Journal of Cleaner Production 236 (2019) 117586

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Wool keratin total solubilisation for recovery and reintegration - An ecological approach

Carmen Gaidau [a, *], Doru-Gabriel Epure [b], Cristina Emanuela Enascuta [c], Cristina Carsote [d], Claudiu Sendrea [a], Noemi Proietti [e], Wuyong Chen [f], Haibin Gu [f]

[a] The Research and Development National Institute for Textiles and Leather-Division Leather and Footwear Research Institute, Leather Research Department, 93, Ion Minulescu Str, Bucharest, 3, Romania
[b] SC Probstdorfer Saatzucht Romania SRL, 20, Siriului Str, 1, 013799, Bucharest, Romania
[c] The National Institute for Research & Development in Chemistry and Petrochemistry, 202, Splaiul Independentei, Bucuresti, 060021, Romania
[d] National Museum of Romanian History, Centre of Research and Scientific Investigation, 12, Calea Victoriei, 030026, Bucharest, Romania
[e] Institute of Chemical Methodologies, Magnetic Resonance Laboratory "Annalaura Segre", CNR Research Area of Rome, Via Salaria Km. 29.300, 00015, Monterotondo, Roma, Italy
[f] Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu, 610065, China

ARTICLE INFO

Article history:
Received 14 March 2019
Received in revised form
14 June 2019
Accepted 7 July 2019
Available online 10 July 2019

Handling editor: Kathleen Aviso

Keywords:
Wool waste
Keratin recovery
Organic pollution
Keratin hydrolysate
Bioactive protein
Total solubilisation

ABSTRACT

Organic waste is a valuable raw material with potential for replacing synthetic products of petroleum origin in a more sustainable economy. Such a material is unmarketable wool, which is a growing resource in many countries and has recently concerned authorities because of the low degree of biodegradation of keratin and the potential for long-term environmental pollution. At the same time keratin represents a smart protein with high compatibility for cell growth and with high sulphur content which can be used for plant treatment in a circular economy. In this respect the aim of this research was to develop a facile and versatile method for total solubilisation of keratin from unmarketable wool which was experimented at pilot level with high potential for reintegration in ecological agriculture. The original investigations indicated that different properties can be tailored by alkaline or alkaline-enzymatic hydrolysis of wool waste with preservation of 9.2–19.2% of cystine sulphur as compared to raw wool. The measurement of particle sizes, zeta potential and contact angle demonstrates that the keratin peptides are able to associate and self assemble with influence on particle size, stability and hydrophilic properties. FTIR-ATR, CP/MAS ^{13}C-NMR and Cryo-STEM-EDX analyses highlighted specific chemical modification of keratin polypeptide with cleavage of disulphide bonds and dehydroalanine generation only in alkaline hydrolysates and with more alkyl species and oxidized sulphur in enzymatic hydrolysates. The results indicated that different compositions of keratin hydrolysates can be modulated through alkaline and enzymatic hydrolysis with release of metabolism precursors for plant growth which can be alternatives to the use of synthetic products. Preliminary tests on foliar fertilization of wheat plants opened the path for large scale application of keratin hydrolysate solubilised with zero waste from wool by-products, in a more ecological agriculture.

1. Introduction

At global level the importance of biobased materials has increased due to the high level of pollution with synthetic polymers of petroleum origin and unmarketable natural by-products. Collagen and keratin based by-products are generated in activities related to animal breeding, meat, hides, leather and textile processing. Leather industry contributes to pollution reduction with an estimated value of 1053 million USD (World Leather, 2018) by processing a by-product generated by farmers and meat industry. At global level, although the research on ecological biotechnologies alternatives is intense (Bin et al., 2017; Ma et al., 2014), bovine hide and sheepskin processing annually generates waste estimated at 200 000 tons of hair and 56 000 tons of wool

* Corresponding author.
E-mail addresses: carmen.gaidau@hotmail.com (C. Gaidau), doru.epure@probstdorfer.ro (D.-G. Epure), cristina.enascuta@gmail.com (C.E. Enascuta), criscarsote@yahoo.com (C. Carsote), noemi.proietti@cnr.it (N. Proietti), wuyong.chen@163.com (W. Chen), guhaibinkong@126.com (H. Gu).

https://doi.org/10.1016/j.jclepro.2019.07.061
0959-6526/

(Thankaswamy et al., 2018). From an annual production of raw wool of 1.2 million tons about 90% is processed by the textile industry and the total keratin waste (wool, feather, hoofs, horns etc) is estimated at 5 million tons annually (Mokrejs et al., 2011a,b). Poultry feathers represent a more abundant and easier to hydrolyse by-product as compared to wool waste and the literature is rich in information regarding the industrial application for animal feed (Gupta and Singh, 2014), soil amendment and fertilizers (Bhange et al., 2016).

In recent years low quality wool has been considered a valuable resource for soluble keratin processing and recycling in textile industry (Du et al., 2018) or other industrial applications like membranes with improved absorption and separation properties (Ma et al., 2017) or for slow-release nitrogen fertilizers (Zoccola et al., 2009). Many studies were dedicated to biomaterials based on wool keratin for wound healing (Loan et al., 2016), tissue engineering (Tachibana et al., 2002), drug delivery (Srisuwan and Srihanam, 2018) and cosmetic products (Worth et al., 2015). The efficiency of wool and hair keratin biomaterials in cell growing and adhesiveness was found to be due to the presence of binding residues of leucine-aspartic acid-valine (LDV) and glutamic acid-aspartic acid-serine (EDS), self-assembling properties and high biocompatibility (Rouse and Van Dyke, 2010). Commercial keratin wound dressings made from oxidized wool were reported to effectively cure chronic wounds (Batzer et al., 2016). Cysteine amino acid with a concentration of 7–20% of the total amino acids represents the fingerprint of keratin proteins like hydroxyproline is for collagen. Cystine is responsible for disulphide bonds in keratin secondary structure with outstanding resistance to physical and chemical environmental factors and insolubility in water, weak acids and alkali as well as to proteolytic digestion. The differences between feather and wool keratin composition and morphology are important and can explain why only few studies are reported related to total solubilisation of the latter. The low reactivity of wool keratin is attributed to higher cysteine content (15.9–12.7 g/100 g protein in wool as compared to 7.8 g/100 g protein in feather) able to generate covalent disulphide bridges and more intra-molecular hydrogen bonds (Tran and Mututuvari, 2016) as compared to inter-chain hydrogen bonds which are characteristic for feather keratin (Mwanza, 2018). The average molecular weight of feather keratin is 10 kDa (Staroń et al., 2011) as compared to wool keratin with a heterogeneous composition of 40–60 kDa intermediate-filament proteins which preserve α-helical structure, amorphous matrix of 11–26 kDa, a high-sulphur protein, and a glycine and tyrosine-rich protein of 6–9 kDa (Marshall and Gillespie, 1977).

The methods for wool solubilisation have been reported in literature but only at laboratory level (1–10 g of wool) and show that the yield of wool keratin reached 30% by sulphitolysis with urea (8 M), metabisulphite (0.5M) and sodium dodecyl-sulphate at pH value of 6.5, after 2 h of strong mechanical shaking (Aluigi et al., 2007) or 78–80% yield by alkaline reduction with urea (6 M) alkylated with iodoacetate (Onifade et al., 1998). Other versions were carried out in alkaline solutions (8M urea with 0.1Nand 1N NaOH, respectively 0.1M $NaBH_4$) for 2–5 h at 65 °C with similar yields of 68%–82% keratin (Jeanette et al., 2009). Enzymatic hydrolysis of wool with 2.6% Savinase, assisted by sodium hydrogen sulphite succeeded the solubilisation of 26% keratin (Eslahi et al., 2013) meanwhile the chemical-enzymatic hydrolysis of wool with 9% calcium hydroxide and 5% Esperase 6.0T reached a maximum yield of 59% soluble keratin (Mokrejs et al., 2011a,b). An economical review of keratin wool solubilisation (Brown et al., 2016) showed that percarbonate oxidation (1% sodium hydroxide, 4.5% sodium percarbonate) and sulphide (6 g/L sodium sulphite and 21 g/L calcium oxide) reduction methods are the most feasible for scaling up in future research with application in textile treatment. Superheated water was also used for wool keratin complete solubilisation at 170 °C in a laboratory-scale reactor at a pressure of 7 bar, for 1 h and at the speed of 25–30 rpm with a consumption of 4 kWh per 1 kg of wool. The resulted keratin hydrolysate with molecular weight of 14 kDa showed low superficial tension and good penetration properties for textile treatment (Bhavsar et al., 2017).

In this present work, a facile and versatile method for wool keratin solubilisation with bioactive properties preservation is presented with experiments at pilot level and addressing the lack of chemical processes with total reclaiming of wool waste. The keratin hydrolysates with different characteristics are proposed to be used in seed growth biostimulation and plant fertilization in ecological agriculture as a source of nitrogen which is known as one of the most important and difficult to manage nutrients in organic agriculture due to the limited resources and the cost of commercially available products (Alaru et al., 2014). The paper proposes a method for total solubilisation of keratin whose application to the entire waste keratin resource could cover 4–5% of total nitrogen consumption for world agriculture demand (Food and Agriculture Organisation, 2015) and which can represent an important source of fertilizers in organic farms.

