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ORIGINAL PAPER

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Cold-adapted yeasts as producers of cold-active polygalacturonases

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Abstract Eight cold-adapted, polygalacturonase-producing yeasts belonging to four species were isolated from frozen environmental samples in Iceland. They were identified as *Cystofilobasidium lari-marini*, *Cystofilobasidium capitatum*, *Cryptococcus macerans* and *Cryptococcus aquaticus* species by sequence analysis of rDNA regions. Growth behavior of the isolates was investigated. All strains could grow at 2°C. Addition of glucose to pectin-containing culture medium had a repressive effect on enzyme production except for *C. aquaticus*, which showed increased polygalacturonase activity. Optimal temperature for enzyme production for the *Cystofilobasidium* strains was 14°C, while that for the *Cryptococcus* strains was lower. Among the isolates, *C. lari-marini* S3B produced highest levels of enzyme activity at pH 3.2. Preliminary characterization of the polygalacturonases in the culture supernatant showed the enzyme from *Cystofilobasidium* strains to be optimally active at 40°C and pH 5, and that from the *Cryptococcus* strains at 50°C and pH 4. The polygalacturonase from *C. macerans* started to lose activity after 1 h of incubation at 40°C, while that from the other strains had already lost activity at 30°C. All the strains except *C. aquaticus* produced isoenzymes of polyglacturonase. In addition to polygalacturonase, the *Cystofilobasidium* strains produced pectin lyase, *C. aquaticus* pectin esterase, and *C. macerans* pectin lyase, pectate lyase and pectin esterase.

Keywords Cold adaptation · *Cryptococcus aquaticus* · *Cryptococcus macerans* · *Cystofilobasidium capitatum* · *Cystofilobasidium lari-marini* · Polygalacturonase

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Introduction

Pectic substances are complex polysaccharides of plant origin and are grouped into pectic acid, which is a polymer of galacturonic acid subunits linked by α-1,4 glycoside linkages, and pectinic acid with the same basic structure but having some of its carboxyl groups methylated. The latter group includes pectins, which are capable of forming gels with sugar and acid under suitable conditions (Kulp 1975; Fogarty and Kelly 1983). Enzymes that degrade pectic substances, are classified into pectin esterases which de-esterify pectinic acid, and depolymerases which cleave the main chain. Depolymerases for both pectinic acid and pectic acid are further divided into polygalacturonases, which hydrolyze the glycosidic bonds by endo- (at random within the chain) and exo- (at terminal end of the chain) action, respectively, and lyases, which break the glycosidic bonds by β-elimination (Kulp 1975).

The main application of pectinases is in the fruit and vegetable processing industry. Today, the main source of pectinases used in these industries is from fungi, mainly *Aspergillus niger*, since it produces high amounts of these enzymes and is a GRAS (generally recognized as safe) microorganism (Alkorta et al. 1998; Kashyap et al. 2001). Scientists have recently been exploring the possibility of using pectinases from yeasts for these applications (Blanco et al. 1999). Yeasts that could be declared GRAS microorganisms and which produce high levels of the enzymes might be an interesting alternative as a pectinase source for these industries. Furthermore, desirable enzymes could be cloned into a GRAS microorganism and overexpressed (Blanco et al. 1999).

A desirable feature of the enzymes for processing fruit juice would be a higher specific activity at lower temperature so as to minimize the risk of microbial growth during processing at higher temperatures. Interest in enzymes from cold-adapted microorganisms has been growing in recent years and various candidates have been identified for application in areas such as food processing and cold washing (Herbert 1992; Margesin and Schinner 1994; Brenchley 1996; Feller and Gerday 1997; Russell 2000). Although a number of cold-adapted bacteria and their enzymes have been isolated and studied, the literature on cold-adapted yeasts is scant.

This paper reports on four cold-adapted, pectinase-producing yeast species isolated in Iceland, and preliminary characterization of the polygalacturonase produced by them.

Materials and methods

Biological materials

The microorganisms studied were isolated from frozen soil, plant leaves and branches in the south-west of Iceland, isolates S from Seltjarnarnes and isolates H from Heidmörk. In the laboratory the samples were incubated at 9°C in liquid medium A for 4 days. Aliquots from these cultures were plated on solid medium A at 9°C for 5 days and the colonies that developed were transferred onto new plates. After incubation as above, the plates were stained with 0.05% Ruthenium Red (Fluka Chemika, Buchs, Switzerland) for 10 min and destained with deionized water for 10 min. Pectinase activity was detected by red zones around the colonies, as described by McKay (1988). The mixed colonies that showed activity were streaked out on new plates so that pure colonies could be isolated and the individual pectinase producers identified.

Media and culture conditions

Medium A contained (in 1 l volume): 1 g yeast extract (Difco, Detroit, USA), 5 g tryptone (Difco) and 10 g pectin from citrus fruit (8.9% esterified; Sigma, St. Louis, MO, USA) as carbon source. The pH of the medium was adjusted to 6.5. Solid medium A contained in addition 12 g bacto-agar (Difco) in 1 l medium.

Medium B was modified from Federici and Petruccioli (1985) and contained (in 1 l volume): 1 g yeast extract, 2 g $(NH_4)_2SO_4$, 1.5 g NaCl, 0.21 g $CaCl_2{\cdot}2H_2O$, 0.32 g $FeCl_3{\cdot}9H_2O$, 0.98 g $MgSO_4{\cdot}7H_2O$, and 0.28 g $ZnSO_4{\cdot}7H_2O$ in 50 mM K_2HPO_4 citric acid buffer. Cultivation experiments were done in medium B. Unless specifically indicated, 1% pectin from citrus fruit (8.9% esterified) was added as carbon source, the pH was adjusted to a desired value, and the cultures were grown at 9°C. For all isolates, 50 ml of medium B was inoculated with 2 ml of the respective cultures grown to stationary phase in the same medium. Cultivation was performed with shaking at 120 rpm. Cell growth was monitored by measuring optical density of the culture samples at 600 nm (OD_{600nm}), and the enzyme activities were measured in the supernatant after spinning down the cells at 10,000 *g* for 5 min.

