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Research Paper

Nutritional regulation of protease production by the feather-degrading bacterium *Chryseobacterium* sp. kr6

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The effects of nutritional conditions on growth and protease production by the feather-degrading *Chryseobacterium* sp. kr6 were investigated. Higher growth was observed on feather-containing or tryptone (TR) medium when compared to casein (CA) or glucose-nitrogen (GN) base medium. Protease production occurred during growth on feather-containing and TR media, whereas no protease activity was detected on CA or GN medium, indicating that protease production is not constitutive, depending on the presence of specific complex nitrogen sources. Supplementation of whole feathers (WF) medium with glucose (WFG) or NH_4Cl (WFN) did not result in major differences in growth and protease production, whereas soluble protein was lower in supplemented media. Glucose consumption and growth were higher on WFG than on GN medium, suggesting that the absence of a specific complex nitrogen source limited bacterial growth. On WF medium, this strain grew closely attached to the feather structures, initially on the barbules and subsequently on the feather rachis. It was observed, through zymogram analysis, that strain kr6 produced diverse proteolytic enzymes in response to different growth substrates. These results were confirmed by the differential behaviors of crude proteases towards protease inhibitors.

Introduction

Protease production is an intrinsic ability of microorganisms and, specifically, extracellular proteases play a crucial role in protein hydrolysis for microbial nutrition [1]. Keratins are proteins possessing high levels of disulfide linkages, hydrogen bonds, and hydrophobic interactions, resulting in its insolubility and stability. Despite its recalcitrance, keratins might be utilized as growth substrates by keratinolytic microorganisms, which produce keratinolytic proteases. Such microorganisms, directly involved in the recycling of carbon, nitrogen and sulphur of keratins, appear to be widespread in nature [2].

Keratinous materials are produced in large amounts as byproducts of agroindustrial activities such as poultry processing. Therefore, the keratinolytic potential of microorganisms is increasingly being investigated for utilization in the bioconversion of keratin-rich wastes, producing protein hydrolysates with commercial value. Additionally, keratinolytic proteases are considered as promising biocatalysts for dehairing processes on the leather industry, and in prion degradation, among other applications [3].

The commercial application of enzymes depends on its production in high levels, at low or moderate costs. Therefore, knowledge of the cultural factors affecting the protease/keratinase yield is of essential importance. *Chryseobacterium* sp. kr6 is a keratinolytic bacterium previously isolated from decomposing feathers, and its biotechnological potential has been extensively investigated [4–9]. However, a detailed examination of nutritional conditions controlling the production of proteolytic enzymes by this strain is lacking. Thus, the present article investigated bacterial growth and protease yield during submerged/liquid culture cultivation of *Chryseobacterium* sp. kr6 on different carbon and nitrogen sources.

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Materials and methods

Microorganism and culture conditions

Chryseobacterium sp. kr6 was cultivated in mineral medium (0.5 g NaCl/L, 0.3 g K_2HPO_4/L, and 0.4 g KH_2PO_4/L) supplemented with different carbon and nitrogen sources. Whole feathers (WF) medium contained 10 g whole feathers/L; WF-glucose (WFG) medium contained 10 g whole feathers/L and 50 mM glucose; WF-NH_4Cl (WFN) medium contained 10 g whole feathers/L and 50 mM NH_4Cl; glucose-nitrogen (GN) base medium contained nitrogen base (Difco) and 50 mM glucose; casein (CA) medium contained 10 g casein/L; and tryptone (TR) medium contained 10 g tryptone/L.

For seed cultures, bacteria were inoculated in WF and incubated at 30°C for 16 h (150 rpm). Ten ml of this culture was centrifuged (3 000 × *g* for 5 min), and the pellet was washed with sterile phosphate buffer (50 mM) and resuspended in 5 mL of the same buffer. The resulting cell suspension was utilized to inoculate the different media, which were incubated at 30°C for up to 44 h in an orbital shaker (150 rpm).

