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Studies on the decomposition of keratin wastes by saprotrophic microfungi. II. Sulphur and nitrogen balance

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In the present work the products of hydrolysis and mineralization of keratin proteins were determined. In addition nitrogen and sulphur balance in the cultures of saprotrophic keratinolytic fungi decomposing keratin wastes (feathers) was carried out.

Key words: decomposition of keratin, saprotrophic micromycetes.

INTRODUCTION

One of the most important tasks in the screening studies on the keratinolytic fungi is apart the selection of active strains, determining the biodegradation and biotransformation products of native keratin. In the studies on transformation of natural keratin most of attention was given to the release of peptide substances, amine bindings and sulphhydryl groups (Chesters, Mathison 1969; Evans, Hose 1975; Hose, Evans 1977; Deshmukh, Agrawal 1982, 1985; Safranek, Goos 1982; Kushwaha 1983). These studies aimed mainly at the evaluation of keratinolytic activity of pathogenic dermatophytes (Weary, Canby, Cawley 1965; Weary, Canby 1967, 1969; Evans, Hose 1975; Hose, Evans 1977; Deshmukh, Agrawal 1982, 1985) and saprotrophic keratinomycetes (Chesters, Mathison 1963; Safranek, Goos 1982; Kushwaha 1983). Studies on the analysis of the products of scleroprotein decomposition by these fungi are less numerous (Kunert 1973, 1976, 1989; Ruffin et al. 1976). There is no information

in literature about the utilization of feather keratin by fungi, even though the these proteins are more susceptible to the microbiological degradation that hair protein (J a i n, A g r a w a l 1980; K u s h w a h a 1983; N i g a m, K u s h w a h a 1989). In the studies conducted hitherto by the author (K o r n i ł ł o w i c z 1994; K o r n i ł ł o w i c z - K o w a l s k a 1997, Part I) it has been shown that in cultures of saprotrophic fungi growing on native feathers, keratinolysis is connected with the loss of substrate mass, release of peptide substances, sulphur amino acids, amine groups, N-NH_4^+, sulphate release and medium alkalization. In the present paper more comprehensive analysis of the catabolic products of native keratin was carried out and the balance of nitrogen and sulphur in the cultures of these microorganisms was made.

MATERIALS AND METHODS

Strains of geophilic dermatophytes and *Chrysosporium* group with strong keratinolytic abilities from the authors collection (K o r n i ł ł o w i c z-K o w a l s k a 1997) were used in the present study. Amino acid composition of the substrate was presenten in Table 1. The content of individual amino acids was determined in the hydrolysates from 6 N HCl by means of an automatic amino acid analyser T339M produced by "Mikrotechna"-Praha. The content of sulphur amino acids was determined after prior oxidation of the samples with performic acid. The total organic content of C, nitrogen, sulphur, and protein were given the previous study (K o r n i ł ł o w i c z - K o w a l s-k a 1977).

Table 1
Amino acid composition of chicken feathers

% of protein*																
Ala	Arg	Asp	Cys	Gli	Glu	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
4.2	4.6	3.7	6.73	16.4	7.1	1.2	4.0	7.2	0.74	0.88	4.0	4.4	11.1	4.6	1.4	8.3

*mean values from 2 repetitions

Fungi cultures were incubated as before using substrate containing feathers as the only source of carbon, nitrogen and energy (K o r n i ł ł o w i c z--K o w a l s k a 1997). The incubation time and study dates were given below or in the documentation material.

Chemical analyses of after-culture fluids comprised determinations of the N-NH_4^+ content by Nessler's method, S-SO_4^{2-} by the nephelometric method at the wave length of 490 nm; the presence of N-NO_3^- by brucine method, and

method, and N-NO_2^- by the Griess reagent. Ammonia release from the culture was determined by means of the absorption method according to K i m (1973) using 4% H_3BO_3. Total nitrogen content in the substrate and mycelium were determined by Kjedahl's method. Nitrogen from the high-molecular compounds was determined after prior dialysis of after-culture fluids. Dialysis was carried out using dialysing tubes "Visking" type 30/32 ("Serva") at +4°C for 48 hours. Nitrogen from the low-molecular compounds was calculated from the difference between nitrogen content in the non-dialysed and dialysed after-culture fluids. Total organic C and S contents were determined using the methods of elemental analysis after complete substrate solubilization.

The amount of peptide substances in the after-culture medium was determined by means of Lowry's method using bovine serum albumin as a standard. High molecular peptide substances were determined after dialysis of after-culture fluids described above. The amount of low-molecular peptides was determined from the difference between the pool of peptide substances in the dialysed and non-dialysed after-culture filtrates. The level of amino groups was analysed according to B a i l e y s (1962) using a standard curve plotted for leucine. Low- and high-molecular amino substances were determined in the same way as the corresponding peptide substances. Amino acid composition of the after-culture medium was determined after liophylisation of the dialysed after-culture fluids (high-molecular fraction) and after-dialysation effluents (low-molecular fraction). Before liophylisation samples were thickened in the vacuum evaporator at 30°C up to the volume of 50 cm^3. In order to determine amino acids in the mycelium the study material was dried up to the constant weight at 50°C. Oxidation of liophylysates and mycelium in order to fix sulphuric amino acids as well as hydrolysis of samples was carried out in 6 N HCl according to the established procedure. Separation of amino acids was conducted in the automatic amino acid analyser Amichrom II (Type OE-914) manufactured by "Labor", Budapest and "Mikrotechna", Praha. The results were presented as the mean value of 5 independent repetitions (amino acid determinations were carried out in 2 repetitions) for which, as a rule, 2-3 measurements were taken.

RESULTS

C o n t e n t o f h i g h- a n d l o w - m o l e c u l a r p e p t i d e s u b s t a n c e s a n d a m i n o a c i d g r o u p s. Analysis of the contents of peptide substances pointed to the nonhomogeneous character of these substances. High molecular peptides (>10 000 daltons) were quantitatively predominant. In the older cultures (42-70 days old) the level of these polymers decreased, and the amount of low-molecular peptides (<10 000 daltons) increased (Fig. 1).

