



Invertase and α -glucosidase production by the endemic Antarctic marine yeast *Leucosporidium antarcticum*

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Abstract: The marine psychrophilic and endemic Antarctic yeast *Leucosporidium antarcticum* strain 171 synthesizes intracellular β -fructofuranosidase, and intra- and extracellular α -glucosidases. Each enzyme is maximally produced at 5°C, while the strain's optimum growth temperature is 15°C. Invertase biosynthesis appeared regulated by catabolic repression, and induced by sucrose; the enzyme was extremely unstable *ex vivo*, and only EDTA, Mn²⁺, and BSA stabilized it for up to 12 h after yeast cell lysis. Thermal stability of the invertase was also low (30 min at temperatures up to 12°C). The optimum temperature for invertase activity was 30°C, and optimum pH was 4.55 to 4.75. The extracellular α -glucosidase was maximally active at 35°C and pH 6.70–7.50, and stable for 30 min up to 20°C.

Key words: Antarctica, invertase, α -glucosidase, psychrophile yeast, *Leucosporidium antarcticum*.

Introduction

Studies of microorganisms in permanently cold environments such as Antarctica have tended to focus on bacteria, although eukaryotes such as cold-adapted yeasts or filamentous fungi also occur in polar regions (Fenice *et al.* 1997; Ray *et al.* 1989). Antarctica hosts many endemic eukaryotes, including the obligately psychrophilic yeast *Leucosporidium antarcticum*. The first *Candida*-like *Leucosporidium* species were isolated in the 1960's from Antarctic soil and seawater (di Menna 1960; Fell *et al.* 1969; Sinclair and Stokes 1965). Most members of the genus *Leucosporidium*, including *L. antarcticum*, are obligate psychrophiles (Ray *et al.* 1992; Watson and Arthur 1976). Among cold-adapted yeasts from polar habitats, psychrophiles form only a relatively small group.

Strains classified as *L. antarcticum* are widespread in permanently cold marine waters below the Antarctic ice, and have been found only south of 62°S (Donachie 1995; Fell 1974). The average temperature in their natural habitat is -1°C but ranges seasonally from -2.2°C to $+4^{\circ}\text{C}$. Probable cold-adaptation renders the species extremely sensitive to temperatures above 20°C . The yeast weakly digests both sucrose and maltose (Fell *et al.* 1969). We considered that characterizing the cell's intracellular β -fructofuranosidase, and extra- and intracellular α -glucosidases, would help to define the cell's enzyme kinetics and potential role of this yeast in the Southern Ocean.

Invertases (β -D-fructofuranoside fructohydrolases, EC 3.2.1.26) which catalyze the hydrolysis of the terminal non-reducing β -fructofuranoside residue in sucrose, raffinose and related β -D-fructofuranosides, are produced by bacteria, fungi, higher plants, and some animals (Belcarz *et al.* 2002). α -Glucosidases (α -D-glucoside glucohydrolases, EC 3.2.1.20) which liberate non-reducing terminal α -glucopyranoside residues from various α -glucosides and related compounds are synthesized by microorganisms, plants and mammals. Based on their diverse substrate specificities, the α -glucosidases have been divided into three groups, those active against synthetic glucosides and sucrose, those active against homogeneous malto-oligosaccharides, and those active against homogeneous malto-oligosaccharides and α -glucans (Sugimoto *et al.* 2003). Extensive research on the synthesis and molecular and kinetic properties of invertases and α -glucosidases has focused principally on mesophilic and thermophilic proteins, *i.e.*, their psychrophilic counterparts have been largely overlooked.

Materials and methods

Strain and culture conditions. — *Leucosporidium antarcticum* 171 was isolated from a seawater sample collected at a depth of 100 m in Admiralty Bay, King George Island, Antarctica (Donachie 1995). For long-term storage the strain was maintained at -70°C in stocks containing 5 mM sodium phosphate buffer (pH 7.20) and 20% glycerol. Prior to submerged cultures it was cultured for 2 weeks at 6°C on 2% agar slants (pH 6.50) with 0.5% bactopectone, 0.3% yeast extract, 0.3% malt extract, 1% glucose and 3.5% artificial marine salt ("Instant Ocean", Aquarium Systems, France). For this study, single colonies were cultivated with shaking for up to 15 days in the liquid medium (pH 6.50) containing 0.5% bactopectone, 0.3% yeast extract, 3.5% marine salt and 1% sucrose (in certain cultures replaced by 1% glucose or maltose) as previously described (Turkiewicz *et al.* 2003). Growth was monitored through the development of colony forming units (CFU) at 6°C for 16 days on the same medium containing 15 g/L agar. Cells were harvested by centrifugation ($500 \times g$, 15 min, 4°C), and stored at -20°C until used in enzyme assays. The supernatant was also stored at -20°C until assayed for extracellular α -glucosidase activity. The

