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ORIGINAL PAPER

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Characteristics of the amylase of *Arthrobacter psychrolactophilus*

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Abstract Properties of the extracellular amylase produced by the psychrotrophic bacterium, *Arthrobacter psychrolactophilus*, were determined for crude preparations and purified enzyme. The hydrolysis of soluble starch by concentrated crude preparations was found to be a non-linear function of time at 30 and 40 °C. Concentrates of supernatant fractions incubated without substrate exhibited poor stability at 30, 40, or 50 °C, with 87% inactivation after 21 h at 30 °C, 45% inactivation after 40 min at 40 °C and 90% inactivation after 10 min at 50 °C. Proteases known to be present in crude preparations had a temperature optimum of 50 °C, but accounted for a small fraction of thermal instability. Inactivation at 30, 40, or 50 °C was not slowed by adding 20 mg/ml bovine serum albumin or protease inhibitor cocktail to the preparations or the assays to protect against proteases. Purified amylase preparations were almost as thermally sensitive in the absence of substrate as crude preparations. The temperature optimum of the amylase in short incubations with Sigma Infinity Amylase Reagent was about 50 °C, and the amylase required Ca^{+2} for activity. The optimal pH for activity was 5.0–9.0 on soluble starch (30 °C), and the amylase exhibited a K_m with 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene of 120 μM at 22 °C. The amylase in crude concentrates initially hydrolyzed raw starch at 30 °C at about the same rate as an equal number of units of barley α-amylase, but lost most of its activity after only a few hours.

Keywords Psychrophile · Psychrotroph · Amylase · Starch · Amylolytic · *Arthrobacter psychrolactophilus*

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Introduction

Amylases produced by plants, animals, bacteria, and fungi find application in commercial processes where starch is to be hydrolyzed to sugars for subsequent fermentation to alcohol, production of high-fructose corn sweeteners and syrups, or for the synthesis of other products [19, 20, 28]. Most processes require that the starch be cooked prior to hydrolysis to make it more easily hydrolyzed by the amylases. Cooking and subsequent cooling of the starch prior to saccharification or fermentation add significantly to the cost, and psychrophilic amylases that efficiently hydrolyze uncooked starch granules at ambient to low temperatures are needed [26]. Bacteria that live in cold environments are being surveyed to recover potentially useful psychrophilic enzymes. However, the amylases from few such bacteria have been surveyed and there is some question as to what exactly constitutes a psychrophilic enzyme [12].

In a previous article, the production of an extracellular α-amylase by the psychrotrophic species [17], *Arthrobacter psychrolactophilus* [16, 27] was described and a few properties of its amylase were reported [24]. *A. psychrolactophilus* produced an intracellular psychrophilic β-galactosidase and a variety of extracellular enzymes whose properties have not been described in detail [15]. In this article, a more complete description of the amylase is presented: the amylase is thermally labile, a property normally associated with psychrophilic enzymes. In spite of thermal instability, the amylase exhibited an optimum temperature for activity of 50 °C on a synthetic substrate, a property associated with mesophilic enzymes rather than with psychrophilic enzymes. It also had an M_r of 105,000. The relatively high temperature optimum was likely induced by the synthetic substrate and might not accurately reflect catalytic efficiency at low temperatures.

Materials and methods

Bacterial strains and culture media

Arthrobacter psychrolactophilus ATCC 700733 was purchased from the American Type Culture Collection, Manassass, VA, USA. Cultures were maintained by bi-weekly transfer on slants of Tryptic Soy Agar (Difco Laboratories, Detroit, MI, USA).

Chemicals

3,5-Dinitrosalicylic acid (DNS), raw corn starch, barley amylase, and Sigma Infinity Amylase reagent were obtained from Sigma Chemical Company, St. Louis, MO, USA. Thermo Infinity Amylase Assay reagent (formerly Sigma Infinity Amylase Assay reagent) was purchased from Thermo DMA, Louiseville, CO, USA because Sigma Chemical Company no longer supplies the amylase reagent. Azocasein used in assays for protease was acquired from Nutritional Biochemicals, Cleveland, OH, USA.

Assays

Assays for amylase activity were performed using soluble starch or the Thermo (or Sigma) Infinity Amylase reagent, which contained 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene as the substrate. Assays using the Thermo Infinity Amylase reagent were carried out according to the instructions supplied with the Sigma Infinity Amylase reagent, except that sample volumes ranged from 20 to 100 µl, and incubations were carried out 22 °C for 4–10 min. Absorbances were automatically read at 405 nm and intervals of 6 s, and graphed using a computerized Cary 100 spectrophotometer and enzyme kinetics software. Activity (U/ml) was calculated from the change in absorbance over the linear portion of the absorbance curve after 2 min of incubation using an extinction coefficient of 10.13 absorbance U/mM for a 1-cm light path. One unit of activity is defined as the quantity of amylase producing 1.0 µmol/min of *p*-nitrophenol.

Assays using soluble starch as substrate were carried out in a total reaction volume of 1.5 ml by adding 300 µl of the supernatant fraction of a culture containing amylase to 1.2 ml assay buffer (50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, 1.0% wt/vol soluble starch, and 0.02% NaN_3). Samples of concentrated supernatant fractions were assayed by adding 100 µl of the sample to 1.4 ml of assay buffer. Incubation was carried out at the indicated temperatures and reactions were stopped at intervals by pipetting 300 µl of reaction mixture into 300 µl DNS color reagent [25], which contained 0.1 N NaOH. The reaction mixtures plus DNS reagent were then placed in a boiling water bath for 15 min for color development, cooled in a cold water bath, and diluted by adding 3.0 ml of water. After dilution, absorbances were read in a spectrophotometer at 540 nm and the hydrolysis products of starch were calculated as glucose from a glucose standard curve. Readings were blanked with samples containing enzyme preparations which had been boiled for 10 min to inactivate amylase or with samples taken at time zero.

Protease was assayed using azocasein as the substrate [13, 23]. Enzyme preparations (60 µl) were added to 240 µl of a 1% (w/v) solution of azocasein in 50 mM MES, pH 6.5, 3 mM $CaCl_2$, and 0.02% NaN_3 and incubated at the indicated temperature (usually 30 °C) for the indicated period of time. Reagent blanks containing 60 µl of water instead of enzyme preparations were included as controls. The reactions were stopped by adding 450 µl of cold 10% (w/v) trichloroacetic acid. The reaction mixtures were then centrifuged for 15 min at 4 °C and 16,000 *g* in an Eppendorf 5415R refrigerated microcentrifuge. Supernatant fractions (700 µl) were collected, and 600 µl of 1 N NaOH was added for color development. Absorbances were read at 440 nm. One unit of activity is here defined as the quantity of enzyme producing a change of 1.0 absorbance unit [23] in 24 h under the conditions of the assay.

