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Original Research

Keratinolytic Potential of Feather-Degrading *Bacillus polymyxa* and *Bacillus cereus*

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Abstract

Keratinolytic abilities of *Bacillus polymyxa* B20 and *B. cereus* B5esz were evaluated in liquid cultures in mineral media containing chicken feathers. Both tested strains were capable of effective liquefying and biodegradation of feather keratin, up to 56.5 – 72.1% in ten-day cultures, releasing considerable amounts of hydrolysis products. Tested bacteria were mesophilic species, producing highest activity of keratinases and proteases in the presence of keratin (1%) as a sole nutrient source or keratin supplemented with yeast extract, at 30ºC. Keratinases of *B. polymyxa* were predominantly highly alkaline serine proteases, with optimum activity at 50ºC, while *B. cereus* produced mainly a mixture of neutral proteases, optimally active at 45ºC. Keratinolytic potential of tested bacterial strains could find a variety of applications, including utilization of keratinous waste from poultry industry and obtaining keratin hydrolysate-based soil fertilizers.

Keywords: *Bacillus polymyxa*, *Bacillus cereus*, feather degradation, keratinases, proteases

Introduction

Keratinases comprise a unique group of proteolytic enzymes involved in biodegradation of keratin, poorly degradable protein that is a constituent of vertebrate skin appendages, like feathers. The capability of extracellular keratinases production is a feature found in several species of filamentous fungi, streptomyces and bacteria, frequently from the genus *Bacillus*. The process of keratinolysis involves the action of specific proteinases, supported by chemical or enzymatic factors reducing disulfide bonds that stabilize keratin structure [1-3]. Bacterial keratinases are mainly serine proteases, but thiol and metalloproteases are also known [4, 5]. Several different strains of *Bacillus*, including *B. pumilus, B. licheniformis, B. subtilis, B. halodurans* or *B. pseudofirmus* are described to possess the ability of keratin biodegradation [6-10]. However, little information concerning keratinolysis performed by *B. polymyxa* and *B. cereus* can be found in literature, although these microorganisms are known producers of a wide variety of hydrolytic enzymes.

The aim of the presented study was to evaluate keratinolytic potential of *Bacillus polymyxa* B20 and *B. cereus* B5esz, focusing on the characteristics of produced proteolytic and keratinolytic enzymes along with the influence of culture conditions on keratinolysis.

Experimental Procedures

Keratinolytic strains of *Bacillus polymyxa* B20 and *B. cereus* B5esz, isolated from soil and keratinous waste, respectively, were used for the present study.

Microbial cultures were carried out in 250 ml Erlenmayer flasks, in 50 ml of medium, at 30-45ºC, with 170 rpm shaking, for ten days. Nutrient broth culture of

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$1.2 \cdot 10^8$ cfu/cm^3 served as inoculum, used in 1 ml per flask. The medium used in the study consisted of (%): $MgSO_4$ 0.1, KH_2PO_4 0.01, $FeSO_4 \cdot 7H_2O$ 0.001, $CaCl_2$ 0.01, yeast extract 0.05, optionally removed or replaced by glucose or peptone. The basic carbon and nitrogen source were whole, degreased, white chicken feathers (1%). The medium was set to pH 7.2 and sterilized by autoclaving at 121ºC for 20 min.

All assays were performed in collected post-culture liquids, after removing feather debris by filtration through medum density filter paper and centrifugation at 10,000 g for 10 min at 4ºC. Protein concentration was determined according to the method of Lowry [11]. The release of amino groups was measured using 2,4,6-trinitrobenzene-sulphonic acid (TNBS) [12]. Bacterial culture growth was estimated by optical density measurement (OD) at λ = 550 nm. Residual feather keratin was weighed after separation and drying at 105ºC.