Analytical determinations

At defined cultivation periods, aliquots of the culture medium were withdrawn and bacterial growth was determined (CFU/mL) by the plate count method on nutrient agar [10]. Proteolytic activity was determined with culture supernatants (crude protease), obtained by centrifugation (12,000 × *g* for 20 min) of culture media, using Hammerstein casein as substrate [5]. The protein concentration on culture supernatants was determined by the method of Lowry *et al.* [11]. Residual glucose on culture supernatants of WFG and GN media was quantified by the Somogyi-Nelson photometric method [12]. The effect of protease inhibitors was assessed by pre-incubating the crude protease with ethylenediaminetetraacetic acid (EDTA; 50 mM) or phenylmethylsulfonyl fluoride (PMSF; 1 mM) at 20°C for one hour; after this period the protease activity assay was performed as described above with the presence of each inhibitor in the reaction mixture. All assays were done in triplicate.

Zymographic analysis was performed as previously described [5] following electrophoresis of crude proteases on 7.5% polyacrylamide gels containing gelatin (1 mg/mL).

Results and discussion

Diverse variables, including growth, protease production, glucose consumption, and release of soluble proteins were evaluated during cultivation of *Chryseobacterium* sp. kr6 in different liquid media. *Chryseobacterium* sp. kr6 reached the stationary growth phase after 15 h on TR, WF, WFG and WFN media (Fig. 1). Although the stationary growth phase was achieved earlier on CA medium (5 h), maximum biomass (approx. 10^9–10^{10} CFU/ml) was detected on TR medium and feather-containing medium. In GN medium, growth was slower and stationary growth phase was not apparently achieved after 44 h of cultivation. The analysis of growth curves on WF and CA media might indicate the higher adaptation and specificity of *Chryseobacterium* sp. kr6 for growth on keratin as a nitrogen source than on casein. This is further supported by the similar growth kinetics observed on WF and TR media, as tryptone is a readily available nitrogen source produced by the enzymatic hydrolysis of casein. Residual glucose on GN and WFG media after 44 h of cultivation was higher in the former (Fig. 2). Addition of glucose to the growth medium did not present

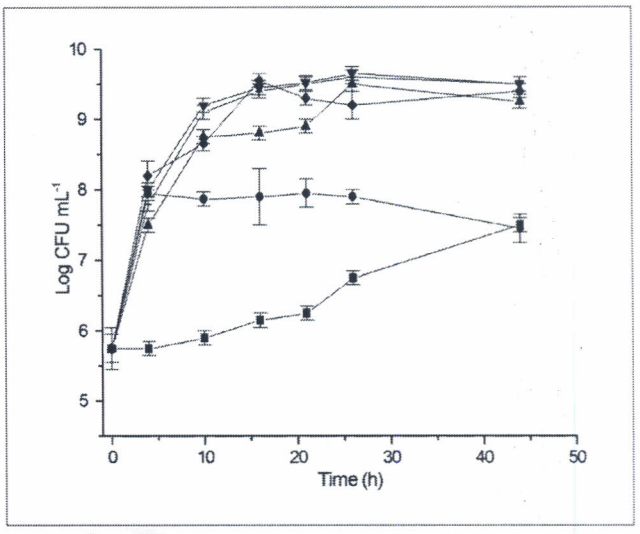

FIGURE 1

Growth curve of *Chryseobacterium* sp. kr6 in different culture media: (●) TR; (◆) WF; (□) WFG; (△) WFN; (×) CA and (▲) GN. The culture conditions were 30°C, 150 rpm, and initial pH 7. Each point is the mean ± S.E.M. of triplicate experiments. Growth on WF and WFG was very similar and the symbols are superimposed.

a beneficial effect on biomass production when comparing WFG and WF media. The absence of a complex nitrogen source might act as a limiting factor for growth in GN medium (Fig. 1).

Protease production was monitored during *Chryseobacterium* sp. kr6 growth. The peak of protease production was achieved after 15–20 h on TR, WF, WFG and WFN media (Fig. 3), coinciding with the stationary growth phase (Fig. 1). Indeed, maximal production of microbial proteases is usually reached at the late exponential or stationary growth phase [3,13]. Proteolytic activity was not detected in CA and GN media, indicating that production of these enzymes is not constitutive. The inability of *Chryseobacterium* sp. kr6 to produce protease during growth on CA medium suggests that specific organic nitrogen sources (keratin and tryptone) are required for protease production. Particularly, keratinase produc-