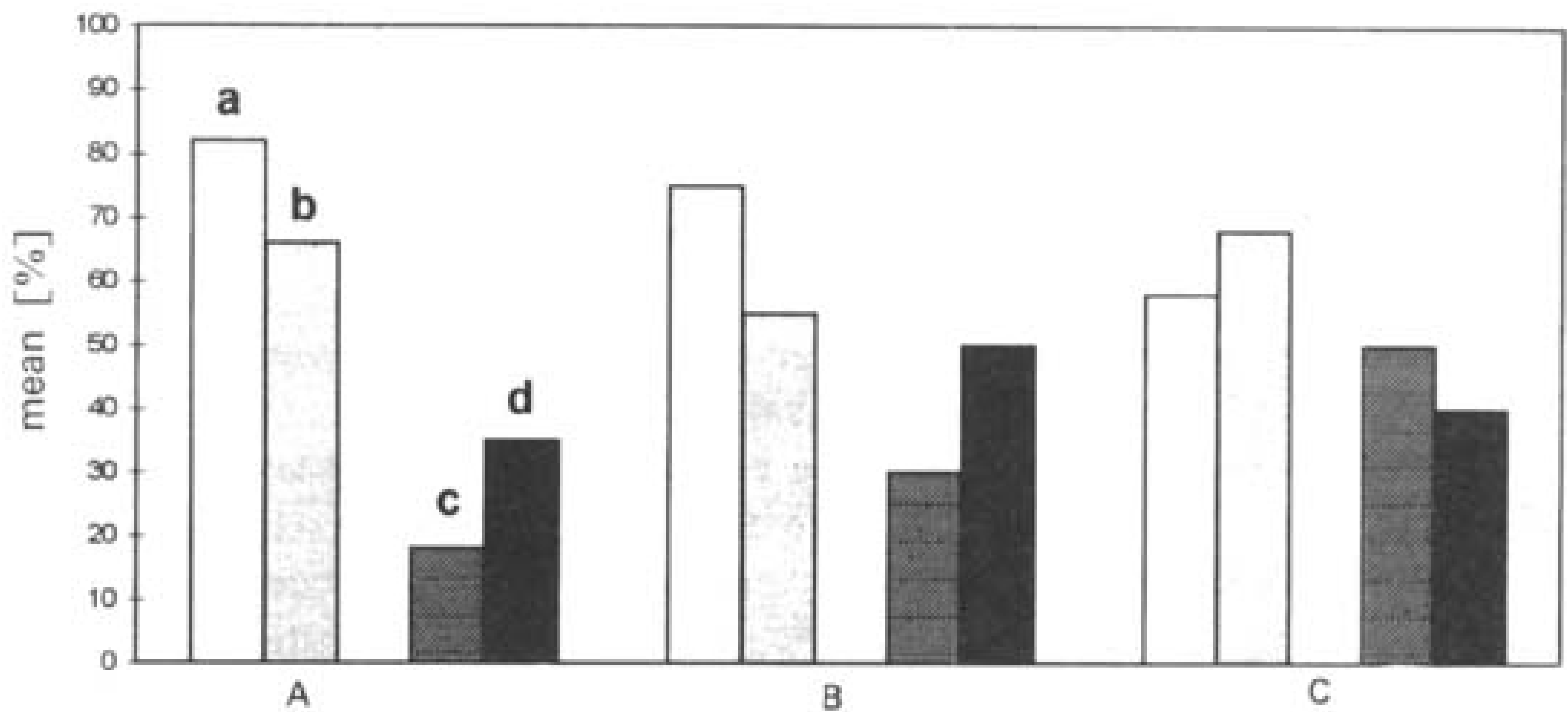

Fig. 1. Content of low- and high-molecular peptide substances in the after-filtrates of fungi decomposing feathers (means values from 5 repetitions)
high-molecular: *a* — 21 days; *b* — 42 days; low-molecular: *c* — 21 days; *d* — 42 days; *A* — *A. quadrifidum* strain 1; *B*—*A. curreyi* str. 2; *C* — *Ch. pruinosum* str. 1