Protein was assayed by the dye-binding method of Bradford [3] using BioRad protein reagent (BioRad Inc., Hercules, CA, USA).

Preparation of amylase

Crude amylase was prepared from 100-ml or 1-l cultures grown at 22 °C in Tryptic Soy Broth without dextrose (TSBWD) which had been supplemented with 0.5–1.0% (w/v) maltose or soluble starch and buffered at pH 7.0 with 50 mM (final concentration) HEPES. When amylase was judged to have reached peak levels (72–96 h), cultures were centrifuged at 13,000× *g* and 4 °C for 20 min and the supernatant fractions were recovered. Cell pellets were discarded. Sodium azide (0.02% w/v) was added to inhibit growth. Supernatant fractions of 100-ml cultures were used without additional treatment. The supernatant fractions of 1-l cultures were concentrated 50- to 100-fold by ultrafiltration (10,000 or 30,000 mw cut-off) before use using a Pellicon XL tangiential flow ultrafilter and Ultracell 10 PLCGC membrane (Millipore Corporation, Bedford, MA, USA).

For some experiments, amylase was purified on a small scale to a high degree of purity by affinity chromatography using uncooked corn starch as the affinity substrate [24]. Uncooked corn starch granules (100 mg) was added to 1.0 ml of concentrated supernatant fractions of cultures and mixed by rotation on an Enviro-Genie mixer (Scientific Industries, Inc., Bohemia, NY, USA) at 4 °C for 15 min to allow the amylase to bind to the corn starch. The suspension was then filtered via syringe through a 10 µm Mobicol

M1002 filter (Mo Bi Tec, LLC, Marco Island, FL, USA). The starch granules on the filter were then washed three times with 3.0 ml each of buffer (50 mM MES, pH 6.5, 3 mM $CaCl_2$). The amylase was eluted from the starch granules with 1.0 ml of a solution containing 50 mM Bis-Tris, pH 6.5, 2 mM $CaCl_2$, 0.02% NaN_3, and 1% (w/v) soluble starch.

Partial purification of the amylase in concentrated supernatant fractions was achieved on a larger scale by anion exchange chromatography using a 4.6- by 100-mm column containing TOSOH TSK-Gel DEAE-5PW anion exchange resin (20 µm bead size, Tosohaas, Montogomeryville, PA, USA) and a Biocad Sprint HPLC system (Perceptive, Framingham, MA, USA). The column was equilibrated with 25 mM MES, pH 6.0, then loaded with 1.0 ml of concentrated supernatant fraction. Amylase was eluted from the column using a linear gradient of NaCl (0–600 mM) in 25 mM MES, pH 6.0 at a flow rate of 2.0 ml/min. Fractions of 1.0 ml were collected in a fraction collector. The fractions were assayed for amylase activity using Infinity amylase reagent. Fractions containing activity (38–40) were pooled from each run. The pooled fractions from multiple runs were subsequently pooled and concentrated using Nanosep centrifugal ultrafilters (10,000 mw cut-off, Pall Filtron, Inc., Northborough, MA, USA).

Results

Calcium requirement

Most bacterial amylases require calcium for activity and the amylase from *A. psychrolactophilus* was not exceptional in that regard (Table 1). Activity was strongly inhibited by the addition of EDTA to 10 mM final concentration to the incubations. No inhibition was observed if incubation mixtures contained 10 mM EDTA plus 30 mM $CaCl_2$. The addition of 30 mM $CaCl_2$ alone had no effect on activity.

Table 1 Calcium requirement

	Activity	
	U/ml	%
Concentrate (3 mM $CaCl_2$)	2.09	100
Concentrate + 30 mM $CaCl_2$	1.96	94
Concentrate + 10 mM EDTA	1.21	57
Concentrate + 30 mM $CaCl_2$ + 10 mM EDTA	1.72	82

A concentrated supernatant fraction (300 µl) from a culture of *A. psychrolactophilus* was incubated with 1.2 ml of a solution containing 50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, and 1% (w/v) soluble starch and the indicated additions at 30 °C for 30 min. The reaction was stopped by pipetting 300 µl of reaction mixture into 300 µl of DNS color reagent, as described in the Materials and Methods section. The concentrate contained 1.3 mg of protein per ml

Optimum pH

Activity on soluble starch was optimal over a wide range (5–9) of pH (Fig. 1). An unconcentrated supernatant fraction (300 µl) was incubated for 3 h at 40 °C in 50 mM buffer at the indicated pH, 3 mM $CaCl_2$, and 1% (w/v) soluble starch then assayed by the DNS method for reducing sugar as described in the Materials and Methods section. The buffers used were sodium acetate (pH 4.0 and 5.0), MES (pH 5.0, 6.0 and 7.0), HEPES (pH 7.0 and 8.0), and TAPS (pH 8.0 and 9.0).

K_m

The K_m at 22 °C for amylolytic activity was estimated as 120 µM using the Infinity amylase reagent and a concentrated supernatant fraction (1.3 mg protein/ml) of a culture of *A. psychrolactophilus* (Fig. 2). The Sigma Infinity amylase reagent was diluted into a solution containing 50 mM HEPES, pH 7.0, 3 mM $CaCl_2$, and 26 U/ml of yeast α-glucosidase. Reactions were started by adding 20 µl of concentrated supernatant fraction to 1.0 ml of Infinity amylase reagent. After mixing, the reaction mixture was placed into a Cary 100 spectrophotometer at 22 °C and the changes in absorbance at 405 nm were recorded at 0.1 min intervals using the enzyme kinetics software provided. The rate of the reaction was estimated from the linear portion of the curve after 2 min of incubation.

