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ORIGINAL PAPER

Biodegradation of native feather keratin by *Bacillus subtilis* recombinant strains

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Abstract A mixed culture containing two recombinant *Bacillus subtilis* strains; was used to hydrolyze 1% chicken feather; both were previously transformed with late-expressed and early expressed alkaline protease (*aprE*) carrying plasmids pS1 and p5.2, respectively. Proteolytic and keratinolytic activities of the mixed culture increased in parallel with those of the culture of *B. subtilis* DB100 (p5.2), and both were higher than that of *B. subtilis* (pS1) cultures. On the other hand, data indicated that degradation of feather by the recombinant strains *B. subtilis* DB100 (p5.2), was greatly enhanced when using a previously optimized medium. High levels of free amino groups as well as soluble proteins were also obtained. The concentration of amino acids was considerably increased during the fermentation process. It was found that, the amino acids Phe, Gly and Tyr were the major amino acids liberated in the cultures initiated by both strains. Results render these recombinant strains suitable for application in feather biodegradation large scale processes.

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Introduction

Feather is composed of over 90% protein; its main component is keratin, an insoluble highly cross-linked fibrous protein. Keratin is characterized by its high mechanical stability and resistance to common proteolytic enzymes (Gessesse et al. 2003; Onifade et al. 1998; Bockle and Muller 1997). Considering its high protein content, this keratinous waste could have a great potential as a source of protein and amino acids for animal feed and for many other applications. However, because of its insoluble nature, use of feather keratin as a source of value-added products has been very limited. Current commercial production of feather meal involves treatment at elevated temperatures and high pressure, this energy intensive process, results in the loss of some essential amino acids (Gessesse et al. 2003). Despite these features; a number of keratinolytic microorganisms, mostly bacteria and fungi, have been reported (Cao et al. 2008; Letourneau et al. 1998; Tamilmani et al. 2008). However, genetic modification for construction of new microbial strains with high yields of enzyme production is essential to allow commercialization. Keratinases of these microorganisms may be useful in

the biotechnological clean up of keratin-containing wastes such as feather, and improving the nutritional value of feather meal (Ramnani and Gupta 2004). Furthermore, keratinases could be applied for waste treatment, textile, medicine, cosmetic, leather, feed and poultry processing industry (Gessesse et al. 2003; Ramnani and Gupta 2004; Zaghloul et al. 1994).

In this paper, we describe feather biodegradation using a combination of two recombinant *B. subtilis* strains, as well as the use of an optimized medium to accelerate feather decomposition process. Amino acid composition of the feather hydrolysate was also evaluated.

Methods and materials

Bacterial strains and plasmids

B. subtilis DB100 *his*$^-$ *met*$^-$, *B. subtilis* DB100 *his*$^-$ *met*$^-$ (pS1) and *B. subtilis* DB100 *his*$^-$ *met*$^-$(p5.2) were used in this study as the source of the extracellular proteolytic and keratinolytic activities. The pS1 plasmid (6.7 kbp) is a pUB110 derivative plasmid, also known as pKWZ; it carries an insert (2.7 kbp) containing the complete *B. subtilis* alkaline protease (*apr E*) gene, as well as a kanamycin resistance gene (selectable marker) (Zaghloul et al. 1994). The p5.2 plasmid (4.7 kbp) is a pUB118 derivative plasmid, where the expression of the alkaline protease gene *aprE* (750 bp) is controlled by an early promoter P^{43}. The plasmid p5.2 carries the kanamycin resistance gene as well (Unpublished data). Transformation was carried out according to Anagnostopoulos and Spizizen (1961).

Media

Bacterial strains were activated and grown on PY medium (Bernhardt et al. 1978) (Bactopeptone, 10 g; Difco yeast extract, 5 g; and NaCl, 5 g/l, pH 7.0) and PA medium (PY medium supplemented with 1.5% agar). Sporulation medium (1X SG) (Leighton and Doi 1971) (beef extract, 3 g; bactopeptone, 5 g; KCl, 1 g; and glucose, 1 g/l; $MgSO_4 \cdot 7H_2O$, 2 mM; $CaCl_2$, 1 mM; $MnCl_2$, 0.1 mM; and $FeSO_4$, 1 mM, pH 7.0).

