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New Thermostable Amylase from *Bacillus cohnii* US147 with a Broad pH Applicability

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Abstract A new thermophilic bacterial strain identified as *Bacillus cohnii* US147 was isolated from the southern Tunisian soil. The identification was based on physiological tests and molecular techniques related to the 16S ribosomal ribonucleic acid. The isolated strain produced amylase, which was purified. This amylase had an apparent molecular mass of 30 kDa as estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Amylase US147 showed K_m and V_{max} values of 0.7 mg/ml and 2.2 U/ml, respectively, with starch as the substrate. The enzyme was active in acid and basic pH and had a maximal activity on starch at pH 9 and 70 °C. The enzyme was stable at pH 9 for 72 h and retained half of its activity after incubation at 70 °C for 150 min. A partially inhibition (15%, 25%, 23%, 20%, and 22%) was obtained with 1 mM SDS, 1 mM $NaBO_3$, 1 mM H_2O_2, 1 mM Zn^{+2}, and 5 mM ethylenediamine tetraacetic acid (EDTA), respectively. The amylase recovered its original activity by the addition of 10 mM Ca^{2+} to the 5 mM EDTA. These properties indicated a possible use of this amylase in starch saccharification, in detergent, and in other industrial applications.

Keywords *Bacillus cohnii* · Amylase · Acid and basic pH activity · Purification · Characterization

Introduction

Amylolytic enzymes such as α-amylase (1, 4-α-D-glucan glucanohydrolases; EC 3.2.1.1) hydrolyze starch molecules to give diverse products including dextrins and progressively

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smaller polymers composed of glucose units. Amylases can be derived from several sources, such as plants, animals, and microorganisms. Enzymes from microbial sources have potential applications in many industrial fields, particularly in food, paper, textile, and detergent industries [1–5]. Amylases that are active at acidic pH are generally used in the glucose syrup industry, whereas those active at basic pH are explored in detergents. Due to the industrial importance of amylases, there is an ongoing interest in the isolation of new bacterial strains producing enzymes suitable for industrial applications, such as alkaline amylases for the detergent industry and starch saccharification [6, 7]. Several *Bacillus* species and few thermostable actinomycetes including *Thermonospora* and *Thermoactinomyces* are valuable producers of these enzymes [1, 8]. Moreover, various extracellular enzymes of alkalophilic microorganisms have been studied.

To our knowledge, there are few amylases that are active at both acidic and basic pH. We report in this paper the purification and the characterization of an alkalophilic and acidophilic thermostable amylase from a newly isolated *Bacillus cohnii* US147 strain.

Materials and Methods

Isolation and Identification of the Strain

A strain having an amylase activity was isolated from the southern Tunisian soil. The cells of this strain were long, Gram positive, mobile, and could grow only in basic pH (8–9.5) at 45 °C. After incubation on the agar plates of a minimal medium [6] at 45 °C for 24 h and at pH 9, the plates were stained with iodine vapor. The colonies with the largest halos were isolated for further investigation. A bacterium designed US147 was retained as a potent amylase producer. The physiological tests were carried out using the API 50CHB system.

The amplification of the 16S ribosomal ribonucleic acid (rRNA) gene was performed using two primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATC CAAGCC-3′ designed from conserved regions within the rRNA operon of *Esherichia coli*. Chromosomal deoxyribonucleic acid (DNA) of the US147 strain was extracted and used as a template [9]. The thermal profile consisted of 40 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 3 min. The polymerase chain reaction (PCR) product was then cloned into a PCR-Blunt vector (Invitrogen) [10].

DNA Sequencing and Analysis

The nucleotide sequence of the 16S rRNA gene was determined using the dideoxy chain-termination method [11]. The homology search was performed using the BLAST algorithm. The nucleotide sequence of the 16S rRNA gene, reported in this article, was submitted to GenBank under the accession number AM287289.