Hydrolysis of raw starch

The amylase of *A. psychrolactophilus* was previously reported to bind to uncooked corn starch granules at 0 °C [24], but its ability to hydrolyze raw starch was not reported. A concentrated supernatant fraction (0.5 ml, 1.3 mg protein/ml, 1.7 U/ml assayed on soluble starch) from a culture of *A. psychrolactophilus* grown on 1% (w/v) maltose was added to 3.0 ml of 50 mM MES, pH 6.5, 3 mM $CaCl_2$, 0.02% (w/v) NaN_3, 0.5% (w/v) uncooked corn starch granules and incubated at 30 °C. For comparison, 0.5 ml of barley α-amylase at a concentration of 1.7 U/ml (assayed on soluble starch) was incubated at 30 °C with 3.0 ml of 0.5% (w/v) uncooked corn starch in 50 mM sodium succinate, pH 5.5, 3 mM $CaCl_2$, 0.02% (w/v) sodium azide, 0.5% (w/v) uncooked corn starch granules. The results (Fig. 3) showed that *A. psychrolactophilus* amylase initially hydrolyzed uncooked corn starch at about the same rate as an equal number of units of barley α-amylase, but unlike barley amylase, the amylase of *A. psychrolactophilus* lost most of its activity within a few hours after the start of the incubation. The initial rates of hydrolysis for both amylases were about one-thousandth the rates for the hydrolysis of soluble starch. The rate of hydrolysis of uncooked starch was not a linear function of time for either of the two enzymes. The concentrates contained high levels of proteolytic activity

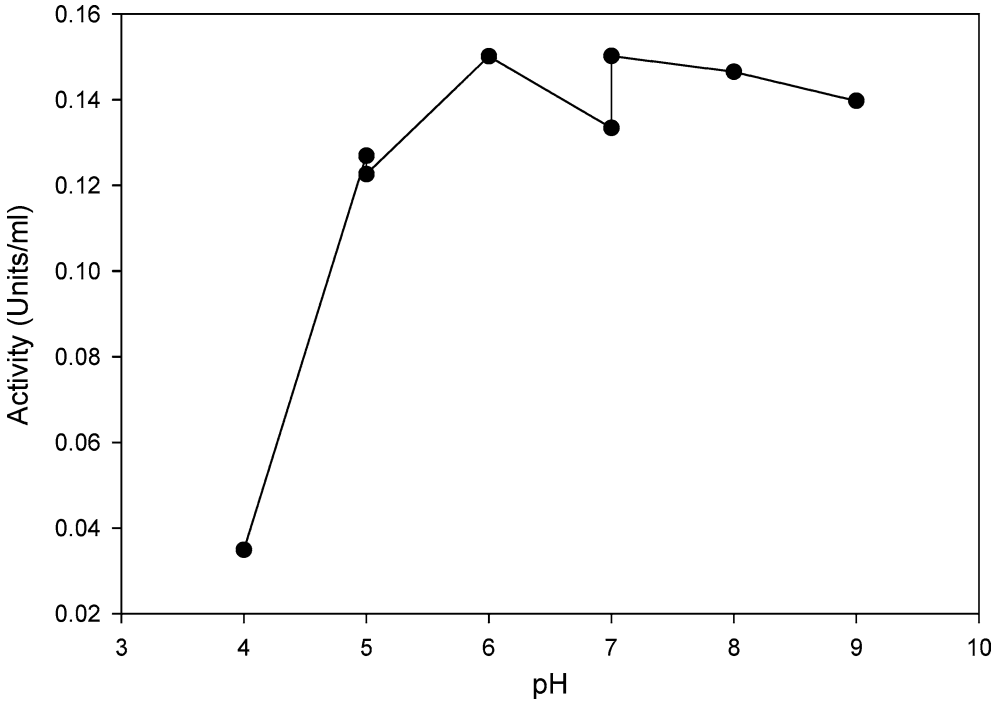

Fig. 1 Effect of pH on amylolytic activity with soluble starch as substrate. Assays were conducted by incubating unconcentrated supernatant fraction (300 µl) from a culture with 1.2 ml of buffer solution at the indicated pH for 3 h at 40 °C, the reactions were stopped with DNS reagent, and color was developed as described in the Materials and Methods section. The buffer solutions contained 50 mM buffer, 3 mM $CaCl_2$, 0.02% (w/v) NaN_3, and 1% (w/v) soluble starch. Buffers used were sodium acetate (pH 4.0–5.0), MES (pH 5.0–7.0), HEPES (pH 7.0–8.0), and TAPS (pH 8.0–9.0)

when assayed with 1% (w/v) azocasein, and it seemed probable that much or all of the activity lost during the incubation could be attributed to proteolytic attack on the amylases by the proteases which were present.

Linearity of assays on soluble starch

Figure 4 shows that assays on soluble starch were also nonlinear with respect to time. In these assays, a concentrated supernatant fraction of a culture (250 µl, 690 µg of protein) grown in 0.5% maltose was diluted with 1,750 µl of buffer (50 mM MES, pH 6.5, 3 mM $CaCl_2$). The diluted supernatant fraction (400 µl) was added to 1.6 ml of 50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, 1% (w/v) soluble starch and the reaction mixtures were incubated in triplicate at 30 and 40 °C. The reaction mixtures were sampled at intervals by removing aliquots of 300 µl and pipetting the aliquots into 300 µl of DNS reagent as described in the Materials and