The ability of the isolates to utilize various carbon sources for growth were explored using an API 20C AUX kit from bioMérieux (Lyon, France). *Candida albicans*, acquired from the Department of Microbiology, University of Iceland, was used as a reference strain. The instructions in the API 20C AUX manual were followed for all five strains, except that the yeast suspension was adjusted to OD_{600} of 1, before inoculating into medium C, instead of measuring McFarland values. Furthermore, isolates *C. lari-marini* S3B, *C. capitatum* S5 and *C. macerans* S3A were grown at 18.5°C for 6 days, and *C. aquaticus* H2 at 5°C for 12 days. *C. albicans* was grown at the recommended temperature of 30°C for 3 days. The composition of medium C is found in the API 20C AUX manual.

Isolation of DNA

Cultures were grown on solid medium A at 9°C for 48 h. Single colonies were scraped from the agar surface, suspended in 500 μl of sterile water, and harvested by centrifugation at 10,000 *g* for 3 min. Chromosomal DNA was isolated using the protocol described by Hoffman and Winston (1987). DNA purity was assessed from the A_{260}/A_{280} absorbance (Johnson 1994).

PCR amplifications

Universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the internal transcribed spacer 1 (ITS1), 5.8S rDNA, and internal transcribed spacer 2 (ITS2) sequences, while primers NL-1 (5′-GCATATCAAAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used to amplify the 26S rDNA D1/D2 domain. PCR assays were performed in a GeneAmp PCR 9700 system (Applied Biosystems). The reactions were carried out in 25 μl of final volume containing 100 ng of DNA as template, 100 nM of each primer, 2.5 µl of STR buffer, and 1 U of *Pfu* DNA polymerase (Promega). In order to check amplifications, 10 μl of PCR-amplified products were subjected to electrophoresis on 1% (w/v) agarose gels (Sambrook et al. 1989).

PCR amplifications of a fragment containing ITS1, 5.8S rDNA, and ITS2 sequences, were performed according to Lott et al (1993), while that of 26S rDNA D1/D2 domain was done according to Kurtzman and Robnett (1998). DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI Prism 3100 DNA analyzer, using the ABI Prism BigDye terminator cycle sequencing ready reactions kit (PE Biosystems) according to the manufacturer's protocol.

The sequences were registered in the GenBank data library under accession numbers AY052479 and AY052487 for strain H1, AY052480 and AY052488 for H2, AY052481 and AY052489 for S1, AY052482 and AY052490 for S3A, AY052483 and AY052491 for S3B, AY052484 and AY052492 for S4, AY052485 and AY052493 for S5, and finally AY052486 and AY052494 for X. Sequences belonging to the same species or closely related species, available through the public databases, were aligned and a similarity matrix was calculated using Similarity Matrix version 1.1

Table 1 Some morphological characteristics of the cold adapted yeasts

Colony characteristic	Yeast species			
	Cystofilobasidium lari-marini S3B	*Cystofilobasidium capitatum* S5	*Cryptococcus macerans* S3A	*Cryptococcus aquaticus* H2
Color	Yellow	Lightpink	Darkpink	White
Viscosity	Botyrous	Semi-viscous	Viscous	Semi-viscous
Diameter (mm)	1.0–2.0	1.0–2.0	1.0–2.0	0.5–1.0
Cellshape	Cylindrical	Cylindrical	Cylindrical/oval	Oval
Cellsize (μm)	3.6–5.6×2.0–2.8	3.6–5.6×2.0–2.8	5.6–6.8×2.8–3.6	3.6–6.4×2.0–2.8

Table 2 Growth of the cold adapted yeast isolates invarious carbon sources

	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF
C. albicans[a]	0	+	+	+	0	+	+	+	+	0	+	+	+	0	0	+	+	+	0	+
C. lari-marini S3B	0	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	+	+	+	+
C. capitatum S5	0	+	+	+	+	+	+	+	0	+	+	0	0	+	+	+	+	+	+	+
C. macerans S3A	0	+	+	+	+	+	0	+	0	+	+	0	0	+	+	+	+	+	+	+
C. aquaticus H2	0	+	+	+	+	+	+	+	+	0	+	0	+	+	+	+	+	+	+	+

GLU: glucose, GLY: glycerol, 2KG: 2-keto-D-gluconate, ARA: L-arabinose, XYL: D-xylose, ADO: adonitol, XLT: xylitol, GAL: galactose, INO: inositol, SOR: sorbitol, MDG: α-methyl-D-glucoside, NAG: N-acetyl-D-glucosamine, CEL: celiobiose, LAC: lactose, MAL: maltose, SAC: saccharose, TRE: trehalose, MLZ: melezitose, RAF: raffinose

[a]*C. albicans* was used as a reference strain

+ + + Detection of growth after 1 day for *C. albicans*, 2 days for isolates S3A, S3B, and S5, and 6 days for H2

+ + Detection of growth after 2 days for *C. albicans*, 4 days for S3A, S3B, and S5, and 9 days for H2

+ Detection of growth after 3 days for *C. albicans*, 6 days for S3A, S3B, and S5, and 12 days for H2

0 No detectable growth

software (Maidak et al. 2000). Only unambiguously aligned positions from all sequences were used to calculate the matrix, and gaps were not included in the match/mismatch count.

Enzyme assays

Polygalacturonic acid from orange (Sigma) was used as substrate for polygalacturonase and pectate lyase assays, while pectin from citrus fruit (90% esterified; Sigma) was used in pectin esterase and pectin lyase assays.