2. Experimental

The aim of the experiments was to develop a facile process for total solubilisation of wool waste which was carried out at pilot level and to demonstrate that the bioactive properties of keratin were preserved in order to be reintegrated in the natural circle of plant metabolism. The methods and materials used for the experiments are presented in the following.

2.1. Keratin hydrolysate preparation and characterization

Raw sheep wool was purchased from sheep breeders and was pre-processed by washing, degreasing and cutting (Fig. 1) with a shredding machine (La Minerva, food service equipment). The chemical materials used for degreasing were technical grade: ammonia (25%) and sodium carbonate were purchased from Chimopar SA, while Borron SE (ethoxylated alkyl derivatives with 65% concentration) from SC Triderma SRL. The other chemical substances (formic acid and sulphuric acid) for pH adjustment were technical grade and were purchased from Chimopar SA. The hydrolysis process was carried out in a single step with sodium hydroxide (Lach-Nersro) for HK1 and HK3 products and in two steps following a chemical-enzymatic process for HK4 and HK2 products. The hydrolysis was performed in a steel vessel with automatic temperature control and agitation system according to the flow process depicted in Table 1. The alkaline hydrolysis process was designed for complete solubilisation of keratin at atmospheric pressure and in a reasonable reaction time for a zero solid waste technology. To obtain keratin hydrolysates with different properties, the alkaline keratin hydrolysate, HK1, was further enzymatically hydrolysed using Alcalase 2.4 L (endopeptidase of *Bacillus licheniformis*) and Protamex (mixture of endo and exopeptidase from *Bacillus* spp. enzymes) from Novozymes with proteolytic activity of 2.4 AU-A/g and 1.786 AU-N/g, respectively.

Physical-chemical characterization of keratin hydrolysates (Fig. 2) in liquid state was carried out for dry substance (ISO 4684), total nitrogen and protein (ISO 5397), amino nitrogen which is correlated with protein break down degree and molecular weight (Sőrensen method), ash content (ISO 4047), cystine and cystine sulphur (ISO 13206), pH (STAS 8619/3). Particle size and zeta potential of keratin hydrolysate dispersions were recorded with Zetasizer Nano-NZ (Malvern). Contact angles of keratin

hydrolysates were measured by using a contact angle analyzer (VGA Optima XE). Keratin hydrolysates were analysed for their sensitivity against bacteria and fungi by dropping 100 μL of material on Petri dishes covered with Sabouraud dextrose agar (Merck, Germany) nutrient medium and by incubation at 30 °C. The observations were collected after 7 and 28 days on 3 parallel Petri dishes. Transmission electron cryomicroscopy (CryoTEM) equipment coupled with EDX detector was used for the tomography studies of HK1 and HK2 samples in liquid state, followed by elemental analyses for O and S (FEI Tecnai F20 G^2 TWIN Crio-TEM and X-MaxN 80T detector). To perform the EDX analysis, STEM mode was used.

2.2. Keratin hydrolysate film preparation

The keratin films were prepared by casting the same dry substance of keratin hydrolysates in Petri dishes and by drying in an oven at 40 °C. The colours of the keratin films are different, from light yellow and yellow for alkaline hydrolysates to brown for alkaline-enzymatic keratin hydrolysates (Fig. 3).

2.3. Keratin film characterizations

Solid state Cross-Polarization Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance (CP/MAS ^{13}C-NMR) spectroscopy was performed on a Bruker AVANCE IIII 400 NMR instrument operating at 400 MHz. The rotor spun at 12 KHz and the number of scans varied from 6000 to 8000.

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) measurements were performed with a portable spectrometer Alpha (Bruker Optics) equipped with a Platinum ATR module. During the experiments, 32 scans were co-added to achieve an acceptable signal-to-noise ratio, with wave number ranging from 4000 to 400 cm^{-1}. All the spectra were recorded at a resolution of 4 cm^{-1} and evaluated using Opus 7.0 software.

2.4. Foliar fertilizer emulsions based on keratin hydrolysate for plant treatment preparation

Foliar fertilizers (Fig. 4) were prepared using HK1 keratin hydrolysate (MED_HK1) and a combination with collagen hydrolysate (MED_HC_HK1) to improve the nutrient value of the new fertilizer and biostimulation of seed germination. The collagen hydrolysate was prepared by alkaline-enzymatic hydrolysis of leather industry by-products (Gaidau et al., 2013) and the main composition was: 9% dry substance, 73% protein, 13% total nitrogen, 6000 Da MW. The keratin and collagen hydrolysates were emulsified using a two-step process (Simion et al., 2018) in view of direct application for wheat plant foliar fertilization. Two types of emulsifiers were used: a hydrophobic one, isopropyl oleate (for W/O emulsion) and a hydrophilic one-a diester of sucrose (for O/W emulsion). In the first step, water and isopropyl oleate surfactant (2%) were added and homogenized by stirring at 60 °C to obtain a water-oil emulsion. In the second step the solution of keratin hydrolysate (MED_HK1) or keratin hydrolysate and collagen hydrolysate (HC_HK1) in equal proportions (MED_HC_HK1) and the diester of sucrose (2%) were added to the water-oil emulsion, and homogenized by stirring at 60 °C, obtaining a multiple water-oil-water emulsion (Fig. 4). The reference fertilizers for plant treatment were water (Control) and a multiple emulsion prepared using the same process and a standard NPK-microelements product (M) instead of keratin and/or collagen hydrolysates.

2.4.1. Biostimulation properties of keratin based emulsions

Different tests were performed on experimental lots of three repetitions of 100 wheat seeds, Mirastar variety, at laboratory level in order to identify the biostimulant effect in comparison with water (Control) or with a NPK-microelements standard foliar fertilizer (M). The influence of keratin hydrolysate on seed germination was evaluated as compared to seed germination in water. The aim of these preliminary experiments was to set the optimum conditions for field experiment scale-up.

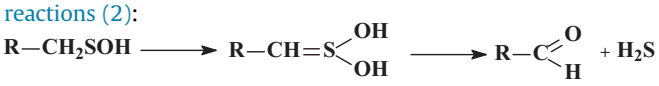
3. Results and discussions

The large number of sulphur bonds confers the keratin molecule outstanding resistance to chemical and enzymatic attack and it is already recognized that the complete hydrolysis of keratin can be reached only after the denaturation by breaking the disulphide bonds which are the main source of high stability (Onifade et al., 1998). The solubilisation of keratin in acidic or enzymatic environment is less likely to occur as compared to alkaline conditions.

The alkaline hydrolysis of wool keratin (Chiego and Silver, 1942) takes place with decomposition of -S-S- bonds according to the reaction (1):

$$R - S - S - R_1 + H_2O \xrightarrow{NaoH} R - SOH + R_1 - SH \quad (1)$$

The reaction can continue in strong alkaline environment by releasing H_2S and generating aldehyde groups according to the reactions (2):



(2)

According to Chiego and Silver the hydrolysis with sodium hydroxide goes with formation of sodium salts or the amino acids (peptides) and small amounts of soluble sulphides.

The proteolytic enzymes, Alcalase 2.4 L and Protamex catalyse proteolysis of peptide bonds of alkaline hydrolysed keratin and selective hydrolysis of carboxylic esters and amino esters (Novoenzymes, 2018) occurs following the reaction (3), presented below:

Fig. 1. Different stages of wool processing in pilot level equipment and the total solubilised keratin.

Table 1
Technological flow for wool keratin solubilisation All percents were reported to raw sheep wool weight.

Weighing:	1200 g raw sheep wool
Degreasing:	in automated drum, Vallero duplex (Fig. 1a)
	300% (w/w) water at 60 °C
	4% ammonia
	1% sodium carbonate
	0.6% Borron SE
	20 min agitation
Drain	
Washing:	300% (w/w) water at 40 °C
	15 min agitation
	pH = 7, control with phenolphthalein
Drain	
Cutting at La Minerva (food service equipment)	(Fig. 1c and d)
Loading into reaction vessel	(Fig. 1d and e)
Alkaline hydrolysis:	300% (w/w) water at 80 °C
	12% NaOH
	4 h agitation
Control: pH, degree of wool solubilisation	(Fig. 1f)
Download reaction vessel content	
Decantation over night in 2 fractions: down (HK1) and upper (HK3) (Fig. 2)	
Filtering through a metal sieve	
Enzymatic hydrolysis of HK1:	
Adjustment of pH at value of 8 with diluted formic acid (1:10)	
	1% Alcalase 2.4 L (HK4) or 0.5% Protamex (HK2)
	3 h agitation at 60 °C
Enzyme deactivation:	90 °C, 10 min
Adjustment of pH value at 7 with diluted sulphuric acid (1:10)	
Download reaction vessel content	
Decantation over night (HK4 or HK2)	(Fig. 2)
Filtering through a metal sieve	

