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ORIGINAL PAPER

Effect of temperature on growth parameters of psychrophilic bacteria and yeasts

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Abstract Three bacterial (*Pedobacter heparinus*, *Pedobacter piscium*, *Pedobacter cryoconitis*) and three yeast strains (*Saccharomyces cerevisiae*, *Leucosporidiella creatinivora*, *Rhodotorula glacialis*) of different thermal classes (mesophiles and psychrophiles) were tested for the effect of temperature on a range of growth parameters, including optical density, viable cell numbers, and cell dry mass, in order to determine the temperature conditions under which maximum biomass formation is obtained. Maximum values of growth parameters obtained at the stationary growth phase of the strains were used for statistical calculation. Temperature had a significant ($P \leq 0.05$) effect on all growth parameters for each strain; correlations between the growth parameters were significant ($P \leq 0.05$–0.01). The maximum growth temperature or the temperature at which microbial growth was fastest was in no case the temperature at which the investigated strains produced the highest amount of biomass. All tested psychrophilic bacteria and yeast strains produced highest amounts of cells (as calculated per mg cell dry mass or per OD_{600} unit) at 1°C, while cell numbers of mesophiles were highest at 20°C. Thus, cultivation temperatures close to the maximum growth temperature are not appropriate for studying psychrophiles.

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Introduction

Temperature is an important factor in governing microbial growth. Psychrophilic microorganisms are adapted to thrive well at low temperatures close to the freezing point of water (Siddiqui and Cavicchioli 2006; Margesin et al. 2008). Microbial activity of psychrophiles has even been reported at subzero temperatures (Panikov and Sizova 2007). Psychrophiles are often grown close to or at the maximum growth temperature, i.e. temperatures at which growth rates are fastest—the so-called "optimal growth temperature", which lead to the somehow questionable distinction between psychrophiles and psychrotrophs (Russell 1990). However, growth rate may not be as relevant as growth yield (Bakermans and Nealson 2004). It has been frequently observed that the growth yield and microbial activity of psychrophilic microorganisms are higher at low temperatures compared to temperatures close to the maximum temperature of growth (Margesin and Schinner 1992; Feller et al.1996; Buchon et al. 2000; Kato et al. 2001; Bergauer et al. 2005). This has been usually explained as successful microbial adaptation to the natural cold environment.

In this study, bacteria and yeasts of different thermal classes (mesophiles and psychrophiles) were tested for the effect of temperature on a range of growth parameters, including optical density, viable cell numbers, and cell dry mass, in order to determine the temperature conditions under which maximum biomass formation is obtained and to determine the relations between the growth parameters tested.

Materials and methods

Microbial strains

The bacterial and yeast strains used in this study, differing in their growth temperature range, are described in Table 1. In this study, the term "psychrophilic" is used for strains able to grow well at 1°C.

Culture media and cultivation

All strains were cultured in a complex medium. Bacterial strains were grown in a pH-neutral complex medium (Standard I) composed of (all figures are g/l) peptone from meat (7.8), peptone from caseine (7.8), yeast extract (3.0), NaCl (6.0), and glucose (1.0). Yeast strains were grown in Sabouraud-2% glucose medium (Merck 49354).

Strains were cultured in 100-ml Erlenmeyer flasks containing 25 ml of bacterial or yeast complex medium (see above), inoculated with a preculture grown in the same medium to give an initial OD_{600} of ca. 0.05. Each strain was cultured over the whole growth temperature range (in 10°C steps; see Table 1) at 200 rpm until the stationary growth phase was reached. At 24-h intervals, three flasks (= replicates) were taken per strain and temperature to monitor growth parameters. The incubation time needed for each strain to reach the stationary growth phase is indicated in Table 1; however, monitoring of growth was also followed during and after this growth phase to ensure the detection of maximum values of biomass formation.

Growth parameters

Culture turbidity (optical density) was measured spectrophotometrically a 600 nm (OD_{600}).

Numbers of viable cells were determined by the plate count method on the bacterial or yeast medium as described above, solidified with agar (15 g/l). Colony-forming units (cfu) were counted after 2 days at 25°C (*Pedobacter heparinus*, *Saccharomyces cerevisiae*), 3 days at 20°C (*Pedobacter piscium*, *P. heparinus*, *Leucosporidiella creatinivora*), or after 5 days at 15°C (*Rhodotorula glacialis*). Only plates containing 30–300 colonies were used for statistically valid enumeration (Koch 1994).

