

A *Bacillus* Strain Able to Hydrolyze Alpha- and Beta-Keratin

Soltana Fellahi^{1,2*}, M Helmy GAD³, Taha I Zaghloul³, Elisabeth Feuk-Lagerstedt² and Mohammad J Taherzadeh²

¹Laboratory of Microbiology and Plant Biology, Department of Biotechnology Faculty of Sciences of Nature and Life, Mostaganem University, Mostaganem, Algeria

²Swedish Centre for Resource Recovery, University of Borås, Borås, Sweden

³Institute of Graduate Studies and Research, University of Alexandria, Alexandria, Egypt

Abstract

The ability to hydrolyze keratin, a rigid and strongly cross-linked protein in the waste of poultry feather and sheep wool, has made keratinase production by microorganisms highly important to the biotechnological industry. A protein-degrading bacterium (*C₄*) was isolated from compost. Based on morphology and biochemical tests, along with 16S rRNA sequencing, the isolated *C₄* was tentatively identified as *Bacillus* sp. *C₄* (2008). The proteolytic activity of the *Bacillus* sp. *C₄* strain was broadly specific; it degraded keratinous and non-keratinous proteins to different degrees. Pea pods as substrate generated the highest protease production, followed by soybean meal and sheep wool. Notwithstanding, using wool keratin as a sole source of carbon and nitrogen yielded the highest level of soluble proteins. Furthermore, the *C₄* bacterium grew well, and produced a significant level of keratinase when using wool and feather as substrates. Supplementing the medium with yeast extract and peptone shortened the time required for feather degradation, but delayed the onset of the wool keratin hydrolysis with two days. The predominant amino acids released in feather hydrolysate were tyrosine, phenylalanine, and histidine. In contrast, the wool lysate was rich in aspartic acid, methionine, tyrosine, phenylalanine, histidine, and lysine. Results established that utilizing the *C₄* strain for keratin degradation in waste management holds considerable potential.

Keywords: Proteolytic enzymes; *Bacillus*; Keratinase; Chicken feather; Sheep wool

Introduction

Every year, large amounts of keratin containing wastes are generated from poultry, leather, and meat processing industries. The annual global feather waste from the poultry processing industry alone reaches 8.5 million tons. At present, the poultry feathers are dumped, buried, used for land filling, or incinerated, resulting in problems in terms of storage, handling, emission control, and ash disposal [1].

Poultry feathers consist to 90% of keratin, which is rich in hydrophobic amino acids, but also contains important amino acids like cysteine, arginine, and threonine [2]. The feather waste can be processed to feather meal, hydrothermal processes or chemical treatments being the most popular methods. These processes result however in the destruction of essential amino acids, yielding a product with poor digestibility and low nutritional value [3,4]. Bioconversion of feather represents an alternative method for improving the nutritional value of feather waste [5].

Also sheep production and wool processing generate a significant amount of keratin waste, and as the world market for wool has dropped dramatically, this has brought about a huge amount of wool waste that cannot be processed [6]. Wool contains a different kind of keratin (α -keratin) than feather, due to the high concentration of cysteine crosslinking in the exocuticle of the wool fiber, and it is consequently not as rigid as feather keratin (β -keratin) [7]. Both types of keratin are intensively cross-linked with disulfide bridges, hampering their degradation by common proteases such as trypsin, pepsin, and papain [8].

In nature, keratin waste is continuously and efficiently decomposed by a large number of bacteria and fungi that produce special proteolytic enzymes, "keratinases" [9]. These enzymes are proteases, and they target insoluble keratin substrates [9,10]. They show high affinity toward hard-to-degrade proteins and have broad substrate specificity in comparison to conventional proteases. They are generally alkaline and thermostable by nature [9,11], and have consequently gained importance not only within various conventional biotechnological sectors, e.g. detergents,

feed, and fertilizers [9,12], but are also applied for environmental cleanup of feather keratin, converting it into feather meal for multiple purposes [13,14]. These proteases are also considered for the leather industry, as they are superior for enzymatic dehairing, and may also be applied in pharmaceutical preparations and in the cosmetic industry as ungual enhancers [15], etc. [16,17]. A recent finding, disclosing that keratinases cause enzymatic breakdown of prion protein PrP^{Sc}, opens the door for novel relevant applications of a broad range of keratinases [18].