Culture Conditions

The amylase production was performed using a minimal medium containing 1% of starch. Its composition was in (g/l): Yeast extract, 1; Na_2HPO_4 ($2H_2O$), 4; KH_2PO_4, 3; NH_4Cl, 1; peptone, 1; NaCl, 0.5; and 1 ml of an oligoelement solution containing $ZnCl_2$=40 mg, $FeSO_4$ ($7H_2O$)=200 mg, H_3BO_3=6.5 mg, and $MoNa_2O_4$ ($2H_2O$)=13.5 mg. The pH was adjusted at 9 with 10 N NaOH.

Enzyme Assays

The enzyme (0.2 ml) was added to 0.5 ml of 1% (*w*/*v*) soluble starch in 0.1 M glycine NaOH buffer pH 9 for 10 min at 70 °C. The produced reducing sugars were measured using the dinitrosalicylic acid method [12]. One activity unit was defined as the amount of enzyme required to liberate 1 μmol min^{-1} ml^{-1} of reducing sugars expressed as glucose equivalents, under the assay conditions.

Protein Determination

Protein concentration was determined by the Bradford method using a Bio-Rad protein assay, with bovine serum albumin as the standard [13].

Purification of the Amylase from Bacillus Strain US147

The *Bacillus* US147 strain was cultivated for 36 h in 1,000-ml Erlenmeyer flasks of minimal M9 medium (200 ml) containing 1% of starch at 45 °C, 250 rpm, and at pH 9. A total of 400 ml of bacterial culture was centrifuged at 10,000×*g* at 4 °C for 15 min to remove the cells. The supernatant was then freeze dried and dialyzed at 4 °C against Tris buffer pH 8 for 1 day with several changes of the buffer. The enzyme solution was then applied to a Q-Sepharose Cl 6B (Pharmacia LKB Biotechnology) column (17×2 cm) previously equilibrated with 20 mM Tris buffer pH (8.0) containing 1 mM phenyl methyl sulfonyl fluoride and 1 mM ethylenediamine tetracetic acid (EDTA). Elution was carried out at 36 ml h^{-1} with the same buffer and a linear NaCl gradient from 0 to 1 M. Fractions of 2 ml per tube were collected, and their amylase activity was determined as described below. The active fractions collected from the Q sepharose column were pooled, freeze dried, dissolved in 20 mM Tris–HCl buffer pH 8, and applied to a Biogel P100 column (2.2× 87 cm) using the same buffer for elution.

Electrophoresis and Molecular Mass Determination

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE; 10%) was carried out as described by Laemmli [14].

Activity Staining of Amylase in the Polyacrylamide Gel Native

The amylase activity band was detected by the activity stain technique (zymogram) on starch agar using an iodine solution as described by Coughlan [15].

Effects of pH and Temperature on the Amylase Activity and Stability

The determination of the optimal temperature of amylase US147 was carried out in the temperature range of 40–90 °C. The optimum pH of amylase US147 was determined by measuring the amylase activity at 70 °C at several pH values (4.0 to 10.0) with 0.1 M citrate phosphate buffer (pH 4 to 6), 0.1 M Na phosphate buffer (pH 7 to 8), and 0.1 M glycine–NaOH buffer (pH 9 to 10). The thermal stability of the enzyme at 70 °C was determined by incubating the purified enzyme at this temperature. After time intervals, the remaining activity was determined under the enzyme assay conditions.

The effect of pH 9 on amylase activity was determined by incubating the enzyme at this pH for 24, 48, and 72 h at 4 °C. Following incubation, the remaining activity was measured under the enzyme assay conditions.

Effect of Some Chemical Ions and Oxidizing Agents on the Amylase Activity

The effect of different oxidizing agents on the purified amylase activity was studied. The agents tested were: 1 mM SDS, sodium perborate ($NaBO_3$), and 1 mM H_2O_2. The influence of some chemical ions was determined by adding 1 mM of each ion (Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}) and 5 mM EDTA to the standard assay. The combination of the chelating agent (5 mM EDTA) and Ca^{2+}(2, 4, 6, 8, and 10 mM) was also evaluated by performing the assay under the same conditions. The activity of the enzyme alone in the glycine–NaOH buffer was taken to be 100%.

