

## Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers

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**The strain *Chryseobacterium* sp. kr6 shown to be useful for biotechnological purposes such as hydrolysis of poultry feathers and de-hairing of bovine pelts. The effect of temperature, initial pH and media composition on protease production by this keratinolytic strain was studied. The enzyme was produced between 25 and 37°C, with maximum activity and yield at 30°C. When protease production was tested in media with different initial pH, maximum activity was observed when cultivation was carried out at 30°C and initial pH ranging from 6.0 to 8.0. Higher activity was observed when feathers or feather meal were used as growth substrates, followed by soybean meal. The addition of carbohydrates or surfactants to feather broth resulted in decrease in keratinolytic activity.**

Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates. Proteases constitute an important fraction of the global enzyme sales, and a relevant part of this market is accounted by bacterial proteases (Rao et al. 1998). Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates, and generally on a broad range of protein substrates (Lin et al. 1995). These enzymes have been studied for de-hairing processes in the leather industry (Raju et al. 1996) and hydrolysis of feather keratin (Lin et al. 1995), which is a by-product generated in huge amounts by the poultry industry. Discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low

nutritional value product (Wang and Parsons, 1997). Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed (Williams et al. 1991). In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids (Dalev and Neitchev, 1991; Choi and Nelson, 1996).

Cultivation conditions are essential in successful production of an enzyme, and optimization of parameters such as pH, temperature and media composition is important in developing the cultivation process. Despite all the work that has been done on production of proteolytic enzymes, relatively little information is available on keratinases (Wang and Shih, 1999). This is even more the case for keratinases of Gram-negative bacteria (Sangali and Brandelli, 2000). The *Chryseobacterium* sp. strain kr6 was isolated from waste of a poultry industry and was capable to completely degrade chicken feathers. Its extracellular keratinase is a metalloprotease with great potential for biotechnological applications (Riffel et al. 2003a). This work describes the effect of temperature, initial pH and substrates on keratinase production by *Chryseobacterium* sp. kr6 during growth on native feathers.

### MATERIALS AND METHODS

#### Bacterial strain and media

The bacterium *Chryseobacterium* sp. strain kr6 (LBM 9006) was isolated and characterized as described by Riffel

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et al. (2003a). Brain heart infusion broth (BHI, Difco) medium was used for maintenance of strain with 20% (v/v) glycerol at -20°C.

### Enzyme activity and protein determination

The proteolytic activity was monitored as describe previously (Sangali and Brandelli, 2000). Briefly, 100 µl of enzyme extract was added to 400 µl of 10 mg ml<sup>-1</sup> azokeratin in 0.1 M phosphate pH 8.0. The reaction mixture was incubated for 15 min at 50°C and then stopped by addition of trichloroacetic acid to reach a final concentration of 10% (w/v). Absorbance at 440 nm was measured after centrifugation at 10,000 g for 5 min. One unit of activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm in 15 min at 50°C. Azokeratin was synthesized as described by Riffel et al. (2003b). The protein concentration was measured by the Folin phenol reagent method (Lowry et al. 1951).

### Growth conditions

The inoculum was prepared by streaking a feather meal agar (10 g l<sup>-1</sup> feather meal, 15 g l<sup>-1</sup> agar, 5 g l<sup>-1</sup> NaCl, 0.4 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 0.3 g l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>) plate with cells from a stock culture of kr6 strain. A single colony was transferred to 10 ml of feather meal broth (FMB; 10 g l<sup>-1</sup> feather meal, 5 g l<sup>-1</sup> NaCl, 0.4 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 0.3 g l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>), incubated at 30°C until the cell density reached about 10<sup>8</sup> cells ml<sup>-1</sup>. Then 1.0 ml was transferred to 500 ml Erlenmeyer flasks containing 100 ml of feather keratin broth (FKB; washed, whole feathers are used instead of feather meal) and cultivated in a shaker at 180 cycles min<sup>-1</sup> for desired times. Bacterial growth was monitored by viable cell counts (CFU ml<sup>-1</sup>) and related to biomass dry weight with a calibration curve. The culture supernatants were used for assays of proteolytic activity.

The influence of temperature on growth and production of protease was studied at 25°C, 30°C, 37°C and 42°C. Protease production was also investigated in FKB medium with initial pH adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0. The cultivation was carried out at 30°C.