effect of temperature on *L. antarcticum* 171 growth and enzyme synthesis was examined in non-shaken cultures in liquid medium between 0°C and 30°C.

To determine if *L. antarcticum* 171 can convert glucose to ethanol, the strain was cultured under anaerobic conditions at 5 and 15°C in liquid medium containing 10% (w/v) glucose, 2% (w/v) (NH₄)₂SO₄, 0.5% (w/v) K₂HPO₄ and 0.1% (w/v) MgCl₂. The amount of ethanol in the medium during the incubation was determined with a commercial kit (Sigma, USA).

Cell-free extracts from yeast. — Frozen yeast cells (1 g wet weight) were thawed in either 10 ml of 10 mM sodium acetate buffer (pH 4.55), or 10 ml of 10 mM sodium phosphate buffer (pH 7.0), and homogenized with glass beads (0.25–0.50 mm, 2 g of beads per gram wet weight of yeast cell pellet) at 0°C in a MPW-302 homogenizer equipped with a glass ball mill (2000 rpm, 3 × 2 min). Cell debris was pelleted by centrifugation (5000 × g, 30 min, 4°C), and the supernatant retained for assays of β -fructofuranosidase and α -glucosidase determinations, respectively.

Invertase activity assays. — β -fructofuranosidase was assayed as previously described (Goldstein and Lampen 1975). Sucrose hydrolysis was carried out for 15 min. Activity was expressed in μ moles of glucose released from a 125 mM solution of sucrose through 1 min at 30°C and pH 4.55.

α -Glucosidase activity assays. — α -Glucosidase activity was determined in the same way as that of invertase (15 min, 30°C) but using a 146 mM solution of maltose (pH 7.00) rather than sucrose. Activity was expressed in μ moles of glucose liberated in 1 min. A second method used 100 μ l of 10 mM *p*-nitro- α -D-glucopyranoside (PNPG) solution with 10 mM sodium phosphate buffer (pH 7.00) (2.8 ml), and enzyme (100 μ l) in a glass cuvette. The increase in Abs₄₀₀ at 30°C was determined with time (15 min). Activity was expressed in μ moles of *p*-nitrophenol released in 1 min.

Proteolytic activity assays. — Proteolytic activity was determined as previously described (Anson 1938). One unit of activity was the amount of enzyme required to liberate TCA-soluble products equivalent to 1 μ mole of L-Tyr (detection with Folin-Ciocalteu reagent) through 1 min under standard reaction conditions (2% urea-denatured hemoglobin solution, 30°C, pH 6.50, 15 min).

Polyacrylamide gel electrophoresis (PAGE) and detection of invertase. — PAGE of crude cell-free extracts was carried out under non-denaturing conditions (4°C, 10 mM sodium acetate buffer pH 5.60) in 10% polyacrylamide gel. Gels were washed twice with 10 mM sodium acetate buffer pH 4.50, and incubated for 30 min at 30°C in 125 mM sucrose solution in fresh buffer. After incubation, gels were washed with distilled water and immersed in 0.1% fuchsin in 0.5 M NaOH (Cairns and Ashton 1991); a pink formozan band indicated invertase activity during the 1 min. incubation. PAGE under non-denaturing conditions does not deacti-

vate the enzyme. Since the enzyme was not purified due to its inherent lability, the total protein pattern after denaturation differed from that of mixed native proteins mixture. Thus, the location of the denatured invertase and its molecular weight could not be determined (Fig. 3).