The effect of metal ions and chelating and oxidizing agents on the amylase was determined by preincubating the enzyme in the presence of the inhibitor for 15 min at 37 °C and then performing the assay in the presence of the same inhibitor concentration at an optimal temperature for 60 min [16, 17]

Kinetic Properties

K_m and V_{max} values were estimated from Lineweaver–Burk equation.

Results and Discussion

Identification of the Isolated Bacterial Strain

The bacterium US147 was isolated from alkaline soil samples collected in the Tunisian Sahara. The identification of this new isolated bacterium was based on both catabolic and molecular methods. Morphological and physiological characteristics showed that the isolated strain belonged to the *Bacillus* genus. It was a spore-forming and Gram-positive bacterium. It could metabolize the starch and the amylopectin better than other simple sugars. The optimum culture conditions were 45 °C and pH 9–10 with quick and abundant growth on the M9 medium containing 1% of starch as the carbon source.

A PCR using two primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGT GATCCAAGCC-3′ designed from conserved regions within the rRNA operon of *E. coli* was performed on the genomic DNA extracted from the isolated strain. A fragment of 1,500 bp was amplified. The sequence showed a similarity of 99% with the 16S rRNA genes of two alkalophilic strains: *B. cohnii YN-2000* (accession number AB023412) and *B. cohnii DSM 6307* T (accession number X76437). Based on the obtained data, the strain US147 seemed to be closely related to the *B. cohnii* species. Therefore, we proposed the alignment of this organism as the *B. cohnii* US147 strain.

Amylase Production by US147

As shown in Fig. 2, the amylase activity produced by *Bacillus* US147 increased with the cell growth and reached a maximum level at 36 h (2.1 U/ml). The maximum amount of extracellular amylase was produced at the end of the exponential growth phase, as was previously reported for several other *Bacillus* strains, for instance the *Bacillus* sp.GM80901

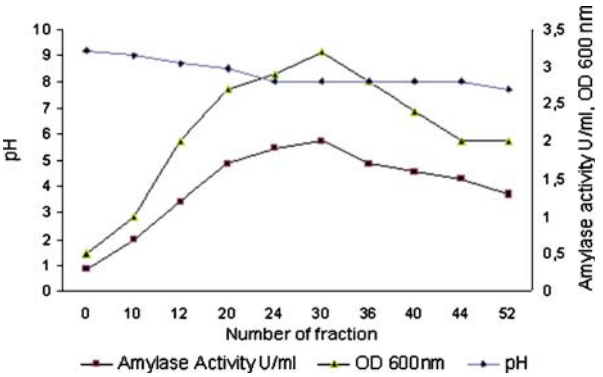

Fig. 1 Times courses of cell growth, amylase production, and pH change in the culture medium. *Bacillus* US147 strain was cultivated in 200 ml of medium in 1l Erlenmeyer flasks at 45 °C, 200 rpm and at pH 9

strain [5]. The pH of the cell-free supernatant always remained in the alkaline region and decreased after 43 h in the culture (Fig. 1).

Purification of the Amylase US147

Amylase US147 was purified by a combination of ion exchange and size exclusion chromatography. As shown in Fig. 2, the enzyme was eluted in the washing fractions suggesting that it had an isoelectric point (p*I*) more than 8 (pH of the buffer used for elution). The analysis of the active fractions eluted from Biogel P100 column by SDS-PAGE showed a single band with an apparent molecular mass of 30 kDa (Fig. 3). Compared to other described bacterial amylases, this enzyme was the smallest [18, 19]. The native polyacrylamide gel stained with an iodine solution showed a single alkaline amylase band, which coincided with that detected in SDS-PAGE (Figs. 4 and 5). Using these two steps of purification, the enzyme was purified five folds with a yield of 35% of the total activity. The results of these two chromatographic steps are summarized in Table 1.The purified enzyme had a specific activity of 86.8 U/mg using a solution of starch 1% as the substrate.