When different protein sources were used as substrates, a concentration of 10 g l<sup>-1</sup> was used, replacing the raw feathers. The effect of carbohydrates on keratinase production was tested by adding 10 g l<sup>-1</sup> of glucose, sucrose or lactose to FKB. The effect of surfactants was tested by the addition of 10 g l<sup>-1</sup> of Tween 80 or Triton X-100.

All experiments were done in triplicate.

### Kinetic parameters

Kinetic data were analyzed as described elsewhere (Sinclair and Cantero, 1990; Doran, 1995). The maximal specific growth rate ( $\mu_{max}$ ) was calculated from the equation:

$$\mu = dX/Xdt$$

where  $X$  is the cell concentration in g l<sup>-1</sup> and  $t$  the time (h).

Specific production rates ( $q_p$ ), defined as the amount of activity produced per gram of cell dry mass and per hour (U g<sup>-1</sup> h<sup>-1</sup>) were calculated during growth from the relation:

$$q_p = dP/Xdt$$

where  $P$  is the activity expressed in U l<sup>-1</sup>,  $X$  the biomass concentration on g l<sup>-1</sup> and  $t$  the time (h).

## RESULTS

The strain of *Chryseobacterium* sp. kr6 grew well and completely degraded chicken feathers in the medium (Figure 1). This intense feather-degrading activity was achieved in the range of 25-37°C and with initial pH adjusted from 6.0 to 8.0. Similar growth curves were observed within this range of temperature and pH. A typical curve is shown in Figure 2. The stationary growth phase was reached after 24 h, coinciding with maximum proteolytic activity. In all cases, the pH values increased up to 8.3 during cultivation, and maximum specific production rate was achieved at 24 h, coinciding with the end of exponential growth phase. No important growth neither proteolytic activity were observed during cultivation of the strain at 42°C or higher and pH 9.0 or higher (not shown).

The maximum specific growth rate ( $\mu_{max}$ ), the doubling time ( $t_d$ ), the yield of specific product formation ( $Y_{P/X}$ ), and specific production rate ( $q_p$ ), calculated at the final growth phase are shown in Table 1. Maximum enzyme activity and enzyme yields were observed at 30°C and pH ranging from 6.0 to 7.0. Maximum biomass was obtained with more alkaline pH values (Table 1). Although the production pattern of keratinase was similar for the different pH values tested, lower activities were observed at pH 5.0. The  $q_p$  values were higher at 30°C and pH 6.0-7.0.

The effect of various substrates on keratinase production was investigated. Production of keratinase activity was similar when the strain kr6 was grown in raw feathers or feather meal, but decreased with other proteinaceous substrates (Figure 3). The FKB medium was supplemented with carbohydrates and the production of keratinase was then evaluated. The addition of glucose, and markedly sucrose or lactose, resulted in strong inhibition of keratinase synthesis (Figure 3). Other additives such as the surfactants Tween 80 and Triton X-100 also caused reduction in protease yields of about 90% and 75%, respectively.

The effect of concentration of three growth substrates on keratinase production was tested. Keratinase was produced at a similar level at different concentration of raw feathers and feather meal (Figure 4). Increased amounts of feather and feather meal resulted in higher bacterial growth, but the cultivation with 50 g l<sup>-1</sup> feathers resulted in a share of fragmented quills in solution. When soy meal was used as substrate, a decrease in keratinase activity was observed at

elevated concentrations, although this effect was less evident at longer cultivation times (Figure 4).

The yields for the production of keratinase using three inexpensive common substrates were determined (Table 2). The most effective production was achieved by using feather meal, which is 1.6 fold higher than that in soy meal.

## DISCUSSION

A keratinase was produced by *Chryseobacterium* sp. kr6 under different growth conditions. This enzyme has been shown to be useful for biotechnological purposes such as hydrolysis of poultry feathers (Riffel et al. 2003a) and de-hairing of bovine pelts (Riffel et al. 2003b).