Polygalacturonase activity was determined by incubating the enzyme with polygalacturonic acid and measuring the increase of reducing ends with DNS reagent (3,5-dinitrosalicylic acid; Sigma) by a slight modification of the method from Gupta et al. (1993). Fifty microliters of appropriately diluted culture supernatant was added to 450 μl of 0.5% (w/v) polygalacturonic acid (Sigma) in 0.1 M potassium acetate buffer, pH 4.9. After incubation at 23°C for 10 min, the reaction was stopped by adding 750 μl of DNS prepared according to Bernfeld (1955). The samples were heated in a boiling water bath for 10 min and then spun at 10,000 *g* for 10 min. Absorbance was measured at 590 nm. The standard curve was prepared with D-galacturonic acid (0.58.0 μmol). One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 μmol of reducing ends per minute under the standard conditions.

Detection of pectate- and pectin-lyase activity was achieved by applying a modified method of Kamimiya et al. (1977). Fifty microliters of culture supernatant was added to 1.45 ml of 0.2% (w/v) substrate solution, which was polygalacturonic acid for pectate lyase, and pectin for pectin lyase analysis. The assays were performed in 0.1 M potassium acetate buffer at pH 5.0 and in 0.1 M TrisHCl buffer at pH 7.6, respectively. In case of pectate lyase, the assay was also performed in the presence of 0.1–1.0 mM $CaCl_2$ (Truong et al. 2001). Increase in OD_{235nm} over a period of 20 h at 23°C was measured.

Detection of pectin esterase activity was achieved by applying a method modified from Christensen et al. (1998) by measuring the drop in pH from the initial value over a period of 20 h at 23°C. Twenty five microliters of culture supernatant was incubated with 0.5% (w/v) citrus pectin, 0.15 M NaCl, and 0.1 M buffer in a total volume of 1.5 ml. The buffers used were potassium acetate at pH 5.0, potassium phosphate at pH 7.0, and 0.1 M TrisHCl at pH 8.0, respectively.

Activity staining

Culture supernatants from all the isolates, containing 30–100 U/ml of polygalacturonase, were run on a ready made isoelectric focusing gel (pH 3–9) in a PhastSystem (Pharmacia, Piscataway, NJ, USA). Overlays, to detect polygalacturonase activity in the gel, contained 1% (w/v) agarose, 0.2% (w/v) polygalacturonic acid (Sigma), 0.1 mM potassium acetate (pH 5), and 5 mM EDTA to inhibit any pectate lyase activity (McGuire et al. 1991). The isoelectric focusing gel was then laid on the overlay and incubated at 30°C for 30 min. The gel was discarded and the overlay was flooded with 0.05% Ruthenium Red for 8 min, followed by destaining with deionized water for 15 min.

Results

Characterization and identification of isolates

Eight pectinase-producing yeast isolates, H1, H2, S1, S3A, S3B, S4, S5 and X, were isolated from the samples collected in Iceland. For their identification, sequence analysis of the rDNA region in the genome, including ITS1, 5.8S rDNA, ITS2, and 26S rDNA D1/D2 domain,