To determine cell dry mass, cultures were centrifuged at 7,500 rpm and 10°C for 15 min, and the pellet was dried overnight at 105°C.

Table 1 Strains and cultivation conditions used in this study

Strain	Reference	Growth temperature range	Cultivation temperature and time[a]
Bacteria			
Pedobacter heparinus DSM 2366	Steyn et al. 1998	10–30°C (mesophilic)	10°C: 6 days
			20°C: 5 days
			30°C: 2 days
Pedobacter piscium DSM 11725	Steyn et al. 1998	1–30°C (psychrophilic)	1°C: 8 days
			10°C: 5 days
			20°C: 2 days
			30°C: 1 day
Pedobacter cryoconitis DSM 14825	Margesin et al. 2003b	1–25°C (psychrophilic)	1°C: 8 days
			10°C: 5 days
			20°C: 2 days
Yeasts			
Saccharomyces cerevisiae		10–30°C (mesophilic)	10°C: 8 days
			20°C: 3 days
			30°C: 3 days
Leucosporidiella creatinivora[b] PB20	Bergauer et al. 2005	1–25°C (psychrophilic)	1°C: 8 days
			10°C: 5 days
			20°C: 3 days
Rhodotorula glacialis CBS10437	Margesin et al. 2007	1–20°C (psychrophilic)	1°C: 7 days
			10°C: 7 days
			20°C: 5 days

[a] Cultivation time required to obtain maximum values in stationary growth phases of the strains

[b] Originally described as *Rhodotorula creatinivora*; this species was renamed by Sampaio et al. (2003) as *Leucosporidiella creatinivora*

Statistical evaluation

Normal distribution of data was confirmed by the Kolmogorov–Smirnov test. Data were analyzed by ANOVA ($P \leq 0.05$), followed by multiple range analysis (Fisher LSD test, $P \leq 0.05$). Differences between temperatures were considered significant when $P \leq 0.05$. Correlations were analyzed by the Pearson product–moment correlation technique.

Results

Maximum values of growth parameters obtained at the stationary growth phase of the strains were used for statistical calculation and for interpretation of the data. Temperature had a significant ($P \leq 0.05$) effect on all growth parameters (OD_{600}, viable counts, and dry mass) for each strain.

For the bacteria *P. heparinus*, *P. piscium* and *P. cryoconitis*, maximum OD_{600} values were obtained at 20, 1–20, and 10°C, respectively (Fig. 1, Table 2). For the yeasts *S. cerevisiae*, *L. creatinivora* and *Rh. glacialis*, these values were at 20, 1, and 1°C, respectively (Fig. 1, Table 2). The trends noted with OD_{600} values corresponded well with values for viable counts and dry mass, both for bacteria and yeast strains (Fig. 1, Table 2), which was also confirmed by significant correlations ($P \leq 0.05$–0.01) between the growth parameters. This shows that each of these parameters is appropriate to monitor