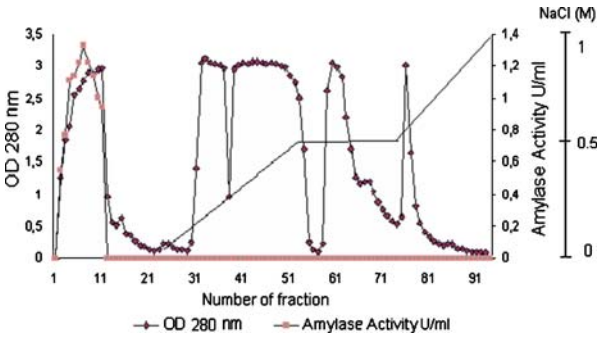

Fig. 2 Elution profile of amylase US 147 on Mono-Q sepharose Cl 6B chromatography (Pharmacia LKB Biotechnology) column (17×2 cm) previously equilibrated with 20 mM Tris buffer pH (8.0) containing 1 mM phenyl methyl sulfonyl fluoride and 1 mM ethylenediamine tetracetic acid. Elution was carried out at 36 ml h^{-1} with the same buffer and a linear NaCl gradient from 0 to 1 M. Fractions (2 ml per tube) were collected

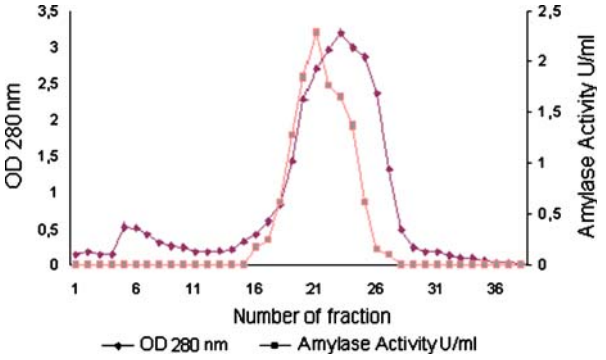

Fig. 3 Elution profile of amylase US 147 on Biogel P100 chromatography (Bio-Rad) column (2.2×87 cm) previously equilibrated with 20 mM Tris buffer pH (8.0) containing 1 mM phenyl methyl sulfonyl fluoride and 1 mM ethylenediamine tetracetic acid. Elution was carried out at 36 ml h^{-1} with the same buffer. Fractions (5 ml per tube) were collected

Properties of the Purified α-Amylase

The activity of the enzyme was assayed in buffers of various pH values, and the relative activities are shown in Fig. 6a. These results indicated that the US147 amylase was more active at pH 9. The same optimum pH was obtained with the amylase of *Baclilus* sp.TS-23 [20] (Table 2), but our results showed that the enzyme retained 85% of its activity in the pH range 4 to 10. The stability of the US147amylase was examined at 70 °C. As shown in Fig. 6b, this amylase booked approximately 98% and 85% of activity after incubation for 15 and 72 h, respectively. The studied amylase was more stable than the enzyme produced by *Bacillus* sp.ANT-6 at pH 9 and at 37 °C, which lost 42% of its activity after 15 h of incubation [18].

The enzyme activity of amylase US147 buffered at pH 9 was measured at various temperatures. As shown in Fig. 7a, the enzyme had an optimal temperature of 70 °C and reserved approximately 100% of activity after 1 h of incubation (Fig. 7b). A similar result at the same temperature was reported with *Bacillus* sp. PN5 [21]. After 2 h of incubation at 70 °C, this enzyme retained nearly 70% of its activity.

The amylase of US147 had K_m and V_{max} values of 0.7 mg/ml and 2.2 U/ml, respectively, with 1% starch as the substrate**.** As shown in Table 2, the amylase US147 exhibited a lower apparent K_m value than amylases from *Bacillus* sp. L1711 [22] and *Bacillus* sp. TS-*23* [20].