A large number of microorganisms have been reported to produce keratinases [9-11]. So far, the vast majority of the identified keratinase producing organisms appear to be able to hydrolyze only β -keratin in chicken feather [19]. Few organisms, e.g. *Bacillus subtilis* and *Stenotrophomonas*, are known to be able to hydrolyze both α - and β -keratin. The most likely explanation for this is that β -keratin is more susceptible to the enzyme, as it contains less cysteine residues, and thus also fewer disulfide bonds than α -keratin.

In spite of the broad range of applications for keratinases, only a few commercial preparations of these enzymes are available on the market. Research needs to be focused on screening keratinases in order to succeed in extending the range of their applications, ultimately bridging the gap between demand and availability.

Owing to the complexity and variability of substrates, specific keratinases are required for each application. Advances in keratinase

***Corresponding author:** Soltana Fellahi, Swedish Centre for Resource Recovery, University of Borås, Borås, Sweden, Tel: 213-772-384-393; E-mail: Soltana.Fellahi@hb.se

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research should therefore be directed toward searching for novel keratinases with broader substrate specificity and a higher catalytic efficiency. Hence, we seized different aquatic and terrestrial bacteria in an attempt to obtain a bacterial strain that produced a keratinase with broad substrate specificity and with good catalytic efficiency.

Materials and Methods

Media

Bacterial isolates were cultivated on Peptone Yeast extract (PY) medium [20] (Bacto-peptone 10 g/l, Difco yeast extract 5 g/l, NaCl 5 g/l, pH 7.0), PA (PY supplemented with 15 g/l agar), milk agar (yeast extract 0.5 g/l, skimmed milk 10 g/l, agar 15 g/l, pH 7.0), basal medium II [5] (NH_4Cl 0.5 g/l, NaCl 0.5 g/l, K_2HPO_4 0.5 g/l, KH_2PO_4 0.4 g/l, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 g/l, yeast extract 0.1 g/l, pH 7.0), and modified basal medium II (basal medium II supplemented with yeast extract 1.5 g/l and peptone 1g/l). Basal medium II and modified basal medium II (the latter supplemented with 10 g/l chicken feather, sheep wool, soybean meal, or pea pods) were used for protease production.

Isolation, screening, and identification of the most potent protease-producing microorganism

Egyptian aquatic and terrestrial samples were used for the experiments. Thirty-one bacterial isolates were obtained from the material, twenty from the Red Sea, two from Wadi Natrun (EL HAMRA spring), one from olive oil waste, one from compost, five from Sohag soil, and two from Aswan soil.

Protease-producing bacterial isolates were obtained by using the spread plate method on PA and milk agar. The ratio (X/Y) was inferred to indicate protease activity, (X) being the diameter of the bacterial colony and (Y) the diameter of the clearing zone.

The bacterial isolate displaying the highest protease production, based on the ratio (X/Y), was identified by means of morphological, physiological, and biochemical characteristics, and by conducting 16S rRNA gene sequencing (Ribotyping). The ensuing data were compared with the standard descriptions in Bergey's Manual of Determinative Bacteriology [21].