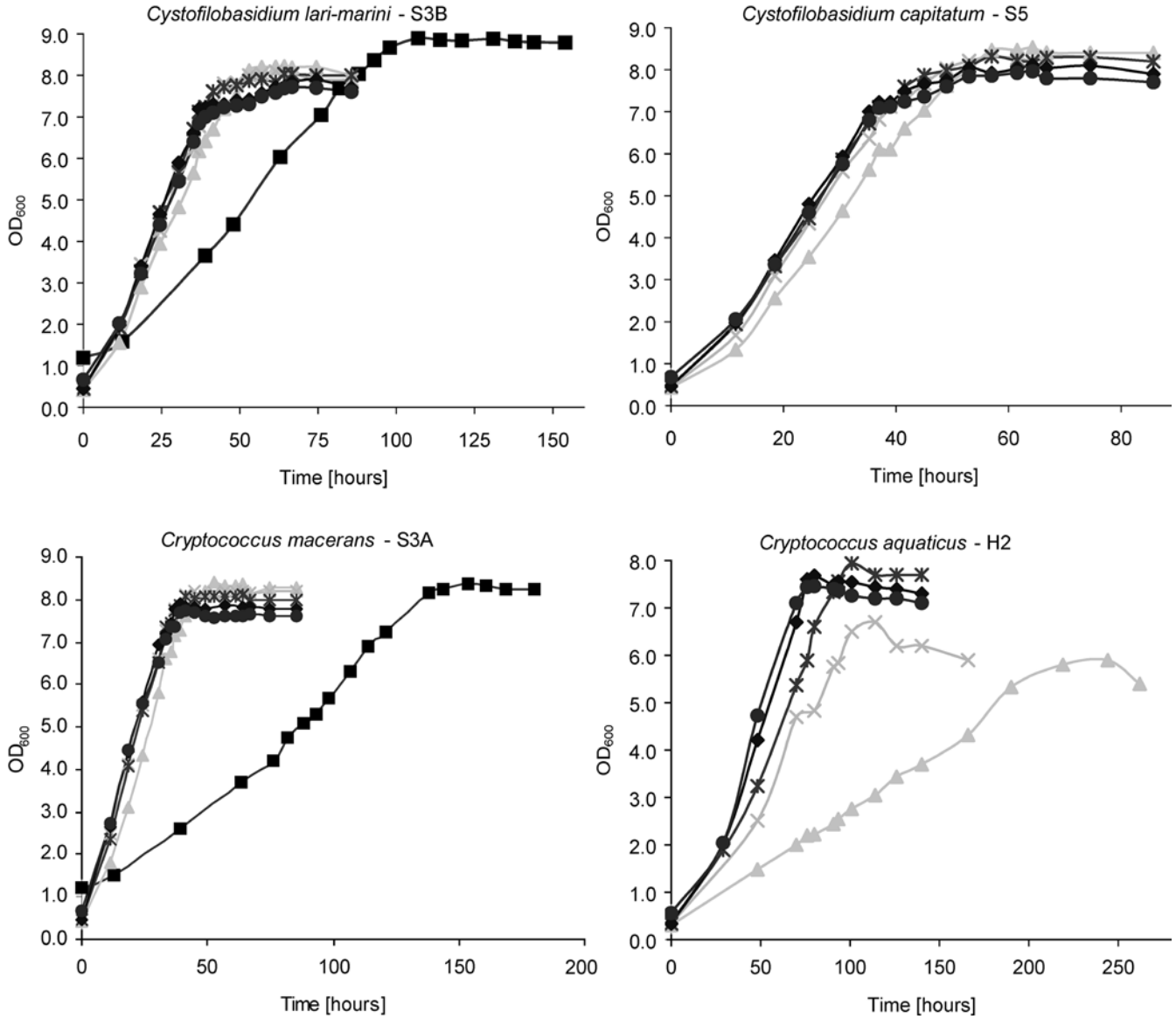

Fig. 1 Effect of initial pH of the culture medium on growth of the yeast isolates. All cultivations were done at 9°C in medium B with 1% pectin as carbon source. Symbols: ■ pH 2.6, ▲ pH 3.2, × pH 3.9, ✳ pH 4.7, ◆ pH 5.6, ● pH 6.4

was performed. Comparison of the determined sequences of 5.8S and D1/D2 domains with sequences from related taxa available in public databases revealed that the eight isolates belong to four different species. The sequences for other areas were not available in the databases. The isolate S3A shared more than 0.99 sequence similarity (*S*) with *Cryptococcus macerans*, while H1 and H2 isolates exhibited 0.997 *S* values with *Cryptococcus aquaticus* strains. The remaining isolates showed *S* values of 0.99 with both *Cystofilobasidium lari-marini* and *Cystofilobasidium capitatum*, which have a close taxonomic relationship, but presented different culture pigmentation, as described by Shu and Sugiyama (1993). *C. lari-marini* presents buff or yellow colored colonies, as was seen for isolates S3B and X, while *C. capitatum* presents reddish colored colonies, which was the case for S1, S4, and S5.

Isolates S3B, S3A, S5, and H2, representing the four yeast species, were chosen for further investigations. When grown at 9°C for 5 days on solid medium A, all the isolates showed well developed colonies, which were smooth and circular, presenting convex elevation and entire margins. Additional morphological characteristics of the isolates are presented in Table 1. All the isolates showed a similar pattern of carbon source utilization for growth, but none had the same pattern. All the strains differed clearly from the reference strain, *C. albicans* in utilizing a wider variety of substrates (Table 2).

Effect of culture conditions on cell growth and polygalacturonase production

The isolates were cultivated in liquid medium B under varying conditions of pH, temperature, and carbon

source to determine the effect on cell growth and polygalacturonase production.

pH

Figure 1 shows growth curves of the isolates at 9°C in the medium adjusted to different pH values that was subsequently not controlled during cultivation. All the isolates exhibited the highest growth rate around pH 5.6–6.4. The *Cystofilobasidium* isolates and *C. macerans* required about 37 h to reach the stationary phase. Further reduction in pH down to 3.2 led to a gradual decrease in growth rate, which was significantly affected at pH 2.6. On the other hand, *C. aquaticus* H2 had slower growth requiring a minimum of 76 h to reach the stationary phase and growth was also more sensitive to decrease in pH (Fig. 1). The pH in the medium increased up to values between 6 and 7 in all cultivations except those performed at the lowest pH values corresponding to poor cell growth, where the pH increased by less than half a unit.

Figure 2 shows the maximal polygalacturonase activity produced by the yeasts grown under different pH conditions. The highest enzyme production was attained for *C. lari-marini* S3B at pH 3.2. Interestingly, maximal yield of enzyme by *C. macerans* was obtained at pH 2.6 but after 5 days of cultivation. *C. aquaticus* H2 produced very low enzyme levels at all pH values.

Temperature

All four strains could grow at 2°C on solid medium A. For determination of optimum temperature of growth, the pH of the medium was set to the value optimal for enzyme production (Fig. 2), i.e., 3.9 for *C. aquaticus* H2 and *C. capitatum* S5, 3.2 for *C. lari-marini* S3B and 2.6 for *C. macerans* S3A. The optimal

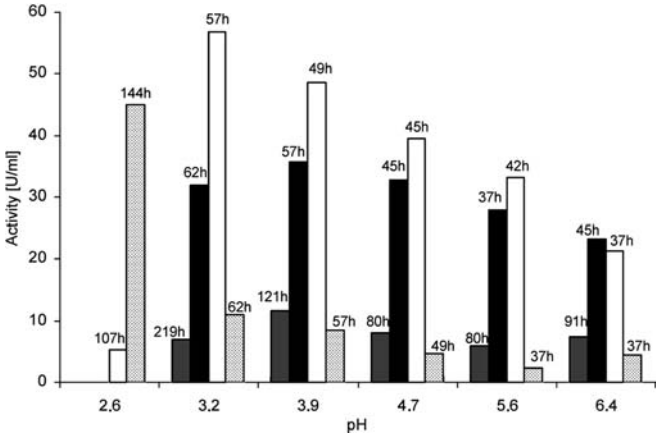

Fig. 2 Maximal polygalacturonase activity produced by the yeast isolates; *C. aquaticus* H2 (*dark shaded bars*), *C. capitatum* S5 (*black bars*), *C. lari-marini* S3B (*white bars*), *C. macerans* S3A (*light shaded bars*), grown in medium with varying initial pH. The cultivation time at which these values were reached is given in hours above each column

temperature of growth was about 18°C for *C. lari-marini* sp. and *C. macerans* S3A, 14°C for *C. capitatum* S5, and 9°C for *C. aquaticus* (Fig. 3). The *Cystofilobasidium* strains reached stationary phase in 42 h while *C. macerans* and *C. aquaticus* in 72 h and 101 h, respectively. Practically no growth was detected for *Cystofilobasidium* species at 30°C, for *C. macerans* at 25°C, and for *C. aquaticus* H2 already around 18°C. The effect of cultivation temperature on production of polygalacturonase activity is seen in Fig. 4. Highest enzyme levels (75 U/ml) were attained for *C. lari-marini* S3B at 14°C.