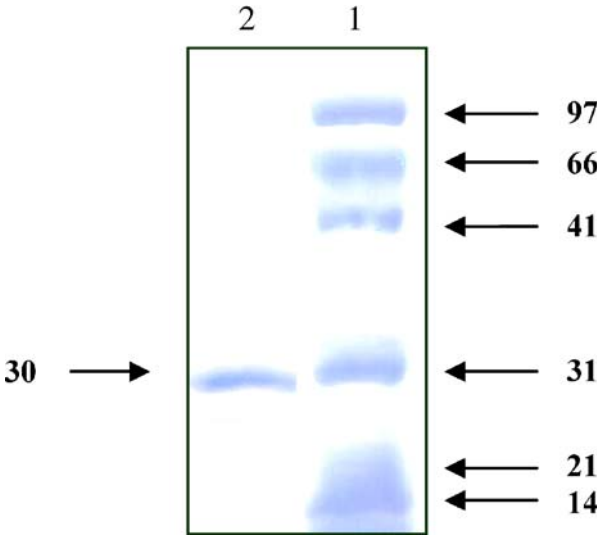

Fig. 4 SDS-PAGE analysis of the purified amylase on 10% polyacrylamide gel. *Lane 1*: marker protein. *Lane 2*: purified amylase from US147

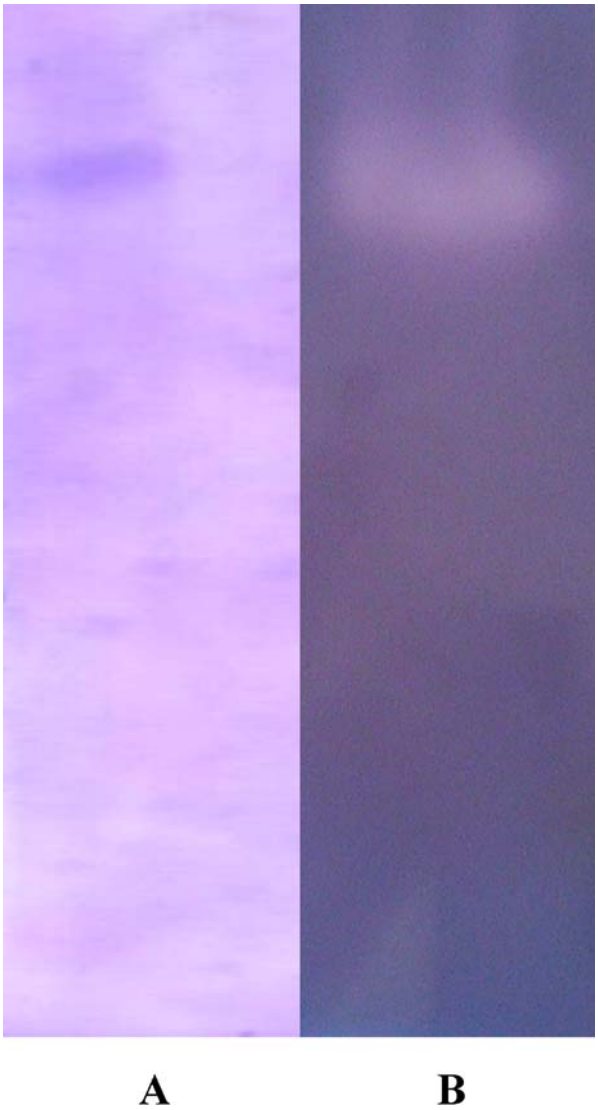

Fig. 5 Electrophoretic characterization of the purified amylase under nondenaturating conditions. **a** Stained gel by Coomassie brilliant blue G 250. **b** The amylase activity band was detected by zymogram on starch agar using iodine solution

Table 1 Purification of *Bacillus* US147 amylase.