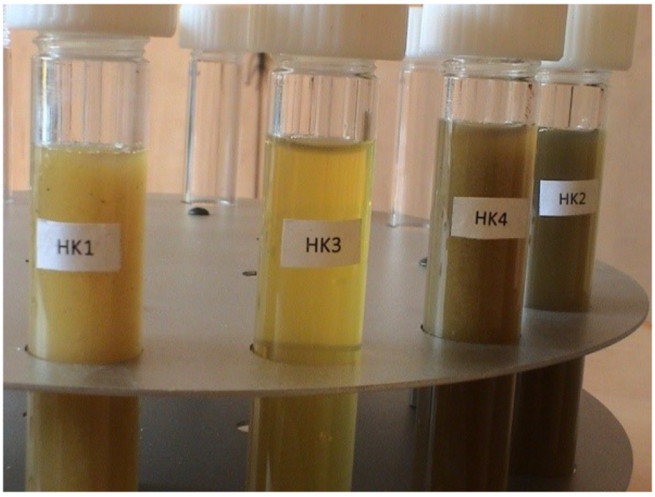


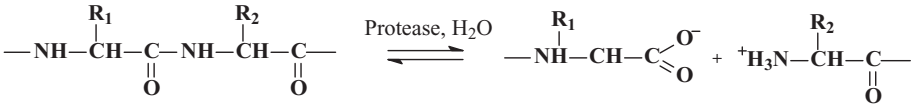
Fig. 2. Keratin hydrolysates: alkaline hydrolysates (HK1 and HK3) and alkaline-enzymatic hydrolysates (HK4 and HK2).

The main characteristics of alkaline and alkaline-enzymatic keratin hydrolysates are presented further and showed that proposed process for wool solubilisation with zero solid waste is facile and versatile. Starting from a material with a composition of 60% hydrophobic chemical groups and, 40% hydrophilic chemical groups, respectively (Barone and Schmidt, 2006), we show that keratin can be sequenced in products with different properties for diverse applications.

The proposed method for the solubilisation of raw wool keratin, one of the most inert keratin biomass forms, can also be successfully applied to the solubilisation of feathers, wool from sheepskin or textile industries, especially as the latter have already undergone denaturation processes.

3.1. Keratin hydrolysate characteristics

Table 2 indicates that using chemical or chemical-enzymatic hydrolysis, different characteristics can be tailored: higher molecular weights for alkaline hydrolysates (HK1 and HK3) and lower molecular weights for chemical-enzymatic hydrolysates (HK2 and HK4). The lower concentration of Protamex was more effective for the keratin sequencing compared to Alcalase that was used in higher concentration. The total nitrogen content represents a valuable source of organic nitrogen for plant fertilization and growth stimulation. The protein concentration is high (67.2–81.1%) and represents a long lasting source of nitrogen (Choi and Nelson, 1996), able to be released in time and to protect the plants against variation of fertilization and climate change effects. As compared to commercial foliar fertilizers, the presence of proteins (keratin or collagen) with low contact angle, chelating action for microelements and film forming properties can add durability to



(3)

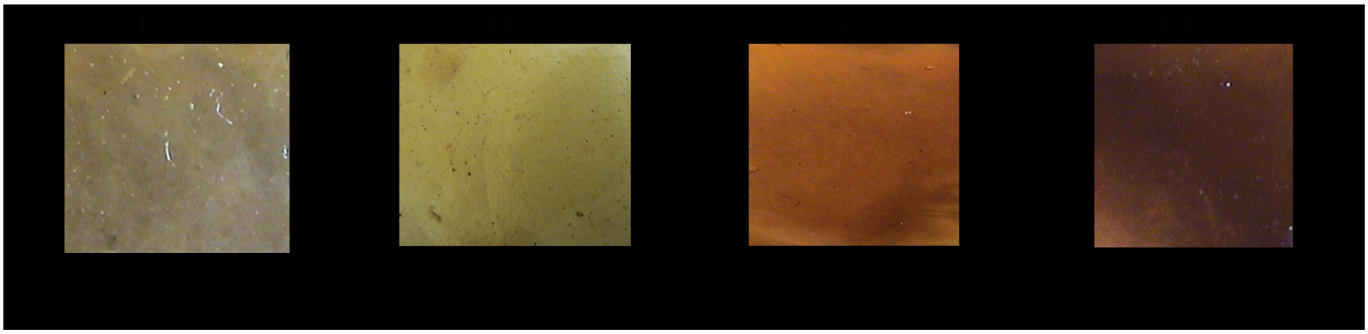


Fig. 3. Keratin hydrolysate films prepared from alkaline (HK1, HK3) and alkaline-enzymatic keratin hydrolysates (HK4, HK2).

the foliar fertilization treatment.

Cystine and cystine sulphur contents of keratin hydrolysates are between 9.6-19.5% and 9.2−19.2%, respectively as compared to wool, with higher concentrations in HK4 keratin (Fig. 5), probably due to the keratin ability to self-assemble and polymerize (Rouse and Van Dyke, 2010).

The lowest concentrations of cystine and cystine sulphur were found in HK2, the most hydrolysed keratin product where probably the sulphur was transformed in oxidized groups.

The measurement of particle size and zeta potential of keratin hydrolysates showed that the particles are highest and the most stable in HK1 product as compared to the other keratin hydrolysates. The particle size of HK2 showed a higher size as compared to HK3 and HK4 products (Fig. 6 and Fig. 7), probably due to the higher tendency of particle aggregation, confirmed by lowest values of zeta potential.

The contact angle measurements (Fig. 8) revealed that all keratin hydrolysates are hydrophilic (with values under 42°) and the most hydrophilic product is HK2, with most ionized groups due to the high peptide cleavage as compared to HK1, HK3 and HK 4.

The contact angle value of HK4 suggests slightly more hydrophobic properties as compared to HK3 which shows that the enzymatic hydrolysis can release more hydrophobic chemical groups as compared to chemical hydrolysis. The conclusion is supported also by the higher concentration in cystine content of HK4 product (Fig. 5). Decreased contact angles values and lower superficial tensions were also found after hair or textile treatments with keratin hydrolysates due to their hydrophilic properties (Tsuda and Nomura, 2014).

CP/MAS ^{13}C-NMR analyses spectra are presented in Fig. 9 and show a peak with a maximum around 175 ppm which is attributed to the amide carbonyl carbons of the keratin protein. The secondary structure of keratin containing α-helix and β-sheet resulted in a slightly different chemical shift of C=O in spectra.

It is worth noting that C=O peak between 165 and 190 ppm was further resolved to obtain the fraction of α-helix, β-sheet, and random coil. The α-helix structure was associated with the peak at 178 ppm, and the peak with a maximum at 173 ppm was ascribed to

Table 2
Physical-chemical characteristics of keratin hydrolysates and degreased raw wool.

Characteristics	Keratin hydrolysates				Wool
	HK1	HK3	HK4	HK2	
Dry substance, %	10.1	9.2	11.3	11.3	78.8
Total nitrogen*,%	13.9	14.4	12.1	12.0	13.6
Protein, %	78.3	81.1	68.2	67.5	76.6
Amino nitrogen**, %	1.0	1.3	1.4	2.4	
Molecular weight, Da	11000	6800	5600	2000	
Ash*, %	16	13.4	11.4	12.8	7.1
pH, pH units	12.3	12.3	8.2	8.0	
Cystine*, %	1.34	1.5	1.81	0.89	9.3
Cystine sulphur*, %	0.36	0.4	0.48	0.24	2.5

Percentages based on: *dry substance weight ** protein weight.

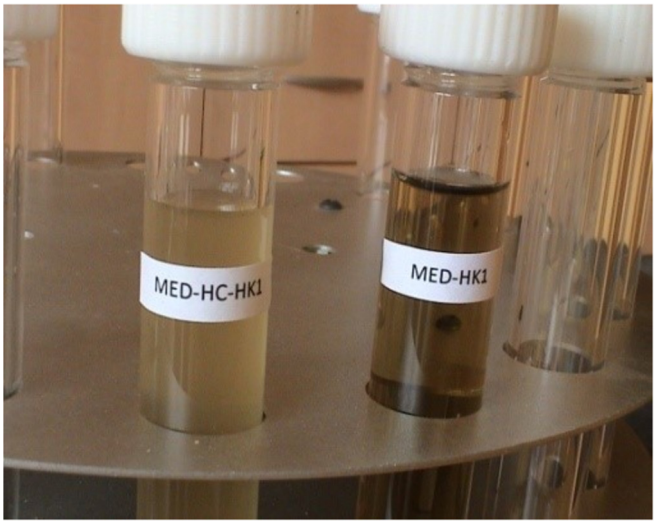


Fig. 4. New fertilizers based on keratin and collagen hydrolysates (MED_HC_HK1 and MED_HK1).