Carbon source

Yet another parameter that was varied during cultivation was the carbon source. The media were supplemented with 1% (w/v) pectin, 1% (w/v) glucose, and both 1% (w/v) glucose and 1% (w/v) pectin, respectively, and cultivation was performed at 9°C and at the respective optimal pH for enzyme production. In accordance with the observations in Table 2, all the isolates were able to grow in the medium with only glucose, although the growth of *C. capitatum* was significantly lower (Table 3). However, the enzyme production was almost negligible. Addition of glucose to the pectin-containing medium resulted in a higher cell density but enzyme production was reduced except for *C. aquaticus* H2. In this case the cell density was much higher than with only pectin as carbon source (Table 3).

Profile of pectinase activities produced by the cold-adapted yeasts

The presence of isoenzymes of polygalacturonase produced by the four yeast isolates was determined by activity staining of an isoelectric focusing gel. Both *Cystofilobasidium* strains produced four isoenzymes, all having isoelectric point above pH 5. Each of the *Cryptococcus* strains produced one polygalacturonase with a pI value in the basic region and *C. macerans* S3A produced, in addition, three polygalacturonases with their pI values in the acidic region.

Besides polygalacturonase, presence of other pectin-degrading enzymes in the culture supernatant of all the four isolates was investigated. All strains except *C. aquaticus* H2 showed clear pectin lyase activity. On the other hand, *C. macerans* S3A was the only isolate showing positive pectate lyase activity in the presence of $CaCl_2$; no activity was detected without the calcium salt. In all the above cases, higher enzyme activity was obtained at pH 7.6 than at pH 5.0.

Differences were also observed among the isolates regarding pectin esterase activity. *C. aquaticus* H2 showed clear pectin esterase activity, reducing the pH of the reaction medium from 7 to 4 in 2 h, while *C. macerans* S3A had marginal activity, needing 3 days to attain similar pH reduction. The *Cystofilobasidium* strains