Purification step	Total activity (UT)	Total protein (mg)	Specific activity (UI/mg)	Yields (%)	Purification (*X*-fold)
Culture supernatant	388	20	18.86	100	1
Mono Q	280	10	26.8	73	1.39
Biogel P100	136.6	1.56	86.6	35	5

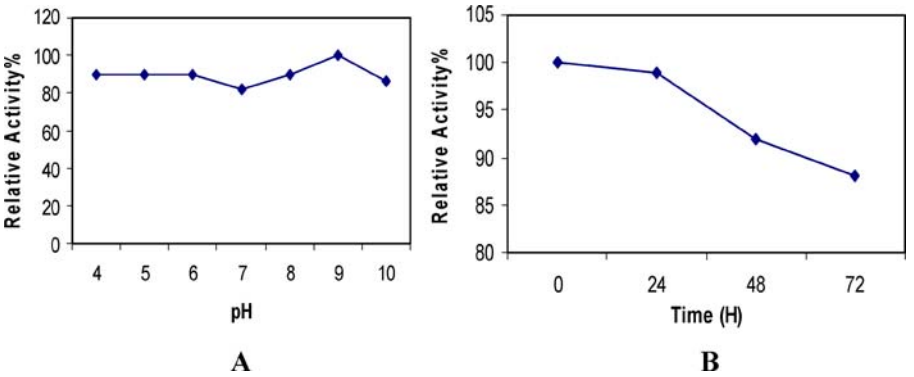

Fig. 6 Effect of pH on amylase US147 activity (**a**) and stability at pH 9 (**b**). The optimum pH of amylase US147 was determined by measuring the amylase activity at 70 °C at several pH values (4.0 to 10.0) with 0.1 M citrate phosphate buffer (pH 4 to 7), 0.1 M phosphate buffer (pH 8), and 0.1 M glycine–NaOH buffer (pH 9 to 10).The effect of pH 9 on amylase activity was determined by incubating the enzyme at this pH for 24, 48, and 72 h at 4 °C. The remaining activity was then achieved under the enzyme assay conditions

Effect of Some Chemical Ions and Oxidizing Agents on the Amylase Activity

The majority of α-amylases were inhibited by metal ions, and alkaline amylases varied in their response to the chelator EDTA [18, 24]. The stability of the amylase US147 toward some ions and detergent compounds was also studied. In fact, the activity of US147 amylase buffered with 0.1 M glycine NaOH pH 9 was assayed in the presence of various metal ions and chemical reagents. As shown in Table 3, amylase US147 was activated in the presence of 1 mM Mg^{2+} and Cu^{2+}. In fact, the relative activity increased to 120% and 110%, respectively. The thermostable amylase of *Bacillus* US147 reached an activity of 130% with 1 mM $CaCl_2$. Similar results were obtained with the thermostable amylase of *Bacillus* sp. TS-23 [20].

Strong inhibitory effects of EDTA were reported for alkaliphilic amylases from *Bacillus* isolates TS-23, GM8901, and KSM-1378 [21, 5, 24]. However, amylases from others *Bacillus* isolates including ANT-6, KSM-K38, and L1711 were found to be indifferent to EDTA [20, 22, 25]. However, no significant inhibition was observed (22% of inhibition) for the amylase US147 with 5 mM EDTA. The enzyme recovered its original activity following the addition of 10 mM Ca^{2+} after incubation with 5 mM EDTA (Table 3).

Table 2 Some Characteristics of alkaline amylases produced by *Bacillus* species.

Sources	T_{op} (°C)	pH_{op}	K_m (mg/ml)	References
Bacillus US147	70	4–10 (max at 9)	0.7	this paper
Bacillus sp. L1711	35–45	9.5–10; 7–7.5	1.9	[22]
Bacillus sp. KSM1378	55	8–8, 5		[23]
Bacillus sp. GM8901	60	10–12		[5]
Bacillus sp. KSM-K38	55–60	8–9.5		[25]
Bacillus sp. TS-23	70	9	2.7	[20]
Bacillus sp. ANT-6	80	10.5		[18]
Bacillus sp. 17-1		4.5; 10		[26]
Bacillus sp. PN5	90	55–6		[21]