DNA isolation was carried out in accordance with a method previously described by Zaghloul et al. [22], and the 16S rRNA gene was amplified by PCR in a thermocycler (Progene, England). The forward and reverse primers were:

Fw: 5'AGAGTTTGATCMTGGCTCAG-3'

Rv: 5'TACGGYTACCCTGTTACGACTT-3'

The primers were designed based on the conserved zones within the rRNA operon in *E. coli*. The PCR reaction was executed with 30 pmole of each primer, 10 μM dNTPs, and 2 units of *Taq* polymerase enzyme, producing a final volume of 50 μl . The process comprised an initial denaturation at 95°C for 5 min, followed by 30 cycles, each comprising 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, followed by a final extended period at 72°C for 10 min. The PCR product was purified by using the spin column (The Wizard[®] SV Gel & PCR Clean-Up System, Promega, Madison, USA).

The nucleotide sequences of the 16S rDNA gene were determined by Eurofins Genomics, Germany. Similarity to other bacteria was investigated by using the nucleotide search engine "Blastn", accessible at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The 16S rDNA gene sequence was subsequently submitted to the NCBI GenBank. A

dendrogram was generated by the neighbor-joining method, using the BioEdit software (<http://bioedit.software.informer.com/>).

Measurement of enzyme activity

Proteolytic activity was measured as described by Cliffe and Law [23], using Hide Powder Azure (HPA, Sigma) as substrate. The amount of enzyme causing a change in absorbance of 0.1 (in comparison with a control at 595 nm) after 30 minutes at 37°C equals 1 unit. Keratinolytic activity of the bacterial cells, as indicated by free amino ($-\text{NH}_2$) groups being released during biodegradation of feather and wool, was determined by using ninhydrin [24]. Soluble proteins were determined by the method of Bradford [25].

Ability to hydrolyze different protein-based substrates

The isolate showing the highest protease production in the screening process was further tested for its ability to hydrolyze the following protein substrates: Chicken feathers, sheep wool, pea pods, and soybean. Chicken feathers (collected from medium sized white hens), sheep wool, and pea pods were washed with tap water, followed by distilled water, and then dried overnight at 60°C. Chicken feathers, sheep wool, and pea pods were chopped into smaller particles. The dried pea husks were finely powdered [26].

For enzyme production 500 ml Erlenmeyer flasks containing 50 ml basal medium II supplemented with 10 g/l of the following: pea pods, soybean, chicken feathers, or sheep wool were used. In addition, 500 ml Erlenmeyer flasks containing 50 ml of modified basal medium II were used for the keratin substrates (Chicken feather and sheep wool). The batch cultures were inoculated with 3.16×10^9 CFU/ml and maintained at 37°C under shaking conditions (160 rpm; New Brunswick Scientific, USA) for three days. Protein content, Colony-Forming Units (CFU/ml) as well as proteolytic activity (for the four substrates in basal medium II) and keratinolytic activity (for the substrates in the modified basal medium II) were determined daily.

Analysis of the released amino acids

The amino acids released from feather and wool keratin into the modified basal medium II upon the action of bacterial isolate were analyzed (Beckman 119 CL AAA, Palo Alto, USA) [27], and the concentration of each amino acid (with the exception of tryptophan) was calculated on day 0 and day 3.

Results and Discussion

Selection of the most potent protease-producer

In accordance with the ratio X/Y (diameter of bacterial colony/diameter of the clearing zone) resulting from the hydrolysis on milk agar, three bacterial isolates (NS_1 , NS_2 , and C_4) were deemed to be high protease producers. In spite of using several liquid media for cultivating the bacterial isolates NS_1 and NS_2 , neither growth nor proteolytic activity were successful. Being the most potent producer of extracellular protease, the isolate C_4 was thus selected for further experimental studies. Our results concur with bacteria being the most dominant group of alkaline protease producers, with the genus *Bacillus* as the most prominent one [28-30].

Identification of the isolate C_4

Electron microscopic examination disclosed that the selected isolate was a spore-forming, Gram-positive bacterium. Cells were shaped as short rods, and the endospores were oval and located centrally in a slightly swollen sporangium (Figure 1). Morphological and



Figure 1: Transmission Electron Microscope (TEM) micrograph of the protease-producing isolate of the selected C₄ strain, showing a longitudinal section of a vegetative cell at division stage, and a spore. (40,000 X).