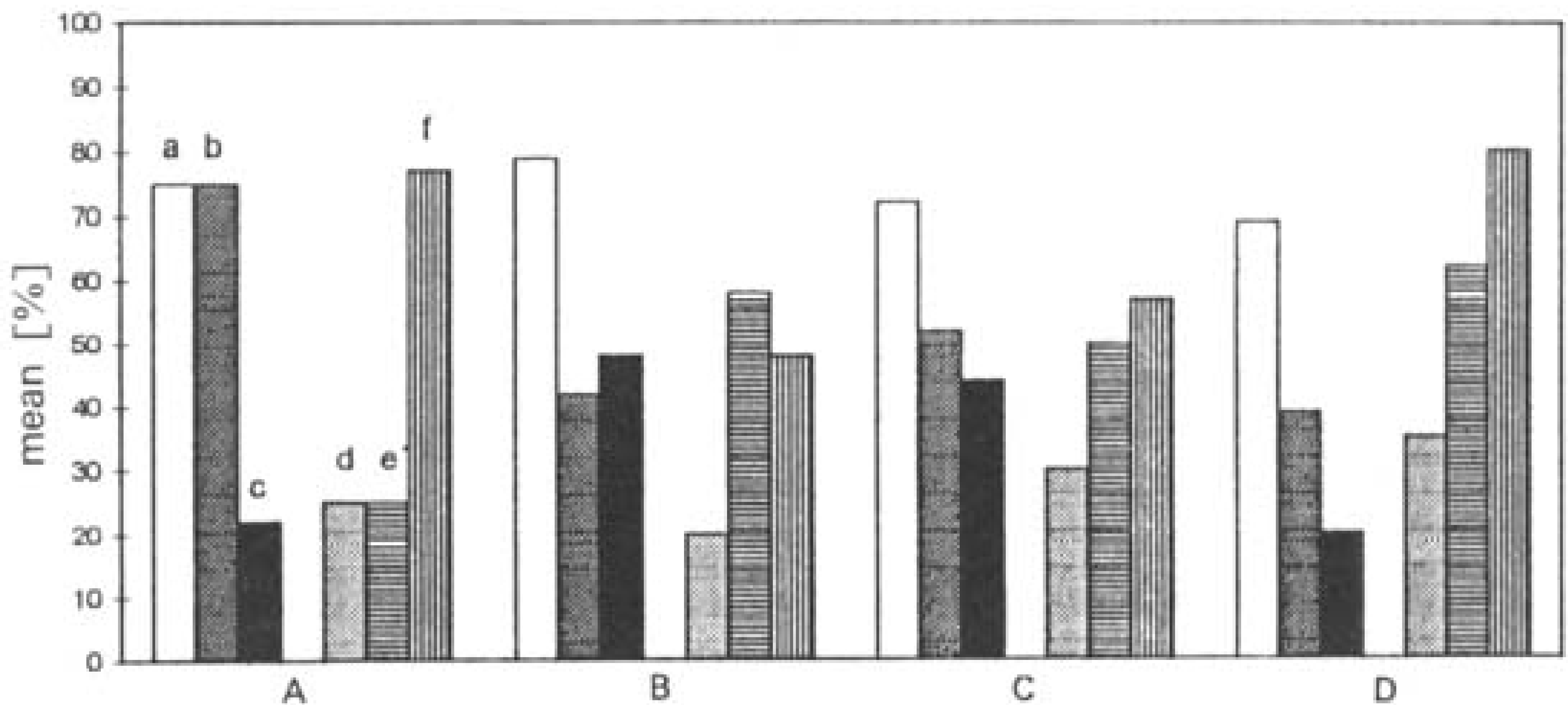

Fig. 2. Changes in the N-amine content in he after-culture filtrates of fungi (mean values from 5 repetitions
high-molecular peptide: *a* — 21 days; *b* — 42 days; *c* — 70 days; low-molecular peptide: *d* — 21 days; *e* — 42 days; *f* —70 days; *A* — *T. terrestre* strain 1; *B* — *Ch. asperatum* str. 2; *C* — *Ch. pruinosum* str. 1; *D* — *Ct. serratus* str. 1

Changes observed in the composition of peptide substances corresponded to the changes in the content of high- and low-molecular N-amine substances and were manifested in the predominance of high-molecular amine substances in the young cultures and low-molecular substances in the older cultures (Fig. 2).

Amino acid composition of feather lysates. As feather keratin contains a range of valuable amino acids, including those which are exogenous for animals, it was advisable to apropriate determine the amino acid composition of fungi feather lysates. The studies were conducted on 21-, 42-, and 70-day old cultures of several varieties. Due to the fact that on different study dates differences in individual amino acids of lysates were only quantitative the values for the 21-day old culture were only presented (Table 2). They indicated the presence of 17 of these monomers in the fungi lysates. The most frequently detected were endogenous amino acids: serine and cystine/cysteine, which constituted 10-20% and 20-30% of the total pool of these compounds in the substrate respectively. Out of the 8 essential amino acids mainly phenyloalanine, leucine, lysine, and methionine were detected. The level of these amino acids did not exceed 10% of the total amino acid content in the substrate.

It is noteworthy that the amount of lysine and methionine detected in the feather lysates was often higher than it could be gathered from their level in feathers (Table 1). It appeared that the level of these amino acids, as well as that of tyrosine was higher in the mycelium (Table 3) than in the feathers (Table 1). This suggests that some amino acids detected in the substrate originate not only from feather decomposition but also from the biotransformation process.

$N\text{-}NH_3$ volatilization. In order to balance nitrogen in fungi cultures decomposing feathers the release of this element in gasous form was also determined. Due to the necessity to take measurements very frequently and danger of infecting fungi cultures, only 3 strains were subjected to this analysis. These strains were incubated during the period of quick substrate lysis only (21 days). (Korniłłowicz-Kowalska 1997). Nitrogen losses after complete feather solubilization were calculated from the difference between the content of nitrogen in the substrate and the total amount of nitrogen in the substrate and mycelium.

The results of this experiment (Fig. 3) showed that ammonia volatilization started in the 2nd week of feather decomposition and increased quickly reaching the equivalent of 23-24% of the total nitrogen content in the substrate after 21 days.

Additional experiment with the use of glucose as a soure of easily available carbon (Fig. 4) showed limitation or even inhibition of $N\text{-}NH_3$ volatilization

T a b l e 2

Amino acid composition of fungi feather lysates (mg g^{-1} of protein – mean values from 2 repetitions)