Other analytical methods. — The total amount of protein in enzyme preparations (both cell free extracts and culture medium supernatant), was determined after Lowry *et al.* (1951) using bovine serum albumin as a standard. Sucrose concentrations in the culture broths were estimated with a commercial diagnostic kit (Boehringer-Mannheim, Germany). Temperature-dependence of each enzyme's activity was determined from 0°C to 65°C at pH 4.55 (β -fructofuranosidase) or pH 7.00 (α -glucosidase). Each enzyme's thermal stability was estimated through 30 min and one-hour incubations of cell-free extracts (β -fructofuranosidase, pH 4.55) or culture medium supernatants (α -glucosidase, pH 7.0) 0 to 80°C. The effect of pH on the activity and stability (30 min and one-hour incubation) was determined at 30°C and 4°C, respectively, and at pH 2.00–9.00 (0.1 M Britton-Robinson buffer solutions). Residual activity was determined under standard conditions, described above.

Statistical analysis. — Triplicate samples were prepared for each determination described above, and the Student's *t*-test was used to analyze the data distribution. Experimental results are presented as mean \pm SD. SD ranged from 1 to 3%. Statistical analyses were carried in the Origin 6.0 statistical software.

Results and discussion

As with *L. antarcticum* strains characterized by Fell *et al.* (1969), *L. antarcticum* 171 is a strictly aerobic, budding yeast (Fig. 1). The strain does not produce ethanol, and grows only below 21°C. In contrast to Fell *et al.* (1969) who found that *L. antarcticum* strains did not assimilate sucrose as a sole carbon source, our experiments revealed that *L. antarcticum* 171 weakly digested this disaccharide. The strain's highest growth rates were observed between 5°C and 15°C, with the latter being the optimum. Growth at 20°C was weak; after 15 days incubation at 20°C, CFU ml⁻¹ equaled just 8% of those determined at 15°C. At 18–20°C, the lag phase increased from 2 to 8 days, and the final pH of culture broth was close to 4.00; at 15°C the final pH of the culture broth was 7.40. The strain preferred media that contained 0.5–2.5% (w/v) marine salt (concentrations to 9% were tolerated), though the salinity of seawater from which *L. antarcticum* was isolated is about 3.5% (Tokarczyk 1987). Glucose (1%) was the preferred carbon source, with stationary phase attained in ten days. In the presence of 1% maltose as the sole carbon source, stationary phase was attained after thirteen days and CFU ml⁻¹ was the same as in the 1% glucose medium above. In the case of both glucose and maltose, maximum CFU ml⁻¹ was twice that in the presence of 1% sucrose alone.

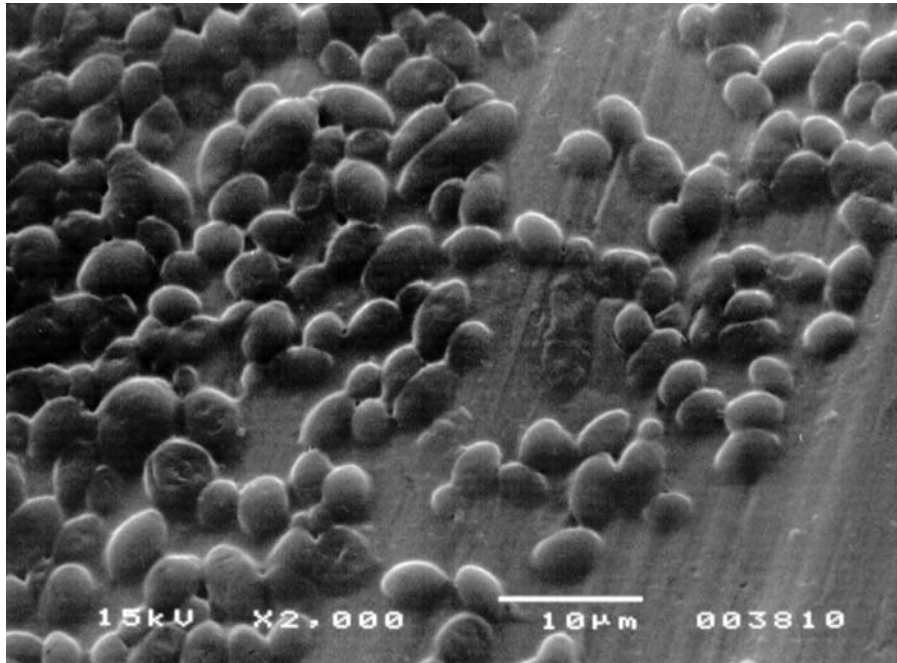


Fig. 1. Electron micrograph of *Leucosporidium antarcticum* 171 cells.