Character	Reaction
Morphological characteristics	
Gram stain	+
Cell shape	Short rods
Spore	+ (Central)
Salinity tolerance:	
5 g/l	+
30 g/l	+
70 g/l	+
Growth at:	
55°C	+
60°C	-
Hydrolysis of:	
Casein	+
Gelatin	+
Starch	-
Biochemical characteristics:	
Voges-Proskauer (VP) test	-
Nitrate reductase	+
Methyl red (MR)	-
Catalase	+
Gas from glucose	-
Egg yolk lecithinase	-
Formation of indole	-
Acid from:	
Glucose	+
Arabinose	-
Xylose	-
Mannitol	+
Maltose	-
Lactose	-
Sucrose	+
Motility	Motile
O-F test	Fermentative
Gelatin liquefaction	+
Urease	-
Citrate utilization	+

Table 1: Some morphological, physiological, and biochemical characteristics of the most potent protease-producing isolate.

biochemical characteristics of the bacterial isolate, summarized in table 1, suggested that the bacterial isolate C₄ belongs to family Bacillaceae, and is a member of the genus *Bacillus*. The genus was confirmed by a

phylogenetic analysis of the 16S rDNA gene. The 16S rDNA sequence of the isolate C₄ were highly similar to the sequences of a group comprising several *Bacillus* strains (Figure 2), e.g. agreeing to 96% with *Bacillus aerophilus* sp. nov. and *Bacillus stratosphericus* sp. nov. Morphological, biochemical, and physiological characteristics, along with the results from the comparative sequence analysis of the 16S rDNA (RNA) gene of the isolate C₄ with that of other 16S rDNA sequences (available in the GenBank database), indicate that our bacterial isolate should be categorized as a *Bacillus* species. We have hence tentatively named our bacterial isolate "*Bacillus* sp. C₄ (2008)", submitting its 16S rDNA gene sequence to the NCBI GenBank (accession number FJ214667).

Ability of *Bacillus* sp. C₄ to hydrolyze protein substrates

Proteolytic activity and release of soluble proteins proceeded throughout the cultivation of *Bacillus* sp. C₄ in basal medium II (Figure 3), pea pods displaying the highest protease activity of the substrates, and peaking on day 2 (10.36 U/Log CFU). The soybean meal substrate showed the second highest activity, and peaked on day 1, but declined only after 2 days (6.10 U/Log CFU). Proteolytic activity showed a linear increase in sheep wool, with no sign of decline on day 3 (5.94 U/Log CFU). The least successful substrate was chicken feather, showing a small increase that declined after day 2. The release of soluble proteins did not follow the proteolytic activity curves for the four substrates; the release from soybean, for instance, dropped abruptly after one day. It did however gradually recover as the cultivation period continued, and the level of soluble proteins from soybean eventually approximated the level emitted on day 3 by chicken feather, which reached 0.208 mg/ml. The level of soluble proteins from pea pods did not change much during incubation, the maximum level reaching 0.337 mg/ml at the end of the experiment (three days). Sheep wool appeared to be the most effective substrate, with soluble protein levels reaching 0.595 mg/ml on day 3 after a rather steep rise (Figure 4).