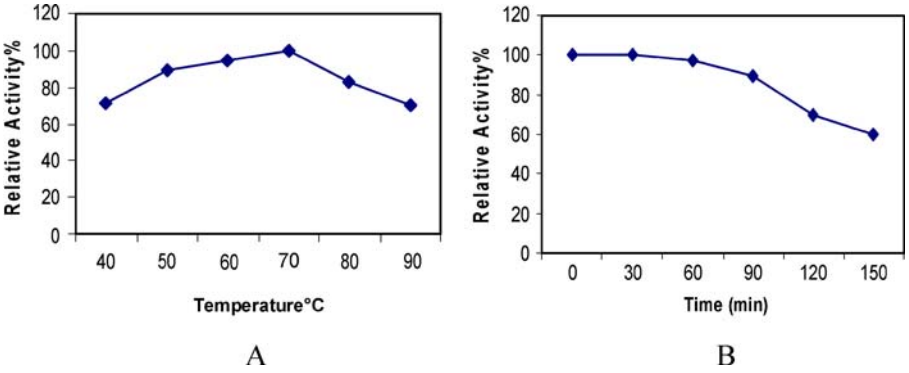

Fig. 7 Effect of temperature on amylase US147 activity (**a**) and stability of the enzyme at 70 °C (**b**). Enzyme activity was assayed in the temperature range of 40–90 °C. The thermal stability of the enzyme at 70 °C was determined by incubating the purified enzyme at this temperature. After the time intervals, the remaining activity was determined under the enzyme assay conditions

The inhibition with Zn^{2+} was found to be an important parameter determining the thermostability of the amylase [1]. In fact, it had a potent inhibitory effect on the amylases from *Schwanniomyces alluvius* and *Bacillus cereus* NY 14, whereas it could have no effect at all on the enzyme of *Aspergillus kowachii* [21]. In comparison to thermostable α-amylases from the thermophilic *Bacillus* sp. TS-23 [20] and *Baclilus* spANT-6 [18], which showed, respectively, 46% and 37% of inhibition, the amylase of US 147 had a more favorable rate (20%). Moreover, the inhibition of amylase US 147 in the presence of the Zn^{+2} (1 mM) ion indicated that this enzyme was highly thermostable.

The addition of 1 mM SDS, 1 mM $NaBO_3$, 1 mM H_2O_2, and 1 mM Zn^{+2} did not significantly affect the enzyme activity. The relative activities were, respectively, 85%, 75%, and 77%. The enzyme buffered at pH 9 was assayed in the presence of organic solvents (30% concentration) such as methanol and acetonitrile for 24 h. Under these conditions, the enzyme retained about 60% of its initial activity**.** This suggests that this

Table 3 Effect of metal ions on the activity of the purified amylase US147.

Treatment	Relative activity (%)	Magnitude of the errors (%)
None	100	
Metal ions (1 mM)		
$CaCl_2$	130	1
$MgCl_2$	120	3
$CuCl_2$	110	2
$FeCl_2$	90	0.5
$ZnCl_2$	80	2
$CoCl_2$	60	1
EDTA (5 mM)	78	3
EDTA(5 mM)+		
$CaCl_2$(2 mM)	82	1
$CaCl_2$(4 mM)	83	2
$CaCl_2$(6 mM)	84	4
$CaCl_2$(8 mM)	86	3
$CaCl_2$(10 mM)	100	1

enzyme may be used as an additive in detergents containing organic solvents employed for washing colored stains from crocking and cutlery.

Conclusion

In this paper, we described a new isolated *Bacillus*US147 strain producing thermostable amylase. According to the metabolic fingerprint (API 50 CHB) of this strain and its 16S rRNA gene nucleotide sequence, the strain is likely to be a new *B. cohnii* strain.

Amylase US147 showed a high thermostability and a large range of pH activity. The optimum temperature and pH of this amylase were 70 °C and 9, respectively. This enzyme retained 80% of its activity in the pH range from 4 to 10, so it could be used either for the production of glucose syrup from starch or in foodshiffs and as an additive in the laundry detergents. The resistance of the amylase US147 to ions ($CaCl_2$, $ZnCl_2$, and SDS) was similar to other amylases produced by *Bacillus* sp. Ant 6 and *Bacillus* sp. PN5. These properties confirmed the usefulness of this amylase in detergents.