Amino acid	*A. quadrifidum* strain 1			*T. terrestre* strain 1			*A. curreyi* strain 2			*Ch. asperatum* strain 2			*Ch. pannicola* strain 1			*Ch. pruinosum* strain 1			*Ct. serratus* strain 1		
	H	L	T	H	L	T	H	L	T	H	L	T	H	L	T	H	L	T	H	L	T
Ala	2,6	0.8	3.4	2.2	0.7	2.9	1.72	2.86	4.58	1.4	1.6	3.0	1.9	1.55	3.45	1.2	0.9	2.1	3.45	1.9	5.35
Arg	5.0	4.4	9.4	2.4	1.1	3.5	1.34	0.0	1.34	5.5	2.0	7.5	2.5	1.2	3.7	1.75	2.1	3.85	2.3	1.35	3.65
Asp	10.5	6.5	17.0	5.2	2.65	7.85	7.8	7.25	15.06	7.5	2.0	9.5	17.7	6.4	24.1	3.7	1.6	5.3	4.8	2.9	7.7
Cys	35.8	20.8	56.4	19.3	6.75	26.05	33.6	9.7	43.3	11.5	10.5	22.0	21.8	12.8	34.6	20.3	5.6	25.9	17.5	17.5	35.0
Glu	8.4	3.4	11.8	3.65	3.4	7.05	4.2	4.1	8.3	5.14	3.0	8.14	9.3	2.14	11.44	3.4	2.1	5.5	6.55	6.0	12.55
Gly	6.1	3.0	9.1	0.66	1.55	2.21	2.3	5.2	7.5	2.75	3.0	5.75	5.6	4.5	10.1	3.3	1.75	5.05	5.0	3.3	8.3
His	2.0	1.0	3.0	–	–	–	4.05	1.3	5.35	–	–	–	4.7	3.3	8.0	–	–	–	–	–	–
Ile	2.3	5.0	7.3	1.3	0.88	2.18	1.15	1.3	2.45	4.35	1.4	5.75	1.4	1.0	2.4	2.0	0.7	2.7	0.8	4.4	5.2
Leu	3.2	1.0	4.2	4.6	1.3	5. 9	2.6	6.1	8.7	5.5	3.2	8.7	3.1	1.2	4.3	1.8	2.3	4.1	5.8	2.1	7.9
Lys	6.6	4.1	10.7	4.3	1.3	5.6	4.2	6.5	10.7	2.4	2.4	4.8	3.5	2.5	6.0	1.16	1.57	2.67	3.08	1.93	5.01
Met	2.1	0.0	2.1	0.85	0.0	0.85	1.55	2.9	4.45	7.53	2.94	10.47	4.9	3.1	8.0	2.56	2.33	4.89	3.85	0.96	4.81
Phe	4.5	9.25	13.75	9.6	1.0	10.06	3.0	0.6	3.6	1.42	2.0	16.2	1.4	4.7	6.1	4.5	1.2	5.7	1.0	17.0	18.0
Pro	0.6	0.4	1.0	–	–	–	0.0	0.95	0.95	–	–	–	1.0	0.6	1.6	–	–	–	–	–	–
Ser	33.0	14.3	47.3	13.8	4.4	18.2	16.8	10.7	27.5	10.7	8.3	19.0	11.0	5.4	16.4	8.9	3.4	12.3	17.3	11.0	28.3
Thr	3.75	2,0	5.75	1.88	0.77	2.65	2.1	2.1	4.2	3.0	2.0	5.0	3.3	2.3	5.6	2.3	1.5	3.8	4.3	3.5	7.8
Tyr	1.0	0.36	1.36	–	–	–	4.2	0.0	4.2	0.8	0.0	0.8	3.3	214	5.44	–	–	–	–	–	–
Val	5.0	1.0	6.0	0.39	0.88	1.27	0.94	1.5	2.44	3.0	1.6	4.6	1.0	1.2	2.2	0.7	1.4	2.1	7.9	6.2	14.1
total	1.3	2.25	77.31	70.13		26.68	91.55		63.06	85.3	45.94	131.21	97.4		56.03	57.57		28.39	83.63		80.04
		209.56			96.81			154.61						153.43			85.96			163.67	

H – high-molecular fraction; L – low-molecular fraction; T – total

Table 3
Amino acid composition of mycelium of some fungi strains after solubilization of feather (mg g^{-1} protein of mycelium — mean values from 2 repetitions)

Amino acid	*A. quadrifidum* strain 1	*A. curreyi* strain 2	*Ch. asperatum* strain 2
Ala	77	51.3	47.4
Arg	24	98.2	91
Asp	84	91.2	89
Cys	23	31.5	27
Phe	56	24	32
Gli	87	91	76
Glu	92	73	65
His	87	80	127
Ile	29	71	70
Leu	116	78	93
Lys	33	56	37
Met	24	21	13,4
Pro	28	25	18
Ser	39	21	26
Thr	53	30	41
Tyr	50	27	20
Val	38	66	43,3
total	936	935.2	916.1

in the resence of higher sugar concentrations (2%). Glucose concentration (1-2%) counteracting this phenomenon slowed down the rate of keratinolysis measured as thet loss of substrate mass (Fig. 5).

Nitrogen balance in the fungi cultures after complete solubilization of feathers. The culture of these fungi which completed feather lysis in the shortest time (70 days) were subjected to analysis (Korniłłowicz-Kowalska 1997). High losses of nitrogen, even up to 60%, in the form of gasous ammonia were observed (Fig. 6). The remaining nitrogen of the substrate was captured by fungi in the mycelium (<10%) and was changed into a soluble form of which 25% constituted easily hydrolysing organic nitrogen and 75% $N\text{-}NH_4^+$ (Fig. 7)

Sulphur balance. In the first part of the study it was stated that different varieties of keratinophylic fungi in the period of most intensive mineralization of organic S released about 50% of this element into the substrate.

On the basis of the content of sulphuric amino acids in crude feathers it was calculated that the amount of organic S introduced with this substrate was 17.2 mg. Ninety-five percent of this organic sulphur was transferred to the