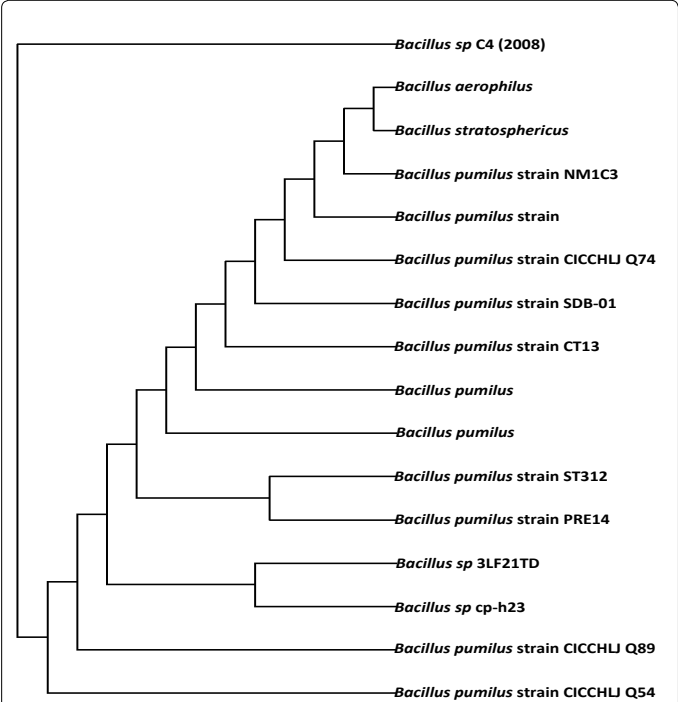
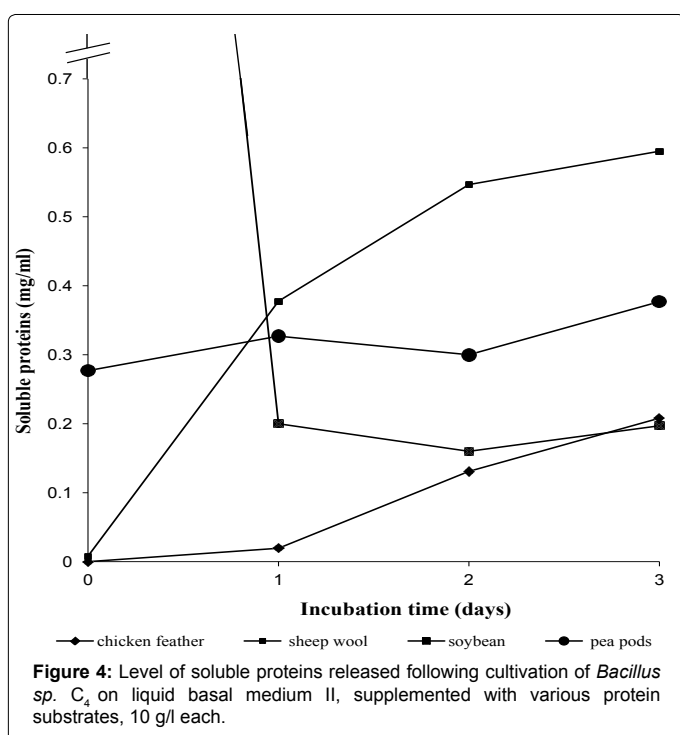
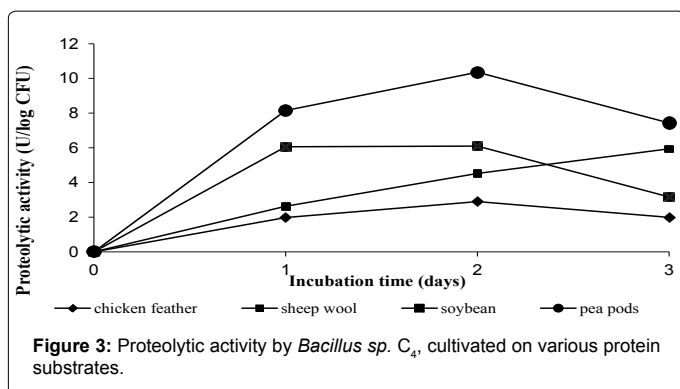


Figure 2: Phylogenetic relation of the 16S rDNA sequence of the *Bacillus* sp. C₄ (2008) strain to the 16S rDNA sequence of the 15 bacteria displaying the highest similarity.



