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DEGRADATION OF KERATIN CONTAINING WASTES BY BACTERIA WITH KERATINOLYTIC ACTIVITY

KERATĪNU SATUROŠO ATKRITUMU DEGRADĒŠANA AR BAKTĒRIJU

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Abstract. *The aim of this study was to select keratin-degrading bacteria from JSC "Biocentras" collection and poultry processing plant wastewater, and to study their ability to degrade chicken feathers. Isolated from poultry processing plant wastewater bacteria was grown in basal media with feathers meal and showed high keratinolytic activity and protein content throughout the cultivation time. Bacterial strains B. licheniformis 511, B. subtilis I1, B. subtilis 717, and B. subtilis 103 suggested strongly of bacteria that produces keratinolytic activity in the cell free culture supernatants. The obtained results showed that maximum activity of keratinase is a function of cultivation time by the bacteria tested. B. subtilis 103 reached to its maximum level of keratinase production (152 U/mL) after 24 hrs, when over bacteria (148-242 U/mL) after 48 hrs. The good ability of selected bacteria to degrade feathers was detected. The best biodegradation of feathers was obtained using B. subtilis I-1. Over bacillus good degraded feathers as well.*

Keywords: *Feather, feather degrading bacterium, keratins, keratinolytic activity, poultry wastes*.

Introduction

Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather, and wool. The protein chains are packed tightly either in α-helix (α-keratins) or in β-sheet (β-keratins) structures, which fold into final 3-dimensional form [1; 2; 3]. Keratins are grouped into hard keratins (feather, hair, hoof and nail) and soft keratins (skin and callus) according to sulphur content [4]. These proteins belonging to the scleropeptides group are compounds that are extremely resistant to the action of physical, chemical and biological agents. One of the main characteristics of keratins is that they have high mechanical stability and resistance to proteolytic degradation, which depends on the disulfide and hydrogen bonds, salt linkages and other crosslinkings [5; 6]. Therefore, keratinous material is water insoluble and extremely resistant to degradation by common proteolytic enzymes such as trypsin, papain and pepsin [4; 6; 7].

The leather and fur plants as well as slaughterhouses throw away considerable amounts of materials containing keratin; such as wool, bristle, horns, feathers, hoof, etc. Until recent years, these materials along with other animal wastes were treated at high temperatures and then milled in order to produce the so called "animal flour" and used as "protein supplement" into the feed mixtures of domestic animals. However, it was established that this flour is the carrier of the enigmatic cause (Called prion) of some related disease (mad cow, swine fever, bovine spongiform encephalopathy, Creutzfeldt-Jacob disease, etc.) [8; 9].

Wold-wide poultry processing plants produce millions of tons of feathers as a waste product annually, which consists of approximately 90% keratin. Feathers represent 5-7% of the total weight of mature chickens. These feathers constitute a sizable waste disposal problem. Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed [8; 10; 11]. Most feather waste is land filled or burned which involves expense and can cause contamination of air, soil and water [10; 11]. Feathers hydrolysed by mechanical or chemical treatment can be converted to feedstuffs, fertilizers, glues and foils or used for the production of amino acids

and peptides [5]. An alternative to decrease this pollution is the utilisation of feather constitutes that can use as animal feed, preventing accumulation in the environment and the development of some types of pathogens. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids (methionine, lysine, histidine) but also consume large amounts of energy [12; 13]. Utilizing poultry feathers as a fermentation substrate in conjunction with keratin-degrading microorganism or enzymatic biodegradation may be a better alternative to improve nutritional value of poultry feathers and reduce environmental waste.

A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases produced by some microorganisms [7; 12]. Keratinase properties depend upon its producers. It is usually a serine protease [7; 13]. Occasionally, it has been found to be a serine protease with a cysteine protease [13; 14] and a metallo protease [13; 15]. Keratinolytic enzymes have important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline [4; 12; 16].

Many keratinases from species of *Bacillus* [5; 6; 11; 12; 13; 17], fungi [1; 18; 19] and *Actinomycetes* [2; 7] has been reported and some of them were purified and characterized.

The aim of this study was to select keratin-degrading bacteria from JSC "Biocentras" collection and poultry processing plant wastewater, and to study their possibility to degrade chicken feathers.

