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**Comparison of the growth kinetics and proteolytic activities
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2 *Chryseobacterium* **species and** *Pseudomonas fluorescens*

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15 Running head: Growth and proteolytic activities of *Chryseobacterium* and
16 *Pseudomonas fluorescens*

17

18 **Abstract:** The effect of temperature on the growth kinetics and proteolytic activity of
19 *Chryseobacterium joostei* and *Chryseobacterium bovis* was determined during this
20 study. The results were compared to the activities of *Pseudomonas fluorescens*
21 which is regarded to be a major food spoilage psychrotolerant microorganism. For
22 the growth studies, cultures were incubated in nutrient broth in a Temperature
23 Gradient Incubator (from 9 to 50 °C) and separately at 4 °C and the optical density
24 was measured at different time intervals. Growth temperature profiles for each
25 organism were constructed. For determination of proteolytic activity, the cultures
26 were incubated in fat-free UHT processed milk in the Temperature Gradient
27 Incubator for 72 hours (temperature range as above). Cell free extracts were used to
28 determine the proteolytic activity using the azocasein method. Results of the growth
29 studies showed that *C. joostei* had the ability to grow over a wider temperature range
30 than *C. joostei* and *Ps. fluorescens* without being affected by changes in the
31 temperature. For the proteolytic activity, *C. joostei* had significantly ($p < 0.001$) higher
32 activity per mg protein at 15.5 °C, followed by *C. bovis* and *Ps. fluorescens*. The
33 results showed that *C. joostei* potentially has an even greater spoilage capacity in
34 milk on the basis of growth rate and proteolytic activity than *Ps. fluorescens*.

35

36 **Key words:** *Chryseobacterium*, *Pseudomonas*, temperature, growth kinetics,
37 proteolytic activity

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39 The genus *Chryseobacterium*, formerly known as *Flavobacterium*, has been
40 isolated from a wide variety of habitats including soil, freshwater, marine
41 environments, humans, hospital equipment, and diseased animals and fish (Hugo et
42 al. 2003; Bernardet et al. 2005, 2011; Hugo and Jooste 2012). The psychrotolerant
43 ability of some of these species provides them with the capacity to grow in, and
44 cause spoilage of, refrigerated food products, especially milk and dairy products
45 (Cousin 1982). Species that have been isolated from milk include: *C. bovis* (Hantsis-
46 Zacharov et al. 2008a), *C. haifense* (Hantsis-Zacharov and Halpern, 2007), *C. joostei*
47 (Hugo et al. 2003) and *C. oranimense* (Hantsis-Zacharov et al. 2008b).

48 The growth and activity of spoilage microorganisms is mostly described and
49 studied as a function of a substrate base and of chemical and physical parameters
50 such as temperature, pH, a_w and gaseous atmosphere (Gram et al. 2002). Even
51 though *Chryseobacterium* spp. have long been recognized as a psychrotolerant food
52 spoilage organisms (Champagne et al. 1994), the comparative spoilage capacity of
53 *Chryseobacterium* in terms of growth and proteolytic activity over a range of
54 temperatures has, however, never been studied in detail before.

55 The production of proteolytic and lipolytic enzymes by *Chryseobacterium* spp.
56 contributes to the spoilage potential of these organisms (Bernardet et al. 2006). Such
57 activity can occur by growth in raw milk or as biofilms in pipelines conveying the raw
58 milk (Jooste and Britz 1986b; Koutzayiotis et al. 1993). Proteolytic enzymes of a
59 *Chryseobacterium* species have been shown to be pasteurisation resistant (Venter et
60 al. 1999). The activity of proteolytic enzymes can cause off-odours and off-flavours in
61 the processed milk as well as gelation of milk and coagulation of milk proteins in long
62 life milks (Harwalker et al. 1993; Tondo et al. 2004). It has also been reported that

the major cause of bitterness in milk can be ascribed to the formation of bitter peptides due to the action of proteolytic enzymes (Huis in't Veld 1996). Datta and Deeth (2003) have also reported that proteolysis of UHT milk, may lead to the development of bitter flavours and an increase of viscosity in this product, with eventual gel formation. Such proteolytic activity by *Chryseobacterium/Flavobacterium* spp. has also been reported to result in the spoilage of butter (Wolochow et al. 1942; Jooste et al. 1986a), creamed rice (Everton et al. 1968) and canned vegetables (Bean and Everton 1969).