Several studies report on the use of agro-industrial residues for the production of proteases, such as the following combinations: nug meal and *Bacillus* sp. AR 009 [31], pigeon pea and *Bacillus* sp. JB-99 [26], wheat bran and *Rhizopus oryzae* [32], green gram husk and *Bacillus* sp. [33], soybean meal and *Bacillus* sp. L21 [34]. The proven ability of bacteria to grow and to produce sizable levels of protease from various wastes and agro-industrial substrates offers tremendous potential for developing biotechnological methods for hydrolysis of such by-products. An aspect of great significance is that these natural materials are cheap and readily available substrates, and might thus be of interest for industrial-level production of protease. In the production of industrial enzymes, the growth substrate accounts for up to 30-40% of the production cost [35]. Utilizing natural residues, particularly in countries where they are generated in abundance, might hence considerably reduce the cost of enzyme production [36]. Furthermore, using agro-industrial substrates or environmental wastes might greatly bring down pollution problems [37].

The 3-day monitoring of *Bacillus* sp. C₄ hydrolyzing keratin-rich materials, such as chicken feather and sheep wool (10 g/l each), manifested bacterial growth and protease production, using both

substrates as sole source of carbon, energy, nitrogen, and sulfur. The modified basal medium generated a higher level of proteolytic activity than the unmodified basal medium. Furthermore, when supplementing the medium with yeast extract and peptone, the proteolytic activity of feather and sheep wool displayed a 4.3- and 2.7-fold increase, respectively (Figure 5a), which conformed with previously reported results [38,39].

Growing *Bacillus* sp. C₄ on feather keratin in modified basal medium enhanced the keratinolytic activity 1.53 times on day 2, while in the basal medium, a similar enhancement occurred already on day 1 (Figure 5b). However, while the activity in the modified basal medium continued to rise on day 3, it showed a dramatic drop in the unmodified medium. In contrast, keratinolytic activity of sheep wool in modified basal medium did not rise until day 3, and was not prominent, while in the unmodified basal medium, it increased steeply already on day 1, although figures fluctuated after that. Moreover, as illustrated in Figure 6, yeast extract and peptone supplementation shortened the time required for feather degradation from three days to two. The time required for wool decomposition was however not shortened (data not shown). Our results are in good agreement with previous research [40,41].

The degradation of feather and wool keratin by *Bacillus* sp. C₄ was accompanied by a release of soluble proteins in the culture medium. The protein release from feather degradation varied between the two media, but showed consistency in the wool incubation. Soluble proteins, liberated at the end of the experiment, exceeded 0.2 and 0.5 mg/ml in basal medium II containing feather and wool, respectively (Figure 5c), the latter producing as much as 0.475 mg/ml already on day 1, but in the modified basal medium. Our results disclose that *Bacillus* sp. C₄ favored yeast extract and peptone as nutrients, and thus wool keratin hydrolysis started only on day 2 in the supplemented modified medium. Interestingly, this strain of bacterium appears to develop considerable amounts of soluble proteins in comparison with other feather-degrading microorganisms. For instance, *Bacillus subtilis* DB100 (p5.2) released a maximum of 0.2 mg/ml soluble proteins after four days of incubation on 10 g/l feather [39], *Streptomyces fradiae* yielded a maximum of 0.12 mg/ml soluble proteins after 5 days [40,42], while *Trichoderma atroviride* strain F6 and a *Streptomyces* strain produced somewhat more, maximum values reaching 0.323 and 0.423 mg/ml after 8 and 3 days, respectively, when cultivated in 10 g/l feathers [43,44]. Growing a recombinant strain of keratinase-producing *Bacillus subtilis* on sheep wool-based distilled water medium yielded however as much as 1 mg/ml soluble protein after 3 days [45].