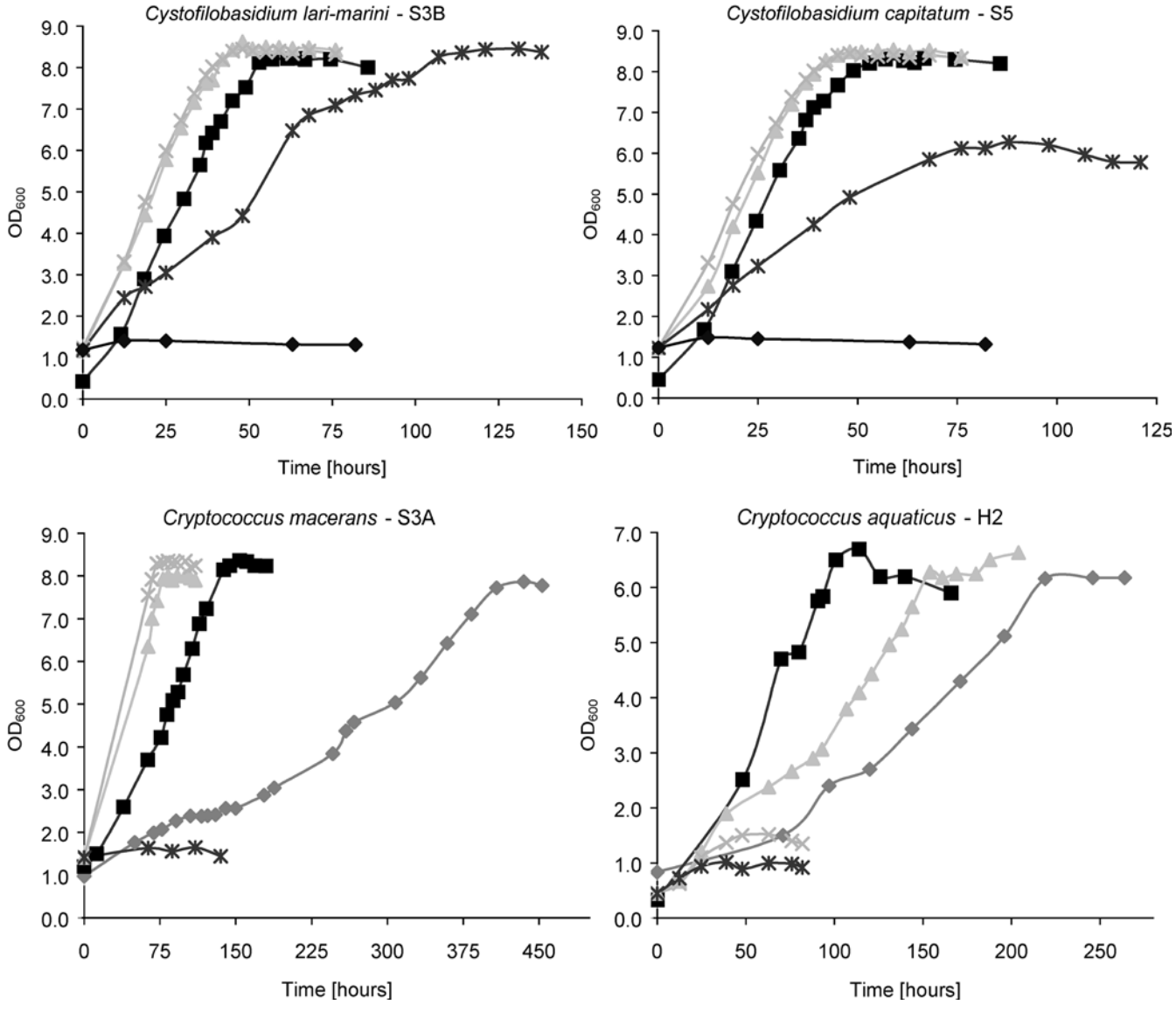

Fig. 3 Effect of temperature on growth of the four yeast isolates. The pH was set to 3.9 for *C. aquaticus* H2 and *C. capitatum* S5, 3.2 for *C. lari-marini* S3B, and 2.6 for *C. macerans* S3A. All cultivations were done in medium B with 1% pectin (9% esterified) as carbon source. Symbols: ◆ 4°C, ■ 9°C, ▲ 14°C, × 18.5°C, ✳ 25°C

showed no sign of pectin esterase production under any of the pH conditions used for the assay.

Preliminary characterization of polygalacturonase activity

A preliminary evaluation of the effect of pH and temperature on activity and stability of polygalacturonases in the cell-free culture supernatants was done. The enzyme activity of both *Cystofilobasidium* strains exhibited an optimum at pH 5, while that of the *Cryptococcus* strains occurred at pH 4 (Fig. 5). No activity was detected at pH 3 and pH 7 in all cases. However, when the enzyme samples were stored at

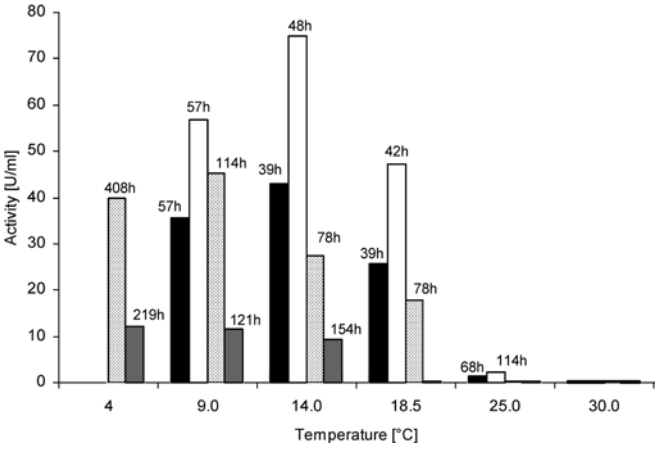

Fig. 4 Polygalacturonase activity produced during cultivation of yeast isolates; *C. aquaticus* H2 (*dark shaded bars*), *C. capitatum* S5 (*black bars*), *C. lari-marini* S3B (*white bars*), *C. macerans* S3A (*light shaded bars*), at different temperatures. The time at which maximal enzyme production was reached is given in hours above each column

Table 3 Effect of carbon source on cellgrowth and polygalacturonase production during cultivation of the cold-adaptedyeasts

All cultivations were done at 9°C in medium B. The pH was set to 3.9 for *C. aquaticus* H2 and *C. capitatum* S5, 3.2 for *C. lari-marini* S3B and 2.6 for *C. macerans* S3A

Isolate	Carbonsource					
	1% glucose		1% pectin		1% glucose + 1% pectin	
	(OD_{600})	(U/ml)	(OD_{600})	(U/ml)	(OD_{600})	(U/ml)
Cystofilobasidium lari-marini S3B	8.1	0.5	7.7	56.8	8.6	40.6
Cystofilobasidium capitatum S5	1.3	0.1	6.9	44.6	7.5	24.9
Cryptococcus macerans S3A	7.5	0.4	7.7	35.7	8.6	15.2
Cryptococcus aquaticus H2	7.5	0.3	5.2	11.1	7.5	15.3