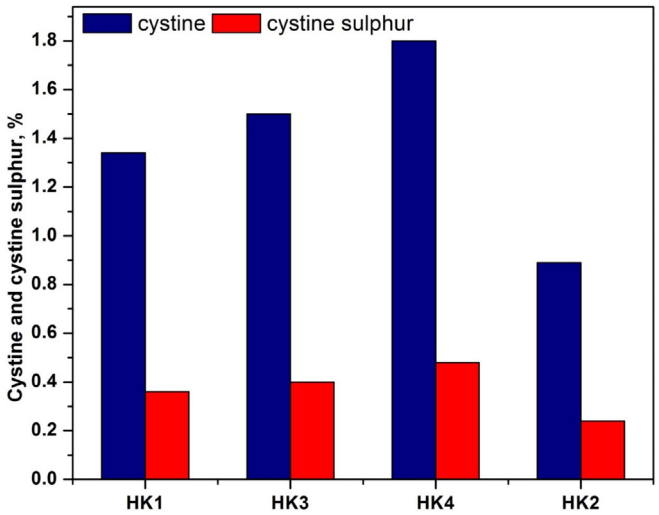


Fig. 5. Cystine and cystine sulphur content of alkaline (HK1, HK3) and alkaline-enzymatic (HK4, HK2) keratin hydrolysates.

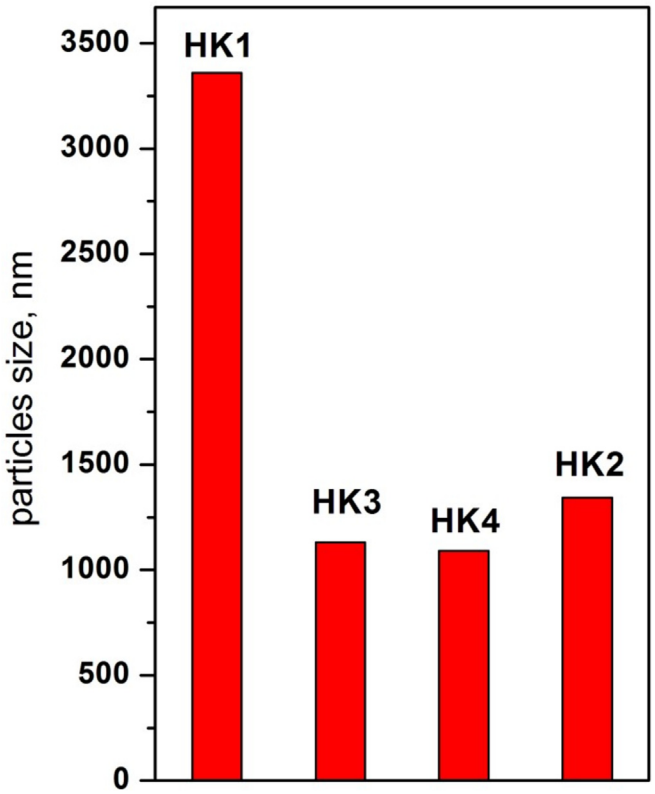


Fig. 6. Average particle size of keratin hydrolysates.

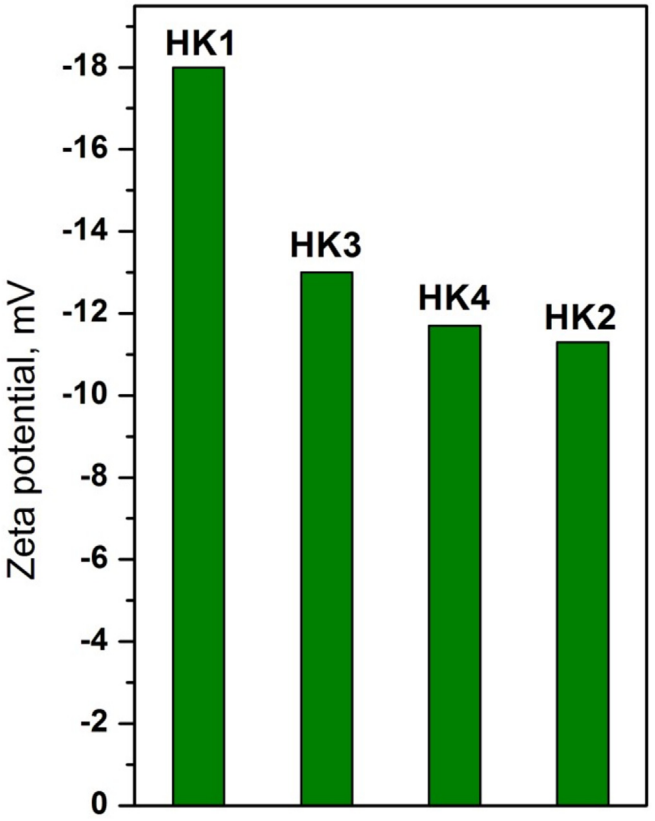


Fig. 7. Zeta potential of keratin hydrolysates.

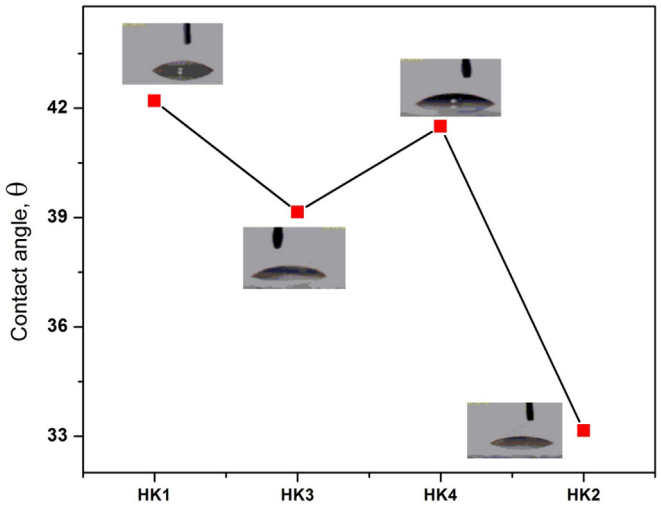


Fig. 8. Contact angle of keratin hydrolysates.

β-sheet and random coil conformations. The peak at about 180.5 ppm, observed as shoulder peak in HK4 and HK2 can be due to the carbonyl group from side chain carbonyl carbons of aspartic and glutamic acids.

The peak at around 130 ppm indicates the presence of aromatic group containing amino acids in the keratin (phenylalanine and tyrosine). The α-carbons were recorded between 52 ppm and 56 ppm (leucine, glutamic acid, arginine), while the β-carbons were observed at around 40 ppm (present in phenylalanine, tyrosine, leucine, cross-linked cysteine residues). The carbon peaks recorded between 30 ppm and 40 ppm indicated the presence of proline and glutamic acid residues. The ^{13}C-NMR signal at low chemical shifts is associated with the alkyl groups of the side chains. The cleavage of disulphide bridges associated with thiol group formation was expected to reduce the β-carbon signal at 40 ppm and produce a signal around 25–29 ppm (Duer et al., 2003). It is difficult to find the signal around 25 ppm–29 ppm in the NMR spectra as they overlap with the chemical shift of other side chain resonances.

For alkaline hydrolysates (HK1, HK3) the ^{13}C-NMR signal of the alkyl groups at 25 ppm substantially decreases compared to alkaline-enzymatic hydrolysates (HK4, HK2). More alkyl groups in keratin hydrolysates prepared by alkaline-enzymatic hydrolysis confirm the cleavage of keratin molecule at peptide level which is in agreement with reaction (3) and with more hydrophilic properties of HK2.

ATR-FTIR analyses of keratin hydrolysates in comparison with degreased wool (Ref) exhibit characteristic absorption bands of proteins (Fig. 10). The broad band from 3500 cm^{-1} to 3200 cm^{-1} is attributed to stretching vibrations of -O-H (bonded water) and -N-H (Amide A). The amide A band at ~3300 cm^{-1}, corresponding to the stretching vibration of N-H bonds, depends on the strength of the hydrogen bond, whereas the band at ~3400 cm^{-1}, attributed to the stretching vibration of -O-H bonds depend on the moisture of the sample. Bands which fall in 3000–2800 cm^{-1} region are related to C-H stretching bonds. Amide I band (1700-1600 cm^{-1}) is mainly due to the CO stretching vibration being directly related to the backbone conformation (Barth, 2007). Amide II band (1580-1480 cm^{-1}) is attributed to the NH bending vibration and CN stretching vibration and is predominantly known for its sensitivity to the protonation state of the peptide unit (De Flores et al., 2009). Amide III band (1300-1220 cm^{-1}) results in phase combination of CN stretching and NH in plane bending (Mokrejs et al., 2011a,b) and depends on the nature of side chains (Barth, 2007).