Analysis of amino acids released during feather and wool bioconversion

The amino acid profile in feather and wool hydrolysates is presented in table 2. Feather hydrolysate was rich in tyrosine, phenylalanine, and histidine residues, while aspartic acid, methionine, tyrosine, phenylalanine, histidine, and lysine were the predominant amino acids released in wool hydrolysate. Aspartic acid was the most abundant amino acid liberated in wool hydrolysate, but the nutritionally essential amino acids, such as methionine, tyrosine, histidine, and lysine were (as mentioned above) also present in this lysate. The levels of the other amino acids were rather modest in both feather and wool lysate, suggesting the possibility of these amino acids being consumed by *Bacillus* sp. C₄ during the fermentation process.

The two amino acid profiles mapped in the present study were compared with those obtained by the action of various keratinases

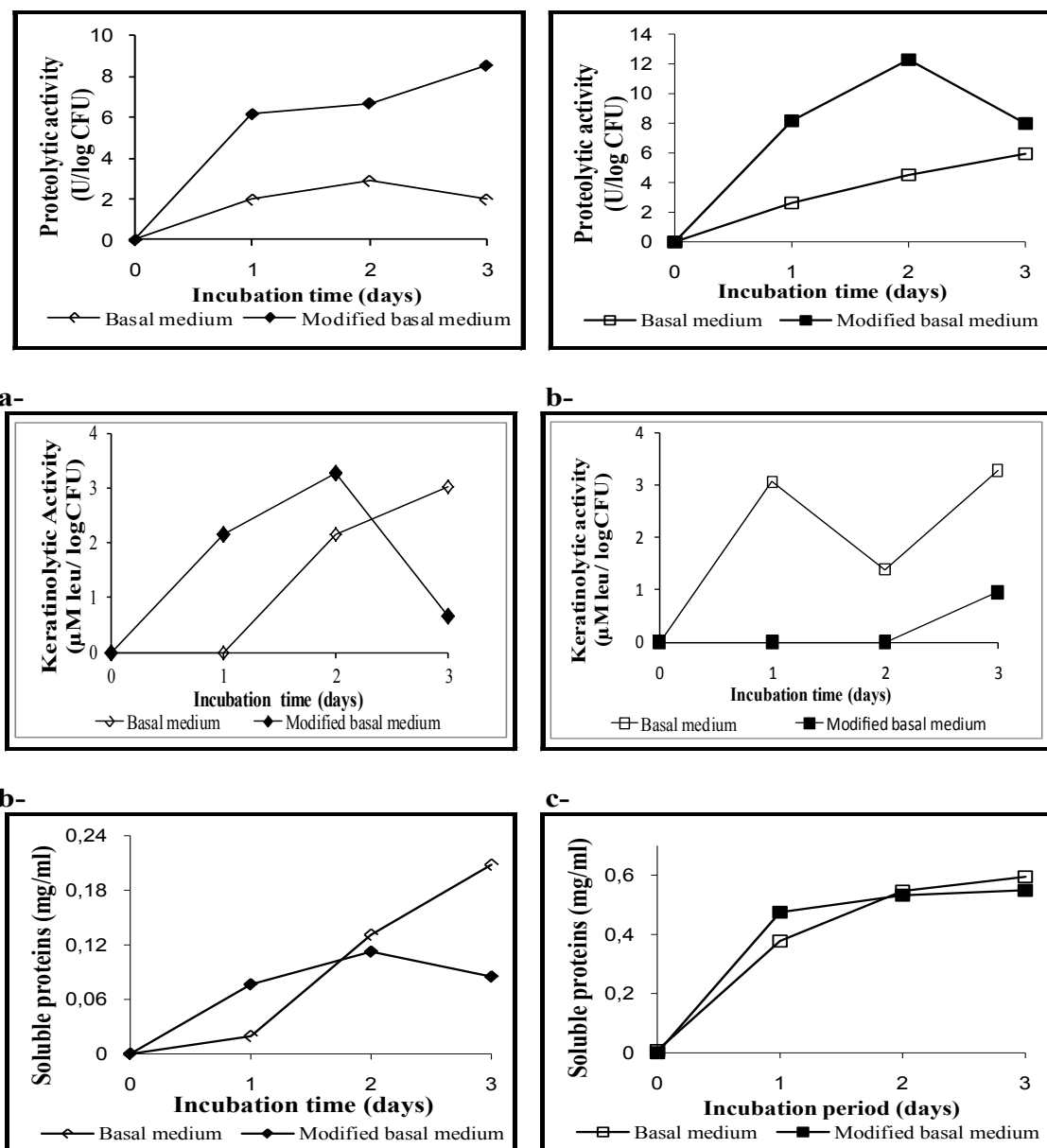


Figure 5: Proteolytic (a) and keratinolytic (b) activity as a result of cultivating *Bacillus* sp. C₄ on basal medium II (empty marks) and on modified basal medium II (filled marks), supplemented with chicken feather (diamond shape) and sheep wool (square shape), 10 g/l each. (c) Illustrates the levels of soluble proteins produced under the same conditions.



Figure 6: Physical appearance of chicken feather at three different stages: (A) before incubation, (B) on day 3 after the action of a *Bacillus* sp. C₄ strain on basal medium II, and (C) after 2 days of incubation on modified basal medium II.

Free Amino acids	Feather-keratin			Wool-keratin		
	Day 0	Day 3	Day 3/Day 0	Day 0	Day 3	Day 3/Day 0
Aspartic acid	170.65	0.75	0.004	1.00	67.86	67.86
Threonine	101.10	36.77	0.007	105.88	8.24	0.08
Serine	75.27	27.37	0.36	26.28	14.01	0.53
Glutamic acid	288.05	8.81	0.03	32.95	18.24	0.55
Proline	29.29	18.45	0.63	8.86	7.38	0.83