Proteolytic activity (PA) was determined using a modified method of Anson, after 20 minutes of reaction on soluble casein, at 30ºC and optimum pH. The unit of proteolytic activity (1 PU) was expressed as 0.01 increase of absorbance of TCA-soluble products at 280 nm, released by 1 cm^3 of enzyme (post-culture medium) in 1 minute. Keratinolytic activity was determined in 20 minutes reaction on soluble keratin preparation, according to Wawrzkiewicz et al. [13], at 40ºC and optimum pH. The unit of keratinolytic activity (1 KU) was expressed as 0.01 increase of absorbance of TCA-soluble products at 280 nm, released by 1 cm^3 of enzyme in 1 minute [14].

Keratinolytic activity against native keratin (KA) was measured as above. Finely cut, degreased feathers were used as a substrate. Reaction mixtures included fluids from two and three-day cultures of *B. polymyxa* B20 and *B. cereus* B5esz, respectively. The activity unit (1 NKU) was expressed as 0.01 increase of absorbance of TCA-soluble products at 280 nm, released by 1 cm^3 of enzyme in 4 hours reaction time.

Optimum temperature for proteases and keratineses was determined over a 30-60ºC range with 5ºC interval in 0.1 M borate buffer pH 7.4. The influence of pH was tested over a range of pH 5-11 using 0.1 M Britton-Robinson universal buffer, at optimum temperature. To determine a dominating class of proteases and keratinases in crude culture fluids, 20 minutes pretreatment was performed with the following inhibitors: PMSF (phenylmethylsulphonyl fluoride), NEM (N-ethyl maleimide), EDTA (ethylenediaminetetraacetic acid, disodium salt) and activators: cysteine, $CaCl_2$.

All assays were performed in triplicate. Mean values and standard deviation were given.

Results

Feather Degradation in Bacterial Cultures

Due to the ability of keratin degradation, both tested bacterial strains were capable of growth in the medium containing chicken feathers as a main source of carbon, nitrogen and sulphur, as confirmed by OD rise and biochemical indices. The direct effect of keratinolytic proteases hydrolytic action was accumulation of hydrolysis products, such as soluble proteins and peptides, as well as free amino groups of amino acids. In the culture of *B. polymyxa,* maximum level of the first was 2.10 mg·cm^{-3} and the concentration of the latter was 3.05 mM (Fig. 1). In the case of *B. cereus* a larger amount of high molecular weight hydrolysis products was observed (2.35 mg·cm^{-3}) at the expense of released amino acids (2.71 mM) (Fig. 2). Moreover, the concentration of amino groups began to decline right after the major reduction of hydrolytic activities on the 6th day of cultures, while the release of souble proteins remained constantly increasing. Deamination of amino acids derived from keratin resulted in alkalinization of culture media, reaching pH 9.19-9.22.

Keratinolytic enzymes, responsible for hydrolysis of feather keratin, were detected in culture media at levels reaching 31.5 KU (specific activity 34.6 KU/mg) and 9.5 KU (12.4 KU/mg) for *Bacillus polymyxa* B20 (Fig. 3) and *B. cereus* B5esz (Fig. 4), respectively. Less keratin-specific proteases were also synthesized by tested strains. Exceptionally high activity of proteases, 229 PU (200 PU/mg), was produced by *B. cereus* B5esz, in contrast to 23.5 PU (19.4 PU/mg) of proteolytic activity of *B. polymyxa* B20. The peak of keratinolytic and proteolytic activity of *B. polymyxa* was observed on the 2nd and 3rd days of cultures,

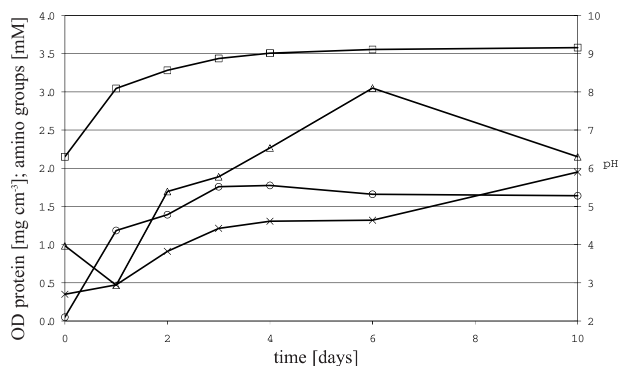

Fig. 1. Bacterial growth (OD) O, pH -, protein concentration ×, amino groups concentration △ during *B. polymyxa* B20 culture in synthetic medium with chicken feathers.