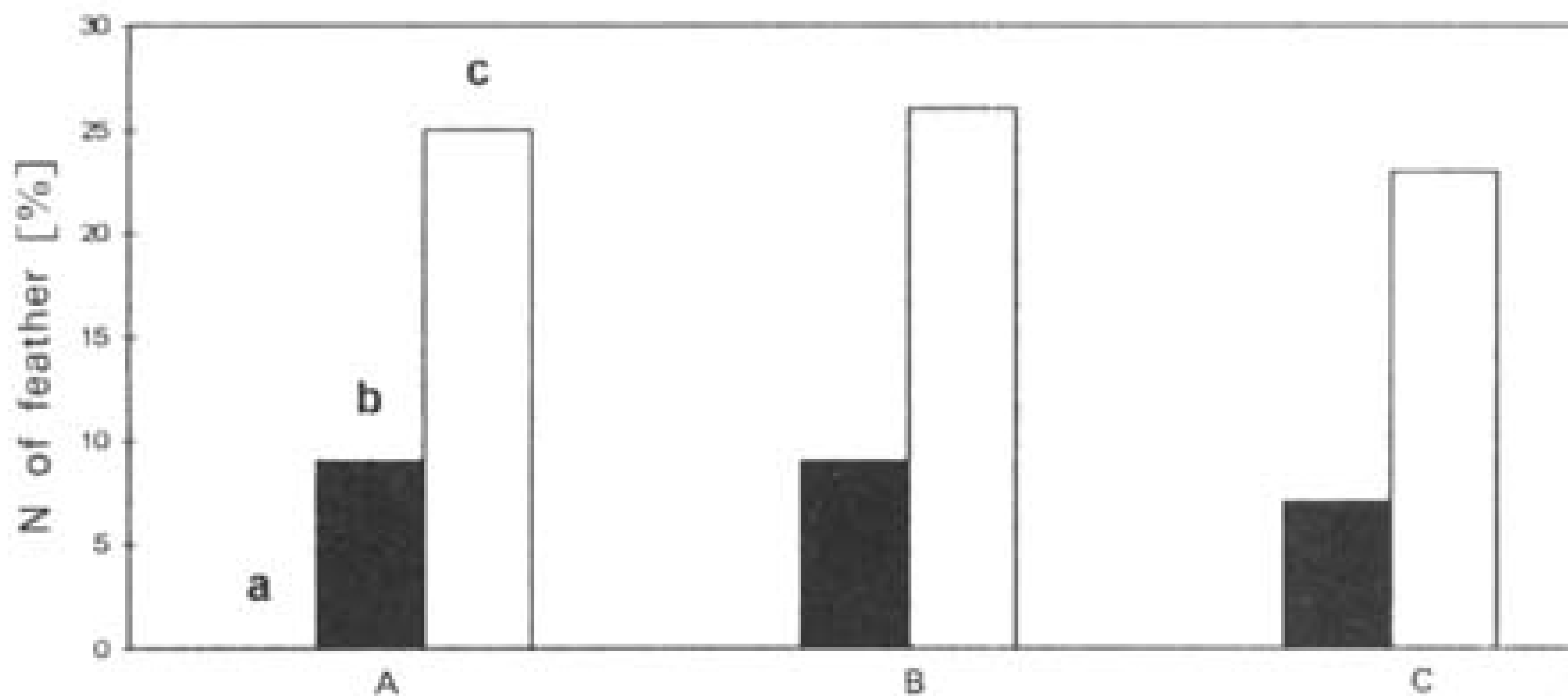

Fig. 3. $N-NH_3$ release during feather decomposition by fungi (mean values from 5 repetitions) *a* — 7 days; *b* — 14 days; *c* — 21 days; *A* — *A. quadrifidum* strain 1; *A. curreyi* str. 2; *C* — *Ch. pruinosum* str. 3

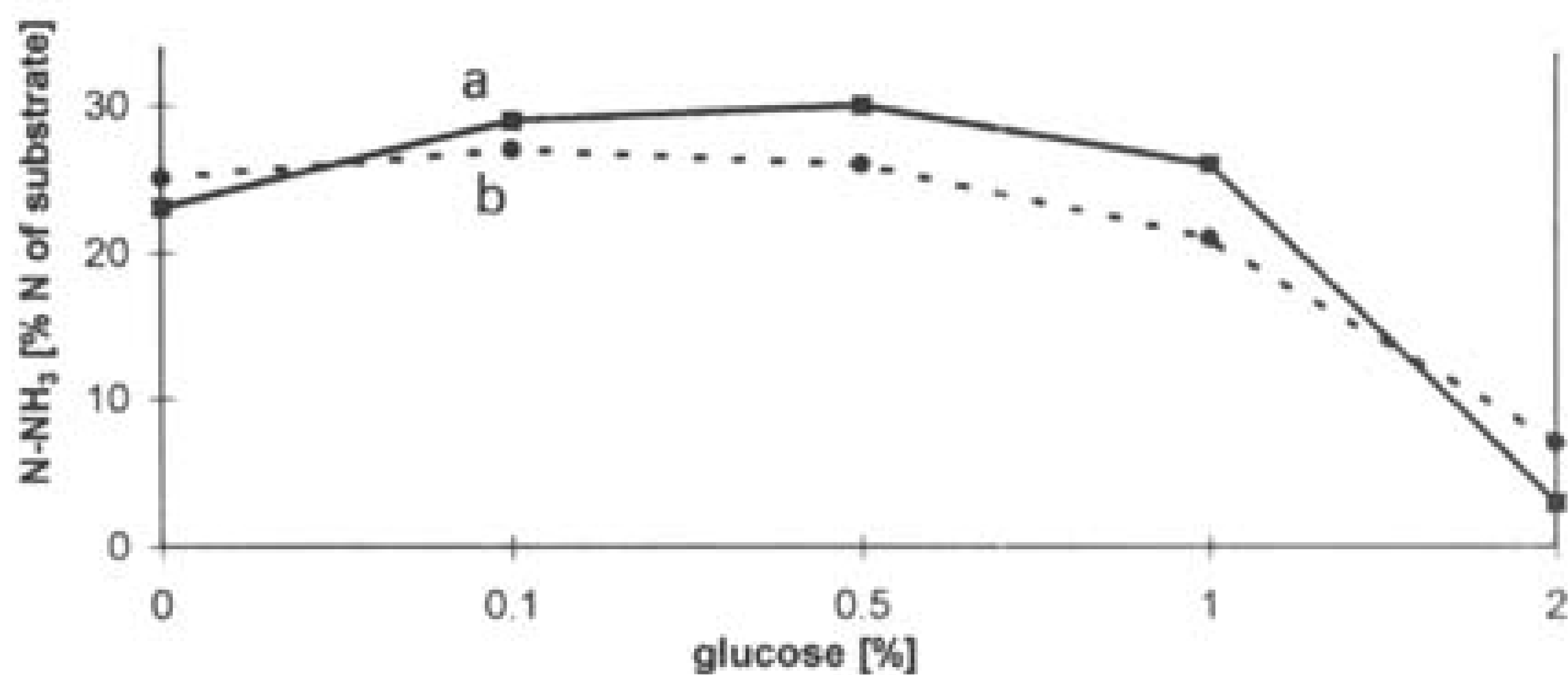

Fig. 4. Effect of glucose on $N-NH_3$ volatization from culture of fungi decomposing feathers (21 days)
a — *A. quadrifidum* strain 1; *b* — *Ch. pruinosum* str. 1