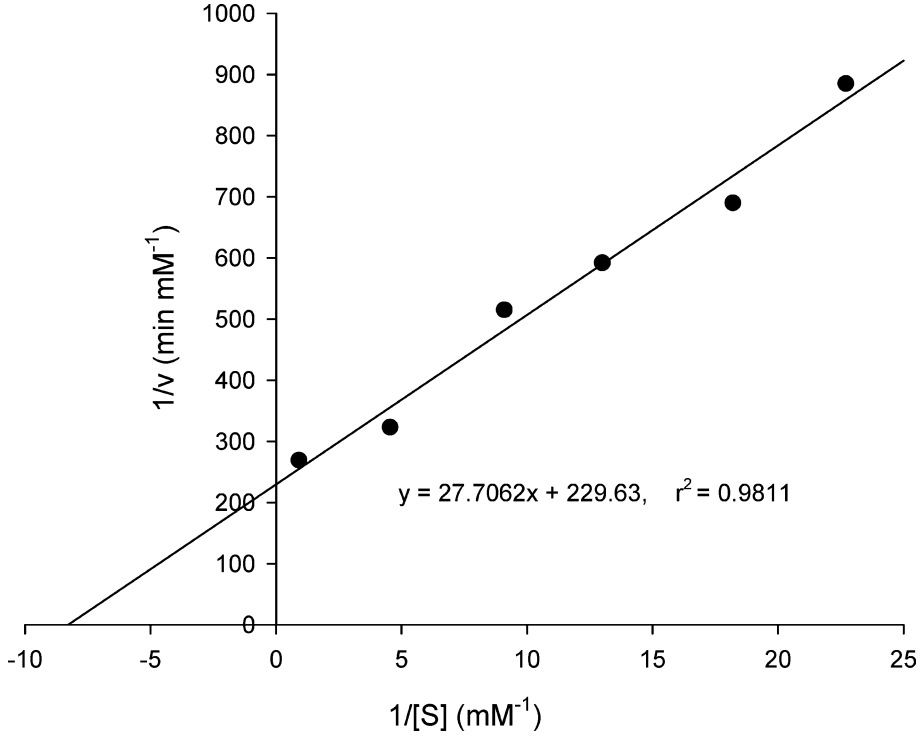

Fig. 2 Double-reciprocal plot of substrate concentration versus velocity of hydrolysis of 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene. Concentrated supernatant fraction (20 µl, 1.2 mg protein/ml) was incubated at 22 °C with 1.0 ml of Sigma Infinity amylase reagent (1.1 mM substrate) or Sigma Infinity amylase reagent which had been diluted with 50 mM HEPES, pH 7.0, 3 mM $CaCl_2$, 0.02% NaN_3, and 26 U/ml yeast α-glucosidase to contain 44, 55, 77, 110, and 220 µM substrates. Reaction rates were obtained with a computerized spectrophotometer and enzyme kinetics software

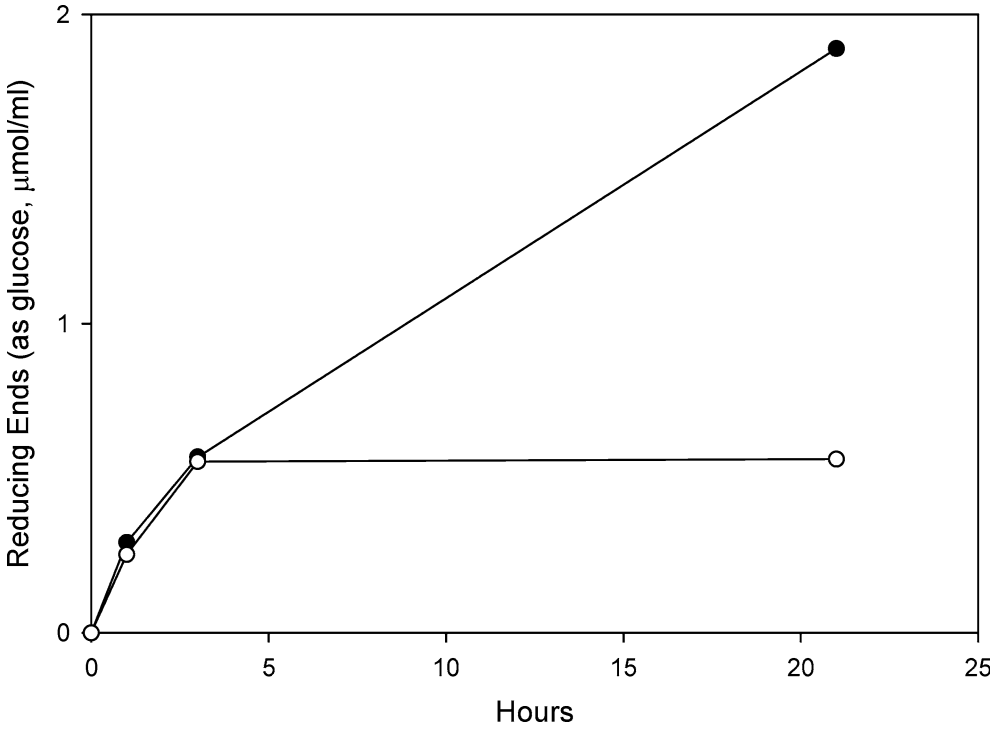

Fig. 3 Time-course for the hydrolysis of uncooked corn starch by barley α-amylase and crude concentrate of a supernatant fraction of a culture of *Arthrobacter psychrolactophilus* at 30 °C. A concentrated supernatant fraction from a culture of *A. psychrolactophilus* (0.5 ml) containing 1.7 U/ml was added to 3.0 ml of reaction solution and incubated at 30 °C for 21 h. The reaction solution consisted of 50 mM MES, pH 6.5, 3 mM $CaCl_2$, 0.025 (w/v) NaN_3, and 0.5% (w/v) of uncooked corn starch granules. Barley α-amylase was freshly prepared by dissolving 17 mg of barley amylase (Sigma Chemical Company) in 10 ml of buffer solution containing 50 mM sodium succinate, pH 5.5, 3 mM $CaCl_2$, 0.02% (w/v) NaN_3. β-amylase was inactivated by heating at 70 °C for 10 min. The resulting barley α-amylase solution assayed at 2.1 U/ml on soluble starch. Barley α-amylase solution (500 µl) was incubated with 3.0 ml of 0.5% (w/v) uncooked corn starch granules in 50 mM sodium succinate, pH 5.5, 3 mM $CaCl_2$, 0.02% (w/v) NaN_3 at 30 °C for 21 h. Reaction mixtures were mixed by rotation on an Enviro-Genie mixer. Starch hydrolysis was monitored by DNS assay, as described in the Materials and Methods section. Barley α-amylase (●), *A. psychrolactophilus* supernatant fraction (○)

Methods section to stop the reactions and begin color development for the measurement of reducing sugar. At 30 °C the hydrolysis of soluble starch continued for the entire 3 h of incubation and the rate was approximately linear for the first 30–60 min. Mean activities computed for 2 and 3 h of incubation were 74 and 91% of initial activity based on the first 15 min of incubation.

Deviation from linearity was greater at 40 °C than at 30 °C. At 40 °C the rate of hydrolysis of soluble starch was approximately linear for the first 30 min, decreased after 1 h of incubation, and hydrolysis essentially ceased after 2 h. Mean activities computed for 1, 2, and 3 h of incubation were respectively 92, 77, and 59% of activity computed for 30 min of incubation.

These temperature-dependent deviations from linearity indicated that the amylase was not stable in crude preparations over extended periods of time. The instability could have been the result of contaminating proteases or an inherent instability in the amylase itself.

Optimum temperature

The deviations from linearity observed in assays on soluble starch suggested that former measurements of the optimum temperature for activity [24] might contain error because they were based on mean activities measured over an interval of 3 h instead of initial activities, and it assumed that the hydrolysis of starch was a linear function of time. Temperature optimum for activity was therefore reestimated using the Sigma Infinity amylase reagent. The Sigma assay relied on short (<10 min) incubations and was therefore less sensitive to slow inactivation caused by either proteases or thermal effects. The results (Fig. 5) showed a somewhat higher temperature maximum (ca. 50 °C) than the results we reported for soluble starch [24]. The amylase retained 40% of its activity at 20 °C compared to its activity at 48 °C, suggesting a low energy of activation. Arrhenius plots (not shown) also suggest a low energy of activation (30–40 kJ/mol) over the range temperatures.