Glycine	116.53	1.99	0.01	20.09	9.42	0.46
Alanine	169.44	4.03	0.02	29.93	12.41	0.41
Cysteine	8.09	3.23	0.40	13.09	3.08	0.23
Valine	103.64	13.84	0.13	18.93	3.32	0.17
Methionine	79.05	11.03	0.14	26.87	97.61	3.63
Isoleucine	79.44	7.03	0.09	11.92	10.69	0.89
Leucine	137.12	8.23	0.06	26.00	2.57	0.09
Tyrosine	34.12	249.09	7.30	34.80	204.73	5.88
Phenylalanine	78.25	684.07	8.74	40.57	305.05	7.51
Histidine	19.38	62.88	3.24	30.53	86.79	2.84
Lysine	56.85	32.49	0.57	10.83	17.26	1.59
Arginine	86.45	5.40	0.06	27.99	8.65	0.31
Ammonia	118.06	214.07	1.81	167.25	184.17	1.10

Table 2: Levels of released amino acids and ammonia in cell-free supernatants of *Bacillus* sp. C₄ on day 0 and day 3 of cultivation in modified basal liquid medium II. Values are given in mg/l.

derived from other bacteria, and it appears that the amino acid profile depends on the bacterial strain. For instance, the amino acid composition of feather lysate generated by the action of *Fervidobacterium islandicum* AW-1 was rich in alanine, proline, serine, and cysteine [46], while serine, leucine, and glutamate residues dominated among the amino acids released in feather hydrolysate of *Vibrio* sp. strain kr2 [47]. In contrast, the action of *B. cereus* protease on wool keratin generated an amino acid profile rich in glutamate, serine, leucine, proline, arginine, aspartic acid, and threonine residues [48], while phenylalanine, tyrosine, and lysine were the main amino acids produced in wool lysate of a recombinant strain of *Bacillus subtilis* [45].

Conclusion

We have in the present study accomplished the isolation of a strain of *Bacillus* sp., capable of hydrolyzing both α - and β -keratin in three days. The strain proved suitable for degradation of avian feathers and sheep wool, and its keratin hydrolysates hold a strong potential for future applications in biotechnological processes.

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