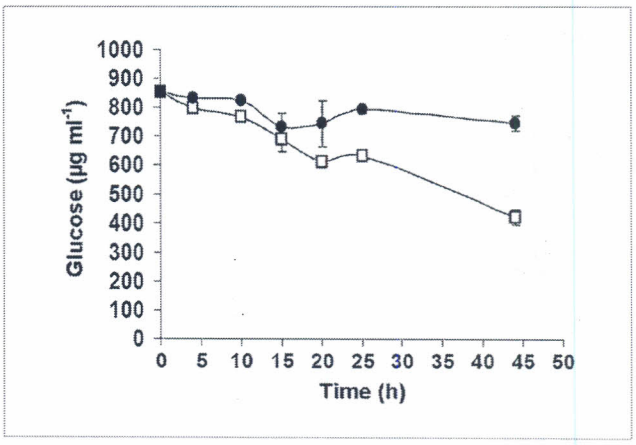

FIGURE 2

Residual glucose concentration during growth of *Chryseobacterium* sp. kr6 in the (□) WFG and (●) GN media at 30°C, 150 rpm, and initial pH 7. Each point is the mean ± S.E.M. of triplicate experiments.

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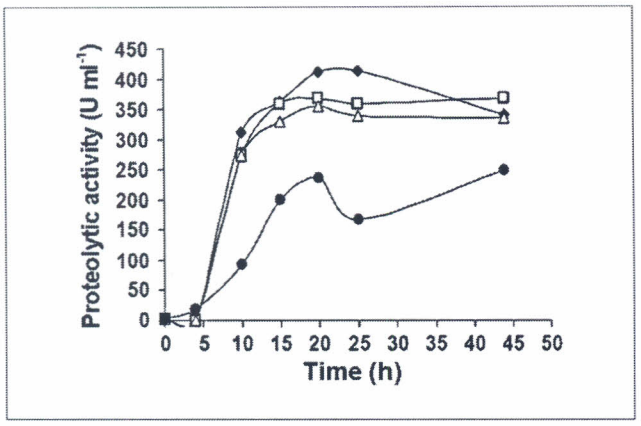

FIGURE 3

Protease activity during growth of *Chryseobacterium* sp. kr6 in different culture media: (●) TR; (◆) WF; (□) WFG and (△) WFN. The strain was cultivated at 30°C, 150 rpm, and initial pH 7. Each point is the mean ± S.E.M. of triplicate experiments.

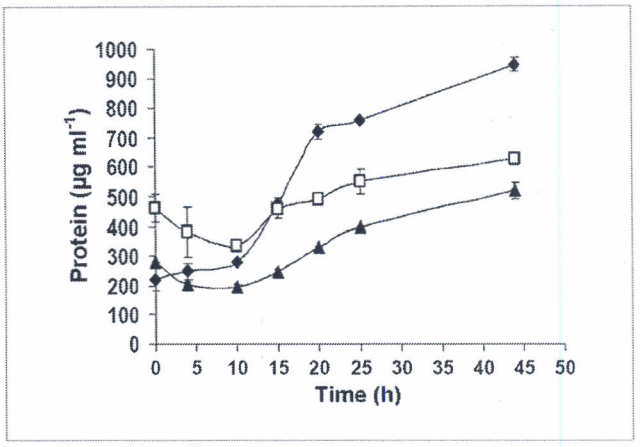

FIGURE 4

Soluble protein during growth of *Chryseobacterium* sp. kr6 at 30°C, 150 rpm, and initial pH 7 in the following media: (◆) WF; (□) WFG and (▲) WFN. Each point is the mean ± S.E.M. of triplicate experiments.

tion is reported to be mainly induced by keratins and, hence, production of this enzyme is usually achieved by the addition of keratin-rich materials (feathers and hair) to culture media [2].

Production of protease appeared to be partially controlled only by catabolite repression mechanisms, as glucose and NH_4Cl provoked a slight decrease in the protease yield during growth on feather-containing medium (Fig. 3). The effect of supplementary carbon and/or nitrogen sources on protease production is highly variable, depending on the microorganism and the substrates utilized. Son *et al.* [14] observed that the addition of glucose to feather medium resulted in enhanced growth and keratinase production by *Bacillus pumilis* F3-4, whereas the addition of NH_4Cl slightly increased cell growth but drastically decreased keratinolytic activity. Glucose increased protease production by *Bacillus pseudofirmus* AL-89 on feather medium; nevertheless, *Nesterenkonia* sp. AL-20 protease production was suppressed in the presence of glucose [15]. Glucose and NH_4Cl inhibited keratinase production by *Bacillus subtilis* KD-N2 on human hair medium [16]; however, the presence of an inorganic nitrogen source (ammonium phosphate) increased protease production by *Bacillus licheniformis* ATCC 21415 [17]. In *Bacillus cereus* MCM B-326, the protease activity decreased in the absence of a carbon source [13].

