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Identification and properties of a keratinase from *Stenotrophomonas maltophilia* N4 with potential application in biotechnology

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Abstract. The bacterium *Stenotrophomonas maltophilia* N4 produces different extracellular proteases when cultured in a mineral medium with 1% of bird feathers. One of the enzymes was purified and characterized. The studied enzyme is a peptidase with keratinolytic activity. The optimal temperature for enzyme activity of the purified protein is 60°C, and its pH optimum 8.5. Its thermal stability is approximately 50% after two hours of preincubation at 55°C. Enzymatic activity is strongly inhibited by DFP and EDTA, indicating that the enzyme belongs to the metal-dependent serine proteases. Calcium, magnesium and manganese ions enhance the activity of the studied keratinase. The enzyme has broad substrate specificity; it hydrolyzes not only keratin, but also casein, gelatin and hemoglobin Considering the fact that the N4 bacteria are capable of using bird feathers as a source of organic nitrogen and carbon, and bearing in mind the stability and the broad substrate specificity of the studied enzyme, it appears that it may find application in various branches of biotechnology.

Key words: extracellular protease, feather degradation, keratinase, enzyme purification, *Stenotrophomonas maltophilia.*

1. Introduction

Proteolytic enzymes, also known as peptidases, proteases or proteinases, have always been a relevant and valid study subject. The considerable interest in bacterial proteolytic enzymes largely results from the possibilities of their application. Such enzymes are commonly used in biotechnology due to their biochemical diversity, high stability under unfavorable reaction conditions, relatively low cost of obtaining, and the ease of their genetic manipulation (Rao et al., 1998; Sawant & Nagendran, 2014; Vojcic et al., 2015). The enzymes, which hydrolyze peptide bonds that link amino acids together in the polypeptide chain forming a protein, may be used to remove protein waste, particularly including protein waste that is insoluble in water and therefore degrades more slowly in the natural environment. Keratin-rich by-products such as bird feathers, horns, the epidermis and the hair of animals, are an instance of such proteins with low hydrolytic susceptibility. They are a heterogeneous group of fibrous proteins with particularly low susceptibility to chemical and enzymatic hydrolysis (Brandelli et al., 2010; Gupta & Ramnani, 2006; Gupta et al., 2013). These proteins are resistant to hydrolysis because of their tight structure, which is supported primarily by cysteine bridges, and also by hydrogen bonds and hy-

drophobic interactions (Bockle & Muller, 1997; Gopinath et al., 2015). However, the interest of science in keratin hydrolysis relates not only to the effective removal of the waste of insoluble protein polymers from the environment; it is also about the possibility of obtaining keratin, a valuable cosmetic and pharmaceutical ingredient, that is at the center of researchers' interest in this aspect (Lasekan et al., 2013; Fontoura et al., 2014) Attempts have also been made to use keratin wastes as a source of bioactive peptides and agricultural fertilizers (Brandelli et al., 2015). Keratinases [EC 3.4.21/24/99.11] are responsible for the enzymatic hydrolysis of the discussed group of proteins. In addition to hydrolyzing fibrous proteins, keratinolytic enzymes also hydrolyze collagen and non-fibrous proteins such as casein and albumin, thus pointing to the enzymes' broad substrate specificity (Gupta & Ramnani, 2006). A multitude of cysteine bridges in the keratin molecule suggests that sulfite reductases or reducing compounds participate in the initial stage of hydrolysis (Korniłłowicz-Kowalska & Bohacz, 2011; Prakash et al., 2010). Keratinolytic microorganisms include many types of microorganisms, including a large number of species from the genera: *Bacillus, Streptomyces*, *Lysobacter* and *Vibrio* (Łaba et al., 2015; Brandelli, 2008). The bacterium *Stenotrophomonas maltophilia* is also capable of the effective degradation of bird feathers and depilation of animal skins (Yamamura et al., 2002; Cao et al., 2009; Jeong et al., 2010). *Stenotrophomonas maltophilia* N4 is one of its strains; it has high proteolytic activity and is the source of the enzyme that is discussed in this paper.