Invertase and α -glucosidase biosynthesis. — *L. antarcticum* 171 synthesized two glycoside hydrolases; an intracellular β -fructofuranosidase was ca. three times less active towards raffinose as compared to sucrose, and undetectable in culture medium supernatant, while an α -glucosidase was detected in both the cell-free extract and in culture broth (both fractions displayed similar activity against maltose and PNPG). These enzymes were produced maximally at 5°C (almost double the amount produced at 15°C), and thus 10°C below the optimum growth temperature of the strain. The specific activity of each enzyme halved as the temperature increased from 5°C to 15°C. Recall that the yeast's natural habitat is closer to 5°C than 15°C. Elevated enzyme expression rates at low temperatures have been described in other organisms from high latitudes and are believed to allow cells to compensate for the low enthalpy and, otherwise, slow metabolic reactions at prevailing temperatures (Feller *et al.* 1996).

Extracellular α -glucosidase synthesis in *L. antarcticum* 171 commenced on the fourth day of incubation at 5°C, and peaked at the end of the exponential phase (ca. 0.1 U mg s.s.⁻¹, days 12 to 14). Intracellular α -glucosidase was synthesized from the sixth day, and its activity amounted to 0.27 U mg s.s.⁻¹ from the ninth day onward. Intracellular invertase was detectable from day 4, and attained the highest level of activity at the end of the exponential phase, a period that was dependent on culture medium composition and particularly on the nature of the carbon source.

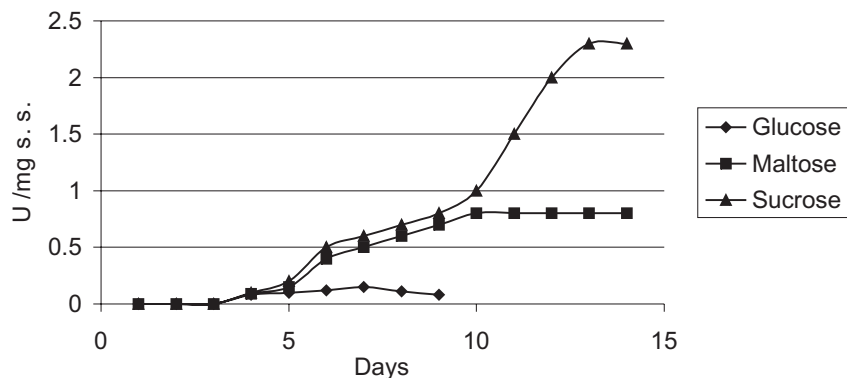


Fig.2. The effect of the type of carbon source on dynamics of intracellular *L. antarcticum* 171 invertase biosynthesis.

The effect of the nature of carbon source was more significant in the case of β -fructofuranosidase because extracellular α -glucosidase activity was only 21% greater when glucose was replaced by sucrose or maltose (an increase from 0.100 to 0.121 U ml⁻¹). This indicates that enzyme synthesis is not inducible, and that it is not repressed by glucose, in contrast to intracellular invertase. The latter enzyme's activity was low in the presence of glucose as the sole carbon source (ca. 0.15 U mg s.s.⁻¹, 0.25% glucose, maximum on the 7th day, Fig. 2). In the presence of maltose only, however, the enzyme's activity exceeded by 5 times that in the presence of glucose only (0.8 U mg s.s.⁻¹, 0.25% maltose, maximum from 10th to 14th day); when sucrose only was available, enzyme activity increased by a factor of 15 (2.3 U mg s.s.⁻¹, 0.25% sucrose, maximum on 13th and 14th day). Optimization of sucrose concentration in the culture medium further enhanced activity, to 4.0 U mg s.s.⁻¹ with 1% (w/v) sucrose in the medium. Activity decreased when the sucrose concentration was raised from 1 to 2% (to 3.0 U mg s.s.⁻¹).