References

1. Mamo, G., & Gessesse, A. (1999). *Enzyme and Microbial Technology*, *25*, 433–438.
2. Pandey, A., Nigam, P., Socrol, C. R., Socrol, V. T., Singh, D., & Mohan, R. (2000). *Biotechnology and Applied Biochemistry*, *31*, 135–152.
3. Oudjeriouat, N., Moreau, Y., Santimone, M., Svensson, B., Marchis-Mouren, G., & Desseaux, V. (2003). *European Journal of Biochemistry*, *270*, 3871–3879.
4. Peixoto, S. C., Jorge, J. A., Terenzi, H. F., Delourdes, M., & Polizeli, T. M. (2003). *International Microbiology*, *6*, 269–273.
5. Kim, T. U., Gu, B. G., Joeng, J. Y., Byum, S. M., & Shin, Y. C. (1995). *Applied and Environmental Microbiology*, *61*, 3105–3112.
6. Mc. Tigue, M. A., Kelly, C. T., Fogarty, W. M., & Doyle, E. M. (1994). *Biotechnology Letters*, *16*, 569–574.
7. Kobayashi, T., Kamni, H., Hayashi, T., Akiba, T., Akaboshi, R., & Horikoshi, K. (1992). *Journal of Bacteriology*, *174*, 3439–3444.
8. Ben Ali, M., Mezghani, M., & Bejar, S. (1999). *Enzyme and Microbial Technology*, *21*, 570–573.
9. Gurtler, V., & Stanisich, V. A. (1996). *Microbiology*, *142* (1), 3-16.
10. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *A laboratory manual*. New York: Cold Spring Harbor Laboratory.
11. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). *Proceedings of the National Academy of Sciences of the United States of America*, *74*, 5463–5467.
12. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–428.
13. Bradford, M. M. (1976). *Analytical Chemistry*, *72*, 248–254.
14. Laemmli, U. K., & Favre, M. (1973). *Journal of Molecular Biology*, *80*, 575–590.
15. Coughlan, M. P. (1988). *Methods in Enzymology*, *160*, 135–144.
16. Egas, M. C. V., da Costa, M. S., Cowan, D. A., & Pires, E. M. V. (1998). *Extremophiles*, *2*, 23–32.
17. Lo, H. F., Lin, L. L., Chen, H. L., Hsu, H. H., & Chang, C. T. (2001). *Process Biochemistry*, *36*, 743–750.
18. Burhan, A., Nisa, U., Gökhan, C., Ömer, C., Ashabil, A., & Osman, G. (2003). *Process Biochemistry*, *38*, 1397–1403.
19. Sidhu, G. S., Sharma, P., Chakrabarti, T., & Gupta, J. K. (1997). *Enzyme and Microbial Technology*, *21*, 525–530.
20. Lin, L.-L., Chyau, C. C., & Hsu, W.-H. (1998). *Biotechnology and Applied Biochemistry*, *28*, 61–68.
21. Saxena, R. K., Dutt, K., Agarwal, L., & Nayyar, P. (2007). *Bioresource Technology*, *98*, 260–265.
22. Bernhardsdotter, E. C. M., Owen, J. D. N., & Pusey, M. L. (2005). *Process Biochemistry*, *40*, 2401–2408.

23. Igarasshi, K., Hatada, Y., Hagibara, H., Saeki, K., Takaiwa, M., Uemura, T., et al. (1998). *Applied and Environmental Microbiology*, *64*, 3282–3289.
24. Mc. Tigue, M. A., Kelly, C. T., Fogarty, W. M., & Doyle, E. M. (1995). *Enzyme and Microbial Technology*, *17*, 570–573.
25. Hagibara, H., Igarachi, K., Hayashi, Y., Endo, K., Ikawa, K., Itayama, K., et al. (2001). *Applied and Environmental Microbiology*, *67*, 1744–1750.
26. Horokoshi, K. (1996). *FEMS Microbiology Reviews*, *18*, 259–270.