Basal medium II (Williams et al. 1990) (NH_4Cl, 0.5 g; NaCl, 0.5 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; and yeast extract, 0.1 g/l, pH 7.0) supplemented with 1% (w/v) chicken feather was used in the fermentation process. Kanamycin was added when necessary to a final concentration of 5 μg/ml.

Monitoring proteolytic and keratinolytic activity

The proteolytic and keratinolytic activity was monitored throughout the growth of the bacterial strains. Cells were activated by growing them overnight on PA plates supplemented with kanamycin when necessary at 37°C. Fresh colonies were then transferred to 10 ml PY with kanamycin for the recombinant strains, and the culture was allowed to grow at 37°C with shaking for 2 h. Hundred milliliter of pre-autoclaved basal medium II, supplemented with 1% chicken feathers and kanamycin (when necessary) was inoculated with 4 ml (containing 10^6 CFU/ml) of the above culture. Cells were allowed to grow at 37°C with shaking at 180 rpm, during 4 days or more. One milliliter of culture was taken to determine bacterial growth. Another 2 ml culture was taken and centrifuged at 4,500 g in a microcentrifuge for 2 min. The supernatant was used as a crude solution to determine the extracellular alkaline protease activity, as well as the keratinolytic activity.

Proteolytic activity

Proteolytic activity was measured according to the method of Cliffe and Law (1982), using hide powder azure (HPA) as a substrate. One unit of alkaline protease was equal to the amount of enzyme that develops a change of absorbance against the control at 595 nm per 30 min at 37°C using HPA as substrate. Measures were carried out in triplicates.

Keratinolytic activity

Keratinolytic activity was determined based on the free amino (NH_2 groups) that were released as a result of the biodegradation of feather by bacterial cells. NH_2 free amino groups were determined using ninhydrin as described by Pearce et al. (1988). A standard curve for leucine was established. Measures were carried out in triplicates.

Soluble protein determination

Soluble proteins released as a result of the biodegradation of chicken feathers were determined as described by Bradford (1976). Measures were carried out in triplicates.

Effect of using mixed culture of *B. subtilis* DB100 (p5.2) and *B. subtilis* DB100 (pS1) on the biodegradation process

In an attempt to maximize the action of the keratinolytic enzyme on chicken feather biodegradation and to minimize the fermentation time, a mixed culture of equal ratio, of the two recombinant strains *B. subtilis* DB100 (p5.2) and *B. subtilis* DB100 (pS1) (10^6 CFU/ml) was used to inoculate either 1XSG and basal medium II supplemented with 1% chicken feather. The proteolytic activity of the culture in the sporulation medium was monitored during 24 h. Proteolytic, keratinolytic as well as soluble proteins were analyzed in the basal medium II culture through 4 days of incubation.

Effect of using an optimized medium on the biodegradation process

The recombinant strain *B. subtilis* DB100 (p5.2) was allowed to grow on a previously optimized medium, which consists of a basal medium II supplemented with yeast extract (0.1%), corn oil (0.5%) and chicken feathers (2%) (Zaghloul et al. 2004). Media were inoculated with 5% freshly prepared inoculums (about 10^8 CFU/ml). Proteolytic and keratinolytic activities were monitored during the incubation period.

Analysis of the produced amino acids

After 4 days of incubation, the undegraded insoluble feathers were removed by centrifugation at 7,000 rpm for 5 min in a microcentrifuge. The free amino acids, except tryptophan, which are produced as a result of the hydrolysis of feather keratin using the recombinant strains, were determined using Beckman 119 CL amino acid analyzer (Spackman et al. 1958).

Results and discussion

Synergistic action of *B. subtilis* DB100 (p5.2) and *B. subtilis* DB100 (pS1) on the biodegradation of chicken feather

As it was stated earlier, the *aprE* gene (750 bp) is controlled by an early promoter in *B. subtilis* DB100 (p5.2). This promoter can be transcribed by RNA polymerase with sigma σ^{43} factor, and expressed early (that is to say 2–3 h before the beginning of stationary phase) (Wang and Doi 1987). This is expected to enhance the biodegradation process. On the other hand *aprE* gene (2.7 Kbp) in *B. subtilis* DB100 (pS1) is expressed 2 h after the beginning of stationary phase (Zaghloul et al. 1994).

The presence of *aprE* gene in two different forms (different promoters and different size vectors) in the same culture medium is expected to enhance the level of its expression and consequently could minimize the time necessary for feather hydrolysis.