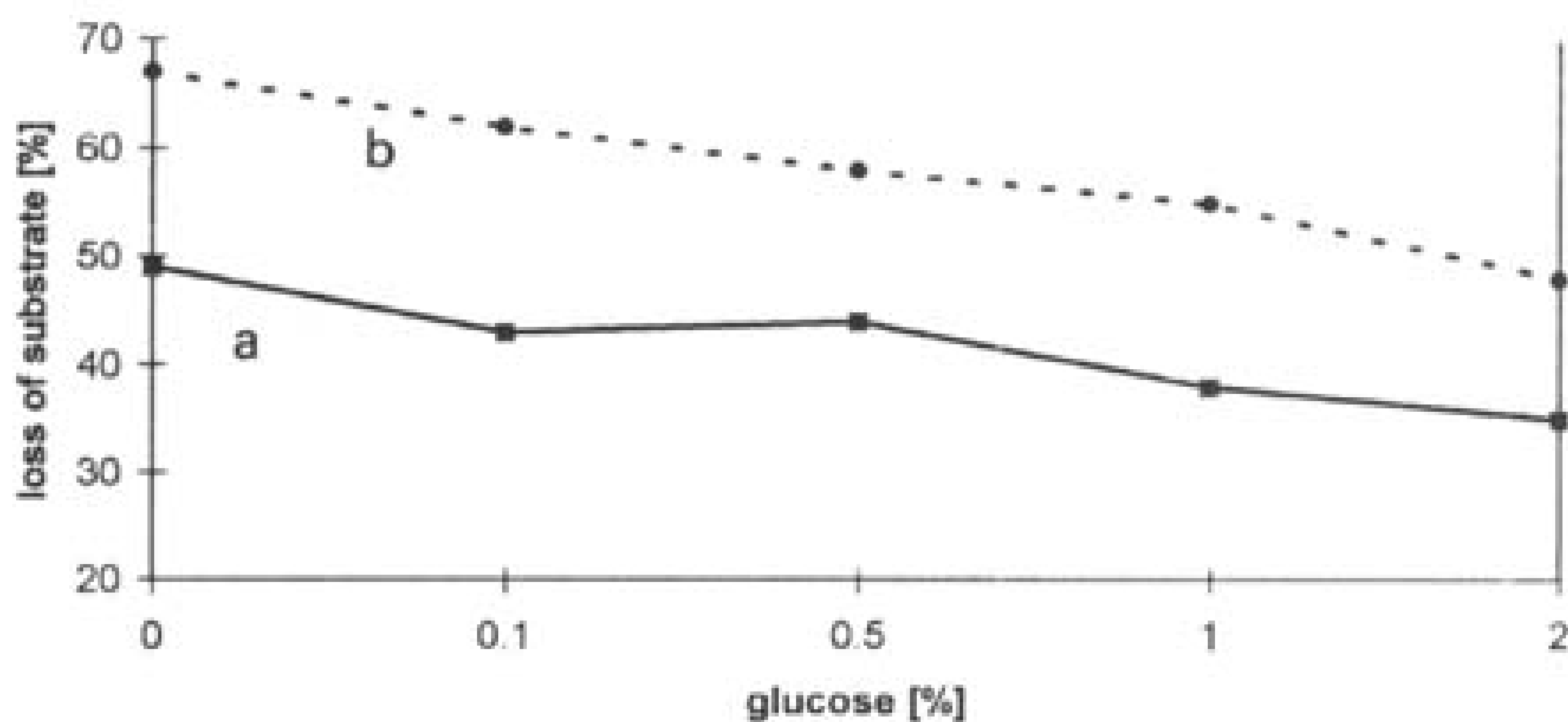

Fig. 5. Effect of glucose on degree of utilization of native feather keratin by *A. quadrifidum* and *Ch. pruinosum* (21 days)-old of culture)
a — *A. quadrifidum* strain 1; *b* — *Ch. pruinosum* str. 1

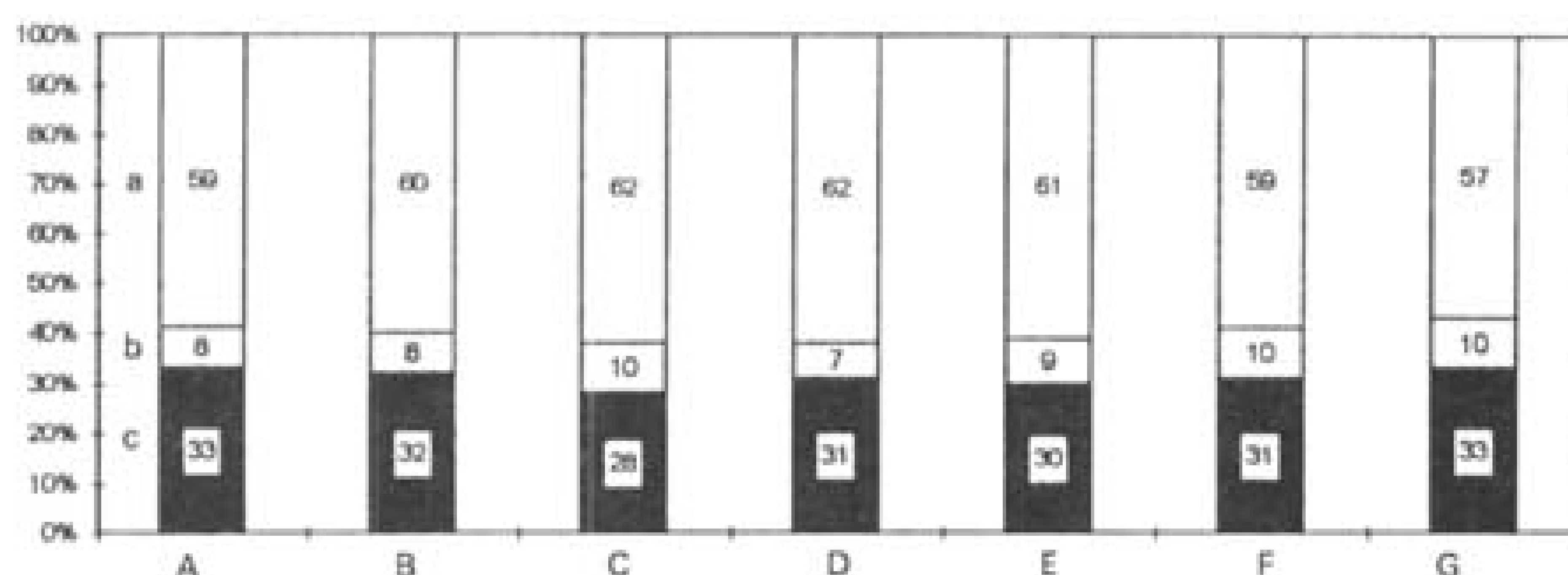

Fig. 6. Nitrogen balance in the 70-day culture of fungi decomposing feathers (mean values from 3 repetitions)
a — nitrogen content in NH_3; *b* — nitrogen content in mycelium; *c* — nitrogen content in the substrate; *A* — *Arthroderma curreyi* strain 1; *B* — *A. curreyi* str 2; *C* — *Chrysosporium keratinophilum* str 1; *D* — *Ch. pannicola* str. 1; *E* — *Ch. pannicola* str. 2; *F* — *Ch. pruinosum* str. 1; *G* — *Ctenomyces serratus* str. 3