Proteases

Proteolytic activity was high in concentrated crude amylase preparations. The aggregate optimum temperature for proteolytic activity on 1% azocasein (Fig. 6) was approximately 50 °C, a temperature where proteases would be most likely to interfere with measurements

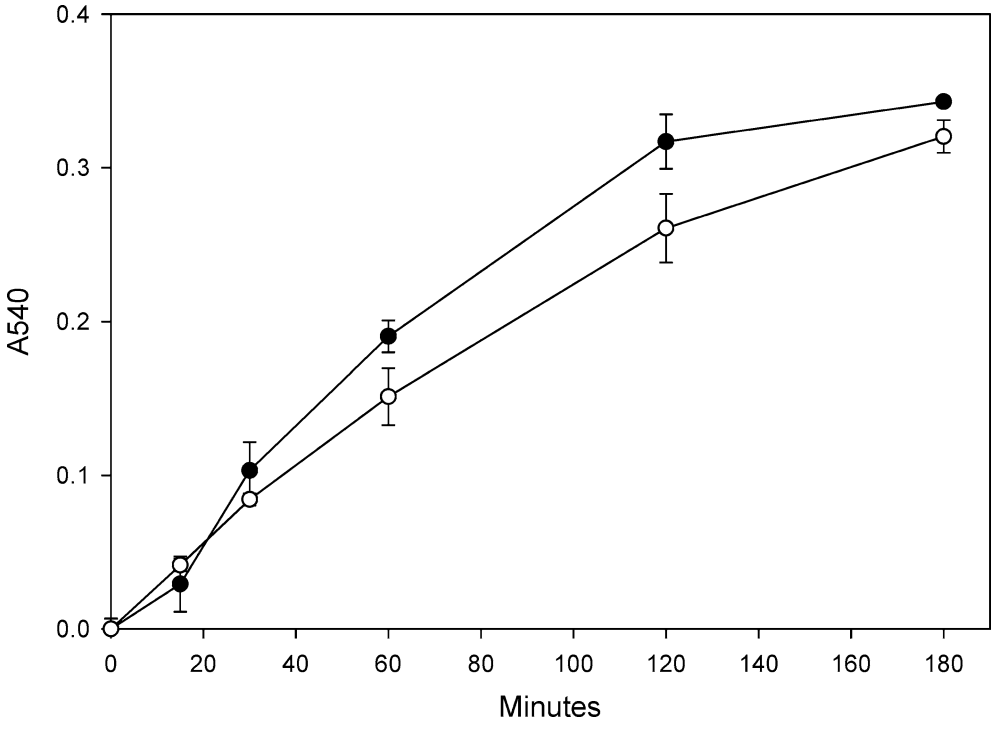

Fig. 4 Time-course for the hydrolysis of soluble starch. Concentrated supernatant fraction from a culture of *A. psychrolactophilus* (250 µl, 690 µg protein/ml) was diluted by adding 1,750 µl of 50 mM MES, pH 6.5, 3 mM $CaCl_2$, 0.02% NaN_3. Assays were performed in triplicate.The diluted enzyme (400 µl) was added to 1.6 ml of 50 mM Bis-Tris buffer, pH 6.5, 3 mM $CaCl_2$, 1% (w/v) soluble starch, and 0.02% NaN_3 and incubated at the indicated temperature. Reaction mixtures were sampled at intervals by removing 300 µl and adding it to 300 µl of DNS reagent. After boiling for 15 min to develop color, the samples were diluted with 3.0 ml of H_2O and read in a spectrophotometer at a wavelength of 540 nm. 30 °C (○), 40 °C (●)

of maximum temperature and stability of amylase activity. Zymograms (see Fig. 7) showed that proteolytic activity was distributed among multiple bands and probably multiple proteases. Since it was possible that temperature-dependent effects could reflect both interference by protease and inherent instability of the amylase, it was necessary to rule out the contribution made by proteases.

Thermal stability of crude and purified amylase preparations

The thermal stability of the amylase in crude, concentrated supernatant fractions was estimated by preincubating aliquots of 300 µl of supernatant fraction without added substrate at 30, 40, and 50 °C for intervals from 0 to 40 min. The fractions were then assayed on soluble starch.

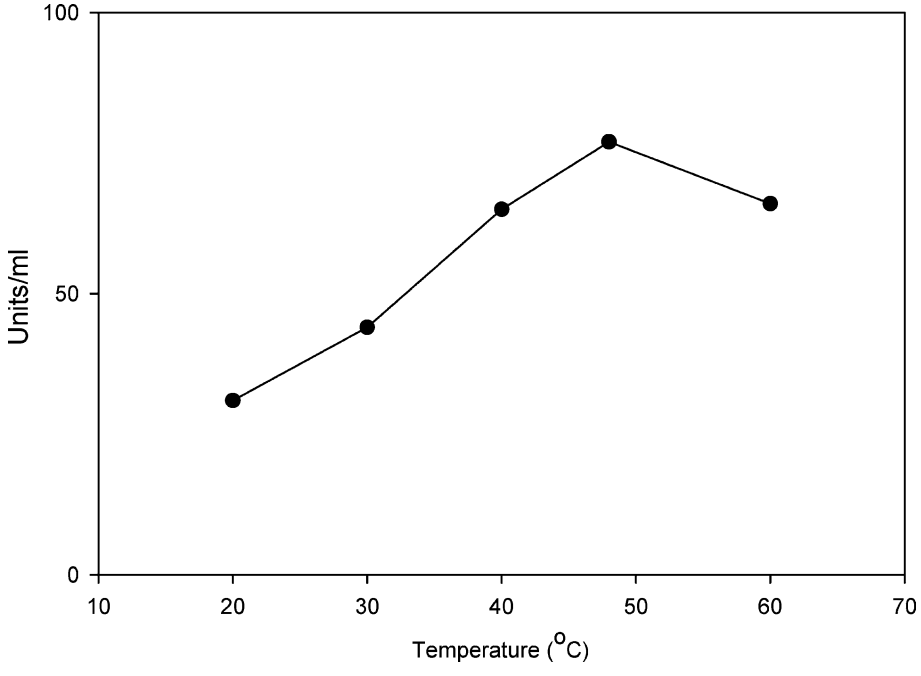

Fig. 5 Temperature optimum of crude amylase preparation with 4-nitrophenyl-α–D-maltoheptaoside-4,6-*O*-ethylidene as substrate. Concentrated supernatant fraction from a culture of *A. psychrolactophilus* (60 µl, 236 µg protein/ml) was incubated with 1.0 ml of Sigma Infinity Amylase Reagent at the indicated temperature as described in the Materials and Methods section