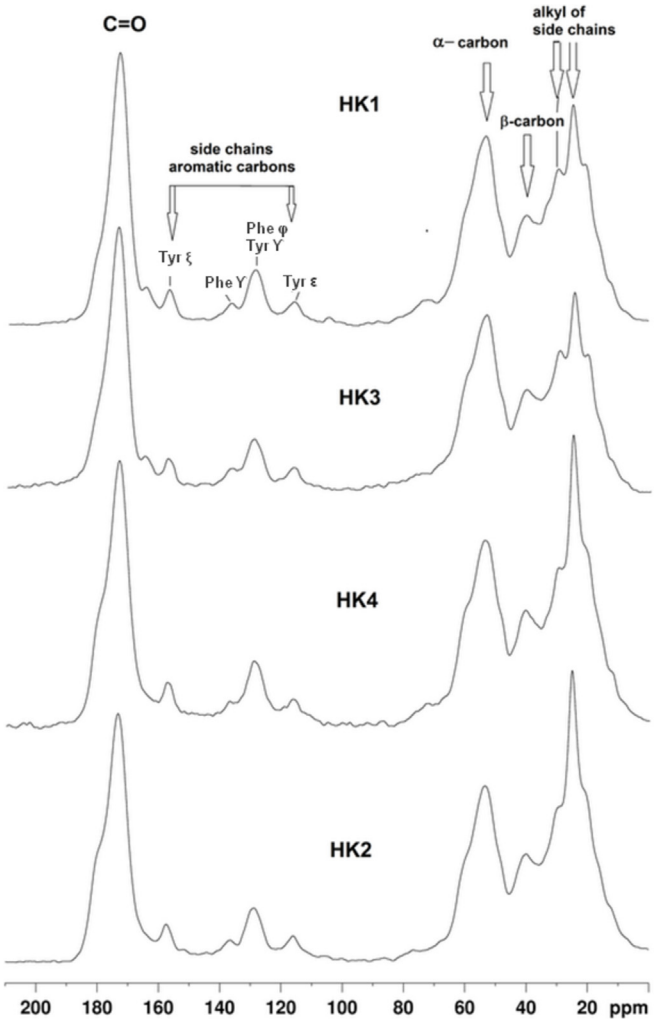


Fig. 9. ^{13}C-NMR spectra of keratin hydrolysates.

According to the literature data, the various secondary-structure components of a protein absorb at different positions in the amide I and amide II region of the IR spectrum. Thus, α-helix conformation absorbs at 1648–1657 cm^{-1} (amide I) and 1540–1550 cm^{-1} (amide II) while the β-sheet absorbs at 1623–1641 cm^{-1} (amide I) and 1515–1535 cm^{-1} (Goormaghtigh et al., 2006). Wool fibres sample (Ref) indicated pronounced peaks at 1636, 1536 and 1515 cm^{-1}, ascribed to β-sheet conformation, probably due to the denaturation conditions of degreasing process. However, the broadness of amide I and amide II bands indicates the coexistence of α-helical and β-sheet conformations. The amide A, amide I and amide III bands of hydrolysed keratins (HK1, HK2, HK3 and HK4) remained similar to that of degreased wool (Ref), whereas the broadness of the amide II band increased by the presence of a shoulder at about 1552–1556 cm^{-1} (Fig. 10). Since the 1400 cm^{-1} band assigned to COO^- symmetric stretch (Fig. 11a and b) has increased in intensity after extraction of keratins, the amide II shoulder at ~ 1556 cm^{-1} (Figs. 11a) and 1552 cm^{-1} (Fig. 11b) could be due to COO^- asymmetric stretch of de-protonated carboxyl. The presence of these prominent bands indicates the strong ionic character of extracted keratin hydrolysates (Cardamon, 2010).

The S-O vibrational absorption bands of oxidized cysteine residues are visible in the 1000–1200 regions (Jeanette et al., 2009). The presence of 1121 cm^{-1} band (S-O symmetric stretch) in all extracted keratins spectra (Fig. 10) suggests the formation of cysteine dioxide (-SO_2-S-). Moreover, the cleavage of disulphide linkages is indicated by the appearance of bands at 1170 cm^{-1} (S-O asymmetric stretch) and 1047 cm^{-1} (S-O symmetric stretch) related to the formation of cysteic acid (-SO_3^-). A higher cysteic acid content was observed for HK2 and HK4 samples (Fig. 5). Besides, only their FTIR spectra exhibit the band at 1021 cm^{-1} (S-O symmetric stretch) assigned to the formation of Bunte salt (–S–SO_3–) which is absent in alkaline hydrolysates (Fig. 10). It is already known that cysteine-s-sulphonate (–S–SO_3–) is a precursor for plant metabolism (Hecklau et al., 2016), which can be an advantage for the utilization of alkaline-enzymatic keratin hydrolysate for plant fertilization.

The results confirm that the cleavage degree of disulphide linkage in keratin is higher after enzymatic hydrolysis of alkaline

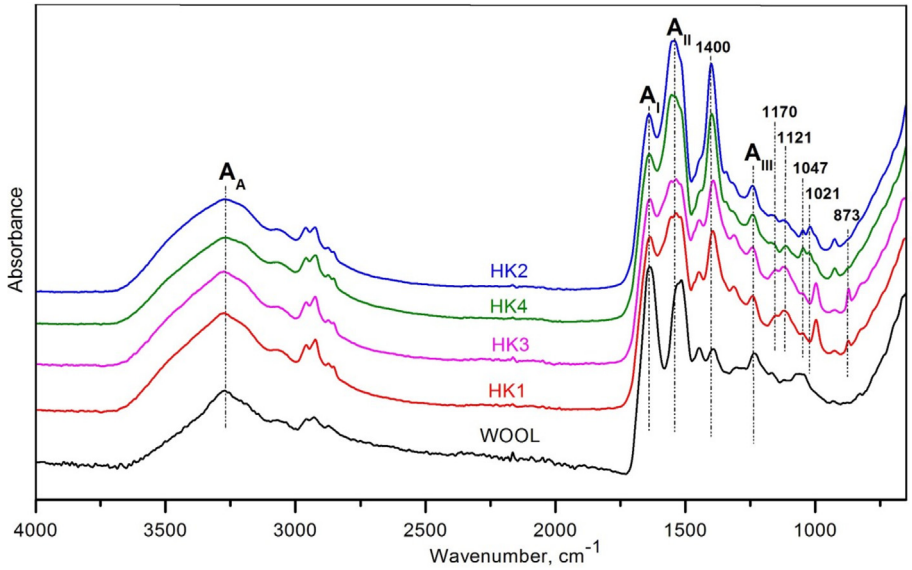


Fig. 10. ATR-FTIR spectra of keratin hydrolysates (HK1, HK4, HK3, HK2) and raw wool (Ref).

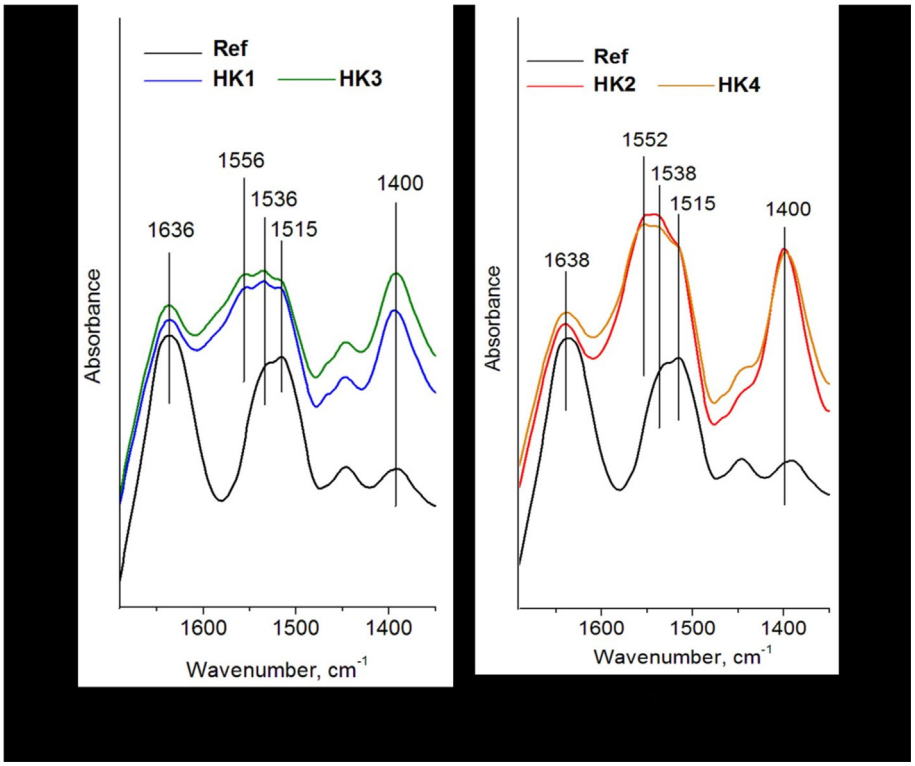


Fig. 11. Amide I and amide II bands of keratin alkaline hydrolysates (a) and alkaline-enzymatic hydrolysates (b) as compared to degreased wool (Ref).

hydrolysate and new ionic species are generated. More alkyl groups were also confirmed in ^{13}C-NMR analysis results for alkaline-enzymatic keratin hydrolysate HK2 and HK4 (Fig. 9).

The band at 873 cm^{-1} related to the dehydroalanine groups was observed only for HK1 and HK3 samples which are alkaline hydrolysates (Fig. 10). According to literature data, the dehydroalanine has antimicrobial properties (Siodłak, 2015) and could be mainly formed by the cleavage of the C–S bond in cystine (4) that in turn results in more oxidized sulphonate groups (Ebrahimgol et al., 2014).