The main purpose of the study was to isolate and characterize one of the proteolytic enzymes released by the bacterium in terms of optimal activity, substrate specificity, the effect of inhibitors and metal ions. In order to evaluate the usefulness of the protein in biotechnology, diverse substrates were used and the enzyme's activity was studied during its reaction in the presence of non-ionic detergents.

2. Materials and methods

2.1. Bacterial culture conditions and enzyme activity analysis

Stenotrophomonas maltophilia N4 (accession number: AB667906) was the source of proteases in the presented study. Bacteria were grown for 72 h with shaking at 120 rpm and temperature 28ºC. Bacterial cultures were grown in mineral medium composed of (g/liter): KH_2PO_4, 3; K_2HPO_4, 3; $MgSO_4$, 0.5; NaCl, 2; $FeCl_3$, 0.005. In order to optimize the composition of the culture medium, the following ingredients were used separately or in combinations as supplements (g/L): feathers 10, yeast extract 2.5, Bacto Peptone 5, glucose 5. Control culture media did not contain the feathers. The covert feathers of the helmeted guineafowl (*Numida meleagris*) were obtained from the Poultry Breeding Division at WULS. Before they were added to the medium and autoclaved, the feathers were cleaned and split into approximately 2 cm fragments.

Keratinolytic activity was measured using a modified protocol by Cai et al. (2008) using 5 mg keratin azure as the substrate suspended in 50 mM Tris-HCl buffer pH 8.5. Enzymatic reaction was carried out for 1 hour at 40°C with constant agitation at 200 rpm. One unit of keratinase activity (U) was the amount of enzyme that caused a change in absorbance of 0.01 at 595 nm after reaction for 1 h.

Caseinolytic activity was determined using the Anson method (1938) a with 1% casein solution in 50 mM Tris-HCl buffer pH 8.0. The enzymatic reaction was carried out for 30 min at the temperature of 40°C. One unit of caseinolytic activity (U) was defined as the amount of enzyme required to liberate 1 µmole of tyrosine within 30 min of the reaction under the specified conditions.

2.2. Enzyme purification and proteomic analysis with mass spectrometry (MS)

All protein purification stages were performed at the temperature of 4°C. In order to obtain a clear supernatant, filtering and subsequent centrifugation (9000 x g for 10 min) were carried out on the third day of bacterial culture in a mineral medium with 1% of bird feathers. The supernatant was precipitated using ammonium sulfate until 85% saturation was reached. The protein precipitate obtained after centrifugation was then dissolved in 20 mM Tris-HCl buffer with pH 7.8 and dialyzed against the same buffer for 12 hours, with two changes of the buffer. The preparation after dialysis was separated on a column of DEAE–Sepharose (Sigma), which was equilibrated with 20 mM Tris-HCl buffer pH of 8.0. Proteins that bound to the anion exchanger were eluted in a gradient of 0 to 0.5 M NaCl. Fractions (5 ml volume) were screened for keratinolytic and caseinolytic activity. The protein fractions that were obtained after ion-exchange chromatography and had the highest activity were concentrated, resulting in approximately 30–50 µg/mL protein in the preparation. The protein samples previously digested with trypsin were separated on a nanoAcquity UPLC (Ultra Performance LC) system and analyzed with an Orbitrap-based mass spectrometer (the Polish Academy of Sciences). The obtained results were analyzed using Blast software.

The fractions with highest keratinolytic activity were concentrated and then subjected to molecular sieve chromatography. The concentrated preparation (10-fold) was applied to a column of Superdex 200 (Sigma), which was equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Active fractions were used for characterizing the enzyme.

2.2.1. Protein determination and zymography analysis

Protein concentration in samples was determined using the method of Bradford (1976) with bovine serum albumin as the protein standard.

Zymograms were obtained after in semi-native SDS-PAGE electrophoretic separation (without thermal denaturation and β – mercaptoethanol) of the samples in 10% polyacrylamide SDS gel with incorporating 0.1% casein (Laemmli, 1970, with modifications). On completion of electrophoresis the gels were incubated for 1 h in 0.5% Triton X-100 solution, and then transferred to 100 mM Tris-HCl buffer, pH 8.5 and stained with 0.1% amide black solution. Proteolytic activity was observed as bright bands where the substrate was digested, against the dark blue background of the gel.