The higher content of soluble protein after 44 h of cultivation was observed in WF medium, followed by WFG and WFN (Fig. 4), somewhat reflecting the levels of protease production by *Chryseobacterium* sp. kr6 (Fig. 3). During growth on feather keratin the medium pH increased from 7.0 to 8.3 after 44 h (result not shown), which results from the ammonia produced by the deamination of peptides and amino acids derived from keratin solubilization [10,18]. Altogether, these results demonstrate the remarkable biotechnologic potential of strain kr6 in connecting the efficient bioprocessing of keratin wastes with the production of microbial biomass (Fig. 1), proteolytic enzymes (Fig. 3) and protein hydrolysates (Fig. 4).

Chryseobacterium sp. kr6 appeared to grow adhering to the feather structure (Fig. 5), as observed with other microorganisms [19,20]. After eight hours of cultivation in WF, little feather colonization was observed; however, the feather surface presented some alterations (Fig. 5b) in comparison to the control (Fig. 5a). At this point (eight hours), strain kr6 was in exponential growth phase (Fig. 1), producing high levels of proteolytic enzymes (Fig. 3), which might have been responsible for initial feather degradation. A substantial increase in feather colonization was observed after 18 h of cultivation (Fig. 5c,d), which coincided with a higher level of protein solubilization (Fig. 4), indicating the relationship between colonization and feather degradation. Feather barbules were first colonized, and only after 36 h of cultivation did the feather rachis become highly degraded. Indeed, bacterial adhesion might play a key role during the degradation process, probably providing suitable redox conditions to break disulfide bridges in the keratinous substrate [20,21]. The reduction

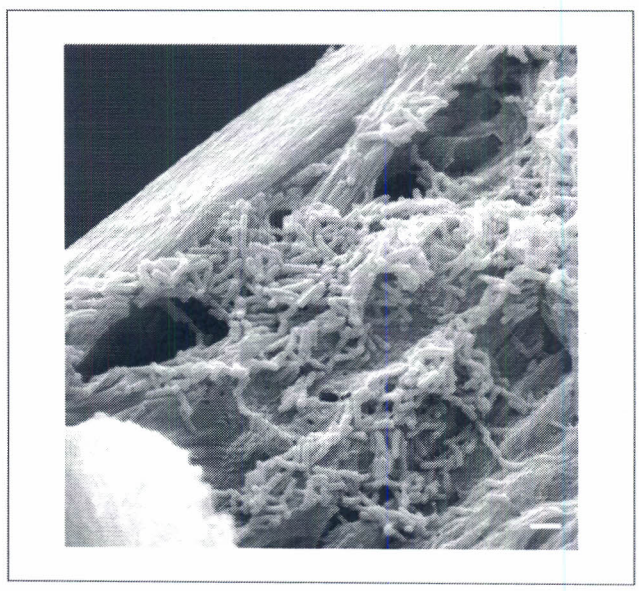

FIGURE 5

Scanning electron micrographs of feather degradation by *Chryseobacterium* sp. kr6 after 0 **(a)**, 8 **(b)**, 18 **(c)** and 36 h **(d)** of cultivation at 30°C in feather (WF) medium. Magnification: ×2000.

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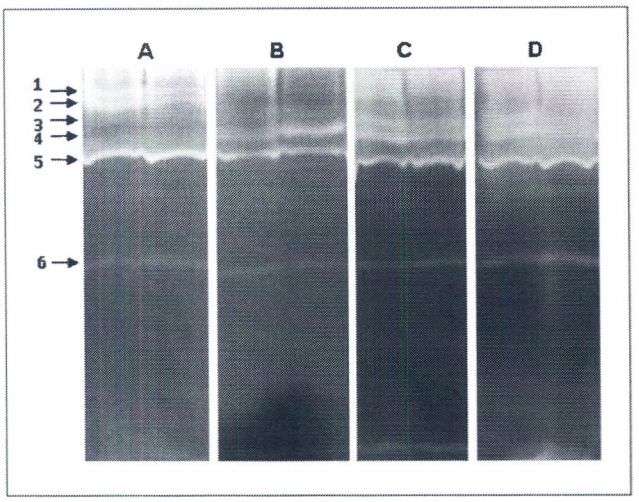