Sporulation medium supplemented with kanamycin was inoculated by a mixed culture of *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2) with an equal number of CFU, in order to evaluate the level of alkaline protease secreted during 24 h of growth. The alkaline protease activity of the mixed culture was produced early, one hour before the end of the logarithmic phase as well as that produced by the culture initiated by *B. subtilis* DB100 (p5.2) cells (Fig. 1). It was increased in function of the time and reached higher levels when compared to the other two cultures. After 24 h of incubation, the activity of the three cultures became comparable and reached about 13 U/ml.

To examine the effect of using a mixed culture on the feather degradation, basal medium II supplemented with 1% chicken feather was inoculated with a mixed culture of the recombinant strains *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2) at a ration of 1/1. Alkaline protease activity, keratinolyic activity as well as the level of soluble proteins and the number of CFU was monitored during 4 days of incubation.

Proteolytic activity of the mixed culture increased in parallel with that of the culture of *B. subtilis* DB100 (p5.2). Both of them reached the same level and both were higher than that of *B. subtilis* (pS1) cultures (Fig. 2a). The level of free amino groups

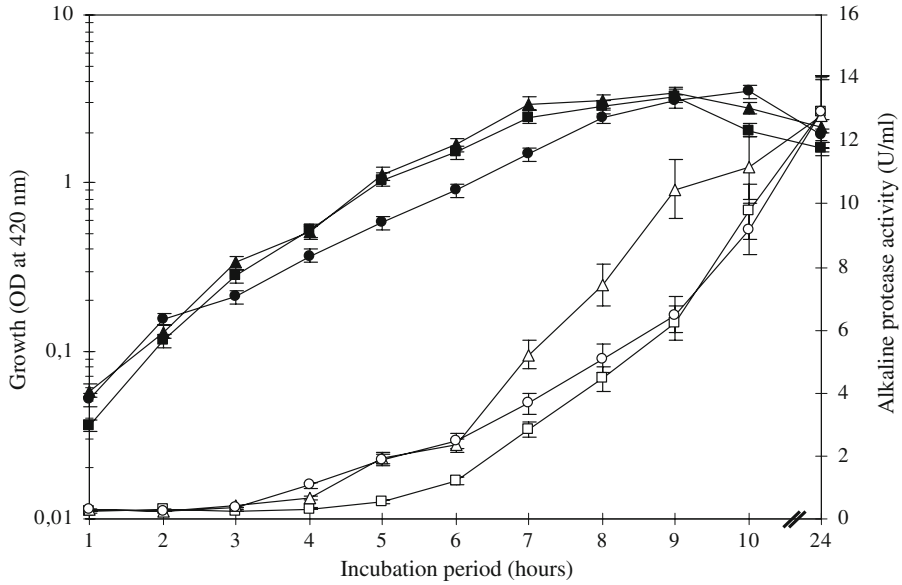

Fig. 1 Synergistic action of a mixed culture of *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2) on the production of alkaline protease. Bacterial cells were grown on sporulation medium. Symbols (□) (Δ) and (○) represent the alkaline protease activity of *B. subtilis* DB100 (pS1), mixed culture and *B. subtilis* DB100 (p5.2) cultures respectively. Symbols (■) (▲), and (●) represent the absorbance at 420 nm of *B. subtilis* DB100 (pS1), mixed and *B. subtilis* DB100 (p5.2) cultures, respectively

released by the action of the mixed culture was similar to that resulting from the action of *B. subtilis* DB100 (p5.2) culture and both were higher than that of *B. subtilis* DB100 (pS1) (Fig. 2b). The level of soluble protein was also determined and results indicated that, there was no significant difference between soluble proteins in mixed culture and those of the other two cultures (Fig. 2c). Results indicated that, the three cultures when grown on the laboratory medium (1XSG, sporulation medium) produced and secreted comparable level of alkaline protease in the cultures. However, when the same cultures were grown on basal medium II supplemented with 1% chicken feather, the level of alkaline protease was higher in either mixed culture or *B. subtilis* DB100 (p5.2) culture. It is recommended to use the mixed culture to gain more enzyme and more NH_2-free amino groups at day 3 of cultivation on basal medium II supplemented with 1% chicken feather (Fig. 2b, c). Several researchers reported the expression of keratinase gene in some *Bacillus* species; like *B. megaterium* MS941 (pWHK3) which displayed high keratinase yield (186.3 U/ml) after 24 h of incubation in LB medium supplemented with starch or xylose (Radha and Gunasekaran 2008), and *B. licheniformis* PWD1, which was extensively studied. In this strain, *ker* A gene was cloned using pUB18-P_{43} and further integrated in multiple copies in the chromosome. When grown in either soy or feather medium, keratinase activity was stable and improved by about 4–6 times (Lin et al. 1995; Wang et al. 2004). The use of mixed cultures containing more than one recombinant strain was not widely applied for the improvement of keratinolysis process it could be advantageous to scale it up for large scale processing after additional studies.