2.3 Characterization of purified protease

The optimal temperature was determined in the range from 30 to 65°C. The optimum pH was determined in the range of 5.5 – 9.5. The following buffer systems were used: 50 mM Britton-Robinson buffer for the pH range 5.5 – 9.5 and 50 mM Tris–HCl buffer for the pH range 6.8 – 8.5.

The substrate specificity of the enzyme was determined using the Anson (1938) method with 1% solutions of substrates (gelatin, hemoglobin, casein, and soluble keratin).

In order to study the effect of specific proteases inhibitors on activity, the purified enzyme was preincubated with an inhibitor solution for 30 min. at 4°C, after which the remaining activity was tested. The effect of metal ions on the activity was determined following preincubation of the enzyme for 30 min at 4°C in the presence of metal ions in final concentration of 5.0 mM, after which the substrate was added and the activity tested.

All results presented in this paper in the form of numerical values were means from 3 independent determinations. The mean error, reflecting maximal deviation of the results of measurements from the mean, did not exceed 5%.

3. Results

3.1 Bacterial culture and composition of growth medium

The results of this study indicate that the bacterium *S. maltophilia* N4 was able to utilize bird feathers as a sole source of organic carbon and nitrogen in mineral medium (Fig. 1).

Enrichment of the culture medium containing 1% feathers in yeast extract, glucose or peptone did not result in enhanced proteolytic activity. At the same time it was observed that the presence of feathers in the medium induced the activity of proteases, particularly in mineral medium with scant peptide components or in mineral medium with glucose (Fig. 2).

3.2 Purification of protease, zymography and MS analysis

The clear culture medium obtained after filtration and centrifugation of a 3 day bacterial culture in mineral medium containing 1% feathers was taken for the purification of proteases. Purification involved the following stages: fractionation with ammonium sulfate at 30–85% satura-

Figure 1. Feather degradation after 24 h of bacterial incubation

tion, ion-exchange chromatography and molecular sieve chromatography (Table 1).

Ion-exchange chromatography yielded three main peaks of caseinolytic and keratinolytic activity as presented in Figure 3.

The three protease fractions obtained as a result of this stage of purification were subject to proteomic analysis using mass spectrometry. The identified proteolytic enzymes included metal-dependent endoproteases and serine endoproteases (details in Table 2, supplementary materials).

In the third fraction, eluted at about 0.35 M NaCl, peptides having a consensus sequence with a serine peptidase