Materials and methods

Organisms. Bacterial strains (*Bacillus licheniformis* 511, *Bacillus subtilis* I-1, *Bacillus subtilis* 717, and *Bacillus subtilis* 103) screened for extracellular keratinolytic activity were obtained from JSC "Biocentras" collection. Also microorganism from poultry processing plant wastewater from JSC "Vilniaus paukštynas" showing keratinolytic activity was isolated.

Microorganism isolation. Water sample was collected from a local poultry industry. The sample were flooded in saline solution 0.9 %, suspension up to 10^{-6} were made and used to streak milk agar plates (5 g/L peptone, 3 g/L yeast extract, 100 mL/L sterile non-fat milk, and 12 g/L agar) which were incubated at 30 ^{o}C for 24 hrs for primary screening of proteolytic activity. Strains that produced clearing zones in this medium were selected.

Growth conditions. Two mediums (pH 7.4) used for keratinase production contained the following constituents (g/L): NaCl 0.5, KH_2PO_4 0.7, K_2HPO_4 1.4, $MgSO_4$ 0.1 and feather meal 10 (Medium 1) or soy flour 10 (Medium 2). Cultivation was performed using 500 mL Erlenmeyer flasks containing 100 mL medium for 24 hrs at 37^{o}C with constant shaking at 200 r/min. As inocula, 4% (v/v) bacteria grew in Oxoid Nutrient broth for 20 hrs. Culture supernatants obtained after centrifugation at 8000×g for 20 min were used for further study.

Keratinolytic activity. Keratin azure (Sigma-Aldrich, USA) was used as the substrate. The 5 mg keratin azure was suspended in 1 mL 50 mmol/L Tris-HCl buffer (pH 8.0). The reaction mixture contained 1 mL keratin azure suspension and 1 mL appropriately diluted enzyme. The reactions were carried out at 50^{o}C with constant agitation of 200 r/min for 1 hr. After incubation, the reactions were stopped by adding 2 mL 0.4 mol/L trichloracetic acid (TCA) and followed by filtration to remove the substrate. The filtrate was spectrophotometrically measured for release of the azo dye at 595 nm. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 increases in absorbance between sample and control at 595 after one hour under the conditions given. The result was taken as an average of three replicates.

Protein determination. Protein concentration was determined by Lowry *et al.* [20], methodology. Bovine serum albumin was used as a standard.

Degradation of keratin wastes. The capacity of degradation of keratin substrates was tested on medium containing 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4 and 10 g/L of raw feathers. Degradation of substrate was visually inspected.

Results and discussion

Ten isolates selected from poultry processing plant wastewater were able to grow on medium containing feather meal as sole carbon and nitrogen source. After being incubated for 24 hrs, a plate containing milk and agar showed the growth of several colonies. The strains produced clearing zones in milk agar plates characterize by proteolytic activity. Only four strains designated as kb1, kb2, kb3, kb4 were characterized by proteolytic activity (Fig. 1). Selected microorganisms weren't identified and it will be done in further study. The largest clearing zone was observed for isolate kb1. This strain was selected and used for keratinolytic activity assay.

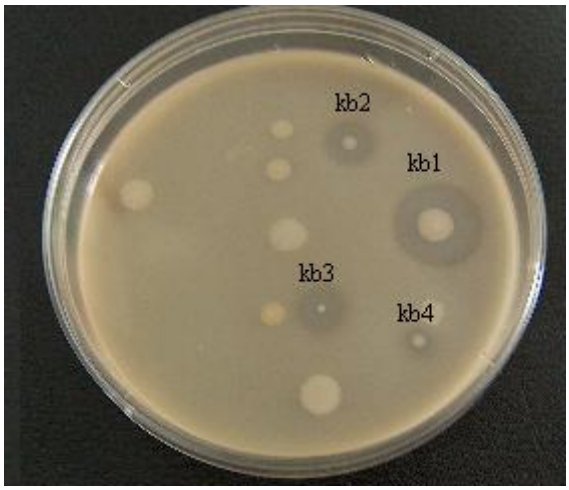

Fig.1. **Production of clearing zones in milk agar plates by keratinolytic bacteria**

Keratinolytic activity of selected bacteria: *Bacillus licheniformis* 511, *Bacillus subtilis* I-1, *Bacillus subtilis* 717, *Bacillus subtilis* 103 and kb1 was monitored during grow in Medium 1 after 24, 48 and 72 hours.

Table 1.