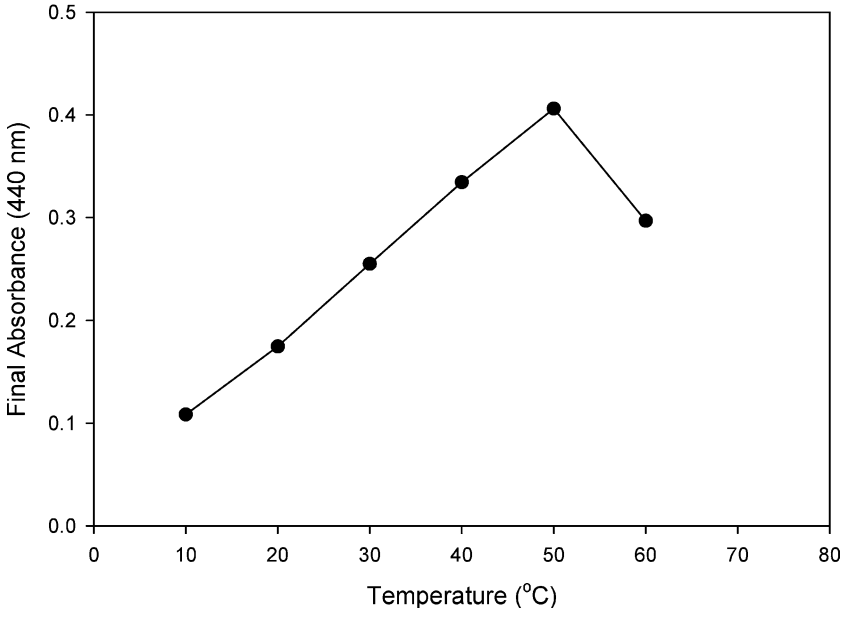

Fig. 6 Temperature optimum of azocaseinase activity in crude concentrate of supernatant fraction of culture. Azocaseinase activity was assayed by incubating 60 µl of concentrated supernatant fraction (690 µg protein/ml) with 240 µl of a 1% (w/v) solution of azocasein (Materials and Methods section) at the indicated temperature for 2 h. The reaction was stopped and the absorbance was read in a spectrophotometer at 440 nm as described in the Materials and Methods section

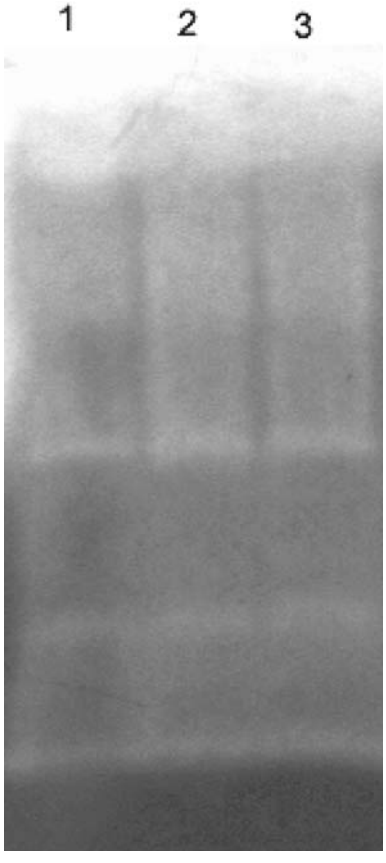

Fig. 7 Zymogram of azocaseinase activity in crude concentrate of supernatant fraction of culture. Samples of a concentrated supernatant fraction of a culture of *A. psychrolactophilus* were loaded onto Novex 4–16% polyacrylamide precast Zymogram gels (Blue casein, 1.0 mm thick, Invitrogen, Inc.) and subjected to SDS PAGE using the Laemmli buffer system. The Zymogram was then developed according to the instructions provided. Lanes (µg protein): 1, 0.9; 2, 1.9; 3, 3.5

The assays were performed by adding 100 µl of supernatant fraction to 1.4 ml 50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, 0.02% (w/v) sodium azide, 1.0% (w/v) soluble starch and incubating at 30 °C for 30 min. Reducing sugar was measured by DNS assay. The supernatant fraction contained 690 µg protein/ml and assayed at 1.61 U/ml on soluble starch. The results (Fig. 8) showed that the crude supernatant fraction was not very stable at any of the temperatures tested, losing 10% of its activity in 40 min at 30 °C, 45% of its activity in 40 min at 40 °C, and 87% of its activity after 10 min at 50 °C. In a separate experiment, the concentrated supernatant fraction lost 70% of its activity after preincubating for 21 h at 30 °C.

To determine if protease was the cause of the thermal instability at temperatures above refrigerator temperatures, 150 µl of a concentrated supernatant fraction of a culture of *A. psychrolactophilus* was added to 1,350 µl a solution containing 50 mM MES, pH 6.5, 3 mM $CaCl_2$, and 20 mg/ml bovine serum albumin (BSA), and aliquots of 300 µl were preincubated at 0, 30, 40, and 50 °C for 0, 40, 40, and 10 min respectively. Control incubations used 150 µl of concentrated supernatant fraction added to 1,350 µl of the same buffer without BSA to control for any effects that dilution might have on the rate of amylase inactivation. The control incubations were also distributed into 300 µl aliquots and the aliquots were incubated for the same length of time as the incubations with BSA. The results (Table 2) showed that dilution did not affect the rate of inactivation of amylolytic activity and that the addition of 20 mg/ml BSA did not slow the rate inactivation relative to controls without BSA. We concluded that the observed thermal instability of concentrates in the absence of substrate was largely an inherent property of the amylase and was not an artifact of protease contamination.