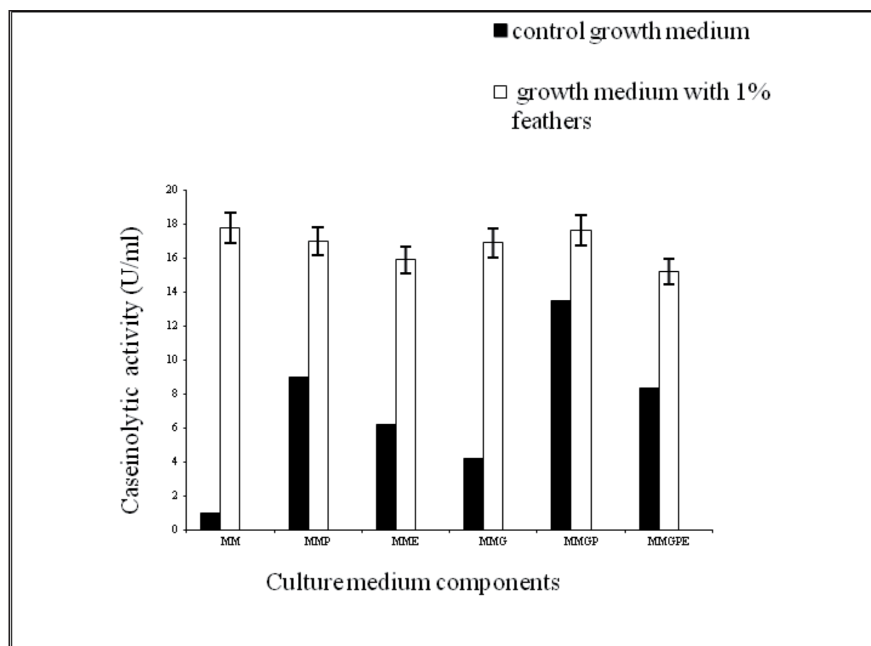

Figure 2. Proteolytic activity on the third day of bacterial culture. The following media were used: MM – mineral medium , MMP – mineral medium supplemented with peptone, MME – mineral medium supplemented with yeast extract, MMG – mineral medium supplemented with glucose, MMGP mineral medium supplemented with peptone and glucose, MMGPE mineral medium supplemented with peptone, glucose and yeast extract. Control culture media did not contain the feathers

Table 1. Purification of keratinase from *S. maltophilia* N4

Purification step	**Total activity [U]**	**Total protein [mg]**	**Specific activity [U/mg]**	**Recovery [%]**	**Purification fold**
Cell free supernatant	8100	454	17.8	100	1
Ammonium sulfate precipitation (30–85%)	5900	290	20.3	72.84	1.14
DEAE Sepharose (0–0.5 M NaCl)	1700	18	94.4	21	4.8
Superdex 200	1000	4.3	232.6	12.3	13.1

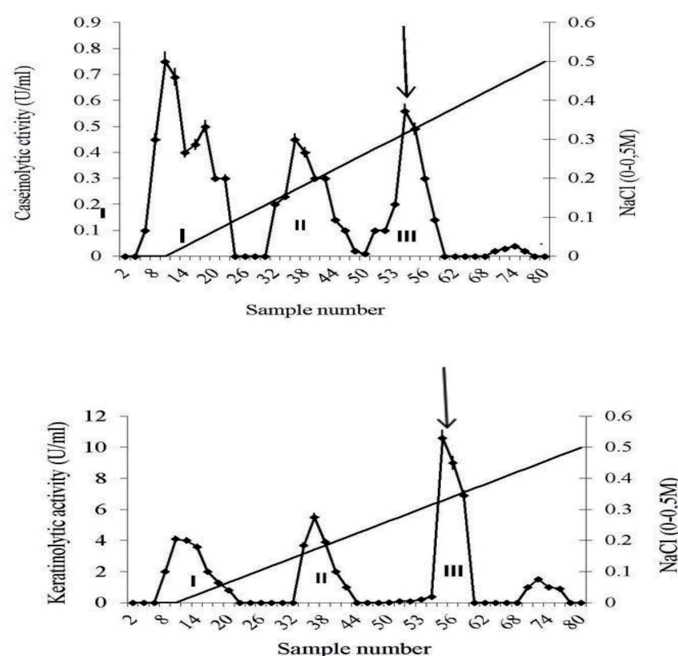

Figure 3. Ion-exchange chromatography of the proteases from *S. maltophilia* N4

Table 2. Mass spectrometry identification of proteases of *S. maltophilia* N4 in Fractions I, II, III after separation by ion exchange chromatography

Fraction of proteases	Identification of the protease	Microorganism /Accession Number in Genbank	Catalytic Type
F I Proteases not binding to the bed, eluted at equilibrium buffer	Peptidyl-Asp metalloendopeptidase	*Stenotrophomonas maltophilia* R551-3/ GenBank/ACF50520,	Metallopeptidase family (M12B family)
	Aminopeptidase	*Stenotrophomonas maltophilia* R551-3]/ ACF50006	Metalloexopeptidase (M28 family)
	Serine Peptidase StmPr1	*Stenotrophomonas maltophilia* R551-3]	Serine protease (S8 family)
F II Proteases binding to the bed; eluted at around 0.22–0.25 M NaCl	Dipeptidyl peptidase IV Alanyl dipeptidyl peptidase	*Stenotrophomonas maltophilia* D457/ CCH11727	Serine prolyl oligopeptidase, (S9 family)
	peptidase S8 (Pr2)	*Stenotrophomonas maltophilia*/ WP_025877979	Serine protease (S8 family)
F III Proteases strongly binding to the bed; eluted at around 0.35 M NaCl	Alkaline serine protease	*Stenotrophomonas maltophilia* OBGTC27/ CBI67281	Serine peptidase (S8 family, S8_53 superfamily)