Effect of using the optimized medium

Many authors have reported the optimization of keratinolytic enzymes production for the hydrolysis of feathers and keratin-containing materials (Ramnani and Gupta 2004; Zaghloul et al. 2004; Suntornsuk and Suntornsuk 2003; El-Refai et al. 2004).

The use of the following optimized medium (basal medium II supplemented with 2% chicken feather, 0.5% corn oil, 0.1% yeast extract) enhanced the hydrolysis of feather by *B. subtilis* DB100 (pS1), the level of proteolytic and keratinolytic activity in cultures was enhanced by 4.8 and 2.1-fold than those of corresponding cultures growing on basal medium II and 1% feather, respectively (Zaghloul et al. 2004).

The same conditions were applied to the recombinant strain *B. subtilis* DB100 (p5.2). Proteolytic and keratinolytic activity were monitored through the growth of the constructed strain harboring the plasmid (p5.2). The biodegradation of feather in the above medium was compared to that in the initially used medium (basal medium II with 1% feather).

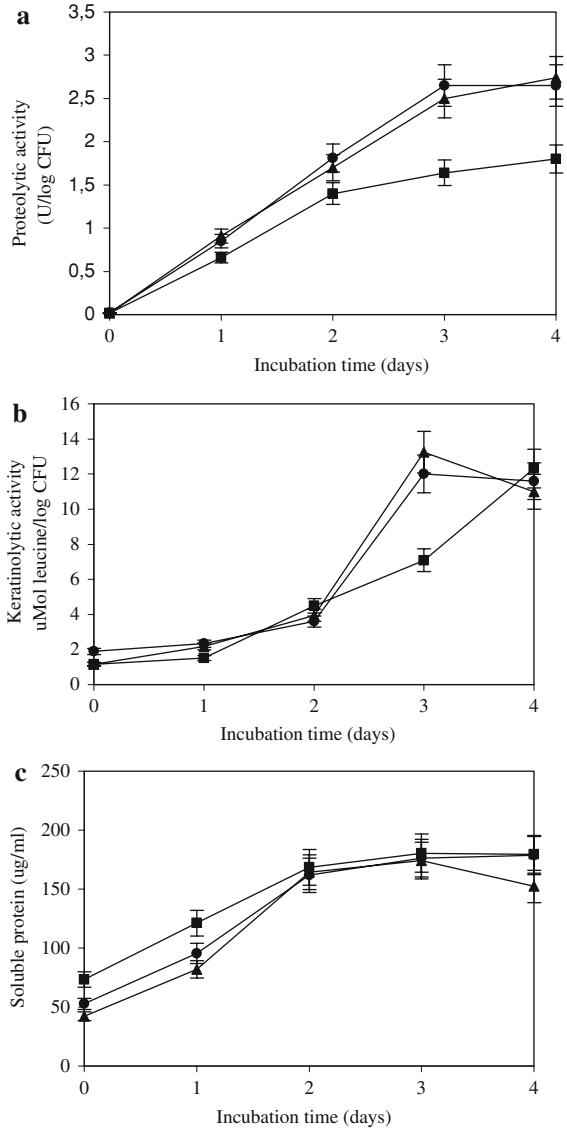

Fig. 2 Proteolytic activity **a** Keratinolytic activity (NH_2- free amino groups); **b** and soluble proteins; **c** of *B. subtilis* DB100 (pS1) (■), a mixed (▲) and *B. subtilis* DB100 (p5.2) (●) cultures grown on basal medium II supplemented with 1% chicken feather