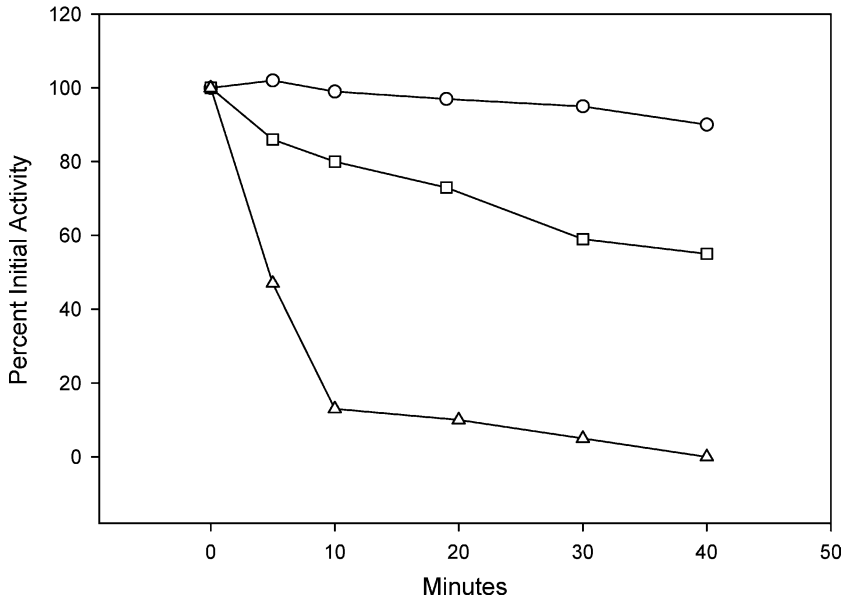

Fig. 8 Thermal stability in the absence of substrate. Samples (100 µl) of a concentrated supernatant fraction of a culture of *A. psychrolactophilus* (690 µg protein/ml) were incubated at 30 and 40 °C for 0, 5, 10, 20, 30, and 40 min then cooled by placing into an ice-water slurry. A solution (1.40 ml) containing 50 mM Bis-Tris, pH 6.5, 3 mM $CaCl_2$, 0.02% NaN_3 and 1% (w/vol) soluble starch was added and the mixtures were then incubated for 30 min at 30 °C. Samples (300 µl) were removed at the end of the incubation period and added to 300 µl of DNS reagent to stop the reaction. Color development was as described in the Materials and Methods section. 30 °C (○), 40 °C (□), 50 °C (△)

The conclusion was confirmed (Table 3) by preincubating at 30, 40, and 50 °C amylase which had been purified by affinity chromatography on corn starch granules and, in a separate experiment, preincubating at 50 °C amylase that had been partially purified by ion exchange chromatography on a DEAE column (see Materials and Methods section). The purified amylases did not exhibit significant proteolytic activity when assayed with 1% azocasein at 30 °C for 24 h. The purified amylase was still thermolabile, losing 54% of its activity in a 22 h incubation at 30 °C, 27% in 40 min at 40 °C, and 64% in 10 min at 50 °C.

Table 2 Effect of addition of bovine serum albumin on thermal stability of crude concentrates

Temperature (°C)	Length of preincubation (min)	Without BSA		With BSA[a]	
		(U/ml)	(%)	(U/ml)	(%)
0	0	13.9	100	13.0	100
30	40	13.3	96	11.6	89
30[b]	1020	7.7	63	6.8	54
40	40	8.8	63	6.2	48
50	10	4.0	29	2.9	22

Crude supernatant fraction prepared from a concentrate was assayed using Sigma Infinity amylase reagent after preincubating without substrate for the indicated length of time. The supernatant fraction contained 75 µg protein/ml[a]Bovine serum albumin (Fraction V) was added to a final concentration of 20 mg/ml[b]Determined in a separate experiment. Controls at 0 °C (100%), which were not preincubated, assayed at 12.3 U/ml without BSA and 12.5 U/ml with 20 mg/ml BSA

Discussion

The psychrotrophic bacterium *A. psychrolactophilus* is adapted to grow over a fairly wide range of temperatures (0–30 °C) [15, 16]. Its production of a cold-active β-galactosidase indicates that at least some of its enzymes are fully adapted to cold environments. *A. psychrolactophilus* produced an extracellular amylase with an optimum temperature for activity near 50 °C when assayed on ethylidene *p*-nitrophenyl maltoheptaoside and an optimum temperature of 40–50 °C on a soluble starch substrate. The amylase was an α-amylase based on its activity in the Phedabas assay [2] for α-amylases. In this paper we provided a more complete description

Table 3 Thermal stability of purified amylase

Temperature (°C)	Length of preincubation (min)	Activity	
		U/ml	%
0	–	29.9	100
30	1,342	13.7	46
40	40	21.8	73
50	10	10.8	36

Amylase was purified in one step by affinity chromatography on raw starch granules. Soluble starch used in eluting the purified enzyme from the raw starch granules was removed by adsorption of the amylase onto DEAE Sephadex A25, washing with buffer solution (50 mM MES, pH 6.5, 3 mM $CaCl_2$), and subsequently eluting the amylase with buffer solution containing 0.5 M NaCl. Samples of purified amylase were heated at the indicated temperatures and lengths of time then assayed at 22 °C using the Sigma Infinity Amylase assay reagent

of the amylase than we provided previously [24]. The amylase appeared to be a fairly large protein (M_r 105,000), but not atypical of bacterial α-amylases in most other respects, including calcium requirement and optimum pH range for activity [19, 20]. The K_m of the amylase at 22–25 °C was comparable to the K_m reported for the psychrophilic amylase from the psychrotrophic Antarctic species, *Pseudoalteromonas haloplanktis* [10] at the same temperature. The amylase possessed a raw starch-binding domain which could make it useful for processes where uncooked starch granules need to be hydrolyzed at ambient temperatures. Most of the psychrophilic amylases that have been studied in detail are from a single bacterial genus, *Pseudoalteromonas* [12]. Hydrolysis of uncooked starch granules has not so far been reported for any psychrophilic amylase.

The most unusual property of *A. psychrolactophilus* amylase was that it was thermally unstable in the absence of added substrate, a property associated with psychrophilic rather than mesophilic enzymes [12]. The amylase lost 20% of its activity within 10 min at 40 °C and over 80% within 10 min at 50 °C, suggesting an optimum temperature below 40 °C and within the range for cold-adapted enzymes [12]. Typical psychrophilic enzymes have temperature optima less than 40 °C, are unstable at mesophilic temperatures, have high catalytic efficiency (k_{cat}/K_m) at low temperature, and have low activation energies relative to mesophilic or thermophilic enzymes at the same temperatures. The apparent discrepancy between the measured optimum temperature of activity of *A. psychrolactophilus* amylase and the thermal stability curves suggests that the substrate protected the amylase from thermal denaturation to some extent. It suggests that the amylase might possess the flexibility needed to function at high specific activity at low temperatures [7, 8], but sufficient pure amylase was not obtained to measure the k_{cat} values at low to moderate temperatures to determine if specific activities are comparable to psychrophilic amylases from *Pseudoalteromonas* sp. Additional research will be needed to determine if the amylase is actually cold-adapted.