from Genbank (accession number CBI67281) were identified. This enzyme fraction was taken for further purification molecular sieve chromatography. Analysis of a zymogram run after this purification stage revealed the presence of a single activity band in the gel (Fig. 4). The final preparation contained a 13-fold purification enzyme, which was used in biochemical characterization steps.

3.3 Biochemical characterization of protease N4

The purified enzyme showed maximal activity at 60°C (Fig. 4).

The activity drastically decreased at higher temperatures and at 65°C only 70% of the maximal activity was observed. Optimal pH was about 8.5, but similar enzymatic activity was observed even at pH 8.0 (Fig. 6).

The data in Table 3 illustrate that the highest activity of the enzyme, that is 2.25 U ml^{-1}, was observed in the presence of casein and keratin, 1.6 U ml^{-1}. This indicates that the K : C ratio was 0.71.

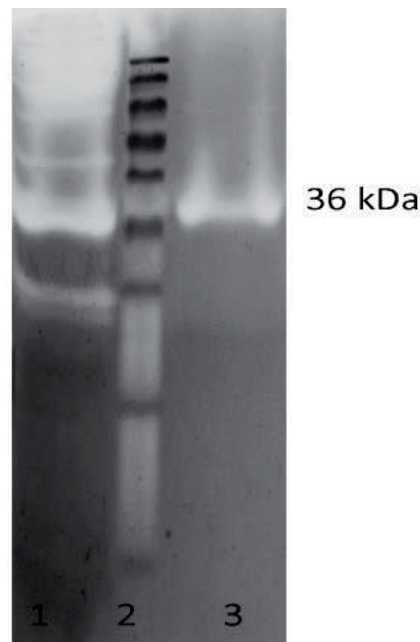

Figure 4. Zymograms analysis of keratinase activity. 1 –crude enzyme preparation, 2 –protein ladder (Thermo Scientific Protein Ladder #26616) 3- purified keratinase

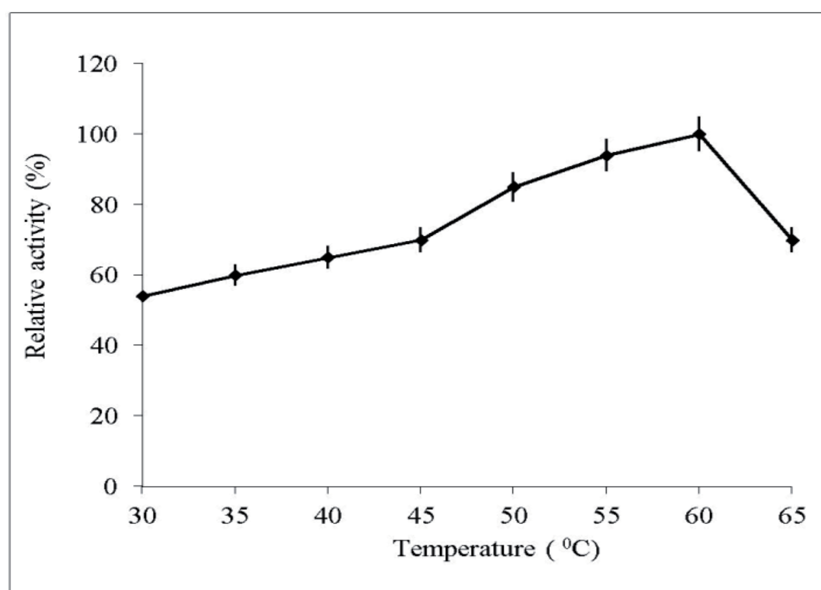

Figure 5. Effect of temperature on the activity of keratinase from *S. maltophilia* N4