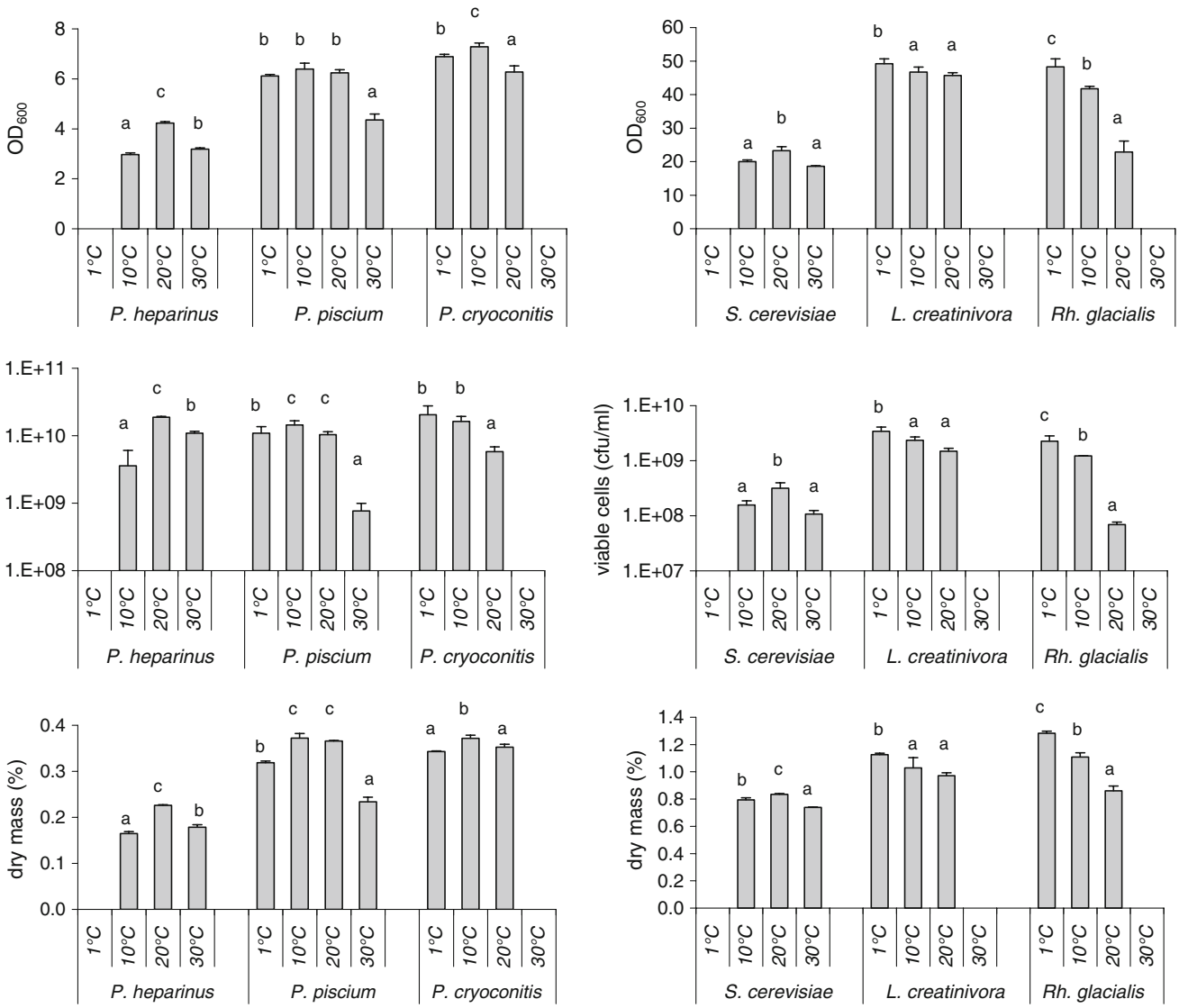

Fig. 1 Effect of temperature on OD_{600} (*top*), viable cell counts (*middle*) and cell dry mass (*bottom*) of bacterial (*left*) and yeast strains (*right*) in the stationary growth phase. Data represent mean values ($n = 3$). Different letters (*a*, *b*, *c*) indicate statistically significant differences (LSD, $P \leq 0.05$) between temperatures for each strain. Values with the same letter are not significantly different

bacterial and yeast growth. Significant correlations were also obtained for each strain, when growth was followed from inoculation until the stationary growth phase was reached (data not shown).

Interestingly, it turned out that maximum values for the growth parameters determined in this study were never obtained at the maximum temperature for growth of the strains, but at considerably lower temperatures. This was especially remarkable for the two psychrophilic yeast strains *L. creatinivora* and *Rh. glacialis*: despite the fact that these strains are able to grow in complex medium at temperatures from 1 to 20°C (*Rh. glacialis*) or from 1 to 25°C (*L. creatinivora*), maximum values for biomass formation (OD_{600}, viable counts, and dry mass) were detected at 1°C, i.e. at least 20°C lower than the maximum temperature for growth. In comparison, mesophilic bacteria and yeasts growing well within 10–30°C (*P. heparinus, S. cerevisiae*) produced highest amounts of biomass at 20°C, i.e. at a temperature lower by 10°C than the maximum growth temperature. *P. piscium*, able to grow over a temperature range of 1–30°C, produced comparable (not significantly different) amounts of biomass at 1–20°C (OD_{600} and viable counts) or 10–20°C (dry mass), and *P. cryoconitis*, growing from 1 to 25°C, showed highest biomass formation at 1–10°C.

These results also show that the temperature for maximum biomass formation tended to be substantially lower for yeasts than for bacterial strains, which corroborates previous findings according to which yeasts are more temperature-sensitive than bacteria (Margesin et al. 2003a, 2005a).