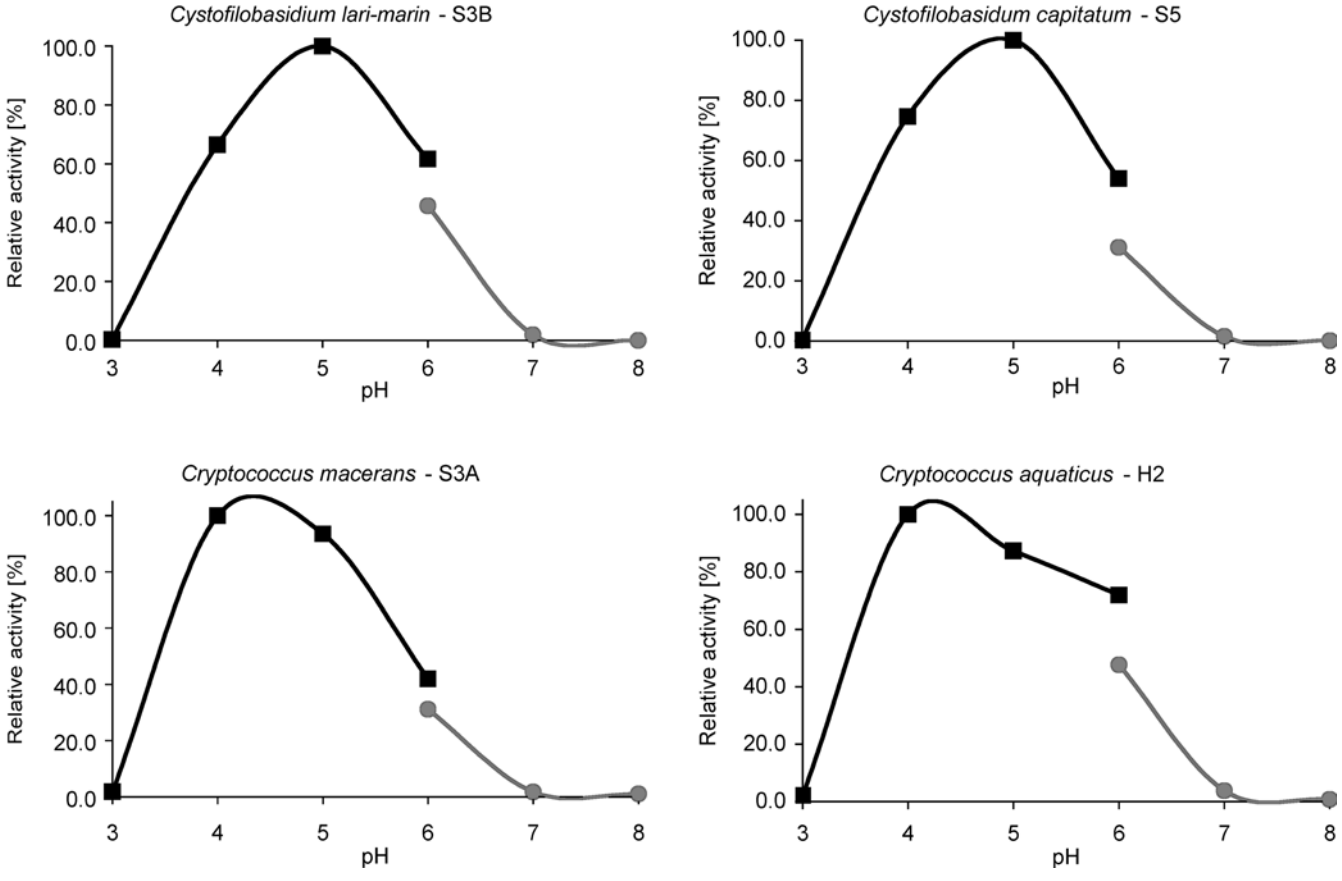

Fig. 5 Effect of pH on the polygalacturonase activity from yeast isolates. The reaction was performed at 23°C for 10 min using culture supernatant. The buffers used were 0.1 M sodium citrate in the pH range 3–6 (■), and 0.1 M potassium phosphate buffer at pH 6–8 (●)

different pH values for 1 h at 10°C and the assay performed under standard conditions, the activity was found to be fully stable over a broad range of pH (at least for pH 4–12).

Optimal temperature of the polygalacturonase activity was determined at pH 4.9. The *Cystofilobasidium* sp. enzymes exhibited maximal activity at 40°C, and *Cryptococcus* sp. enzymes at 50°C (Fig. 6). The former also had higher relative activity at lower temperatures than those from *Cryptococcus* strains, e.g., 18% versus 8% of the maximal activity at 2°C (Fig. 6).

Stability of the enzyme activity towards temperature was determined by incubating undiluted samples of culture supernatant at a particular temperature for 1 h at pH ~5 for *C. aquaticus* H2 and at pH ~7 for the other strains, followed by measuring the activity under standard conditions. Figure 6 shows that *Cystofilobasidium* sp. and *C. aquaticus* H2 enzymes started to lose activity at 30°C and were totally inactive at 50°C, while *C. macerans* S3A retained about 90% activity at 40°C followed by rapid loss to 15% of the original activity at 50°C, and no activity at 60°C.

Discussion

The four yeast strains isolated in this study belong to the order Cystofilobasidiales, which is a group of psychrophilic basidiomycetes (DePriest et al. 2000). Analysis of rDNA sequences allowed us to conclude that S3A is closely related to *Cryptococcus macerans*, H1 and H2 to