Table 3. Substrate preference of the keratinase

Substrates	Activity (U/ml)	Relative activity (%)
Casein	2.25	100
Keratin	1.60	71
Hemoglobina	1.35	60
Gelatin	0.44	20

The activity of the studied enzyme was strongly inhibited in the presence of the specific serine protease inhibitors DFP and Pefabloc (Table 4). What's interesting, the metalloprotease inhibitors EGTA (Ethylene glycol tetraacetic acid), EDTA (Ethylenediaminetetraacetic acid), and 1,10-Phenantroline also decreased the activity of the protease. The inhibitors of aspartyl proteases (Pepstatin A) and cysteine proteases (E-64, iodoacetamide) did not affect the activity of the enzyme. Similarly, the trypsin inhibitor Aprotinin did not inhibit its activity.

The divalent metal ions used in the experiments, such as Mg, Mn or Ca caused either slight stimulation of enzymatic activity (Mn, Mg) or its stabilization (Ca). Ions of cadmium, cobalt and copper strongly inhibited activity (Table 5). The effect of detergents and other chemical compounds on the activity and stability of the studied keratinase was also examined. The presence of SDS and Triton X-100 inhibited the activity of the enzyme by 25 and 5%, respectively.

Table 4. Activity of keratinase in the presence of specific inhibitors

Inhibitor	Concentration (mM)	Residual Activity (%)
none	—	100
DFP	0.1	35
	1	12
Pefabloc	0.1	80
	1	70
PMSF	0.1	60
	1	20
EDTA	1	78
	5	65
EGTA	1	57
	5	45
1,10-Phenantroline	1	80
	5	65
Iodoacetamide	0.1	100
	1	100
E-64	0.1	100
	1	100
Pepstatin A	3	100
Aprotinin	0.1	100

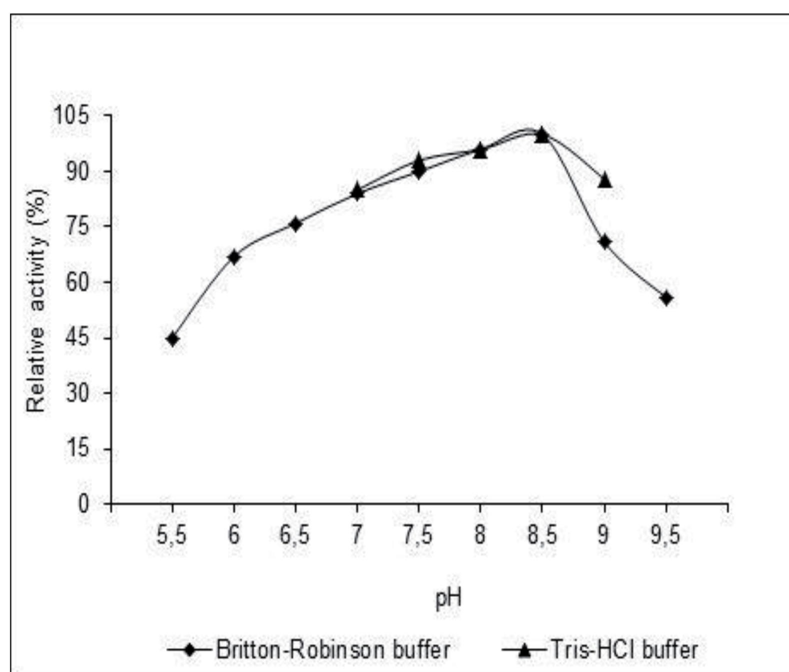

Figure 6. Effect of pH on the activity of keratinase from *S. maltophilia* N4

Table 5. Effect of various reagents on the keratinase activity

Reagents	Concentrations	Residual activity (%)
Control		100
Mg^{2+}	5 mM	120
Mn	5 mM	125
Ca^{2+}	5 mM	105
Zn^{2+}	5 mM	45
Cu^{2+}	5 mM	22
Co^{2+}	5 mM	15
Cd^{2+}	5 mM	21
Triton X-100	1 %	95
Tween 80	1 %	105
SDS	0.1 %	75
2-M EtOH	0.5 %	100