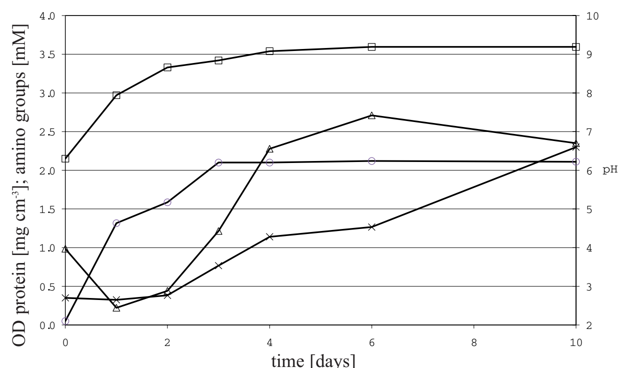

Fig. 2. Bacterial growth (OD) O, pH -, protein concentration ×, amino groups concentration △ during *B. cereus* B5esz culture in synthetic medium with chicken feathers.

Table 1. The effect of temperature on the keratinolytic potential of *B. polymyxa* B20 (maximum values given).

Temperature [ºC]		30	35	40	45
keratinolytic activity	U·cm⁻³·min⁻¹	31.5±5.6	0.2±0.1	0	0
proteolytic activity	U·cm⁻³·min⁻¹	23.4±0.4	18.2±0.7	15.2±0.1	1.17±0.3
protein	mg·cm⁻³	2.10±0.02	1.80±0.04	1.67±0.07	1.09±0.05
amino groups	mM	3.05±0.08	2.05±0.06	2.28±0.04	1.62±0.04

respectively, while maximum biosynthesis of these enzymes by *B. cereus* was delayed to the 3rd and the 4th days. After the 6th day the activity of keratineses and proteases decreased significantly in cases of both tested strains.

Due to solubilization and utilization of feather keratin, the content of residual substrate decreased from 62.4% on the 4th day to 43.5% on the 10-th day of *B. polymyxa* culture, and from 47.0% to 27.9% in the culture, of *B. cereus* (Fig. 5).

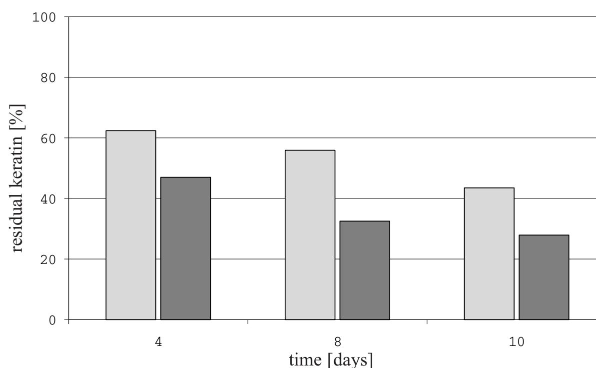

Fig. 5. Feather keratin degradation in cultures of *B. polymyxa* B20 ▢ and *B. cereus* B5esz ■.

Influence of Culture Conditions on Keratinolysis

The influence of culture conditions, like temperature and selected medium supplements, on the production of keratinolytic proteases and the process of keratinolysis was examined. Bacterial cultures carried out at temperatures within 30-45ºC revealed that both tested strains were capable of growing in such conditions. However, decreasing biosynthesis of hydrolytic enzymes at temperatures exceeding 30ºC allowed us to classify these bacteria as mesophilic. *B. polymyxa* cultured at 35ºC decreased its proteolytic activity to 77.7% and practically ceased to produce keratinases (Table 1). Similarly, proteases activity of *B. cereus* lowered to 21.0% and keratinases to 42.1% (Table 2). It is, however, significant that both strains retained at least low activity of proteases up to temperature 45ºC, which allowed them to utilize feather keratin while keratinases activity was undetectable. Lower proteolytic activity resulted in reduced release of feather hydrolysis products, yet no clear correlation was observed.