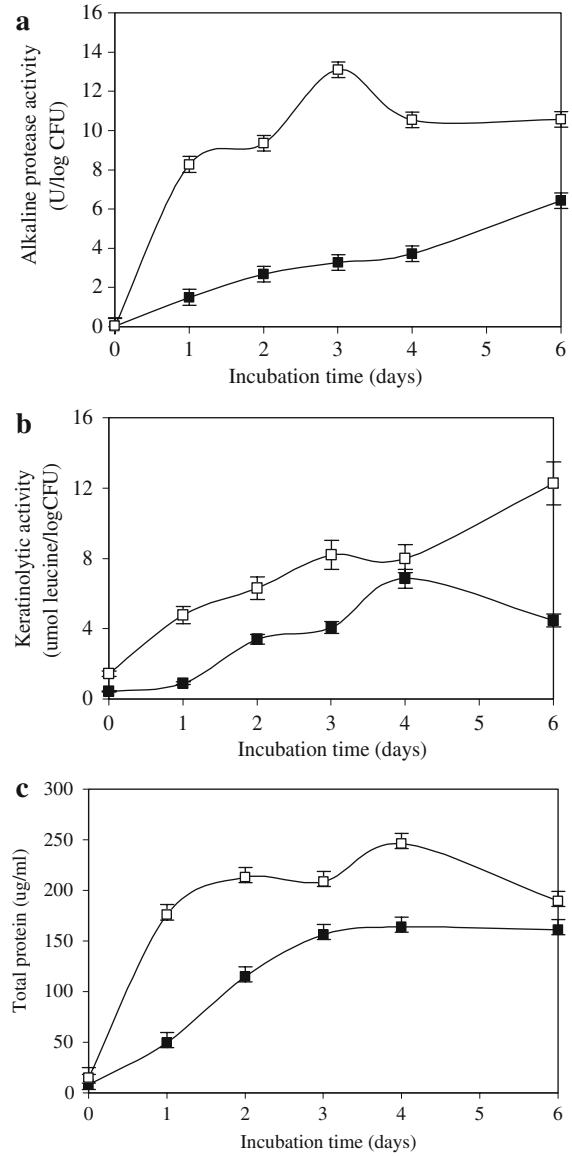

Fig. 3 Effect of the optimized medium on the bioconversion process of chicken feathers by *B. subtilis* DB100 (p5.2). Monitoring of proteolytic activity **a** Keratinolytic activity; **b** and the level of soluble proteins; **c** during 6 days of incubation. Symbols (■) and (□) represent the above parameters for cultures in basal medium II and in the optimized medium, respectively

The level of alkaline protease produced by *B. subtilis* DB100 (p5.2) was increased considerably and reached its maximum (13.1 U/log CFU) at day 3, then it decreased slightly. The protease level of the same cells grown on basal medium II and 1% feather reached about 6.34 U/log CFU at day 6 (Fig. 3a). High levels of amino groups are shown in the optimized culture (12.26 μmol/log CFU) when compared to the basal medium (almost 2.7-fold higher). On the other hand, hydrolysis of feather by *B. subtilis* DB100 (p5.2) in the optimized culture generated higher amounts of soluble proteins (Fig. 3b, c). It is supposed that addition of corn oil and yeast extract in the production medium will support growth of bacteria and consequently increasing the level of keratinolysis. According to reported results on the

optimization of cultural conditions for keratinolysis, it was found that peptone and glutathione highly induced keratinase production by *Bacillus licheniformis* (Ramnani and Gupta 2004), while El-Refai et al. 2004, found that addition of several kinds of nitrogen sources, including yeast extract, did not affect keratinolytic activity of a *B. pumilus* FH9 strain.

Analysis of amino acids released during feather bioconversion

Amino acids play important roles in living organisms. There has been an increased demand for amino acids to be used in many areas such as feed, feed additives and drug pharmaceutical manufacturing. Keratin is the main component of feather, representing nearly 90% of feather weight. It shows an elevated content of the amino acids glycine, alanine, serine, cysteine and valine but lower amounts of lysine, methionine and tryptophan (Grazziotin et al. 2005). Biotechnological applications consider the use of keratin-degrading microorganisms or keratinolytic enzymes in the production of amino acids and peptides. Moreover, amino acids resulting from the utilization of feather or keratin-containing materials are one important product that can promote several new industries. Amino acids released during the biodegradation of feather by the host and the recombinant strains were analyzed.