(4)

Dehydroalanine is known as an electrophilic amino acid with antioxidant properties as compared to other amino acids, and so we can explain the non-oxidized colour of HK1 and HK3 keratin hydrolysate films (Fig. 3) as compared to alkaline-enzymatic keratin hydrolysate film (HK4 and HK2). The experiments showed that dehydroalanine can be removed by enzymatic hydrolysis of alkaline hydrolysate and more cysteic acid is generated. The balance between antimicrobial and growth stimulant properties can be modulated through alkaline and alkaline-enzymatic hydrolysis of wool and keratin hydrolysates.

The results of *sensitivity test against bacteria and fungi* showed that HK1 is resistant after 7 and 28 days of free exposure on a nutrient culture media as compared to other keratin hydrolysates which were invaded after 7 days by *Candida albicans* colonies (Fig. 12). The results are in agreement with other tests regarding the antimicrobial and antioxidant properties of keratin hydrolysate which were attributed to sulphur reductive action. It is recognized that the technological process for keratin solubilisation greatly influences the composition of final products and consequently their chemical and biological properties (Matyašovsky et al., 2017).

In order to understand the different behaviour of keratin hydrolysates against free exposure to fungi and bacteria development, *EDX analyses were* performed for HK1 and HK2 which are extreme products (Fig. 13). The results showed that HK1 which is the most resistant to fungi and bacteria and contains the highest concentration of sulphur and lowest quantities of oxygen suggesting that the sulphur is oxidized in HK2 with less reductive properties. The results are in agreement with other recent studies regarding alkaline keratin hydrolysate antimicrobial properties (Matyašovsky et al., 2017).

3.2. Plant growth biostimulation properties of recovered keratin hydrolysate

The results of wheat plants foliar fertilization treatment with different formulations of keratin hydrolysate and combination of keratin and collagen hydrolysates showed that the plant growth is stimulated and plant seedling length increased as compared to control samples (treated with water) by 8.5% in first stage of fertilization with HK1 based emulsions and by 4.6% in the last stage of fertilization with combinations of keratin and collagen hydrolysates based emulsions. Figs. 14 and 15 show the increase of wheat plant seedling length after foliar fertilization with emulsions based on HK1 product in different stages of vegetation as compared to control samples and NPK-microelements emulsion (M).

The stimulation of root development in the case of seed germination in MED_HK1 emulsion was observed as compared to control samples germinated in water. Hair root development of treated wheat seeds can be seen as compared to control samples in Fig. 16, which is in agreement with other studies regarding the use of keratin as plant growth biostimulant (Bhange et al., 2016). Research is in progress for field scale-up of the wheat biostimulation and foliar fertilization with keratin based emulsions.

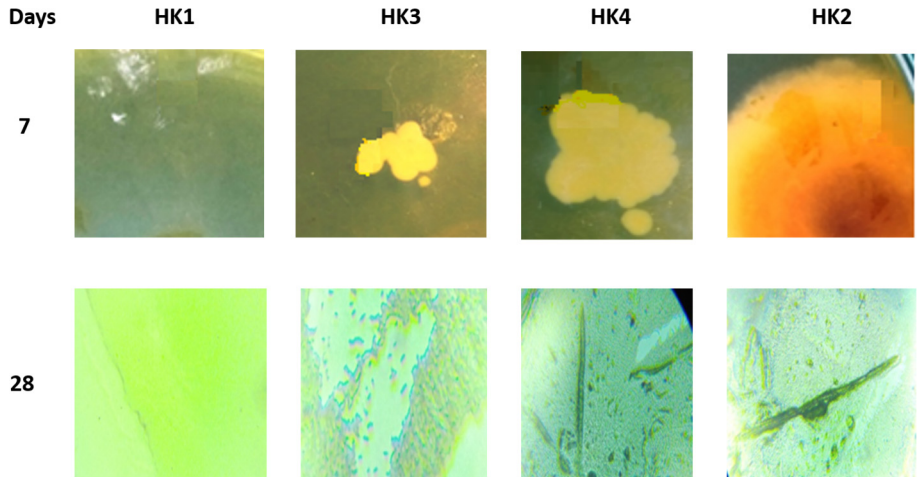


Fig. 12. Optical microscopy images (20× and 10×) of keratin hydrolysates after 7 and 28 days of free exposure to development of bacteria and fungi.

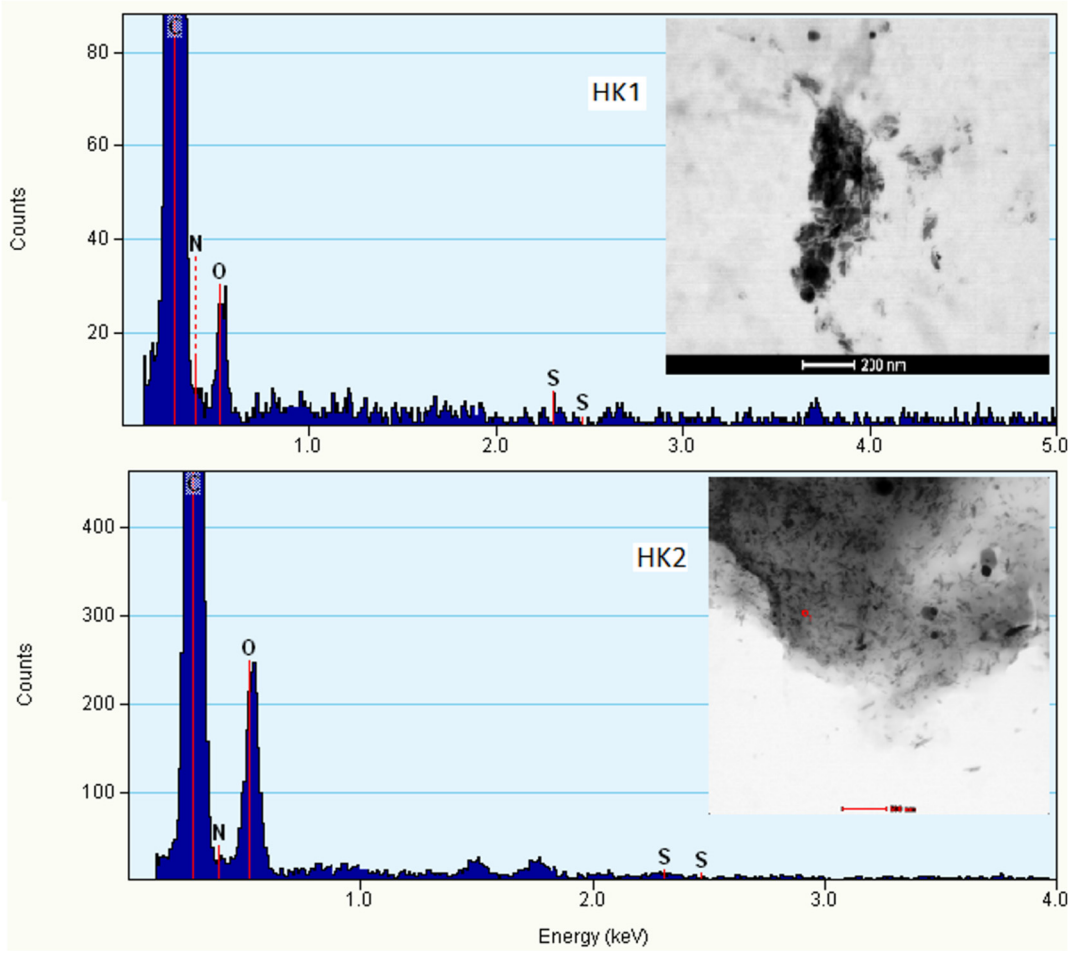


Fig. 13. EDX analysis and TEM images of alkaline (HK1) and alkaline-enzymatic (HK2) keratin hydrolysates.

In our research we found that keratin hydrolysate has natural resistance to bacteria and fungi and biostimulant action for seeds and plant growth and the results represent a contribution to the need for more knowledge regarding the bioactive properties of keratin (Sinkiewicz et al., 2018). The total solubilisation of wool under the condition of keratin bioactive properties preservation was demonstrated at pilot level and the research results open the way for wool by-products reclaiming in a circular economy.

3.3. Mass balance and economic efficiency

The mass balance of chemical materials and the analyses of chemical output are important for industrial scaling of a new process for wool keratin solubilisation. To estimate the economic

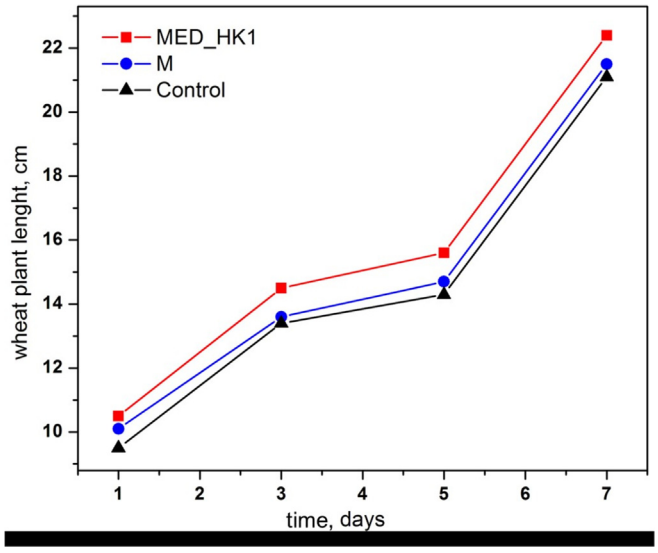


Fig. 14. Wheat plant length increase after foliar treatment with MED_HK1 as compared to Control and NKP-microelements product (M).