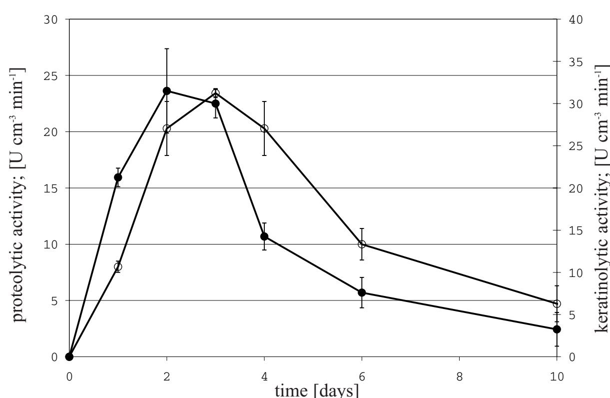

Fig. 3. Biosynthesis of keratinases ● and proteases ○ in a culture of *B. polymyxa* B20 in the presence of chicken feathers.

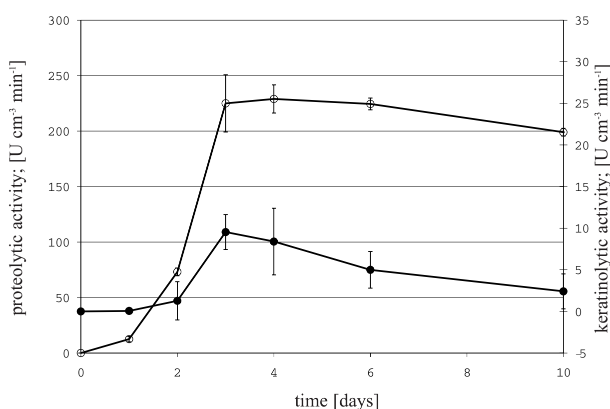

Fig. 4. Biosynthesis of keratinases ● and proteases ○ in a culture of *B. cereus* B5esz in the presence of chicken feathers.

Both *Bacillus polymyxa* B20 and *B. cereus* B5esz synthesized extracellular proteases and keratinases in mineral medium in the presence of chicken feathers as a source of nutrients, while chosen additives influenced enzyme of expression. Easily available glucose almost completely inhibited biosynthesis of keratinases in *B. polymyxa* (Fig. 6a), while it had no negative effect on the synthesis of less keratin-specific proteases. As a result, weak growth of the strain was observed. The addition of yeast extract or peptone sustained growth and biosynthesis of keratinases and proteases. The most significant increase in keratinolytic activity, up to 56 KU, occured in the absence of additional substrates, yet at the expense of slightly decreased proteolytic activity. Similarily, *B. cereus* produced higher keratinolytic activity, reaching 45.9 KU, in a medium with feath-

Table 2. The effect of temperature on the keratinolytic potential of *B. cereus* B5esz (maximum values given).

Temperature [ºC]		30	35	40	45
keratinolytic activity	$U \cdot cm^{-3} \cdot min^{-1}$	9.5±2.1	4.0±0.4	0	0
proteolytic activity	$U \cdot cm^{-3} \cdot min^{-1}$	229.0±12.7	48.2±3.3	44.9±4.0	12.1±2.9
protein	$mg \cdot cm^{-3}$	2.53±0.10	0.79±0.02	0.74±0.04	0.99±0.05
amino groups	mM	2.71±0.09	1.30±0.05	1.25±0.03	2.21±0.06

Table 3. Optimum conditions for keratinases and proteases activity of tested strains.

	Bacillus polymyxa B20		*Bacillus cereus* B5esz	
	keratinolytic activity	proteolytic activity	keratinolytic activity	proteolytic activity
temperature [ºC]	50	50	45	55
pH	10.2	6.6	7.4	7.4

ers as a sole nutrient source, while its proteolytic activity decreased to 50%, as compared to the control medium (Fig. 6b). Unlike in the case of *B. polymyxa*, glucose addition stimulated production of tested enzymes. The control medium intensified protease but not keratinase production. The concentration of keratin hydrolysis products indicated that effective keratinolysis occured either in the presence of yeast extract, peptone or absence of additives, while glucose inhibited the process.