Proteases from *Chryseobacterium* strains are often produced at mesophilic temperatures. A metalloprotease of *C. indologenes* Ix9a (Venter et al. 1999) and an endopeptidase of *Chryseobacterium* sp. (Lijnen et al. 2000) were produced during cultivation in nutrient broth at 25°C and 28°C, respectively. Although those conditions were considered satisfactory to produce proteolytic activity, other settings were not investigated. In the current work, biomass and enzyme activity were found to be higher at 25-30°C than 37°C (Table 1). Although keratinolytic bacteria often display optimal growth and activity at higher temperatures (Lin et al. 1999; Kim et al. 2001), this is consistent with optimum values described for keratinolytic gram-negatives such as *Vibrio* sp. kr2 (Sangali and Brandelli, 2000), *Lysobacter* sp. (Allpress et al. 2002) and *Stenotrophomonas* sp. D-1 (Yamamura et al. 2002), which showed optimum temperature for growth and keratinolytic enzyme production ranging from 20°C to 30°C.

Maximum biomass and keratinase activity were observed from pH 6.0 to 8.0 (Table 1), which agrees with those described for most feather-degrading *Bacillus* (Wang and Shih, 1999; Suntornsukz and Sutornsukz, 2003). For production of keratinase by *B. licheniformis* and a recombinant *B. subtilis*, uncontrolled pH operation was more favorable than the controlled pH operations (Wang and Shih, 1999). The same was observed for alkaline protease production by *B. licheniformis* (Çalik et al. 2002).

Maximum activities were coinciding with maximum biomass and maximum specific production rates were observed at the exponential growth phase, suggesting that the keratinase of strain kr6 was produced as a primary metabolite (Figure 2).

Carbohydrate inhibition of keratinase production was observed (Figure 3), indicating that this strain has a catabolite repression regulatory mechanism, a common control mechanism for biosynthesis of bacterial proteases (Givskov et al. 1991), including keratinases (Wang and Shih, 1999; Thys et al. 2004). Suitable amounts of some surfactants, such as Tween 80 and Triton X-100, are known to stimulate bacterial enzyme production (Reddy et al.

1999). However, the addition of these detergents into the FKB suppressed bacterial growth and keratinase production (Figure 3), similar to that observed for *Bacillus* sp. FK46 during submerged cultivation (Suntornsukz and Sutornsukz, 2003).

Although the production of proteases in complex growth media often promotes exuberant growth and high enzyme yields (Johnvesly and Naik, 2001; Joo et al. 2002), their expensive cost makes them unsuitable for a large-scale production. Some less expensive substrates, such as soybean meal, have been successfully used (Wang and Shih, 1999; Joo et al. 2002). It seems more adequate to use raw materials like some wastes from the food industry as a basis of the culture media. The strain kr6 produced higher yields in feather meal and raw feathers, which have been used as good substrates for production of other keratinolytic enzymes (Wang and Shih, 1999; Sangali and Brandelli, 2000).

High substrate concentrations may cause substrate inhibition or repression of keratinase production. This was observed when soy meal was used as substrate, similar to that described by Joo et al. (2002) during protease production by *Bacillus horikoshii*. This indicates that smaller amounts of substrate are preferred since they result in higher yields of product per substrate ( $U\ g^{-1}$  substrate). It was previously observed for the strain kr6 that substrate and metabolite levels in the extracellular milieu can regulate enzyme secretion (Riffel et al. 2003a). As the strain grows in soy meal faster than in feather substrates, different kinetics for keratinase production would be expected, depending on the type and amount of substrate.

Keratinases have enormous potential applications in processing waste in the poultry and leather industries. The recent finding that *B. licheniformis* PWD-1 keratinase cause enzymatic breakdown of prion protein PrP<sup>Sc</sup> (Langeveld et al. 2003) leave open a novel relevant application for broad range keratinases. In this study, the optimum conditions for keratinase synthesis by the *Chryseobacterium* strain kr6 were determined, which is an essential step for the production of adequate amounts for application in research of feed and other areas.

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## APPENDIX

### Tables

**Table 1.** Kinetic constants of growth and protease production during cultivation of *Chryseobacterium* sp. kr6 in feather keratin broth.

T (°C) /initial pH						
Parameter	25 / 6.0	30 / 6.0	37 / 6.0	30 / 5.0	30 / 7.0	30 / 8.0
$\mu_{\max}$ (h <sup>-1</sup> )	0.020	0.037	0.016	0.033	0.032	0.033
$t_d$ (h)	34.6	18.7	43.3	21.0	21.7	21.0
$X_{\max}$ (g l <sup>-1</sup> ) <sup>a</sup>	0.36	0.35	0.29	0.38	0.39	0.41
$A_{\max}$ (U ml <sup>-1</sup> ) <sup>a</sup>	60	88	42	57	86	82
$Y_{P/X}$ (U mg <sup>-1</sup> )	167	231	145	150	221	191
$q_p$ ( U mg <sup>-1</sup> h <sup>-1</sup> )	6.94	10.5	6.03	6.25	9.18	8.33

<sup>a</sup> Values are the means of three determinations.