In a review by Chen et al. (2003), workers found that it is probable that contaminating bacterial proteases will remain active in processed milk and milk products (even powdered milk) during storage over a broad range of temperatures. Since long life milk products are usually kept at varying ambient temperatures in the retail environment, it was, therefore, deemed important to examine the proteolytic activity of the enzymes produced by psychrotolerant, proteolytic organisms over a wider range of temperatures.

The aim of this study was, therefore, to compare the effect of temperature on the growth rate of two *Chryseobacterium* species to that of a *Pseudomonas fluorescens* strain grown in a broth medium and to determine the minimum, maximum and optimum growth temperatures of these organisms. The proteolytic activities of these species were also compared over a wide range of temperatures.

The test organisms used in this study were *Chryseobacterium bovis* (LMG 34227^T; Hantsis-Zacharov et al. 2008a) and *C. joostei* (UFSBC 256^T; Hugo et al. 2003). Both species were isolated from raw cow's milk and both exhibited protease production in the studies referred to. *Pseudomonas fluorescens* (ATCC[®] 13525TM) was used as the control organism because it is the type strain of the species and

88 proteolytic enzymes from *Ps. fluorescens* are known to cause food spoilage. Master
89 cultures were maintained in freeze-dried form. Freeze dried cultures were reactivated
90 in 10 mL nutrient broth (Oxoid CM0001) and purity was checked by streaking on
91 nutrient agar (Oxoid CM0003). Cultures were incubated at 25 °C for 24–48 h.
92 Working cultures were maintained on nutrient agar slants at 4 °C and were sub-
93 cultured every 2 months.

94 For the determination of the growth kinetics of the organisms, isolates were
95 incubated in 10 mL nutrient broth at 25 °C for 24 h. The 10 mL broth culture was
96 transferred to 100 mL nutrient broth and incubated at 25 °C for 24–48 h. Twenty
97 millilitre volumes of the 100 mL broth culture were used to inoculate 400 mL nutrient
98 broth. Ten millilitres of this broth was transferred immediately after inoculation to
99 sterile Temperature Gradient Incubator (TGI) tubes. The tubes were placed in the
100 TGI (Scientific Industries Inc., New York, USA) consisting of an aluminium bar that
101 was cooled at one end and heated at the other to obtain a stable temperature
102 gradient, following a procedure described previously (du Preez and Toerien, 1978).
103 The bar contained thirty equidistant sample wells on both sides into which L-shaped
104 culture tubes of optically selected glass (40 mL total volume and 17 mm in diameter)
105 were inserted and capped with loose-fitting metal caps. Whereas tubes on the one
106 side contained bacterial cultures, the corresponding tubes on the other side
107 contained water to allow measurement (at least twice during incubation) of the
108 temperature. The bar was rocked through a 30° arc at 60 oscillations min⁻¹ and
109 provided mixing and aeration. The tubes were incubated at temperatures ranging
110 from ca. 9 to 50 °C with 2 ± 0.2 °C increments between the 30 individual positions.
111 The variation in temperature for the individual positions was 0.7 °C in the terminal
112 tubes next to the heated and cooled ends of the aluminium bar. The maximum

variation in the more central tubes was about 0.2 °C. Since the TGI could not attain temperatures lower than ca. 9 °C, inoculated tubes were also placed in a roller drum in a temperature controlled refrigerator at a constant temperature of 4 °C since literature indicated this temperature as the lowest growth temperature applicable to psychrotolerant bacteria (Rhodes 1959; Hugo et al. 2003). This lowest data point ensured an extrapolation point for data analysis. All analyses were done in duplicate and the experiment was repeated once.