Keratinase production is a function of cultivation time by different bacteria species on feather meal

Bacteria	Growth time, h					
	24		48		72	
	Protein content, mg/mL	Keratinase activity, U/mL	Protein content, mg/mL	Keratinase activity, U/mL	Protein content, mg/mL	Keratinase activity, U/mL
B. licheniformis 511	1.13	102	1.32	242	1.17	86
B. subtilis I-1	0.85	96	1.05	198	1.23	76
B. subtilis 717	0.98	89	1.15	180	1.16	70
B. subtilis 103	0.69	152	0.82	136	1.05	38
kb1	1.28	112	1.63	146	1.41	138

All bacteria were grown in basal medium with feather meal as a sole source of carbon, nitrogen and sulphur at 37 ^{o}C, pH 7.4. All microorganisms showed good growth and protein synthesis with a varying level of keratinolytic activity (Table 1) on feather meal. The high activity was demonstrated by the most of the bacillus after 24 hrs of cultivation on feather meal. The highest activity of keratinase was observed after 48 hrs after cultivation on feather meal, except *B. subtilis* 103, which maximum keratinolytic activity was reached after 24 hrs. After 72 hrs keratinolytic activity was strongly decreased by all species of bacillus. Especially *B. subtilis* 103 showed the lowest keratinolytic activity after 72 hrs and the lowest protein content during cultivation on feather meal. Only the strain kb1 isolated from poultry processing plant wastewater showed high keratinolytic activity and protein content throughout the cultivation time.

Strains *B. licheniformis* 511 and *B. subtilis* I-1 were cultivated in Medium 2, where the source of carbon, nitrogen and sulphur was soy flour, and the keratinolytic activity was monitored during cultivation (after 48 and 72 hrs). The obtained results are presented in Table 2. Soy flour as an inducer displayed relatively contradictory results. *B. subtilis* I-1, using soy flour as substrate in basal medium, showed the higher keratinolytic activity as compare with feather meal, while *B. licheniformis* 511 showed the extremely lower keratinolytic activity.

Table 2.

Effect of soy flour in the basal medium on the synthesis of keratinolytic enzymes

Bacteria	Growth time, h			
	48		72	
	Protein content, mg/mL	Keratinase activity, U/mL	Protein content, mg/mL	Keratinase activity, U/mL
B. licheniformis 511	1.14	82	0.49	58
B. subtilis I-1	1.14	216	1.17	118

The selected and isolated cultures suggested strongly of bacteria that produces keratinolytic activity in the cell free culture supernatants. Demonstration of keratinase enzymes on keratinous substrate as the sole source of carbon and nitrogen by *Bacillus* species was also evidenced by other works [5; 6; 8; 10; 12; 15]. In the present study, the all strains of bacillus seem to be promising for keratinase production. However, the maximum activity as obtained here is a function of cultivation time by the bacteria tested. Even all strains of selected bacteria produced maximum amount of enzyme under varied cultivation times. *B. subtilis* 103 reached to its maximum level of keratinase production after 24 hrs, when over bacteria after 48 hrs. The karatinase activity was reduced drastically after 72 hrs in all bacillus species, but in kb1 strain – fractionally. Hence, further studies on the optimization of factors affecting enzyme production by individual bacterium will reveal their real potential as the enzyme producer.

The good ability of selected bacteria to degrade feathers was detected using the cell free culture supernatants (Fig. 2). The visual studies showed the evident degradation of feathers. The best biodegradation of feathers was obtained using *B. subtilis* I-1. The strain *B. subtilis* I-1 grew well and completely degraded feathers in the medium after 72 hrs. Over bacillus good degraded feathers as well, but not all feathers in the medium were digested through. Feathers contains mostly β-keratin and as was described in other works, *Bacillus* species mostly produces β-keratins degrading keratinases [5; 6; 13]. Solubilization of native poultry feathers by the cell free culture supernatants showed the biotechnological potential involving keratin hydrolysis in the processing poultry waste industries.

This work, however, further elucidated that for the evaluation of biotechnological application of the keratinolytic protease from selected bacteria requires more detailed understanding of the factors that enable this enzyme for complete degradation of native keratinous substrates. Therefore, additional researches will be done for the purification and characterization of keratinase, studying the kinetics of enzyme, testing for the range of substrates, effect of inhibitors, enhancing the activity of keratinase, submerged state fermentation and large scale production of keratinase, immobilization of keratinase.