Traditionally, growth parameters are expressed per ml of culture. If these values were transformed and expressed either per mg dry mass or per OD_{600} unit, even lower temperatures turned out to be "optimal" (in terms of highest biomass formation) for microbial growth than those obtained when calculation was done per ml culture (Fig. 2, Table 2). All tested psychrophilic bacteria and yeast strains produced highest amounts of cells (per mg cell dry mass or per OD_{600} unit) at 1°C, while cell numbers of mesophiles were highest at 20°C. Thus, it became even more evident that temperatures close to the maximum growth temperature are not appropriate for studying psychrophiles.

Discussion

All growth parameters measured in this study indicate that psychrophiles, i.e. microorganisms able to grow well around 1°C, are well adapted to low temperatures, as demonstrated by high values for biomass formation at this temperature.

The results obtained in this study clearly show that the maximum growth temperature or the temperature at which microbial growth is fastest (often named the "optimum temperature") was in no case (as tested with three bacterial and three yeast strains) the temperature at which strains produced the highest amount of biomass, as determined with OD_{600}, viable counts and cell dry mass. On the contrary, especially psychrophiles exhibited best performance in terms of biomass production at substantially lower temperatures, i.e. at a temperature lower by 20°C than the so-called optimum "growth temperature". The same effect was noted for mesophilic strains (biomass production was highest at temperatures lower by ca. 10°C than the maximum growth temperature), although to a lower extent than observed with psychrophiles.

Growth properties of cold-adapted microorganisms such as those described in this study (i.e. maximized growth yield at low temperatures) have been reported for a wide range of taxonomic groups of bacteria, e.g. including the

Table 2 Growth temperature range and temperatures for maximum growth rate and maximum values for OD_{600}, viable counts, and cell dry mass (obtained in stationary growth phase) of the investigated bacteria and yeasts, summarizing results shown in Figs. 1 and 2

Strain	Growth range (°C)	Growth rate (°C)	Maximum values obtained in stationary growth phase (°C)				
			OD_{600}	cfu/ml	Dry mass	cfu/mg dry mass	cfu/OD_{600}
Bacteria							
P. heparinus	10–30	$30 = 20 \gg 10$	20	20	20	20	20
P. piscium	1–30	$20 > 30 > 10 > 1$	$1 = 10 = 20$	$10 = 20$	$10 = 20$	1	1
P. cryoconitis	1–25	$20 > 10 \gg 1$	10	$1 = 10$	10	$1 = 10$	1
Yeasts							
S. cerevisiae	10–30	$30 = 20 \gg 10$	20	20	20	20	20
L. creatinivora	1–25	$20 > 10 > 1$	1	1	1	1	1
Rh. glacialis	1–20	$10 \geq 20 > 1$	1	1	1	1	1