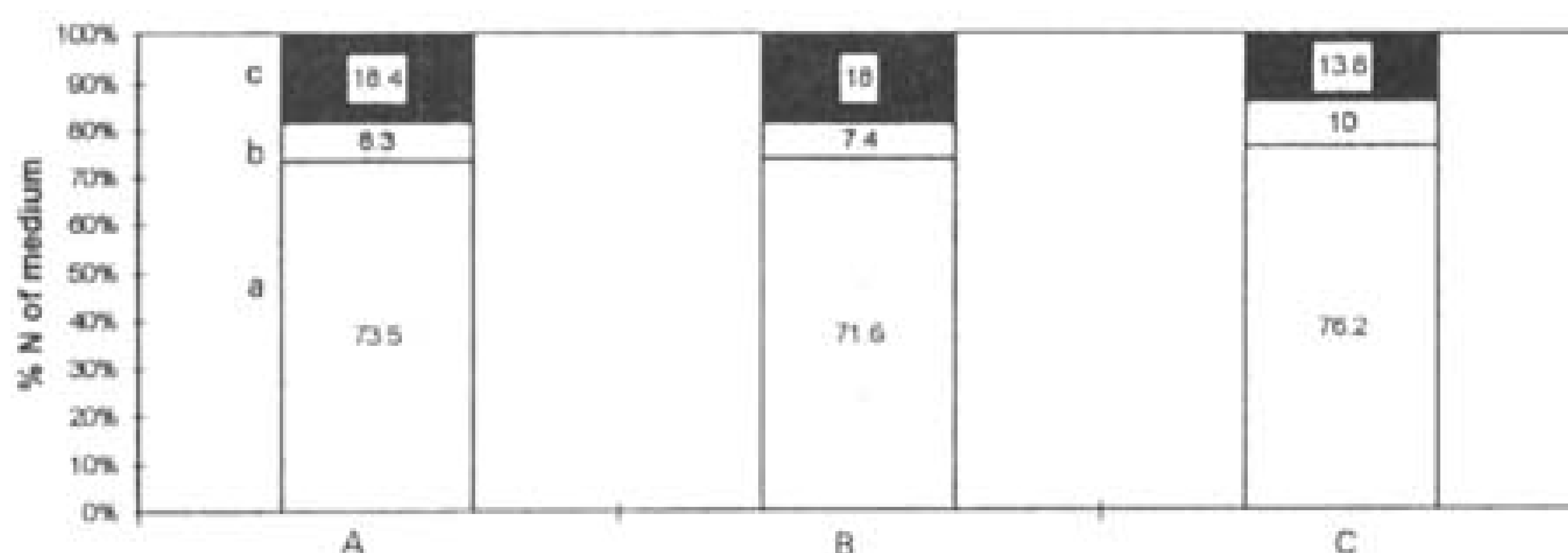

Fig. 7. Composition of the soluble nitrogen fractions (mean values from 5 repetitions) in feather fungi lysates (70-days-old cultures)
a — N-NH_4^+; *b* — N-org high-molecular compounds; *c* — N-org low-molecular compounds
A — *A. quadrifidum* strain 1; *B* — *A. curreyi* str. 2. *C* — *Ch. pruinosum* str. 1

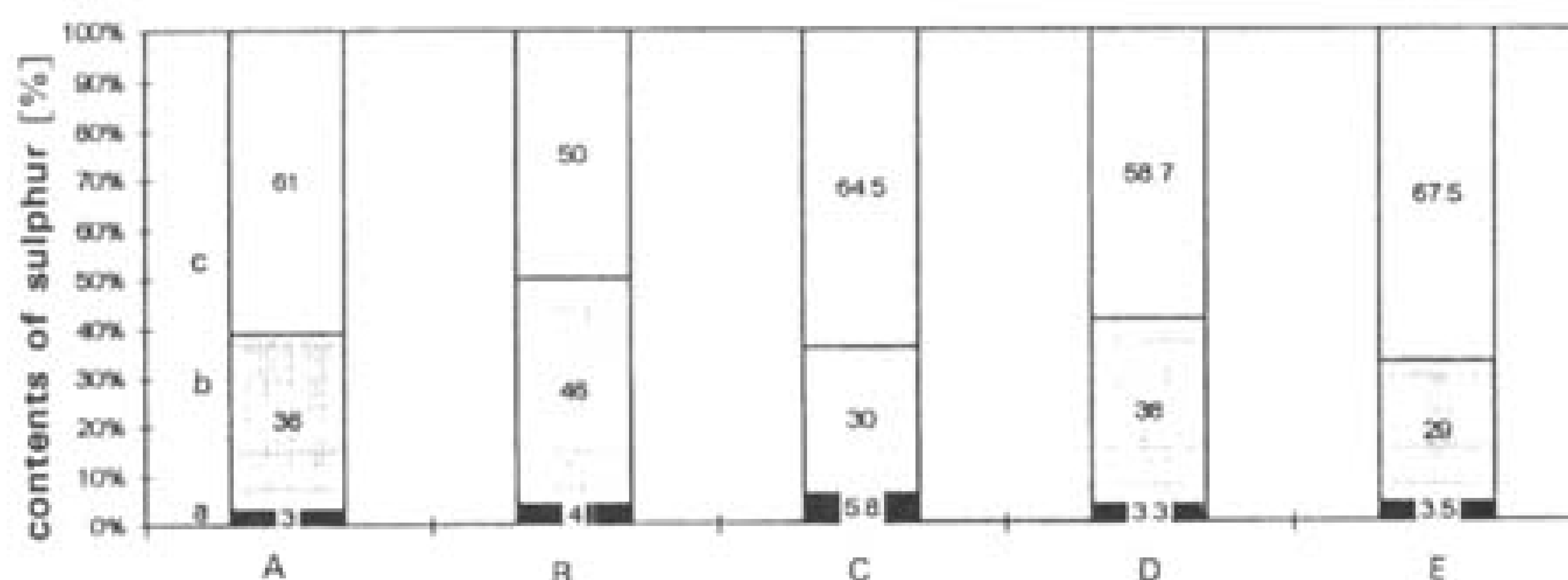

Fig. 8. Sulphfur balance in the cultures of fungi after complete solubilization of feathers (mean values from 3 repetitions)
a — in mycelium; *b* — in sulphate; *c* — in another; *A.* — *A. curreyi* str. 1; *B* — *Ch. keratinophilum* str. 1; *C* — *Ch. pannicola* str. 1; *D* — *Ch. pruinosum* str. 1; *E* — *Ct. serratus* str. 3