**Table 2.** Estimation of keratinase yields with different substrates.

	Raw feathers	Feather meal	Soy meal
Activity (U ml <sup>-1</sup> )	82	95	60
Cell yield (g g <sup>-1</sup> substrate)	0.04	0.05	0.07
Keratinase yield (U g <sup>-1</sup> substrate)	8600	9500	6000

## Figures

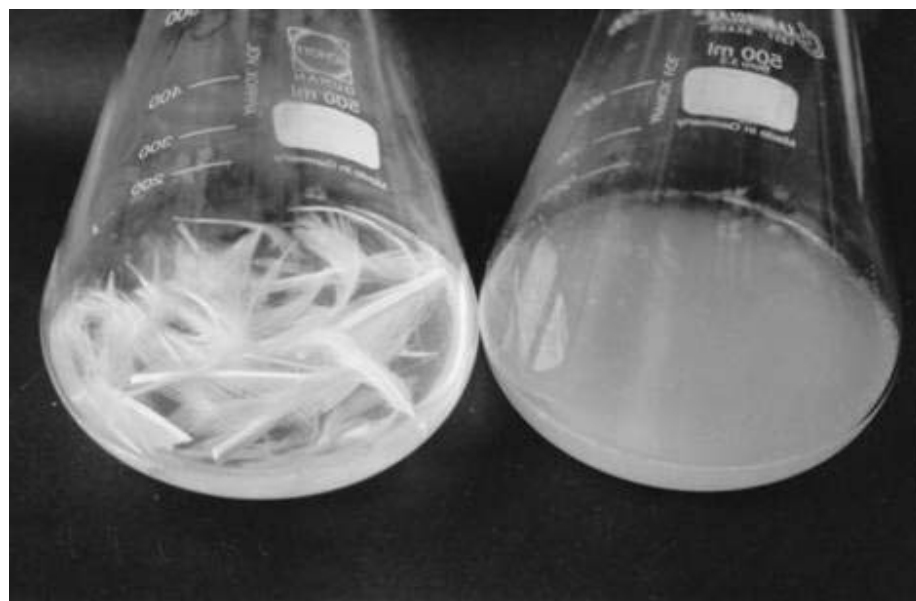


Figure 1. Feather degradation by *Chryseobacterium* sp. kr6. Feathers were incubated for 48 hrs at 30°C with viable cells of the strain kr6 (right flask) and with autoclaved inoculum as control (left flask).