Preliminary Characteristics of Proteases and Keratinases

The optimum pH of *B. polymyxa* keratinase activity, in crude culture fluid, was determined to be higly alkaline (pH = 10.2), while the optimum for caseinolytic activity of proteases was pH=6.6. The optimum temperature for both enzyme types was 50ºC, but keratinase activity remained over 60% of the maximum value within a range of 45-60ºC. In contrast, keratinases and proteases of *B. cereus* were optimally active at pH=7.4, with only 20% loss of activity within a range of pH=6.0-7.8. The optimum temperature was 45ºC and 55ºC, respectively, but, similarly, keratinases showed over 80% of activity between 45ºC and 60ºC (Table 3).

Keratinolytic and proteolytic activity of *B. polymyxa* enzymes in crude culture fluid was largely inhibited by a chelating agent, EDTA, which suggests major participation of metalloproteases in the mechanism of keratinolysis. However, the effect of PMSF showed that serine proteases were also involved. Moreover, the trace of thiol caseinolytic proteases was observed. Almost complete inhibition of both keratinases and proteases of *B. cereus* by EDTA proved that metalloproteases were the dominating class of proteolytic enzymes produced by the tested strain. Nonetheless, thiol and serine proteases were also present (Tables 4, 5).

Table 4. Influence of inhibitors and activators on the activities of keratinases and proteases of *B. polymyxa* B20.

inhibitor / activator	concentration [mM]	residual activity [%]	
		keratinases	proteases
PMSF	10	57.4±3.1	31.5±10.1
NEM	5	86.4±5.9	50.6±4.0
EDTA	5	36.4±3.3	13.7±3.4
cysteine	1	105.0±10.0	95.2±7.7
	5	96.7±6.8	87.1±4.0
$CaCl_2$	10	118.2±7.1	78.3±9.3

Table 5. Influence of inhibitors and activators on the activities of keratinases and proteases of *B. cereus* B5esz.

inhibitor / activator	concentration [mM]	residual activity [%]	
		keratinases	proteases
PMSF	10	64.5±5.8	77.8±6.1
NEM	10	48.9±2.3	81.3±7.2
EDTA	2	5.6±1.4	0.2±2.2
cysteine	1	67.4±1.3	n.d.
	5	42.9±1.2	91.5±1.2
$CaCl_2$	1	n.d.	99.4±8.2
	2	n.d.	102.9±8.4
	10	130.0±9.9	n.d.