FIGURE 6

Gelatin zymogram of crude extract proteases of *Chryseobacterium* sp. kr6 after SDS-PAGE on 7.5% polyacrylamide gels under nonreducing conditions. The strain was cultivated for 20 h in the following media: **(a)** TR; **(b)** WF; **(c)** WFG and **(d)** WFN.

of disulfide bonds (sulfitolysis) was previously reported to occur during the growth of strain kr6 on chicken feathers [4]. The production of intra- and/or extracellular disulfide reductases, release of sulfite and thiosulfate, a cell-bound redox system and membrane potential are among the mechanisms that can lead to sulfitolysis [2,21]. Therefore, an efficient keratinolysis process seems to involve, at least, proteolytic and sulfitolytic mechanisms.

Zymogram analysis of culture supernatants showed that four extracellular proteases were produced on the different culture media (Fig. 6). The production of diverse extracellular proteases during microbial growth is generally reported [18,22]. The proteolytic enzymes detected on culture supernatants of *Chryseobacterium* sp. kr6 grown on WF, WFG and WFN media were analogous (bands 3–6), indicating that the presence of glucose or NH_4Cl did not affect the pattern of the proteases produced. On TR medium, among the four proteases detected, only two (bands 5 and 6) were similar to the proteases produced in feather-containing media; the others (bands 1 and 2) were not produced on WF, WFG or WFN (Fig. 6). At this point it could be argued that bands 5 and 6 correspond to proteases with broad substrate specificity, because they were produced on tryptone and feathers; and that proteolytic bands 3 and 4 are specific enzymes responsible for the degradation of more recalcitrant proteins. Protease production by microorganisms is generally regulated by carbon and nitrogen stress [1]. As keratinous substrates are unable to enter the cell, the production of keratinolytic proteases on keratin-containing media may result from nitrogen limitation rather than keratin induction [23]. The production of different proteolytic enzymes might be adjusted in response to the supply of organic nitrogen, with the sequential secretion of proteases with activities

TABLE 1

Effect of inhibitors on proteolytic activity of culture supernatants of *Chryseobacterium* sp. kr6 grown on different media

Growth medium	Protease inhibitor	Relative activity (%)
WF	None	100
	EDTA (50 mM)	10
	PMSF (1 mM)	90
TR	None	100
	EDTA (50 mM)	67
	PMSF (1 mM)	69

targeted against increasingly inaccessible proteinous substrates as the nutritional availability in the environment deteriorates [23]. Nevertheless, proteases corresponding to bands 5 and 6 might also contribute to keratin degradation, because the action of keratinases and, for instance, disulfide reductases destabilize the keratin molecules, allowing other proteases to act and resulting in extensive keratin degradation [24].

Although each proteolytic band may represent a different product from a distinct gene, such a statement could not be made based on the obtained results because multiple proteolytic bands might originate from non-enzymatic or proteolytic processing of an original enzyme [25]. Thus, in addition to zymogram analysis, the crude extracellular proteases produced by *Chryseobacterium* sp. kr6 on WF and TR media were submitted to inhibition assays (Table 1). The presence of EDTA, a metalloprotease inhibitor, almost completely abolished the protease activity from WF culture supernatants, whereas in TR supernatants the effect was much less pronounced. The inhibitory effect of PMSF, a serine protease inhibitor, was higher in TR than in WF culture supernatants. These results reinforce the idea that different proteases were produced by *Chryseobacterium* sp. kr6 depending on the protein source utilized as growth substrate.

Extracellular proteases and keratinases have important functions in *Chryseobacterium* sp. kr6 metabolism, because this isolate can grow in media containing feather keratin as the sole source of carbon and nitrogen. This feather-degrading isolate and its proteolytic and keratinolytic enzymes have an enormous biotechnological potential involving keratin hydrolysis. As observed in the current investigation, depending on the substrates available for growth, *Chryseobacterium* sp. kr6 may produce (or not) diverse proteases in different yields, demonstrating that the production of such enzymes is controlled by complex mechanism(s). Therefore, medium composition was essential in developing a successful cultivation process for protease/keratinase production.

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