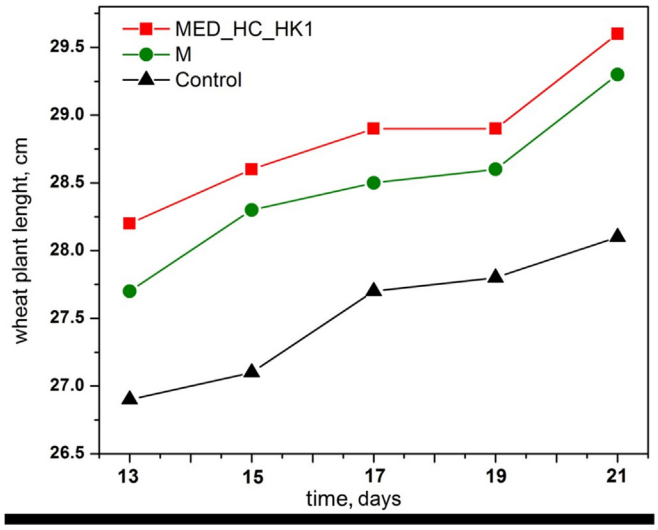


Fig. 15. Wheat plant length increase after foliar fertilization with emulsion based on keratin and collagen hydrolysate as compared to Control and NKP-microelements product (M).

efficiency of the proposed process, consideration was given to the cost, consumption of the chemical materials and the duration of the process and was compared to processes for which similar information exists.

We must mention that the preliminary step of all wool solubilisation processes is the degreasing of wool, a stage involving different degrees of pollution, depending on the type of degreasing agents used. In our process, aqueous degreasing method was selected as a more ecological alternative as compared to dry cleaning based on organic solvent (hexane, ether) use.

The output of total keratin solubilisation is generated only by the chemical materials for raw wool degreasing, with low influence on the total costs (Table 3). We must mention that the proposed process does not generate any solid wool waste as most other processes do.

In the case of the alkaline hydrolysate, which has been successfully tested for use in agriculture, the cost of materials is 0.199 € and the duration of the process is 1 working day. In Table 3 we made the comparison with another process with total solubilisation of keratin after 1 day of treatment with sodium hydroxide and sodium percarbonate, process selected for being the most economically efficient from other five experimented processes (Brown et al., 2016).

The results indicate that the alkaline hydrolysis with sodium hydroxide allows a cost save of 0.143 € (71.8%) per 1 kg of waste wool for chemicals as compared to reference process. When using enzymatic processes, the cost of the chemical materials amounts to 0.783 € for the process with Alcalase 2.4 L and 0.533 € in the case of Protamex use. Since enzymatic products have lower molecular weights than alkaline, they can be used to formulate plant-based biostimulators at competitive prices.

The carbon footprint calculation of waste disposal (VDL Handbook, 2013) indicates that the CO_2 emission amounts to 0.6224 t CO_2/t. We can estimate that at global level the total solubilisation of 5 million tons of keratin based waste is an ecological alternative to waste disposal which at present generates 3.1 million tons of CO_2. The experimented processes are competitive if we take into consideration the increase of cereal plant production by 4–8% and the reduction of ecological impact generated by unmarketable wool landfill. Recycling of wool waste by using keratin hydrolysate in foliar fertilization of cereals can contribute to more sustainable agriculture and to reduction of greenhouse gas emissions by chemical fertilizers production (Spångberg, 2014).

4. Conclusions

Unmarketable wool has become a growing resource in many

Fig. 16. Hair root development of wheat seeds treated with MED_HK1 emulsion (left) as compared to water treated seeds (right).

Table 3
Chemical material mass balance and costs of keratin hydrolysates processed from 1 kg waste wool by alkaline and alkaline-enzymatic hydrolysis as compared to a reference process.

Process	Materials/Chemicals	Cost per kg or L €	Input kg	Output kg or L	Cost €
Degreasing	Raw wool waste	0.213	0.213	greasy matters 0.017–0.033	
	Ammonia	0.691	0.028	0.013	
	Sodium carbonate	0.638	0.006	0.003	
	Detergent	0.787	0.005	0.002	
Waste water treatment		0.0007		6	0.004
Alkaline hydrolysis	Sodium hydroxide	1.183	0.120		0.142
Neutralization	Sulphuric acid	0.264	0.020		0.053
Total					**0.199**
Neutralization	Formic acid	4.255	0.020		0.085
Enzymatic hydrolysis	Alcalase 2.4 L	49.9	0.010		0.499
Total					**0.783**
Neutralization	Sulphuric acid	0.264	0.020		0.053
Enzymatic hydrolysis	Protamex	49.9	0.005		0.249
Total					**0.533**
Reference process					
Alkaline hydrolysis	Sodium hydroxide	1.183	0.090		0.107
	Sodium percarbonate	5.1	0.045		0.230
Neutralization	Sulphuric acid	0.264	0.020		0.005
Total					**0.342**
Cost saving for alkaline hydrolysis of wool waste					**0.143**

countries and preoccupies the authorities due to the low degree of biodegradability and the potential for long-term environmental pollution. The keratin hydrolysates with bioactive properties will contribute to the limited resources of organic nutrients for ecological agriculture in the context of increased demand for sustainable and circular economy.

A facile method for total solubilisation and reclaiming of keratin from wool waste was experimented at pilot level with high potential for reintegration in ecological agriculture as foliar fertilizer. The investigations showed that versatile properties can be tailored by alkaline or alkaline-enzymatic hydrolysis of wool waste, with preservation of 9.2–19.2% of original wool cystine sulphur and with bioactive or antimicrobial components. The heterogeneous composition of wool keratin as compared to keratins of other origin allows it to be easily separated by decantation keratin fractions with higher molecular weight or lower molecular weight and with different average particle sizes of 3360 nm and 1130 nm, respectively, and zeta potentials showing lower stability with molecular weight decrease. ATR-FTIR and CP/MAS ^{13}C-NMR investigations revealed distinct properties of alkaline and alkaline-enzymatic keratin hydrolysates with more alkyl groups (increased signal at 25 ppm in ^{13}C-NMR spectra) due to enzyme action at peptide level, more cysteic acid (1170 cm^{-1}, 1047 cm^{-1}) and cysteine-s-sulphonate content (1021 cm^{-1}) only in alkaline-enzymatic hydrolysates and with dehydroalanine formation (873 cm^{-1} in FTIR spectra) only in alkaline keratin products due to the specific cleavage of disulphide bonds. Cryo-STEM-EDX analyses confirmed more oxidized sulphur in alkaline-enzymatic keratin hydrolysates and higher concentration of sulphur in alkaline hydrolysate which can explain higher antimicrobial resistance.

Different compositions of keratin hydrolysates can be modulated through alkaline and enzymatic hydrolysis with release of metabolism precursors for plant development and without the use of synthetic products. Preliminary field tests on wheat seeds and plants showed biostimulant action and opened the path for large scale application of keratin hydrolysate processed with zero waste in a more ecological agriculture.

Acknowledgements

These works were financially supported by Romanian National Authority for Scientific Research and Innovation (CNCS/CCCDI-UEFISCDI) under project no 55PTE/2016 within PNCDI III; the Ministry of Research and Innovation under project no PN 19 17 01 02/2019 and the Science & Technology Department of Sichuan Province under project no. 2018HH0038.