The growth of the isolates was measured by means of optical density (OD) measurements in a Biowave C0800 cell density meter (Walden Precision Apparatus Ltd., Cambridgeshire, United Kingdom) at 600 nm. To ensure that disruption of growth was kept to a minimum during measurement, the culture tubes were removed sequentially without stopping the shaker. The first measurement was done after 30 min of incubation at the various temperatures to allow the contents of tubes to attain the incubation temperatures. The temperatures were checked before inoculation. The OD was measured at intervals ranging between 30 min and 1 h for temperatures between ca. 9 and 50 °C and incubated until the maximal OD measurement of 2 was reached. Longer time intervals (24 h) were allowed for measurements at 4 °C.

The maximum specific growth rate at each temperature was determined by linear regression analysis with Microsoft Excel (Microsoft Corporation, WA, USA) of the exponential phase of the growth curve. An Arrhenius model was used to describe the linear relationship between the specific growth rate and temperature (Pirt 1975; du Preez and Toerien 1978) as follows: $\mu_{\max} = Ae^{-(E/RT)}$ where μ is the specific growth rate, A an entropy constant, E the activation energy (temperature coefficient), R the universal gas constant (8.314 J·mol⁻¹·K⁻¹) and T the absolute temperature in K. To calculate the slope (i.e. μ), the following equation was used:

138 $\mu_{\max} = (\ln x_t - \ln x_0)/t$ where t = time in hours.

139 The proteolytic activity was assayed by the azocasein method according to
140 Christen and Marshall (1984) and Deeth et al. (2002) with some modification.
141 Microbial cultures of *C. joostei*, *C. bovis* and *Ps. fluorescens* were individually
142 reactivated in 10 mL nutrient broth for 24 h at 25 °C. Six millilitres of each broth
143 culture was then transferred to 60 mL commercial UHT fat-free milk. The inoculated
144 milk was incubated at 25 °C for 24 h. After incubation, 40 mL of the latter milk culture
145 was used to inoculate 150 mL commercial UHT fat-free milk, of which 10 mL was
146 transferred to sterile test tubes immediately after inoculation. The tubes were
147 sequentially incubated in the TGI for 72 h. Activity at 4 °C was measured by
148 incubating the tubes in a roller drum in a temperature controlled refrigerator at a
149 constant temperature of 4 °C. Samples were analysed for proteolytic activity at 0 and
150 72 h. An uninoculated milk sample was included as control at all temperatures.

151 Cell free extracts were prepared by removing 1 mL of milk from each tube and
152 centrifuging for 15 min at 18 080 x g . The supernatant of each sample was used for
153 the determination of proteolytic activity.

154 For determination of proteolytic activity, 250 μ L of the cell free extract was
155 added to 1 ml 1% azocasein (Sigma-Aldrich A2765; 10 $g \cdot L^{-1}$, dissolved in phosphate
156 buffer, pH 7.2). For the control sample, 250 μ L of phosphate buffer was added in the
157 place of the cell free milk extract. The mixtures were incubated for 1 h at 37 °C. After
158 incubation the reaction was terminated by adding 1 mL of 5% trichloroacetic acid
159 (TCA). The mixture was centrifuged for 15 min at 18 080 x g and the absorbance of
160 the supernatant was read at 345 nm (Christen and Marshall 1984; Deeth et al. 2002).
161 Protein determination was done according to the Biuret method (Herbert et al. 1971).
162 The maximum proteolytic activity ($U \cdot mg^{-1}$) was calculated and the cardinal

temperatures for proteolytic activity were determined for each organism. One unit of proteolytic activity was defined as the amount of enzyme required to produce an absorbance increase at 345 nm of 0.01 within 1 hour under the assay conditions (Deeth et al., 2001).

An analysis of variance (ANOVA) procedure was used to determine statistically significant differences in growth kinetics and protease activity between the two *Chryseobacterium* species and *Ps. fluorescens*. The Tukey-Kramer multiple comparison test ($\alpha = 0.05$) was used to identify differences between the treatment means (NCSS, 2007).

Growth rates and cardinal temperatures of the three organisms are shown in Table 1. *Chryseobacterium joostei* and *Ps. fluorescens* had significantly ($p < 0.001$) higher maximum specific growth rates of 0.94 h^{-1} at $31.5 \text{ }^{\circ}\text{C}$ and of 0.93 h^{-1} at $28.9 \text{ }^{\circ}\text{C}$, respectively, than *C. bovis* with 0.65 h^{-1} at $31.2 \text{ }^{\circ}\text{C}$. All three species were able to grow at $4 \text{ }^{\circ}\text{C}$, which was the minimum temperature evaluated for growth.