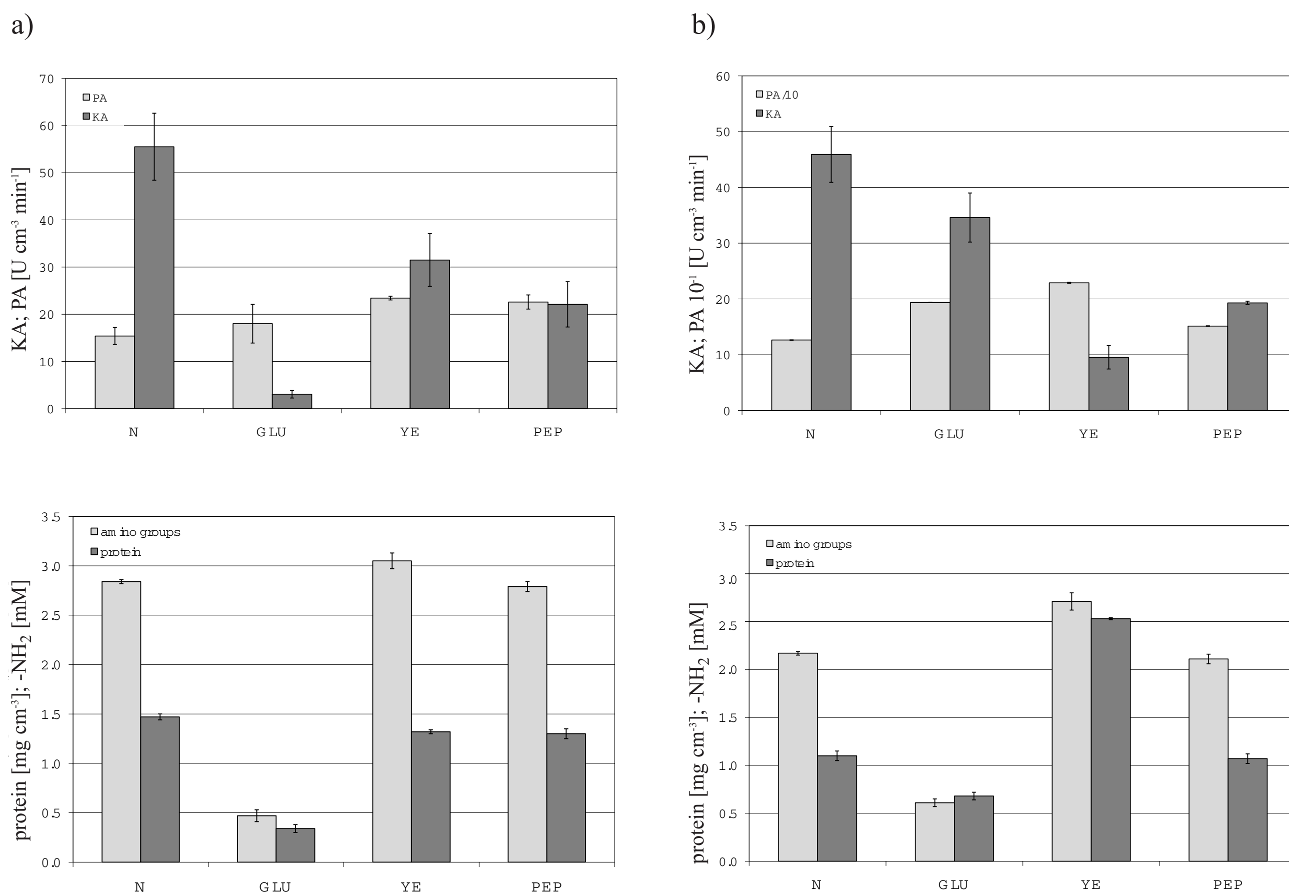

Fig. 6. a) The effect of feather-containing medium supplementation on the release of amino groups and proteins and biosynthesis of proteases and keratinases by *B. polymyxa* B20; b) The effect of feather-containing medium supplementation on the release of amino groups and proteins and biosynthesis of proteases and keratinases by *B. cereus* 5esz.
N – unsupplemented mineral medium with feathers (control), GLU – glucose, YE – yeast extract, PEP – peptone.

In order to verify the role of extracellular keratinases and proteases, enzymatic hydrolysis of untreated feathers with crude culture fluids was performed. The enzymes of both tested strains exhibited hydrolytic action against the native substrate. The measured activity was 2.1 NKU and 8.3 NKU for *B. polymyxa* and *B. cereus*, respectively (Fig. 7).

Discussion

Keratinolytic microorganisms, in contrast to keratinophilic, not only colonize keratinic materials, but also utilize the protein as a source of carbon and nitrogen. Both tested strains, *Bacillus polymyxa* B20 and *B. cereus* B5esz, demonstrated the ability to grow in a medium containing chicken feathers. The direct effects of keratinolysis include: the release of keratin hydrolysis products like soluble proteins, peptides and amino acids, and the presence of reduced thiol groups of liberated cysteine and alkalinization of medium by ammonia derived from amino acids deamination. The level of proteins released by tested strains (2.10-2.53 mg·cm^{-3}) was similar or higher as compared to others: *B. subtilis*, *B. pumilis* and *B. cereus* (0.62-0.75 mg·cm^{-3}) [15], *B. pumilis* F 3-4 (1.6 mg·cm^{-3}) [16], *Vibrio* sp. (2.5 mg·cm^{-3}) [17] or *Streptomyces* sp. (0.8 mg·cm^{-3}) [18]. It is significant that the concentration of soluble proteins was constantly increasing throughout the culture period of tested bacteria, while the content of free amino acids reached its peak on the sixth day (2.71-3.05 mM), three days after maximum value of proteolytic and keratinolytic activity. Increasing alkalinity exceeding a value of pH 9.1 was also an important factor documenting biodegradation processes, as suggested in the research of Lal et al. [19].