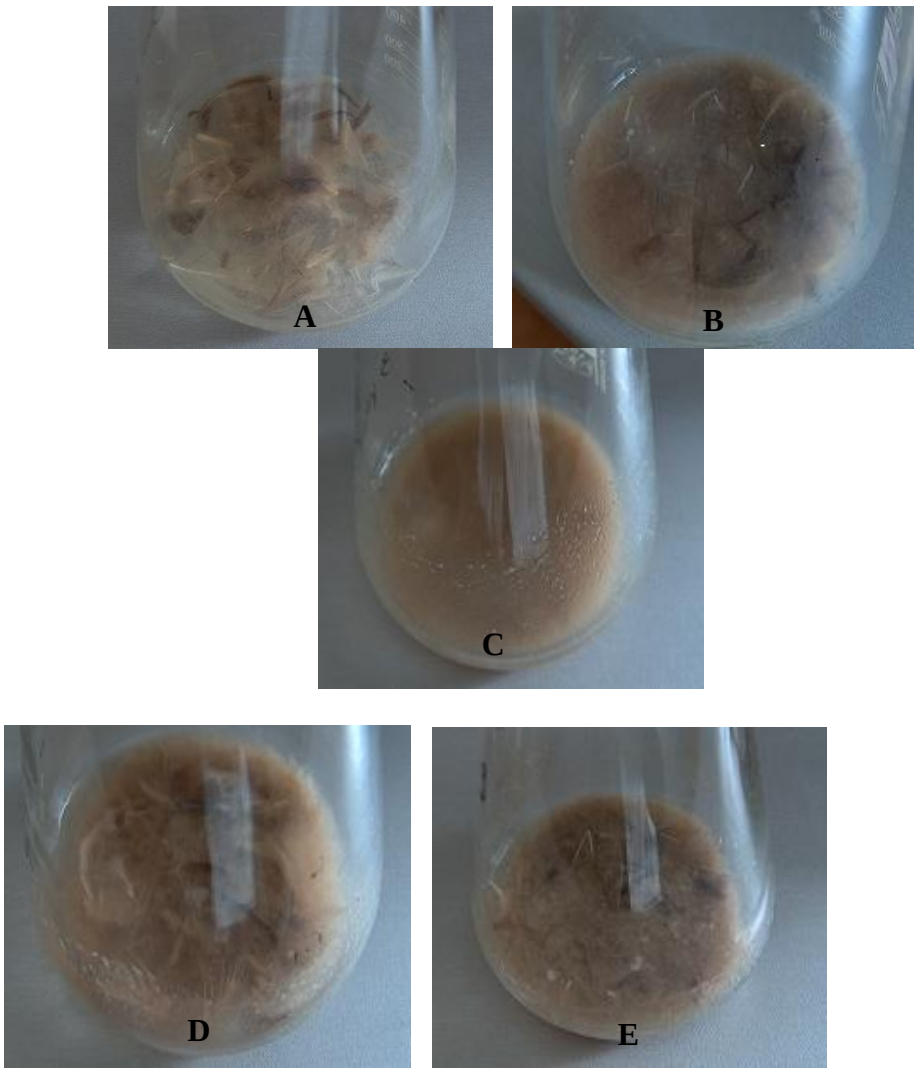

Fig.2. **Feather treatment with the bacteria at 37 ^{o}C for 72 hrs**: A – control, B – *B. licheniformis* 511, C – *B. subtilis* I-1, D – *B. subtilis* 717, E – *B. subtilis* 103

Conclusions

A feather-degrading bacterium was isolated from poultry processing plant wastewater. This bacterium was grown in basal media with feathers meal as its primary source of carbon, nitrogen, sulphur and energy. This strain showed high keratinolytic activity and protein content throughout the cultivation time

Bacterial strains (*Bacillus licheniformis* 511, *Bacillus subtilis* I1, *Bacillus subtilis* 717, and *Bacillus subtilis* 103), obtained from JSC "Biocentras" collection, were screened for extracellular keratinolytic activity. The selected cultures suggested strongly of bacteria that produces keratinolytic activity in the cell free culture supernatants. The maximum activity as obtained here is a function of cultivation time by the bacteria tested. *B. subtilis* 103 reached to

its maximum level of keratinase production (152 U/mL) after 24 hrs, when over bacteria (148-242 U/mL) after 48 hrs.

The good ability of selected bacteria to degrade feathers was detected. The best biodegradation of feathers was obtained using *B. subtilis* I-1. Over bacillus good degraded feathers as well.

Selected bacteria strains have biotechnological potential for degradation and utilization of feather keratin.

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