Chryseobacterium joostei had a maximum growth temperature of $36.9 \text{ }^{\circ}\text{C}$. When this species was first described by Hugo et al. (2003), it was found that the optimum growth temperature was $25 \text{ }^{\circ}\text{C}$ and that the organism could not grow at 37 or $42 \text{ }^{\circ}\text{C}$. This difference in optimum temperature reported in this study could be ascribed to a more accurate method (temperature gradient incubator) used for determining the growth temperatures in this study.

No previous literature is available relating to the specific growth rate of *Chryseobacterium*, except for a study by Jooste and Fischer (1992). The study was done on the growth rate of *Flavobacterium* sp. Group IIb (Strain SPA 39), an organism that was later reclassified as a member of the genus *Chryseobacterium*. It was found that the latter test strain had a μ_{max} of $0.40 \cdot \text{h}^{-1}$ at $30 \text{ }^{\circ}\text{C}$. This growth rate is

lower than the $0.94 \cdot \text{h}^{-1}$ observed for *C. joostei* during the present study. This difference could be ascribed to either different strains and/or different nutritional media used.

The growth results of *C. bovis* were in accordance with a previous study. It was reported during the initial description of *C. bovis* that growth occurred between 7 and 37 °C, with one strain being able to grow at 41 °C (Hantsis-Zacharov et al. 2008a).

According to its description, *Ps. fluorescens* has the ability to grow at 4 °C, but not at 41 °C. The optimum temperature cited for growth is between 25 and 30 °C (Palleroni 2005). Studies done on the growth rate of *Ps. fluorescens* strains have found the maximum growth rate to differ when grown in different media. A rate of $0.56 \cdot \text{h}^{-1}$ at 25 °C was reported by Gügi et al. (1991) in citrate mineral salts medium supplemented with 10% liquid skim milk. Jooste and Fischer (1992) reported a specific growth rate of $0.483 \cdot \text{h}^{-1}$ at 25 °C when grown in skim milk. Makhzoum et al. (1995) reported a specific growth rate at 27 °C of $0.48 \cdot \text{h}^{-1}$ in peptone water and $0.41 \cdot \text{h}^{-1}$ in minimal medium. These differences could be ascribed to strain variability and/or nutritional composition of the media.

Arrhenius plots of the three organisms are shown in Fig. 1. The low activation energy values (Table 2) of *C. joostei* in the temperature range of 22.3 to 38.9 °C, that of *C. bovis* in the range of 17.8 to 30.7 °C and that of *Ps. fluorescens* in the range of 19.0 to 27.7 °C, indicate that the three test strains were least sensitive to temperature changes in these respective temperature ranges.

Chryseobacterium bovis showed the greatest response to temperature change in the range of 9.9 to 16.7 °C. The high overall activation energy also indicated that *C. bovis* was the most sensitive to a change in temperature. *Chryseobacterium*

joostei had the ability to grow over a wider temperature range without being affected by changes in the temperature.

The cardinal temperatures for proteolytic activity ($\text{U} \cdot \text{mg}^{-1}$ of crude enzyme) for each organism are given in Table 3. Amongst the three organisms, *C. joostei* had the significantly ($p < 0.001$) highest activity of $1.15 \text{ U} \cdot \text{mg}^{-1}$ at 15.5°C , followed by *C. bovis* ($0.41 \text{ U} \cdot \text{mg}^{-1}$) and *Ps. fluorescens* ($0.18 \text{ U} \cdot \text{mg}^{-1}$). *Pseudomonas fluorescens* had the highest optimum temperature (23.7°C) for proteolytic activity, followed by *C. bovis* (18.0°C) and *C. joostei* the lowest (15.5°C). All three organisms exhibited some protease activity at 4°C , while the maximum temperatures for protease activity by *C. joostei*, *C. bovis* and *Ps. fluorescens* were 53.4 , 49.7 and 48.5°C , respectively.