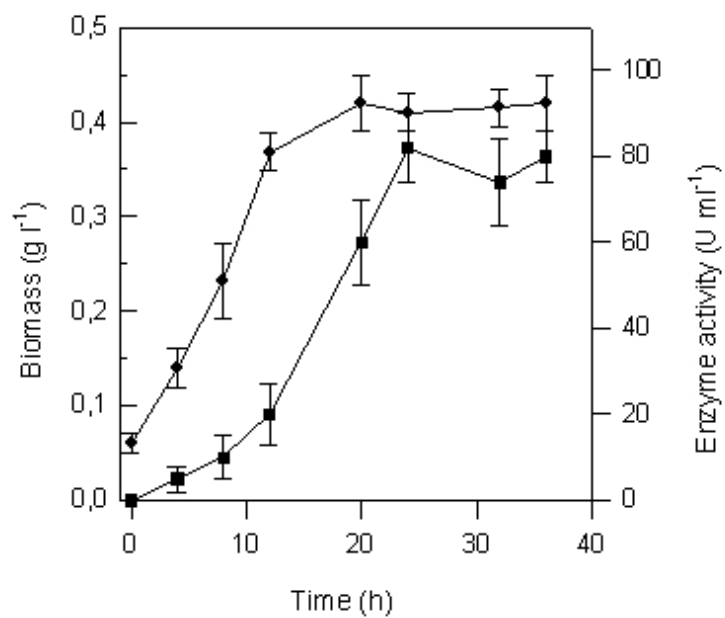
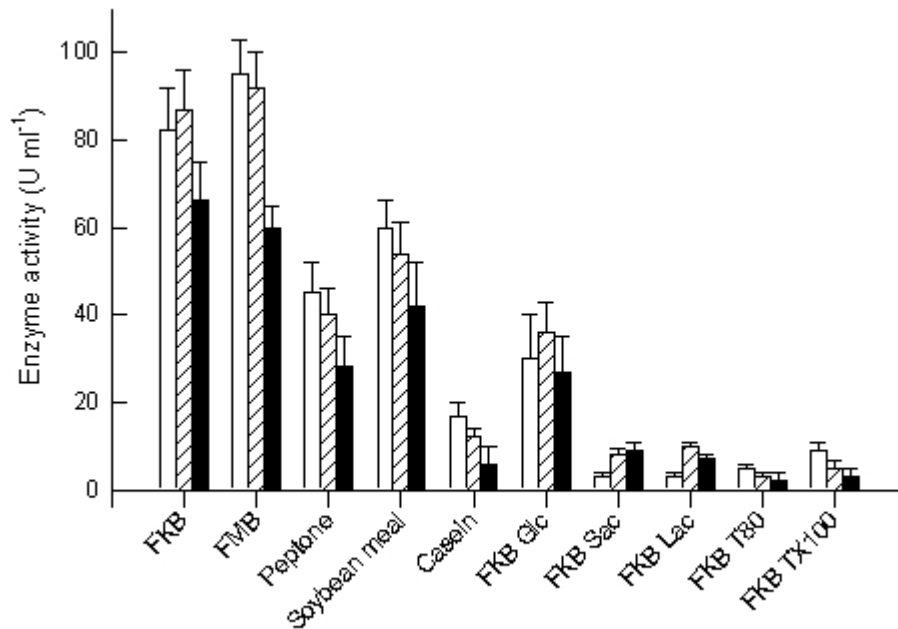
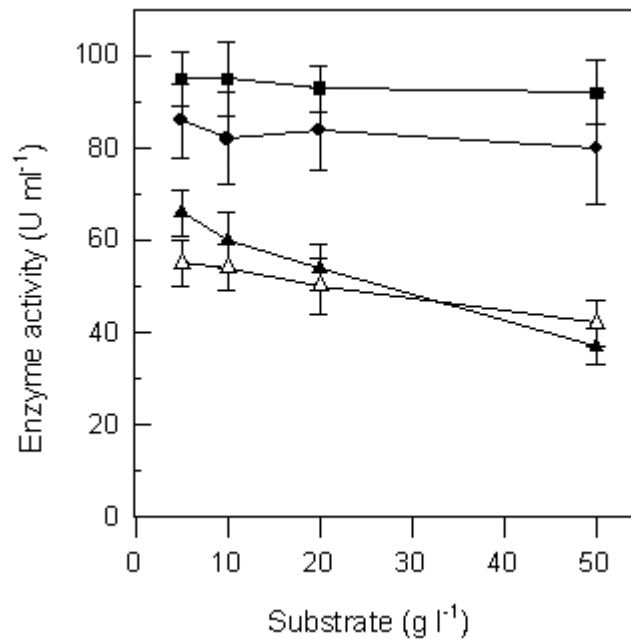


Figure 2. Keratinase activity (squares) during growth (circles) of strain kr6 on 10 g l<sup>-1</sup> raw feathers at 30°C and initial pH 7.0. Points are the means  $\pm$  s.e.m. of three independent experiments.



**Figure 3.** Effect of growth substrates on protease production by *Chryseobacterium* sp. kr6 at 30°C and initial pH 7.0 after 24 hrs (white bars), 48 hrs (dashed bars) and 72 hrs (black bars) cultivation. Bars are the means  $\pm$  s.e.m. of three independent experiments. FKB, feather keratin broth; FMB, feather meal broth; Glc, Sac, Lac, T80, TX100 indicate the FKB contained 10 g l<sup>-1</sup> of glucose, sucrose, lactose, Tween 80 or Triton X-100, respectively.



**Figure 4.** Effect of growth substrate concentration on protease production by *Chryseobacterium* sp. kr6 at 30°C and initial pH 7.0 in media containing different amounts of raw feathers (circles), feather meal (squares) or soy meal (triangles) after 24 hrs (black symbols) and 48 hrs (white symbols) cultivation. Points are the means  $\pm$  s.e.m. of three independent experiments.