The poor stability of typical psychrophilic amylases at ambient temperatures would seem to preclude their usefulness for processes over much of the ambient temperature range. Amylases with an optimum temperature near 40 °C would seem to be better suited to most ambient temperature processes than enzymes with temperature optima of less than 35 °C. Therefore, an amylase having the properties of *A. psychrolactophilus* amylase would be more desirable for most ambient temperature processes than would be most atypical psychrophilic amylases.

There is considerable interest in using cold-adapted enzymes for biotechnological processes where ambient to low temperatures are advantageous or necessary [4, 9, 20, 28], in determining the relationship between protein structure and thermal stability [1, 4–6, 11, 22], and in elucidating the mechanisms whereby enzymes attain high activity at low temperatures [6, 21, 22, 26]. The poor thermal stability of psychrophilic enzymes is thought to be associated with increased local structural flexibility of the active centers at low temperatures, which allows psychrophilic enzymes to accommodate their substrates with low energy cost [7, 8]. However, most of the cold-active enzymes are usually selected for study because they have low temperature optima and are thus thermally labile. The psychrophilic enzymes that have been studied so far might not represent the actual range of properties of cold-active enzymes. Thermal instability is thought not to be an intrinsic property of psychrophilic proteins [12, 18] and a thermally stable aspartase with an optimum temperature of 55 °C from an Antarctic *Cytophaga* sp. was reported to be cold-adapted [14]. Thus optimum temperature might not be a reliable guide to cold-active enzymes. It is thought possible, but not likely, that enzymes can have high temperature optima and high catalytic efficiency at low temperature or to have efficient rates of catalysis that are independent of temperature over a wide range of temperatures [6]. A much larger sample of amylases from cold-adapted bacteria is needed to fully understand adaptation to cold environments and to provide new enzymes for biotechnology. The results reported here are an effort in that direction.

References

1. Aghajari N, Feller G, Gerday C, Haser R (1998) Structures of the psychrophilic *Alteromonas haloplanctis* α-amylase give insights into cold adaptation at a molecular level. Structure 6:1503–1516
2. Barnes WA, Blakeney A (1974) Determination of cereal alpha amylase using a commercially available dye-labelled substrate. Die Starke 26:193–197
3. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–254
4. Caviocchioli R, Siddiqur K, Andrews D, Sowers K (2002) Low temperature extremophiles and their applications. Curr Opin Biotechnol 13:253–261
5. Chessa J, Feller G, Gerday C (1999) Purification and characterization of the heat-labile α-amylase secreted by the psychrophilic bacterium TAC 240B. Can J Microbiol 45:452–457
6. Amico SD, Marx J, Gerday C, Feller G (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278:7891–7896
7. Feller G (2003) Molecular adaptations of cold in psychrophilic enzymes. Cell Mol Life Sci 60:648–662
8. Feller G, Gerday P (1997) Psychrophilic enzymes: molecular basis of cold adaptation. CMLS Cell Mol Life Sci 53:830–841
9. Feller G, Narinx E, Arpigny J, Aittaleb M, Baise E, Genicot S, Gerday C (1996) Enzymes from psychrophilic organisms. FEMS Microbiol Rev 18:189–202
10. Feller G, Lonhiene T, Deroanne C, Libioulle C, Beeumen J, Gerday C (1992) Purification, characterization, and nucleotide sequence of the thermolabile α-amylase from the antarctic psychrotroph *Alteromonas haloplanctis* A23. J Biol Chem 267:5217–5221
11. Feller G, Payan F, Theys F, Quian M, Haser R, Gerday C (1994) Stability and structural analysis of α-amylase from the antarctic psychrophile *Alteromonas haloplanctis* A23. Eur J Biochem 222:441–447

12. Georlette D, Blaise V, Collins T, Amico SD, Gratia E, Hoyoux A, Marx J, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:24–42
13. Girard C, Michaud D (2002) Direct monitoring of extracellular protease activities in microbial cultures. Anal Biochem 308:388–391
14. Kazuoka T, Masuda Y, Oikawa T, Soda K (2003) Thermostable aspartase from a marine psychrophile, *Cytophaga* sp. KUC-1: molecular characterization and primary structure. J Biochem 133:51–58
15. Loveland J, Gutsall K, Kasmir J, Prema P, Brenchley J (1994) Characterization of psychrotrophic microorganisms producing β-galactosidase activities. Appl Environ Microbiol 60:12–18
16. Loveland-Curtze J, Sheridan P, Gutshell K, Brenchley J (1999) Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the *Arthrobacter* subgroup and description of *Arthrobacter psychrolactophilus*, sp. nov. Arch Microbiol 171:355–363
17. Morita R (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167
18. Miyazaki K, Wintrode P, Grayling R, Rubingh D, Arnold F (2000) Directed evolution study of temperature adaptation in a psychrophilic enzyme. J Mol Biol 297:1015–1026
19. Nigam P, Singh D (1995) Enzyme and microbial systems involved in starch processing. Enz Microb Technol 17:770–778
20. Pandey A, Nigam P, Soccol C, Soccol V, Singh D, Mohan R (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31:135–152
21. Russel N (1998) Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. Adv Biochem Eng Biotechnol 61:1–21
22. Russell N (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90
23. Sarath G, de la Motte R, Wagner F (1989) Protease assay methods. In: Beynon RJ, Bond JS (eds) Proteolytic enzymes a practical approach. IRL Press, Oxford, p 28
24. Smith M, Zahnley J (2005) Production of amylase by *Arthrobacter psychrolactophilus*. J Ind Microbiol Biotechnol 32:279–283
25. Sumner J, Howell S (1935) A method for the determination of saccharase activity. J Biol Chem 108:51–54
26. Textor S, Hill A, MacDonald D, St Denis E (1998) Cold enzyme hydrolysis of wheat starch granules. Can J Chem Eng 76:87–93
27. Trimbur D, Gutshall K, Prema P, Brenchley J (1994) Characterization of a psychrotrophic *Arthrobacter* gene and its cold-active β-galactosidase. Appl Environ Microbiol 60: 4544–4552
28. Vihinen M, Mantsala P (1989) Microbial amylolytic enzymes. CRC Crit Rev Biochem Mol Biol 24:329–418