4. Discussion

The studied bacterium *S. maltophilia* N4 is very interesting in view of its wealth of extracellular hydrolases as well as characterized chitinolytic activity (Jankiewicz & Swiontek Brzezinska, 2015; Jankiewicz et al., 2016) and the current study deals with proteases showing keratinolytic activity. The obtained results point to a close dependence between the activity of the bacterial proteases and the composition of the culture medium. A mineral medium supplemented with bird feathers was found to be the optimal growth medium for the production of proteases by this bacterium. This indicates that *S. maltophilia* N4 efficiently utilizes keratin in the feathers as a source of nitrogen and carbon. Of interest is that further enrichment of the growth medium in nutrients readily used by bacteria, such as glucose, peptone or yeast extract did not result in further increase in proteolytic activity and in the case of some of the nutrients used the activity was found to be decreased. Similar observations have been described for cultures of the bacterium *Stenotrophomonas* sp. D1 (Yamamura et al., 2002). On the other hand, enriching the growth medium of *S. maltophilia* R13 in additional, besides feathers, sources of carbon was found to increase the observed level of proteolytic activity (Jeong et al., 2010). A review of the obtained results points to the constitutive nature of the production of proteases by strain N4, but that activity is further induced by non-specific components in the medium, in this case by keratin in the bird feathers. For most of the proteases described so far the inductive nature of their synthesis has been found (Cao et al., 2009; Gupta & Ramnani, 2006). The studied protease demonstrated an unusually high temperature optimum compared to the other *Stenotrophomonas* proteases described. For example, the temperature optimum for the keratinolytic proteases of *S. maltophilia* BBE11-1 was in the 40–50°C range (Fang et al., 2013) and protease S1 at 50°C (Miyaji et al., 2005).

Optimal pH above 8.0 has been described for serine proteases of both *Stenotrophomonas* and other species of bacteria (Raval et al., 2014; Paul et al., 2014; Wang et al., 2016) even though many of these enzymes work better in a neutral environment. The studied protease of strain N4 demonstrated considerable thermal stability at 55°C, similarly to keratinase KerSMD at 50°C (Fang et al., 2014).

The characterized enzyme showed broad substrate specificity with regard to soluble protein substrates but was the most active against casein and keratin, which is confirmed by literature data on the keratinases (Gupta & Ramnani, 2006). In the case of the studied bacterial protease N4 the K : C ratio (keratinolytic : caseinolytic activity), was 0.71, which according to the accepted principles allow to classify the protease to the keratinases (Gupta et al., 2013; Rajput et al., 2010). For example, Fang et al. (2014) gives a K : C for 1.37 keratinase SMD. Studies with the use of specific inhibitors showed that this is a metal-dependent serine protease. This non-classical mechanism of catalysis was also suggested for the keratinases of *S. maltophilia* BBE11-1 (Fang et al., 2013) and the keratinase of *Streptomyces aureofaciens* K13 (Gong et al., 2015). The inhibition of the studied enzyme by metalloprotease inhibitors may point to the participation of metal ions in the enzyme's stabilization or activation. The slight stimulation or stabilization of the activity of the studied keratinase observed in the case of calcium, magnesium and manganese ions was compatible with the results of earlier studies on the keratinase of *S. maltophilia* L1 (Cao et al., 2009) and in part in with the keratinases of strain BBE11-1 (Fang et al., 2014). The opposite was observed for the activity of the alkaline protease of *S. maltophilia* SK1, which was completely inhibited in the presence of calcium ions (Waghmare et al., 2015).

5. Conclusion

The insensitivity of this protein to surfactants, along with high temperature optimum and thermal stability, suggests that the enzyme may find application in the chemical industry. Moreover, it is important from the practical point of view that the studied enzyme demonstrated a broad substrate specificity and hydrolysed raw keratin waste. All things considered, the described protease is an interesting protein and a suitable candidate for industrial applications.

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