Catabolic repression appears to regulate the synthesis of invertase in *L. antarcticum* 171, as in other yeasts (Carlson 1999). As in some other fungi synthesis was induced by sucrose (Cairns *et al.* 1995), and by maltose. Sucrose constituted approximately 77% of the total carbohydrates in the medium containing 1% sucrose, in which *L. antarcticum* 171 was grown in this experiment. The remaining part was glucose, derived from yeast extract and bactopectone. This monosaccharide was consumed during the first 5 days of incubation, and its depletion gave rise to de-repression of β -fructofuranosidase synthesis, and rapid sucrose consumption from day 6.

PAGE and zymograms of the cell-free extract revealed a single protein band with invertase activity among numerous other bands of intracellular proteins (Fig. 3). This strain seems to produce a single intracellular β -fructofuranosidase, in contrast to other yeast species (Belcarz *et al.* 2002). Secretion of this enzyme into the culture medium was not observed in any phase of *L. antarcticum* 171 growth.

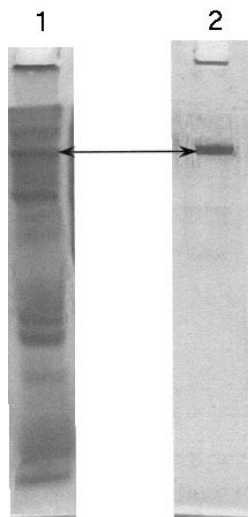


Fig. 3. Protein pattern of the *Leucosporidium antarcticum* 171 cell-free extract (1), and visualization of the invertase activity with basic fuchsin (2) (see methods).

According to Adams *et al.* (1995), entrapment of many hydrolases within their producers' cells is a mechanism through which cells may adapt to harsh conditions, such as those that prevail in the Southern Ocean.

Invertase stabilization. — We initially focused on β -fructofuranosidase because it is a marker enzyme for yeast, and its activity here was several times greater than that of the intracellular α -glucosidase. Regardless of the invertase extraction method (only the most efficient one is reported here), the enzyme was extremely unstable, losing all activity within 24 h at 0°C (in 0.01 M sodium acetate buffer, pH 4.55). Two intracellular proteinases (serine enzymes and metalloproteinases, inhibited by PMSF and EDTA, respectively) with a total activity of approximately 1.1 U mg s.s.⁻¹, were also detected in the cell-free extracts; the influence of their inhibitors (separately and mixed, each at 1 mM) on invertase activity and stability was tested. In the presence of 2 mM EDTA (40% inhibition of total intracellular proteolytic activity), invertase activity was 25% higher than in the control sample, and as much as ~30% of its initial activity was retained after 24 h incubation at 4°C (*versus* almost total inactivation in the EDTA-free control). Although a decrease in proteolytic activity caused by 2 mM PMSF was more pronounced (roughly 80% inhibition), this compound also reduced invertase activity in *L. antarcticum* 171 to one third upon addition of the cell-free extract, and almost completely after 12 h. The mechanism of this inhibition remains obscure since PMSF inactivates serine enzymes, particularly proteinases, and the presence of a Ser residue in the invertase active site has not been reported. A mixture of PMSF and EDTA had the same negative impact on

Table 1
Effect of selected compounds on *L. antarcticum* 171 invertase activity and stability*

Compound	Relative activity (%)		
	Incubation time (h)		
	0	12	24
None	100	40	20
Mn ²⁺	115	90	35
Co ²⁺	113	42	20
Cd ²⁺	55	10	0
BSA	100	60	35
DTT	90	40	5
Reduced glutathione	75	30	0

* 0.25 ml aliquots of cell-free extract in 10 mM sodium acetate buffer, pH 4.55, were incubated with 0.25 ml aliquots of 4 mM solutions of the listed compounds at 0°C, and the residual activity was determined under standard conditions.

β-fructofuranosidase activity and stability. Salting out 86% of the proteolytic activity from the cell-free extracts with (NH₄)₂SO₄ (80% saturation, total invertase activity remained in the solution) did not affect β-fructofuranosidase stability. Thus, the intracellular *L. antarcticum* 171 proteinases seem not to be responsible for the rapid β-fructofuranosidase inactivation in cell-free extracts.