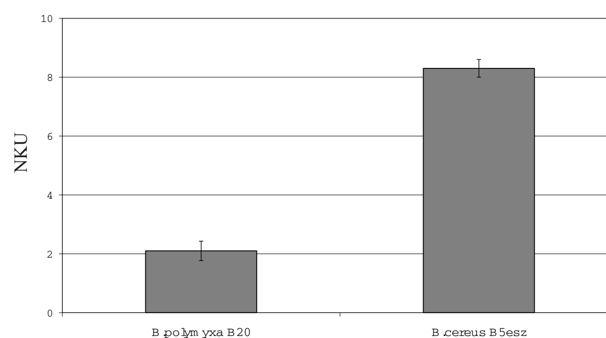

Fig. 7. Keratinolytic activity in crude culture fluids against untreated feathers (maximum values given).

Biosynthesis of bacterial keratinolytic and proteolytic enzymes in periodic cultures usually intensifies in the late logarithmic or early stationary phase. Maximum keratinases and protease production for both tested microorganisms was observed between the second and third days of culture, which is similar to results of Kim et al. [15], Pissuwan and Suntornsuk [20] and Wang and Shih [21] for different *Bacillus* strains. Furthermore, in spore-forming bacteria secretion of proteinases is often associated with the late logarithmic growth phase or the beginning of sporulation, which undoubtedly occured after depletion of easily available nutrients. Production of keratinolytic proteases typically depens on various culture conditions like temperature, concentration of keratinous substrate and medium supplementation. Inoculum size and quality and initial pH of media are also of importance, as suggested by Suntornsuk and Suntornsuk [22] and Kim et al. [15]. Both tested bacterial strains were found to be mesophilic species that preferentially produced keratinolytic enzymes at 30ºC. Nonetheless, limited keratin degradation also occurred at temperatures over 35ºC despite ceased production of keratinases and decreased synthesis of proteases. This might suggest supplementary action of non-specific proteases in biodegradation of keratins. Moreover, keratin degradation is not always directly correlated with keratinase activity, as concluded by Son et al. [16]. A number of keratinolytic isolates from the genus *Bacillus*, mainly of environmental origin, were described to be mesophilic. *Bacillus pseudofirmus* FA30-01 investigated by Kojima et al. [23] was a highly effective mesophilic and alcaliphilic isolate, completely liquefying chicken feathers within three-day cultures. Greater capabilities for keratin biodegradation are also usually assigned to thermophilic species like *B. licheniformis* [24] or *Fervidobacterium pennivorans* [25].

The vast majority of keratinases described for the genus *Bacillus* belongs to serine proteases, like in *B. licheniformis* PWD-1, often classified as subtilizins like in *B. pumilis* [26] or *Bacilus* sp. [7, 27]. Keratinolytic metalloproteases are rarely found in bacteria such as *B. cereus* [5], *Lysobacter* sp. [28] and *Microbacterium* sp. [29]. Some data on thiol keratinase of *B. licheniformis* is also available [4]. In the presented research proteolytic activities in crude culture fluids were studied, therefore the application of specific inhibitors revealed the presence of three protease types to some extent. Keratinases of *B. polymyxa* were partially inhibited by EDTA, NEM and PMSF, which suggests a composition of keratin degrading enzymes require purification prior to detailed description. Participation of serine and thiol-dependent enzymes was also observed, mostly in the proteases fraction. Highly alkaline optimum activity of keratinases as well as low sensitivity to cysteine or Ca^{2+} could indicate their serine character. Both keratinases and proteases produced by *B. cereus* were predominantly metalloenzymes, probably Ca-dependent, of neutral pH optimum. Nonetheless, partial sensitivity to serine and thiol inhibitors was also determined.