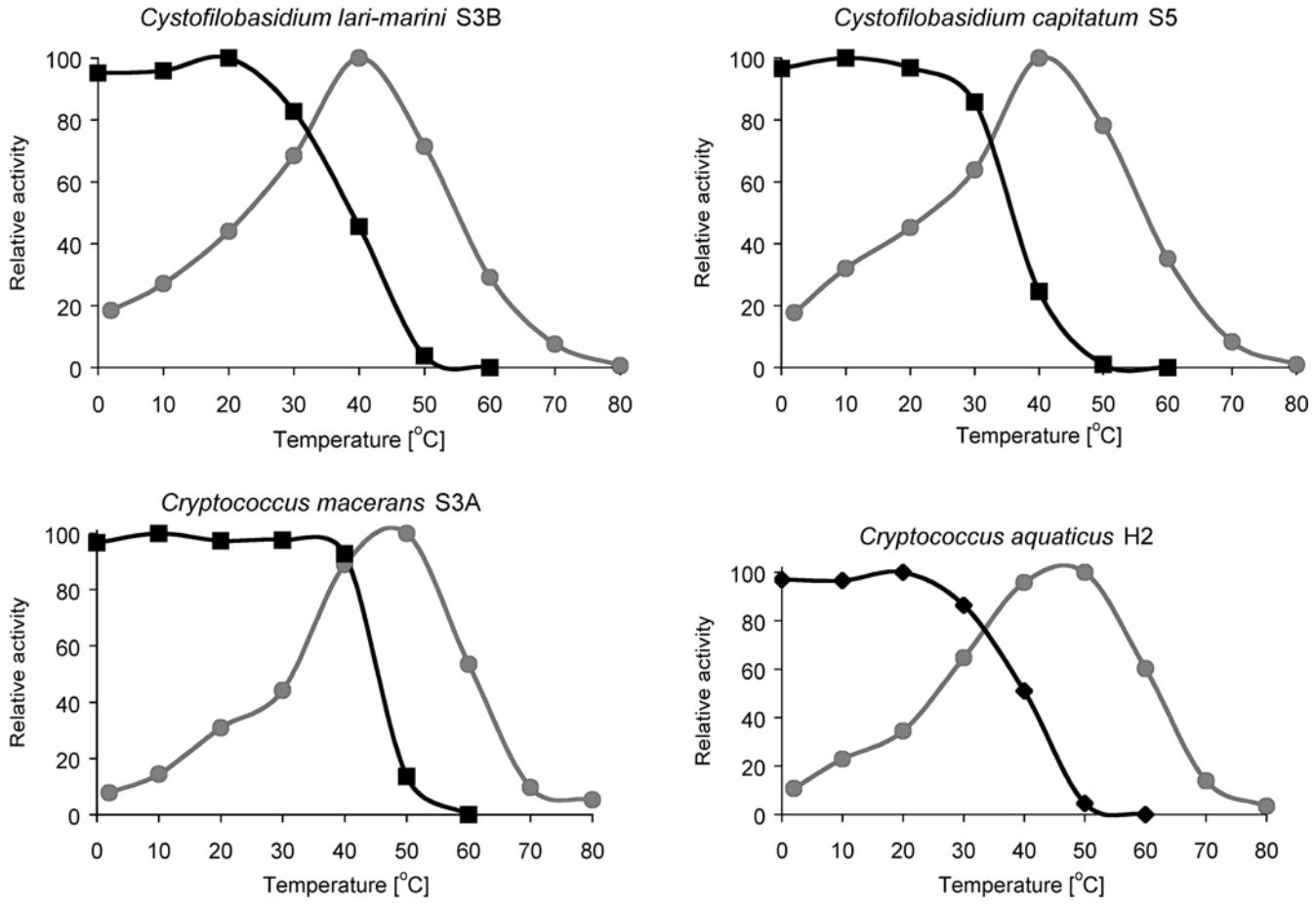

Fig. 6 Effect of temperature on the activity (●) and stability (■) of polygalacturonase in the culture supernatant of the yeast isolates. The effect on activity was determined by performing the reaction at a particular temperature for 10 min in 0.1 M potassium acetate buffer at pH 4.9. For stability measurements, residual activity was determined under standard conditions at 23°C and pH 4.9, after 1 h incubation at a particular temperature

Cryptococcus aquaticus, and finally S1, S3B, S4, S5, and X to both *C. capitatum* and *C. lari-marini*. In spite of different culture pigmentation and some other differences that we found (Tables 1 and 2), a close relationship has been observed between *Cystofilobasidium capitatum* and *Cystofilobasidium lari-marini* in molecular phylogenetic studies using rDNA sequence analysis. Recent taxonomic studies on the genus *Cystofilobasidium* suggested that *C. lari-marini* should be regarded as a synonym of *C. capitatum* and the differences in pigmentation must therefore be considered to be a variable characteristic within the *C. capitatum* species. Yeast species have been known to sometimes give rise to colorless variants (Sampaio et al. 2001).

Pectinase-producing yeasts may be discerned as two types – those that cannot use pectic substances or their hydrolysis products as carbon sources, and those that can. The enzymes from the species of the former type, such as *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*, have been studied more extensively (Blanco et al. 1999). The yeasts studied here belong to the latter type. Pectinases from these yeasts are still relatively unexplored and, to our knowledge, there are no reports on pectinases from any *Cystofilobasidium* or *C. aquaticus* strains. Furthermore, there has only been one report on a polygalacturonase from a cold-adapted organism, i.e., from the psychrophilic fungus *Sclerotinia borealis* (Takasawa et al. 1997).

The growth characteristics of the *Cystofilobasidium* isolates and *Cryptococcus macerans* seemed to be much more similar to each other than to *C. aquaticus* (Fell et al. 1999). Growth under varying temperature conditions suggested the former to be psychrotrophs, while *C. aquaticus* seemed to be at the border between psychrotrophs and psychrophiles according to the classification of the two groups by Russell (1990). The growth of *C. aquaticus* was optimal at 9°C in contrast to 14–18°C for the other isolates, and was also more sensitive to decrease in pH. It also exhibited lowest growth rate and levels of polygalacturonase activity (Figs. 1, 2, 3 and 4).

The yeast isolates could accept a wide range of carbon sources for growth (Table 2); however, pectin is required as an inducer for polygalacturonase production. Almost no polygalacturonase production was attained with only glucose as carbon source (Table 3). Adding glucose along with pectin to the culture medium has been known to repress production of polygalactu-

ronase in certain yeast species including a *Cryptococcus* sp. related to the isolates in this study (Blanco et al. 1999; Federici and Petruccioli 1985). This was also the case for three of the isolates; however, *C. aquaticus* H2 showed higher enzyme production than in the medium with only pectin. The latter effect is probably related to the much higher density of the enzyme-producing H2 cells achieved in the presence of glucose (Table 3), which may compensate for the repressive effect of the sugar on polygalacturonase production.

Cystofilobasidium isolates appeared to be quite similar to each other in terms of the presence of enzyme activities belonging to the pectinase group, and also the polygalacturonase isoenzymes. The two *Cryptococcus* isolates showed definite differences in these aspects. A preliminary evaluation of the activity features of the polygalacturonases was done using culture supernatant of the isolates for the enzyme assays. Interference, if any, by the medium components on the activity profiles of the enzymes should be notably reduced due to dilution of the sample (at least 10-fold) in the assay mixture. The polygalacturonases produced by the cold-adapted yeasts exhibit optimal activity in the lower pH range (Fig. 5), as required for processing of most fruit juices. Furthermore the enzymes, particularly those from *Cystofilobasidium* isolates, possess significant activity at very low temperatures and are inactive at 50–60°C (Fig. 3). These features are useful for low-temperature processing and for terminating the enzyme function subsequent to the processing stage. As the activity and stability profiles shown in Figs. 5 and 6 represent cumulative features of the polygalacturonase isoenzymes or that of the most predominant one, investigation of the individual isoenzymes is required to reveal any differences in their properties and to select the most suitable catalyst for the process application.

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