Apart from EDTA and PMSF, the effects of other compounds as potential invertase stabilizers were determined. Enrichment with 2 mM Co²⁺ and Mn²⁺ (added separately to cell-free extract) increased the enzyme's activity by 13% and 15%, respectively (Table 1). Mn²⁺ ions stabilized the *L. antarcticum* 171 invertase within 12 h incubation at 0°C, with as much as 90% of initial activity retained. BSA protected the enzyme to some extent, with 60% of initial activity remaining after 12 h. After 24 h incubation, activity in samples containing BSA or Mn²⁺ was almost twice that in the control (roughly 35% of the initial activity *versus* 20%). Cd²⁺ ions and reduced glutathione markedly inactivated the invertase, while dithiotreitol had a weaker impact on its activity and stability.

Failure to stabilize the β-fructofuranosidase precluded further characterization of this enzyme. It is presumably bound to cell membranes or the cell wall in *L. antarcticum* 171, as is invertase II in *Solanum tuberosum* tubers (Isla *et al.* 1999).

Effect of temperature and pH on enzyme activity and stability. — The highest activity of *L. antarcticum* 171 invertase was observed at 25–35°C with maximum activity at 30°C (Fig. 4). Slightly more than 20% of the maximum activity was retained at 0°C. The enzyme displayed weak thermal stability (Fig. 4), and its initial activity was retained for only 30 min up to 12°C. A 40% decline in activ-

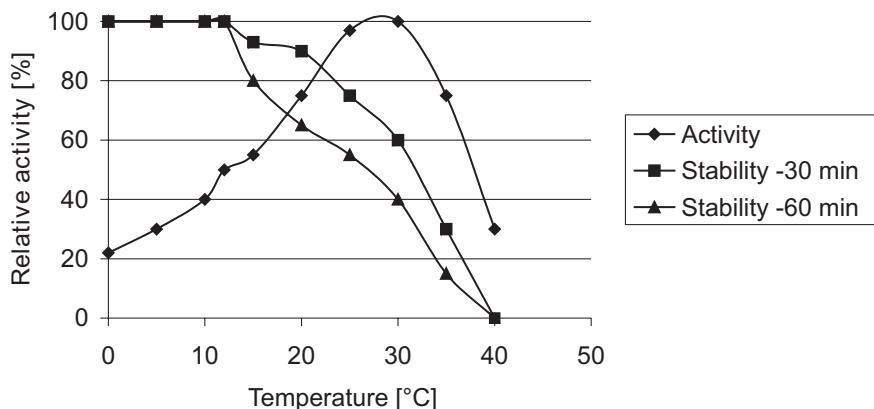


Fig. 4. Effect of temperature on *Leucosporidium antarcticum* 171 invertase activity and stability.

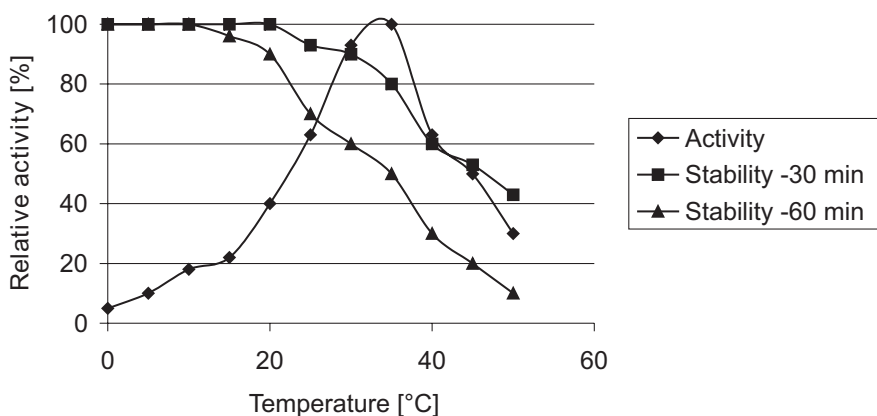


Fig. 5. Effect of temperature on *Leucosporidium antarcticum* 171 extracellular α -glucosidase activity and stability.

ity was observed after 30 min incubation at 30°C, and at 40°C the enzyme was completely inactivated. These properties are typical of true psychrophilic enzymes (Feller *et al.* 1996).