In the limited literature available on proteolytic activity by *Flavobacterium* (*Chryseobacterium*), Roussis et al. (1999) reported that *Flavobacterium* MTR3 proteases were active at 32 to 45°C , and exhibited considerable activity at 7°C . Venter et al. (1999) found that *Chryseobacterium indologenes* lx9a exhibited optimum proteolytic activity at a temperature of 50°C at pH 7.5 . The activity decreased rapidly as the temperature increased above the latter level. Optimum protease activity by *Pseudomonas* usually occurs at 17.5 to 30°C (Braun and Sutherland 2003).

The breakdown of the milk proteins by enzymes results in the formation of bitter peptides and decomposition of the amino acids to produce putrid flavours (Shipe et al. 1978). In a previous study (Bekker 2011), these results were confirmed since putrid odours were only observed for the samples inoculated with *C. joostei*.

The growth rates and levels of proteolytic activity by bacteria can be an indication of the extent and rate of spoilage that may occur in a specific food product. This study has not concentrated on growth and proteolytic activity solely at

refrigeration temperatures. Raw milk pipelines are exposed to a variety of ambient temperatures and contaminating organisms in biofilms, in poorly sanitised pipelines, could grow and produce proteases. Long-life milk products that may contain residual heat-tolerant proteases are offered for sale in retail outlets, usually at ambient temperatures, and spoilage can take place under such conditions.

When considering the growth rate and proteolytic activity, the results of this study clearly indicate that *C. joostei* may have a greater spoilage potential in milk than *Ps. fluorescens* at optimum temperatures.

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Fig. 1. Arrhenius plots of the maximum specific growth rates ($\mu_{\max}$) of *C. joostei* (I; n=31), *C. bovis* (II; n=25) and *Ps. fluorescens* (III; n=26) as a function of cultivation temperature. A to I indicating the activation energy zones (Table 2).

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Table 1. The cardinal temperatures and maximum specific growth rates of *C. joostei*, *C. bovis* and *Ps. fluorescens* at the optimum temperature.

Bacterial test strain	Temperature (°C)			Maximum specific growth rate ($\mu_{\max}$) (n=4)
	Minimum ¹	Optimum	Maximum ²	
<i>C. joostei</i>	4	31.5	36.93	0.94 ^b ± 0.02
<i>C. bovis</i>	4	31.20	37.33	0.65 ^a ± 0.01
<i>Ps. fluorescens</i>	4	28.91	36.99	0.93 ^b ± 0.27
Significance level				p < 0.001

¹ Minimum temperature evaluated for growth
² Maximum temperature at which growth still occurred
³ Means with different superscripts in the same column differ significantly

Table 2. Activation energies for *C. joostei*, *C. bovis* and *Ps. fluorescens* calculated from Arrhenius plots in Figure 1.

Bacterial test strain	Temperature range (°C)	Zone	Slope	Activation energy (kJ/mol)
<i>C. joostei</i>	8.9 – 22.3	A – B	-8724	72.53
	22.3 – 28.9	B – C	-2424	20.15
<i>C. bovis</i>	9.9 – 16.7	D – E	-18177	151.12
	17.8 – 30.7	E – F	-6787	56.43
<i>Ps. fluorescens</i>	9.9 – 18.2	G – H	-13603	113.10
	19.0 – 27.7	H – I	-6228	51.78

Table 3. The cardinal temperatures for proteolytic activity obtained from crude cell-free extracts of *C. joostei*, *C. bovis* and *Ps. fluorescens* at the optimum temperature.

Bacterial test strain	Temperature (°C)			Maximum protease activity (U mg ⁻¹ protein) (n=4)
	Minimum ¹	Optimum	Maximum	
<i>C. joostei</i>	4	15.5	53.4	1.15 ^c ± 0.07
<i>C. bovis</i>	4	18.0	49.7	0.41 ^b ± 0.02
<i>Ps. fluorescens</i>	4	23.7	48.5	0.18 ^a ± 0.09
Significance level				p < 0.001

¹ Minimum temperature evaluated for protease activity

² Means with different superscripts in the same column differ significantly