid bacteria, especially in *Lactobacillus plantarum* (Giraud and Cunny, 1997), *Lactobacillus amylovorus* (Giraud and Cunny, 1997), *Lactobacillus manihotivorans* (Aguilar et al., 2000; Guyot et al., 2000), *L. fermentum* OGI E1 (Talamond et al., 2002), but amylases produced by these strains are not thermostable. Traditionally, high thermostable and thermophiles amylases are found in *Bacillus* and *Thermococcus* genera as: *Bacillus amyloliquefaciens* (Underkoffer, 1976), *B. licheniformis* (Viara et al., 1992), *Bacillus stearothermophilus* (Vihinen and Mantsala, 1990); *Bacillus subtilis* (Canganella et al., 1994), *Thermococcus aggreganes* (Canganella et al., 1994), *Thermococcus profundus* (Kwak et al., 1998), *Bacillus* sp PN5 (Saxena et al., 2007), *Bacillus cohnii* US147 (Ghorbel et al., 2009), and *Chromohalobacter* sp. TVSP 101(Prakash et al., 2009).

Figure 5 shows the thermostability pattern of α-amylase from *L. fermentum* (04BBA19) at 80, 90 and 100°C when the time of heat treatment is beyond 30 min. Table 2 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 (Bayramoglu et al., 2003). The thermal stability was considerably improved by addition of 0.1% (w/v) $CaCl_2.2H_2O$. Goyal et al. (2005) 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 lactic acid bacteria, due to their non pathogen character, are easily used in food industry (Singh et al., 2006). The *L. fermentum* (04BBA19) α-amylase is active and stable in pH range of 4.0 to 7.0 (Figure 4b), which is the pH range of many foods. In this respect, this amylase could be used in starch hydrolysis, brewing and baking. 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 (Figure 6). The behaviour of the enzyme towards metal ions, particularly calcium, indicates its metalloenzyme nature, which is confirmed by the action of EDTA.

Conclusion

L. fermentum (04BBA19) which is a soil isolate, produced very highly thermostable α-amylase. To our knowledge, this is the first study dealing with 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 a single fermentation step, would not only provide a way to make gruels with high energy density, but also improve its safety, since LAB 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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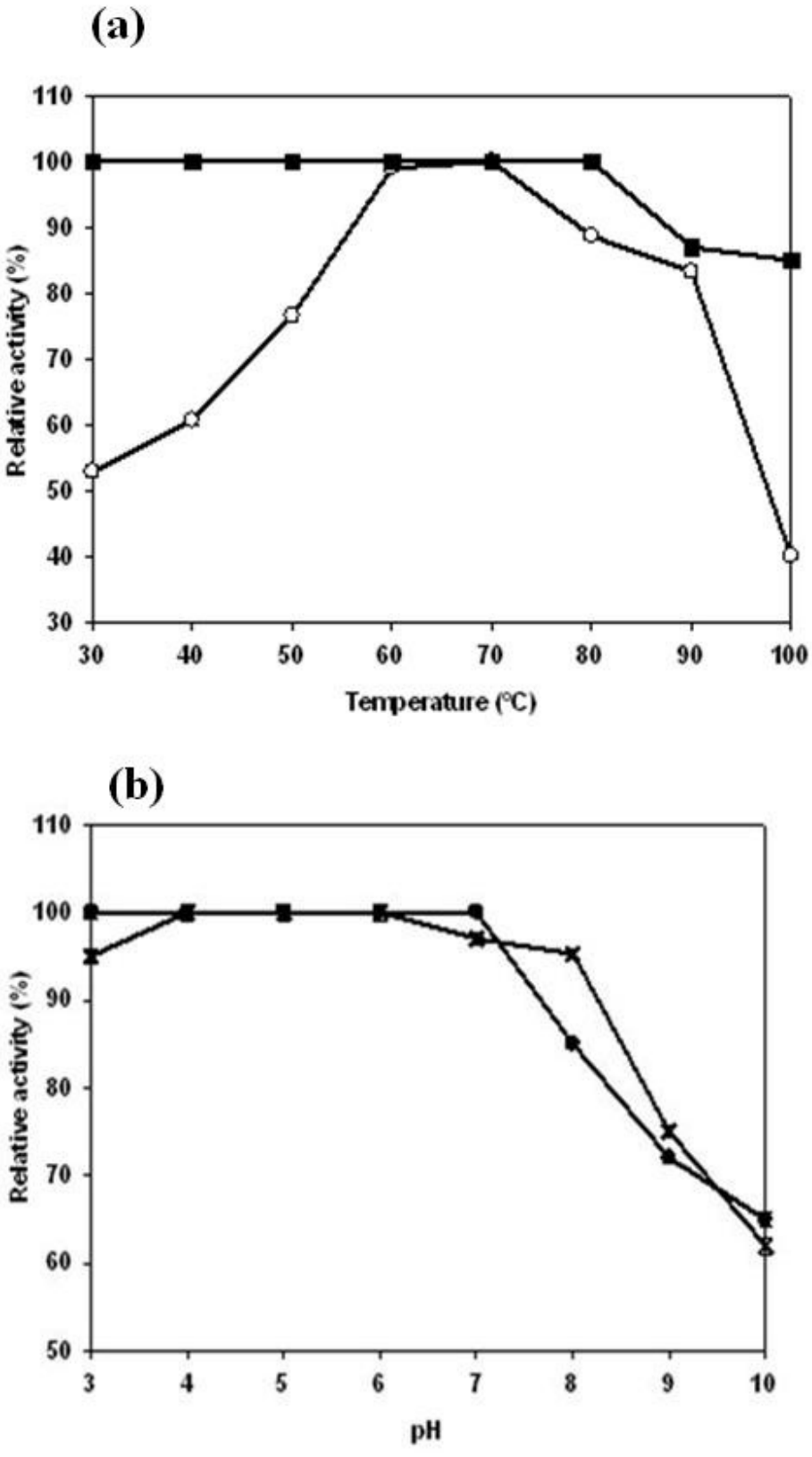