The optimal activity of the extracellular α -glucosidase was observed at 35°C (Fig. 5), and it was relatively weak at 0–15°C (approximately 5% of maximum activity at 0°C, and 20% at 15°C). The enzyme was more thermally stable (Fig. 5) than the invertase, since it was stable for 30 min up to 20°C, retained 60% and 30% of the activity at 40°C after 30 and 60 min, respectively, and was completely inactivated at 60°C in 30 min.

As with the majority of yeast invertases which achieve the highest activity at acidic pH, β -fructofuranosidase from *L. antarcticum* 171 was optimally active towards sucrose at pH 4.55 (Fig. 6), and towards raffinose at pH 4.75. At 4°C it was stable at pH 4.30–4.80 for 30 min, and at pH 4.30–4.60 for 60 min (Fig. 6).

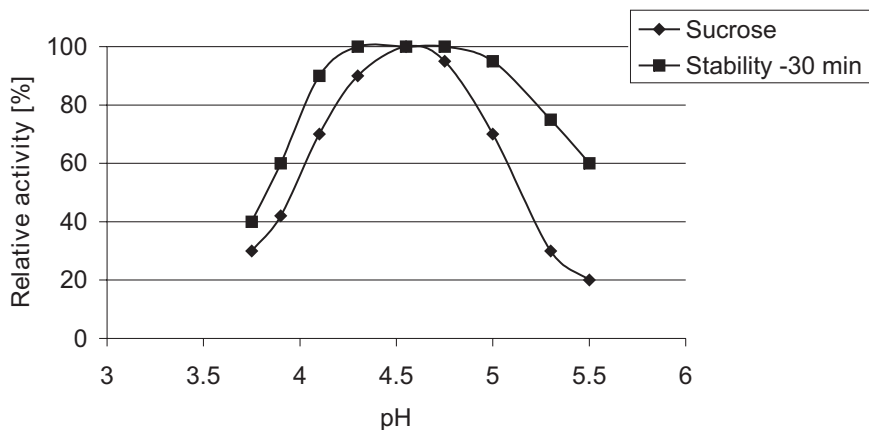


Fig. 6. pH-dependence of *Leucosporidium antarcticum* 171 invertase activity against sucrose, and stability during 30 min incubation at 4°C.

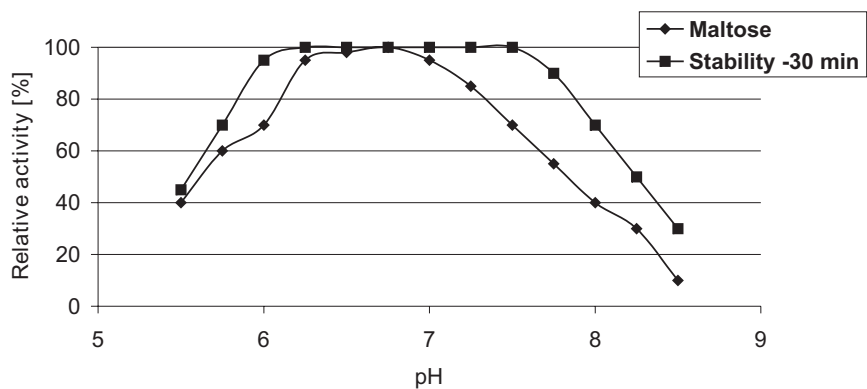


Fig. 7. Effect of pH on extracellular *Leucosporidium antarcticum* 171 α -glucosidase activity against maltose, and stability during 30 min incubation at 4°C.

The extracellular *L. antarcticum* 171 α -glucosidase was most active at neutral pH, with an optimum for maltose hydrolysis of pH 6.70 (Fig. 7), and of pH 7.50 for α -PNPG digestion. At 4°C the enzyme was stable at pH 6.00–7.50 for 30 min, and at pH 6.50–7.50 for 60 min.

Summary

The properties of the two glycoside hydrolases in *L. antarcticum* 171 presented here complement descriptions of the enzyme apparatus in this endemic Antarctic psychrophile (Fell *et al.* 1969). As mentioned above, these authors found that *L. antarcticum* strains did not consume sucrose. Synthesis of the invertase and

α -glucosidase facilitates assimilation by the yeast of β -fructofuranosides and α -glucopyranosides in permanently cold waters of the Southern Ocean, where nutrient availability fluctuates considerably (Misic *et al.* 2002).

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