medium after complete decomposition by the selected strains, in cluding 30-50% as sulphates, and only 3-5% were incorporated into the mycelium. The remaining sulphur was released into the medium in the form of sulphuric amino acids and other intermediate products of sulphitolysis (i.e. hydrolysis of bisulphide bonds) such as sulphite and tiosulphate. The above compounds were detected by other authors who investigated the degradation of hair and wool by dematophytes (K u n e r t 1973, 1978, 1989).

DISCUSSION

The present study showed that the decomposition of waste feathers by strongly keratinolytic strains of saprotrophic fungi first leads to the release of high-molecular peptide substances. It was found out that during the peak of lysis (21-day-old culture) 70-80% of the total pool of these substances was represented by the compounds with a mass > than 10 000 daltons. Even though from the biochemical point of view peptide substances with this kind of weight are classed as proteins, K u n e r t (1976) proved that polypeptides are predominant among the product of fungi keratinolysis of hair with a mass of > 10000 daltons. The author noted the highest amount of low-molecular peptides (< 10 000 daltons). Reversed proportions in the contents of these substances were found in the feather lyses. This probably resulted from a more uniform nature of feather keratin compared to hair keratin, which was ascribed to the presence of uniform, as far as their mass is considered, (10 400 daltons) subunits (F r a s e r, M a c R a e, R o g e r s 1972; L e e, B a d e n 1975). The decrease in of the level of high-molecular peptides in favour of low-molecular ones (observed during the period of lower rate of feather keratinolysis) was probably caused by the split of these polymers. The increase in the content of amine residues in the low-molecular fraction, which accompanied this process, pointed to the alkaline character of these bindings. T a k i u c h i et al. (1984) indicated the release of amino peptides during the process of "digestion" of chemically pure keratin by keratinasis of pathogenic dermatophytes.

In the present study it was indicated that amino acid composition of fungi feather lysates reflected, as a rule, amino acid composition of proteins in the substrate. Most often serine and sulphuric amino acids (except methionine) were found, whereas exogenous amino acids were less frequent. It is noteworthy that some fungi released lysine and methionine in amounts higher than their content in feathers. These amino acids are especially in short supply in poultry feeding (Normy Żywienia Drobiu [Poultry Feeding Norms], 1993). This observation creates a base for studying utilization of keratinolytic fungi, and actinomycetes (E l m a y e r g i, S m i t h, 1971) for the bioconversion of keratin in order to obtain some amino acids essential o animals.

It appeared that, $N\text{-}NH_4^+$ belonged to the inorganic nitrogen products produced during the decomposition of feathers by fungi. In this part of the study it was also shown that gasous ammonia was released. No presence of $N\text{-}NO_2^-$ and $N\text{-}NO^{+}{}_3^-$ was observed. This phenomenon should be cosidered as favourable from the toxicological point of view. Nitrites are, very toxic compounds (they can also be produced from nitrates under the influence of intestine flora). Moreover, the oxidated nitrogen forms are predecossors of nitrozamin – one of the most dangerous poisons of biological origin. However, ver high content of $N\text{-}NH_4^+$ in feather lyses (Part I) excludes the possibility of their direct utilization in fodder. Ammonia volatization from feather lysates lowers their value as fertilizers. Even though an introduction of easily available source of carbon (glucosis) limited and at higher concentrations, eliminated nitrogen losses, it caused slowing down of the keratinolysis rate. Meevootison and Niderpruem (1979) stated that inhibition of decomposition of hair by fungi in the presence of glucose was caused by the caabolic repression of synthesis of proteolytic enzymes. The decrease in the proteolytic activity of fungi that hydrolyse feathers in the medium with glucose was also noted in the present study paper (in preparation).

From the biotechnological point of view, the shortening of the culture time with simultaneous binding of $N\text{-}NH_4^+$ seemed to be more suitable than the introduction of easily available carbon, as it was noted that the proper choice of concentration of phosphates and magnesium ions, both facilitates lysis and induces the precipitations of magnesium-ammonium phosphate ($NH_4MgPO_4 \times 6\,H_2O$). These data will be presented in a separate publication. In order to set directions for the utilization of keratin wastes with the use of saprotrophic micromycetes, it is necessary to learn about the enzymatic apparatus of these microorganisms.

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Badania nad rozkładem odpadów keratynowych przez saprotroficzne mikromycetes. II. Bilans azotu i siarki

Streszczenie

Przeprowadzone badania wykazały, że wśród produktów grzybowej proteolizy odpadów organicznych bogatych w keratynę (pierze kurze) przeważały wysokocząsteczkowe połączenia peptydowe. Poziom tych polimerów obniżał się w hodowlach starszych, w których wzrastała zawartość zasadowych peptydów drobnocząsteczkowych. Skład aminokwasowy uzyskanych lizatów piór odznaczał się przede wszystkim znaczną zawartością aminokwasów siarkowych

(poza metioniną) oraz seryny. Wśród aminokwasów egzogennych lizyna uwalniana była w ilościach większych niż by to wynikało z jej zawartości w substracie. Wąski stosunek C:N w piórach sprzyjał przede wszystkim mineralizacji azotu tego substratu. Nagromadzony w wyniku amonifikacji N-NH_4^+ wywoływał alkalizację podłoża. Czynnikiem przeciwdziałającym stratom azotu w formie gazowej był dodatek glukozy. Nadmiar siarki zawartej w keratynie piór był utleniany głównie do siarczanów. Nie stwierdzono natomiast wydzielania toksycznego siarkowodoru.