cfu colony forming units

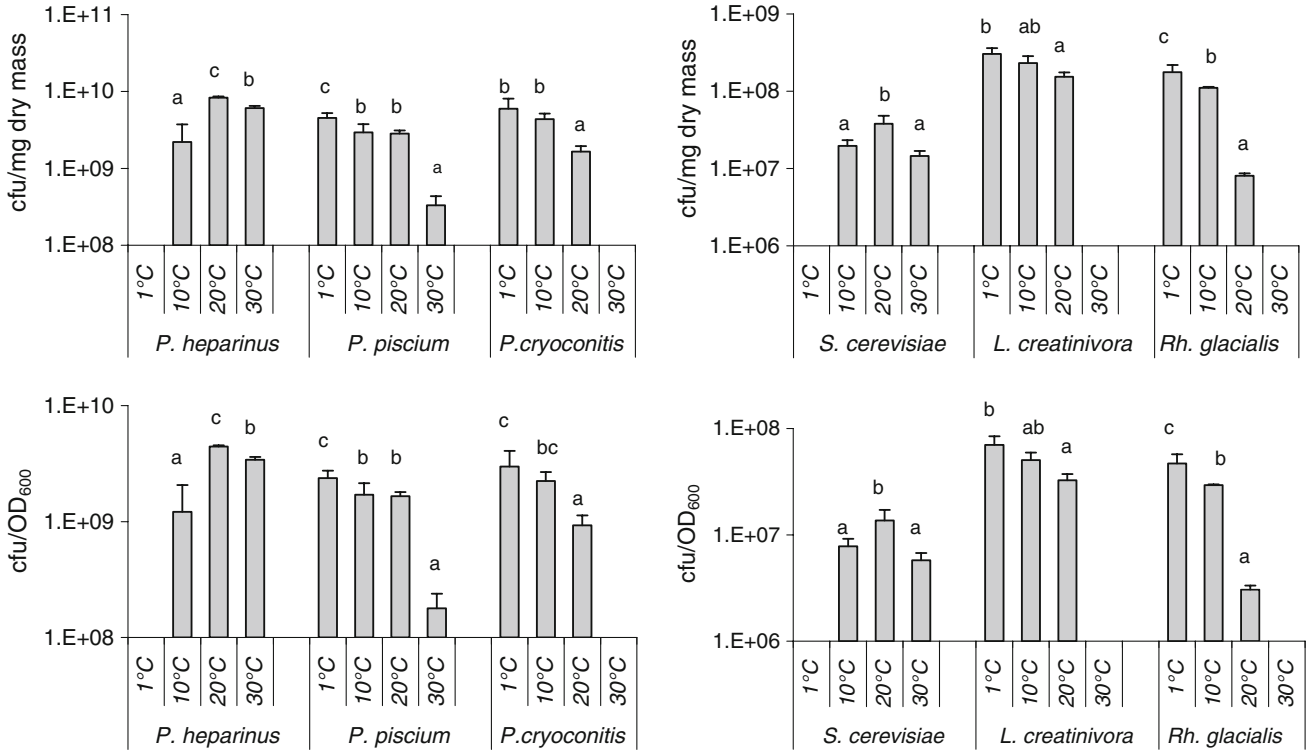

Fig. 2 Effect of temperature on viable cells per dry mass unit (*top*) or per OD_{600} unit (*bottom*) of bacterial (*left*) and yeast strains (*right*) in the stationary growth phase. Data represent mean values ($n = 3$). Different letters (*a*, *b*, *c*) indicate statistically significant differences (LSD, $P \leq 0.05$) between temperatures for each strain. Values with the same letter are not significantly different. Two letters appear when values are not significantly different from two groups (e.g., "*bc*": values are not significantly different from groups *b* and *c* but are significantly different from group *a*)

genera *Pseudomonas*, *Alteromonas*, *Moraxella*, *Psychrobacter*, *Bacillus*, *Arthrobacter* and *Rhodococcus* (Margesin and Schinner 1992; Feller et al. 1994; Guillou and Guespin-Michel 1996; Margesin et al. 2003a, 2005a; Bakermans and Nealson 2004), while comparatively little is know about yeasts (Margesin et al. 2003a, 2005a).

Unfortunately, the temperature resulting in the maximum growth rate is often erroneously termed "optimal growth temperature" (Glansdorff and Xu 2002; Feller and Gerday 2003), although this is a critical temperature due to the deleterious effect of heat (Gerday 2008). It reflects only kinetic effects and occurs above the linear part of the Arrhenius curve, which means that the physiological conditions are not ideal (Gounot and Russell 1999; Glansdorff and Xu 2002; Feller and Gerday 2003), as shown by decreased microbial activity (e.g. enzyme production, biodegradation activity), protein synthesis, membrane permeability, and increased cellular stress (Feller and Gerday 2003; Jaouen et al. 2004; Margesin et al. 2005a, b; D'Amico et al. 2006; Feller 2007). For example, Guillou and Guespin-Michel (1996) reported an increase in the cellular protein degradation rate of *Pseudomonas fluorescens* with increasing temperature, especially above 17°C. *Psychrobacter cryopegella* accomplished to maximize growth yield at low temperatures by streamlining growth processes, including protein and RNA synthesis (Bakermans and Nealson 2004). In addition, microbial growth properties are influenced by a number of factors, such as medium and substrate composition (Chablain et al. 1979) and strain-specific properties.

The ability of psychrophiles to grow slowly at low temperatures may actually be an advantage in nutrient-poor environments, where a rapid exhaustion of available resources would lead to starvation (Russell 1990). So-called "optimal" conditions are never reached in nature. Nonetheless, life under natural conditions is efficient and slow growth rates of psychrophiles at low temperatures are compensated by high growth yield and maximized cellular fitness.

Several studies have shown that temperature has even a more pronounced effect on microbial activity than on growth (Margesin and Schinner 1992; Feller et al. 1994, 1996; Buchon et al. 2000; Huston et al. 2000; Margesin et al. 2005a, b). This is especially important for studies in the field of biotechnology using psychrophiles. Highest yields of psychrophilic cells and their biotechnologically important compounds are generally obtained at cultivation temperatures that correspond to those of the natural

environment of the strains. This should be considered for large-scale production of cold-active compounds as well as for other applied aspects, such as low temperature bioremediation.

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