The level of amino acids produced by the action of *B. subtilis* DB100, *B. subtilis* DB100 (pS1), *B. subtilis* DB100 (p5.2) on feather at day 0 and day 4 of cultivation is shown in Table 1. The concentration of amino acids was considerably increased during the fermentation process. It was found that, the amino acids Phe, Gly and Tyr were the major amino acids liberated in the cultures initiated by *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2). The level of these amino acids liberated by *B. subtilis* DB100 (p5.2) at day 4 of cultivation over that present at day 0 was nearly 463, 190 and 279-fold, respectively (Table 1). A similar profile was generated by *B. subtilis* DB100-

Table 1 Monitoring the level of amino acids in the cell-free supernatant of *B. subtilis* DB100-cells and its newly constructed strains at day 0 and day 4 of cultivation

Amino acid	*B. subtilis* DB100 -			*B. subtilis* DB100 (pS1)			*B. subtilis* DB100 (p5.2)		
	Day 0	Day 4	Day4/Day 0	Day 0	Day 4	Day 4/Day 0	Day 0	Day 4	Day 4/Day 0
Aspartic acid[a]	01.46	00.22	00.15	01.01	022.33	022.10	00.98	020.47	020.88
Threonine	00.73	00.68	00.93	00.38	000.97	002.50	00.71	001.26	001.77
Serine	01.13	00.88	00.78	00.55	007.03	012.80	00.60	005.88	009.80
Glutamic acid[b]	03.51	00.43	00.12	01.98	007.16	003.60	02.02	008.04	004.00
Proline	01.59	01.76	01.10	00.21	004.52	021.50	00.42	004.02	009.57
Glycine	03.07	00.67	00.20	01.28	193.88	151.50	01.15	218.60	190.00
Alanine	03.08	00.73	00.23	00.11	012.45	113.18	01.91	015.91	008.28
Cysteine	00.59	00.00	00.00	00.06	013.18	219.60	00.39	002.96	007.60
Valine	01.48	00.00	00.00	00.49	031.54	064.36	00.10	004.50	045.00
Methionine	02.19	00.39	00.18	00.23	016.19	070.40	00.10	019.43	185.00
Isoleucine	01.39	00.38	00.27	00.37	000.00	000.00	00.22	000.00	000.00
Leucine	00.25	00.24	00.96	00.88	006.40	007.27	01.09	007.33	006.72
Tyrosine	00.90	06.49	07.20	00.40	080.54	201.35	00.22	061.43	279.20
Phenylalanine	01.13	08.26	07.34	00.72	279.22	387.80	00.52	240.89	463.20
Histidine	01.15	00.09	00.07	00.36	001.32	003.60	00.26	003.09	011.88
Lysine	01.14	05.70	05.00	00.73	026.36	036.00	00.53	023.29	044.00
Ammonia	16.06	23.13	01.40	00.13	109.60	843.00	11.77	098.42	008.36
Arginine	01.57	00.00	00.00	01.89	012.55	006.60	00.77	003.84	004.98

Values are expressed as mg amino acid per 100 ml culture

[a] Value for aspartic acid and asparagine

[b] Value for glutamic acid and glutamine

culture, except for the Gly which was apparently consumed during the process of fermentation. Phe was the most abundant amino acid liberated as its concentration after 4 days of incubation reached 8.26, 279 and 241 mg/100 ml in *B. subtilis* DB100-, *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2) cultures, respectively.

However, the amino acids Asp, Val, Met and Lys were present in moderate concentrations in the recombinant strains *B. subtilis* DB100 (pS1) and *B. subtilis* DB100 (p5.2). Some findings indicated that enzyme treatment could improve the nutritional quality of feathers meal, when compared to conventional chemical treatment by which the limiting amino acids His, Met and Lys will be reduced (Kim and Patterson 2000). Conversely, the amino acid composition of a *Vibrio* sp. feather hydrolysate was reported to be rich in Ser, Leu, Ala and Glu residues and contains minor amounts of His and Met (Sangalli and Brandelli 2000).

Results on the composition of feather hydrolysate are with accordance with the results demonstrated previously concerning the recombinant strain *B. subtilis* DB100 (pS1) (Zaghloul et al. 1994), and they contribute mainly to confirm results related to substrate specificity of the cloned alkaline protease enzyme.

These new recombinant strains possess high keratinolytic activity and are very efficient in feather degradation; they offer potential application for biotechnological processes requiring keratin hydrolysis.

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