Figure 4. (a) Effect of temperature on activity (○) and stability (■) of α-amylase from *L. fermentum* (04BBA19). (b) Effect of pH on activity (×) and stability (■) of α-amylase from *L. fermentum* 04BBA19. The data shown are averages of triplicate assays within 10% of the mean value.

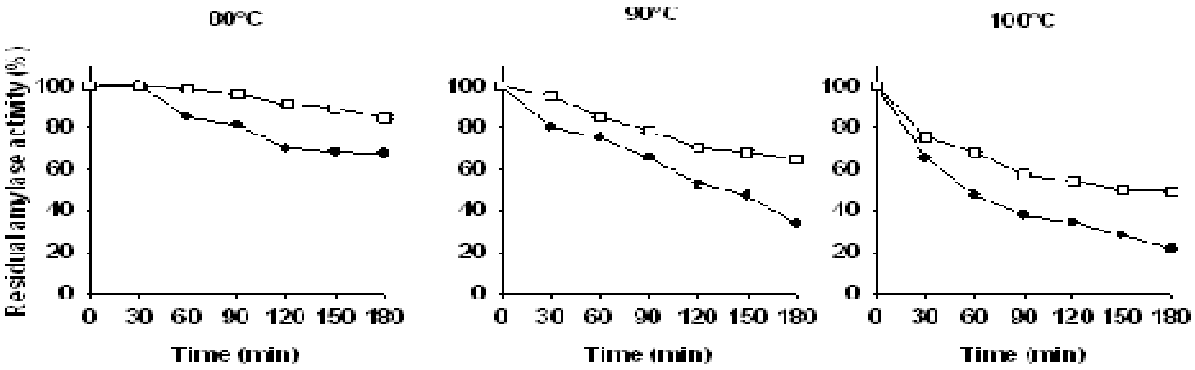

Figure 5. Thermostability pattern of α-amylase from *L. fermentum* (04BBA19), at 80, 90, 100°C without $CaCl_2.2H_2O$ (●) and with 0.1% (w/v) $CaCl_2.2H_2O$ (□). The enzyme was pre-incubated at optimum pH, for 30, 60, 90, 120, 150 and 180 min at temperatures (80, 90 and 100°C). The remaining activity 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.

Table 2. 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) $CaCl_2.2H_2O$.

$CaCl_2.2H_2O$ (%, w/v)	Temperature (°C)					
	80		90		100	
	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

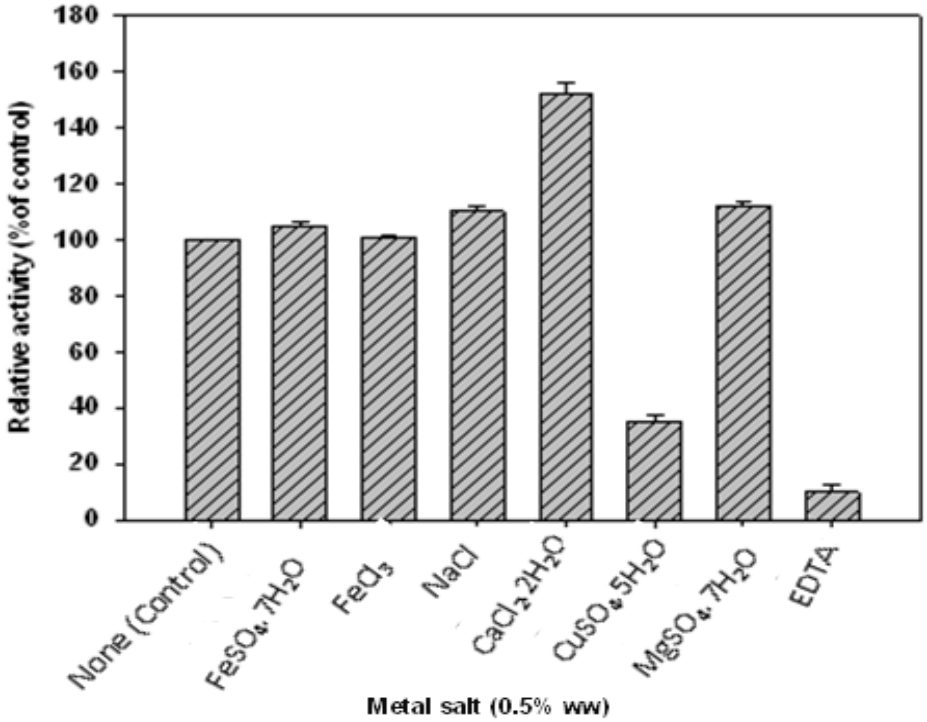

Figure 6. Effect of metal salts and EDTA on the activity of α-amylase from *L. fermentum* 04BBA19. The data shown are averages of triplicate assays with SD within 10% of mean value.

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