The basic action of bacterial cells in order to utilize insoluble substrate-like keratin is biosynthesis of extracellular proteases, dependent on cell density, cell condition, the growth phase and optional induction by the substrate. Therefore, culture media containing keratin are often supplemented with low concentration of additives (up to 0.1%), supporting cell growth in early stages. The abundantly used supplements are: yeast extract [6, 15, 22, 30, 31], peptone or other protein hydrolysates [18, 32] and carbohyrates [2, 33]. *B. cereus* produced higher levels of keratinases and proteases in general, but reached exceptionally high proteolytic activity. The addition of glucose did not considerably inhibit biosynthesis of keratinases and proteases which, resembling the case of *B. polymyxa*, was probably started upon the depletion of easily available carbon. Comparing the results obtained in parallel cultures without feathers, it appears that keratinases are inducible in *B. polymyxa*, but not in *B. cereus,* which synthesized low amount of enzymes (0.6 KU). Both strains expressed much lesser proteolytic enzymes of 7.9 PU and 5.8 PU, for *B. polymyxa* and *B. cereus,* respectively, which is shown to be their basal level of synthesis (data not shown).

B. polymyxa is known to be the species of higher nutritional demands. This probably is the reason why the addition of yeast extract stimulated cell growth, and therefore the production of a higher proteases level, reaching 16.3 PU. A similar effect was observed for *B. cereus* that reached proteolytic activity of 223.3 PU.

The addition of peptone stimulated only proteolytic activity of both strains, as expected. In comparison, for *B. pumilus* FH9 in the research of El-Refai et al. [6] neither ammonium chloride nor yeast extract and peptone showed a favourable effect on keratinase production. Ramnani and Gupta [2] performed optimization of medium composition for *B. licheniformis* RGI and included glucose, peptone, phosphate (1% each) and glutathione (0.05%) as a reducer of liberated thiols. Surprisingly, in cultures of *B. polymyxa,* a higher concentration of released soluble proteins was observed in comparison to *B. cereus*, except for medium with glucose. Nevertheless, protein concentration in culture media was almost twice as high as that achieved by *B. cereus* in the research of Kim et al. [15], in similar conditions. In the unsupplemented medium containing feathers as the sole source of carbon and nitrogen, both strains produced keratinolytic enzymes. However, *B. cereus* synthesized as much as 10.9 KU of keratinolytic activity and this value was of rising tendency, while *B. polymyxa* reached levels of 3.5 U, with a maximum on the third day of the culture. Moreover, the growth and alkalinization were superior for *B. cereus*. This illustrates the exact keratinolytic potential of both tested bacteria, especially *B. cereus*, capable of growth due to keratin utilization as the solitary nutrient source.

Different results could be achieved by modification of C:N ratio. Likewise, Suntornsuk and Suntornsuk [22] found that an addition of 1% glucose to medium containing feathers and yeast extract suppressed growth and keratinase synthesis of *Bacillus* sp. FK46. However, stimulation of keratinase production in the presence of glucose was observed for *B. pseudofirmus* by Gessesse et al. [34] or *B. pseudofirmus* [16].

The examined representants of the genus *Bacillus* expressed considerable amounts of keratinolytic enzymes which allowed them to grow on hardly degradable keratinous material like chicken feathers. Evaluated activity against native feather keratin in cell-free culture fluids demonstrated the capability of keratinolytic proteases to hydrolyse the substrate. Nevertheless, their action might be insufficient for complete digestion in the absence of reducing agents or cells red-ox potential, supporting disulfide bonds cleavage [2].

Keratinolytic potential of *B. polymyxa* and *B. cereus* could find an application in various areas of biotechnology. Microbiological biodegradation of keratinous waste from food industry, e.g. through composting [35], poses a promising alternative for other energy-consuming processes and results in obtaining valuable soil fertilizers.

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