References

Alaru, M., Talgre, L., Eremeev, V., Tein, B., Luik, A., NemvaltsA, Loit, E., 2014. Crop yields and supply of nitrogen compared in conventional and organic farming systems. Agric. Food Sci. 23 (4), 317–326.
Aluigi, A., Zoccola, M., Vineis, C., Tonin, C., Ferrero, F., Canetti, M., 2007. Study on the structure and properties of wool keratin regenerated from formic acid. Int. J. Biol. Macromol. 41, 266–273.
Barone, J., Schmidt, W.,F., 2006. Effect of formic acid exposure on keratin fiber derived from poultry feather biomass. Bioresour. Technol. 97, 233–242.
Barth, A., 2007. Infrared spectroscopy of proteins. Biochim. Biophys. Acta 1767, 1073–1101.
Batzer, A.,T., Marsh, C., Kirsner, R.,S., 2016. The use of keratin-based wound products on refractory wounds. Int. Wound J. 13 (1), 110–115.
Bhange, K., Chaturvedi, V., Bhatt, R., 2016. Ameliorating effects of chicken feathers in plant growth promotion activity by a keratinolytic strain of *Bacillus subtilis* PF1. Bioresour. Bioprocess. 3, 13.
Bhavsar, P.,S., Zoccola, M., Patrucco, A., Montarsolo, A., Mossotti, R., Giansetti, M., Rovero, G., Muresan, A., Maier, S.,S., Tonin, C., 2017. Superheated water hydrolyzed keratin: a new application as a foaming agent in foam dyeing of cotton and wool fabrics. ACS Sustain. Chem. Eng. 5 (10), 9150–9159.
Bin, L., Kun, Ch., Jianzhong, M., Xueyan, H., Dangge, G., He, G., Jing, Z., Yuliang, Q., 2017. A cleaning and efficient approach to improve wet-blue sheepleather quality by enzymatic degreasing. J. Clean. Prod. 148, 701–708.
Brown, E.,M., Pandya, K., Taylor, M.,M., Liu, C.,K., 2016. Comparison of methods for extraction of keratin from waste wool. Agric. Sci. 7, 670–679.
Cardamon, J.,M., 2010. Investigating the microstructure of keratin extracted from wool: peptide sequence (MALDI-TOF/TOF) and protein conformation (FTIR). J. Mol. Struct. 969 (1–3), 97–105.
Chiego, B., Silver, H., 1942. The effect of alkalis on the stability of keratins. J. Investig. Dermatol. 5, 95–103.
Choi, J.,M., Nelson, P.,V., 1996. Developing a slow-release nitrogen fertilizer from organic sources: II. Using poultry feathers. J. Am. Soc. Hortic. Sci. 121 (4), 634–638.
De Flores, L.,P., Ganim, Z., Nicodemus, R.,A., Tokmakoff, A., 2009. Amide I'-II' 2D IR spectroscopy provides enhanced protein secondary structural sensitivity. J. Am. Chem. Soc. 131 (9), 3385–3391.
Du, Z., Ji, B., Yan, K., 2018. Recycling keratin polypeptides for anti-felting treatment of wool based on L-cysteine pretreatment. J. Clean. Prod. 183, 810–817.
Duer, M.J., McDougal, N., Murray, R.C., 2003. A solid-state NMR study of the structure and molecular mobility of α-keratin. Phys. Chem. Chem. Phys. 5, 2894–2899.
Ebrahimgol, F., Tavanai, H., Alihosseini, F., Khayamian, T., 2014. Electrosprayed recovered wool keratin nanoparticles. Polym. Adv. Technol. 25 (9), 1001–1007.
Eslahi, N., Dadashian, F., Nejad, N.,H., 2013. An investigation on keratin extraction

from wool and feather waste by enzymatic hydrolysis. Prep. Biochem. Biotechnol. 43 (7), 624–648.
Food and Agriculture Organisation, 2015. World Fertilizer Trends and Outlook to 2018, Rome. http://www.fao.org/3/a-i4324e.pdf. (Accessed 3 November 2019).
Gaidau, C., Niculescu, M., Stepan, E., Epure, D.,G., Gidea, M., 2013. New mixes based on collagen extracts with bioactive properties, for treatment of seeds in sustainable agriculture. Curr. Pharmaceut. Biotechnol. J. 14 (9), 792–801.
Goormaghtigh, E., Ruysschaert, J., Raussens, M., 2006. Evaluation of the information content in infrared spectra for protein secondary structure determination. Biophys. J. (8), 2946–2957, 15, 90.
Gupta, S., Singh, R., 2014. Hydrolyzing proficiency of keratinases in feather degradation. Indian J. Microbiol. 54 (4), 466–470.
Hecklau, C., Pering, S., Seibel, R., Schnellbaecher, A., Wehsling, M., Eichhorn, T., Hagen, J., Zimmer, A., 2016. S-Sulfocysteine simplifies fed-batch processes and increases the CHO specific productivity via anti-oxidant activity. J. Biotechnol. 218, 53–63.
Jeanette, M., Nunez, C.,A., Garcia, R.,A., Aldema-Ramos, M., 2009. Characterizing wool keratin. Research Letters in Materials Science. https://doi.org/10.1155/2009/147175. Article ID 147175.
Loan, F., Marsh, C., Cassidy, S., Simcock, J., 2016. Keratin-based products for effective wound care management in superficial and partial thickness burns injuries. Burns 2 (3), 541–547.
Ma, J., Hou, X., Gao, D., Lv, B., Zhang, J., 2014. Greener approach to efficient leather soaking process: role of enzymes and their synergistic effect. J. Clean. Prod. 78, 226–232.
Ma, H., Shen, J., Cao, J., Wang, D., Yue, B., Mao, Z., Wu, W., Zhang, H., 2017. Fabrication of wool keratin/polyethylene oxide nano-membrane from wool fabric waste. J. Clean. Prod. 161, 357–361.
Marshall, R.,C., Gillespie, J.,M., 1977. The keratin proteins of wool, horn and hoof from sheep. Aus. J. Bioi. Sci 30, 389–400.
Matyašovsky, J., Sedliačik, J., Valachova, K., Novak, I., Jurkovič, P., Duchovič, P., Mičušik, M., Kleinova, A., Šoltes, L., 2017. Antioxidant effects of keratin hydrolysates. JALCA (J. Am. Leather Chem. Assoc.) 112, 327–337.
Mokrejs, P., Krejci, O., Svoboda, P., 2011a. Producing keratin hydrolysates from sheep wool. Orient. J. Chem. 27 (4), 1303–1309.
Mokrejs, P., Krejci, O., Svoboda, P., Vasek, V., 2011b. Modeling technological conditions for breakdown waste sheep wool. Rasayan J. Chem. 4 (4), 728–735.
Mwanza, E.,P., 2018. Identification, Purification and Characterisation of a Keratolytic Enzyme of *Chryseobacterium, Carnipullorum*. PhD thesis. http://scholar.ufs.ac.za.
Novoenzymes, 2018. Proteases for Biocatalysis for Smarter Chemical Synthesis (accessed in 2019). https://www.novozymes.com/-/.../Novozymes/.../Biocatalysis_brochure_Proteases.pdf.
Onifade, A.,A., A1-Sane, N.,A., Al-Musallam, A.A., Al-Zarban, S., 1998. A review: potentials for biotechnological applications of keratin-degrating microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour. Technol. 66, 1–11.
Rouse, J.,G., Van Dyke, M.,E., 2010. A review of keratin-based biomaterials for biomedical applications. Materials 3, 999–1014.
Spångberg, J., 2014. Recycling plant nutrients from waste and by-products. Diss. Uppsala: sveriges lantbruksuniv. Acta Universitatis Agriculturae Sueciae 20, 1652–6880.
Simion, D., Gaidau, C., Berechet, D., 2018. New micro and nano-structured emulsions based on collagen and keratin hydrolysates. In: International Scientific Conference "Innovative solutions for sustainable development of textiles and leather industry", Fascicle of Textile, Leatherwork, pp. 149–154. XIX, (1).
Sinkiewicz, I., Staroszczyk, H., Sliwinska, A., 2018. Solubilization of keratins and functional properties of their isolates and hydrolysates. J. Food Biochem. 42 (2), e12494.
Siodłak, D., 2015. α,β-Dehydroamino acids in naturally occurring peptides. Amino Acids 47, 1–17.
Srisuwan, Y., Srihanam, P., 2018. Preparation and characterization of keratin/alginate blend microparticles. Ann. Mater. Sci. Eng. https://doi.org/10.1155/2018/8129218. Article ID 8129218.
Staroń, P., Banach, M., Kowalski, Z., 2011. Keratin - origins, properties. application. Chemik 65 (10), 1019–1026.
Tachibana, A., Furuta, Y., Takeshima, H., Tanabe, T., Yamauchi, K., 2002. Fabrication of wool keratin sponge scaffolds for long-term cell cultivation. J. Biotechnol. 93, 165–170.
Thankaswamy, S.R., Sundaramoorthy, S., Palanivel, S., Ramudu, K.,N., 2018. Improved microbial degradation of animal hair waste from leather industry using brevibacteriumluteolum (MTCC 5982). J. Clean. Prod. 189, 701–708.
Tran, C.,D., Mututuvari, T.,M., 2016. Cellulose, chitosan and keratin composite materials: facile and recyclable synthesis, conformation and properties. ACS Sustain. Chem. Eng. 4 (3), 1850–1861.
Tsuda, Y., Nomura, Y., 2014. Properties of alkaline-hydrolyzed water fowl feather keratin. Anim. Sci. J. 85, 180–185.
VDL Handbook, 2013. http://www.eco2l-leather.com/wp-content/uploads/2013/01/ECO2L-Leitfaden. Handbook-complete-1-2013_GB1.pdf. (Accessed 13 June 2019).
World Leather, 2018. Raw Material-Use it or Lose it. http://nothing-to-hide.org/LeatherFacts/Hides_&_skins:_use_or_lose. (Accessed 17 January 2019).
Worth, G., Kelly, R., Krsinic, G., Scott, S., Roddick-Lanzilotta, A., 2015. Topically applied keratins for hair and skin care. The Science of Beauty 5 (1), 53–59.
Zoccola, M., Aluigi, A., Tonin, C., 2009. Characterisation of keratin biomass from butchery and wool industry wastes. J. Mol. Struct. 938 (1–3), 35–40.