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Methods for determining keratinolytic activity of saprophytic fungi

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In order to evaluate the keratinolytic activity of saprophytic microfungi the following values were determined; the percentage keratin substrate decomposition (native feathers), the changes of pH of medium the liberation of peptidic substances in this process, as well as the liberation of amine nitrogen and cystein. The presence of keratinase was examined in culture filtrates and the proteolytic activity in the presence of casein. It was noted that among the above mentioned indices the percentage decrease of substrate mass was the most useful criterion of keratinolytic activity evaluation.

INTRODUCTION

Feathers belong to the most troublesome keratine and poorly utilised westes. Mainly physical and chemical methods are applied in disposing or processing these organic wastes with high protein content (Jeske et al., 1976; Wolski, 1985). At the same time this is a biological process in nature. First of all some fungi play an important role in such process especially keratinophilic fungi as well as actinomycetes with keratinolytic abilities (Noval, Nickerson, 1959; de Vries, 1962; Domsch et al., 1980; Oorschot, 1980). These are mainly saprophytic microorganisms (Otčenašek, Dvořak, 1975; Oorschot, 1980). This is very important from the point of view of keratine by-products utilization.

Most works dealing with the keratinolytic activity of microorganisms are based on studies concerning hair decomposition by pathogenic dermatophytes (Weary, et al., 1965; Weary, Canby, 1967, 1969; Yu, Harmon, Blank, 1968, 1969; Evans, Hose, 1975; Kunert, 1973, 1976; Meevootison, Niedepruem, 1976; Takiuchi et al., 1982; Wawrzkiewicz et al., 1987). In recent years considerable interest has been focused on the degradation of the above mentioned keratine substrate by non-pathogenic fungi (Chesters, Mathison,

1963; Safranek, Goos, 1982; Deshmukh, Agrawal, 1982, 1985). However, little attention has been given to the decomposition of native feather keratine fungi (Jain, Agrawal, 1980; Kushwaba, 1983). There are no indices determining the keratinolytic activity of these microorganisms as well.

The present paper is an attempt at selecting methods for determinig the keratinolytic activity of saprophytic fungi which decompose feather keratine. The methods described in literature were used in this study adjusted to the subject of research.

MATERIALS AND METHODS

Fungi strains. Two fungi strains isolated from arable soils were used in the study: *Ctenomyces serratus* Eidam (chernozem formed from loess) and *Chrysosporium tropicum* Carmichael (black earth formed loamy sand). Both species are often isolated from soils rich in feathers or directly form bird feathers (Domsch et al., 1980; Oorschot, 1980).

Fungi were isolated with the method of keratine baiting (chicken feathers) which had been described by Korniłłowicz (1993).

The preparation of Leghorn chicken feathers obtained from the Poultry Plant in Lublin were used as a substrate. The feathers were washed in a detergent and subsequently after rinsed with tap water and dried at room temperature. Dry feathers were mechanically crumbled into 0.5-1 cm fragments. After degreasing in ether (24 hours) the feathers were rinsed several times in distilled water and dried again. The material prepared in this way was sterilized with the method of gassing with ethylene oxide in chambers under the pressure of 0.5 atm for 6 hours (in Polfa Works, Lublin). Before sterilization the material was kept in wet chambers (39°C, 90 % water vapour saturation) for 1-2 days.

Fungi inoculum was prepared from 10-14 day old cultures in scarfs with Sabouraud's glucose agar: the spores were rinsed with 5 cm^3 of NaCl (0.9 %) with Tween 80 (0.02 %). The spores were diluted in mineral medium with the composition given below. Their density was about 1000 spores in 1 cm^3. Thom's chamber was used for determining the number of spores.

Culture methods. The experiment was carried out in liquid media containing feathers as the only source of C, N and S. With the exception of the keratinase activity experiment, in all the remaining cases (I) medium with the following composition (g) was used: K_2HPO_4 – 1.5; NaCl – 0.01; $MgCl_2 \cdot 7H_2O$ – 0.05; chicken feathers – 5 distilled H_2O – 1000 cm^3; pH – 6.5. The fungi cultures were grown in Erlenmayers flasks with the capacity of 300 cm^3 containing 100 cm^3 of mineral medium. The dosed 0.5 g of feathers was introduced separately. The 1 cm^3 suspension of spores was used as inoculum. The medium of Nickerson, Norval and Robinson (1963) with pH 7.8 was used for keratinase induction (2 %

of crumbled feathers were introduced instead of hair – medium II). 200 cm^3 of medium in Legroux flasks after the addition of 4 g of feathers was grafted with 2 cm^3 of spore suspension. In all the cases, keratin salts broth was used as control, to which a proper amount of mineral medium I or II was introduced instead of inoculum.

Fungi cultures incubated at 20°C ± 2°C for about 100 days with the addition of kanamycin (from Sigma) for the inhibition of bacterial contamination. Kanamycin was applied in the amount of 40 mcg in 1 cm^3 of medium (W e a r y, C a n b y, 1967). The same amount of antibiotic was used for securing the control.

All the analyses were carried aut directly after the experiment had started and after 3, 7, 14, 21, (28), 42, 70, 98, (126) days of incubation applying 3-5-fold repetitions.

The indices of keratinolytic activity of fungi. The percentage loss in the weight of feathers was determined with C h e s t e r s' and M a t h i n s o n's method (1963), which had been applied so far for the estimation of the degree of hair decay when applying the above mentioned method the index of spawn loss was separately determined. It amounted to 1.66 for *Ct. serratus* and 1.73 for *Ch. tropicum*.

The pH of liquids was measured by means of a potentiometer.

The amount of peptidic substances in medium was determined with Lowry's method (L o w r y et al., 1951) applying bovine serum albumin as standard. The results were given in mcg of released peptidic substances in 1 cm^3 of after-culture filtrate.

The content of ninhydrin-positive substances in medium was analysed with Bailey's method (B a i l e y, 1962). The results were determined using the calibration curve for leucine.

The content of cystein was determined with the method of liquid chromathography in dialysates of culture filtrates. Dialysing tubes "Visking" type 30/32 of "Serva" firm were used for dialysis. It was carried out for 48 hours at 4°C in 300 cm^3 of redistilled water changing it once after 24 hours. The obtained dialysates were condensed at 30°C in a vacuum evaporator to the capacity of 50 cm^3 and they were lyophilized. The oxidation of lyophilizates with the aim of sulphur amino acid fixing and their hydrolysis was carried out according to the method described by W i e r c i ń s k i (1984). The separation of amino acids was carried out in cirtate buffer solution with pH 2.2 in norleucine as a standard applying the automatic amino acid analyzer, Aminchrom II type OE-914 of "Labor" firm, Budapest. The sulphur amino-acids content was determined from the curve of amino acids separation. The amount of cystein was calculated from the content of cystein acid applying the calculation index 0.71. The final results were given in mcg of cystein in 100 cm^3 of medium.

The total proteolytic activity of after-culture filtrates was determined in casein as substrate. 1 cm^3 of 1 % casein solution with pH 7.6 was mixed with 1 cm^3 of clear after-culture filtrate. The reaction mixture was incubated for 3 hours in 35.6°C in water bath with shaking. The reaction was stopped by the addition of 3 cm^3 of 10 %

TCA and left for 30 minutes at room temperature. The precipitate was removed by filtration in Whatman 1 filters and the amount of released aromatic amino acids was determined with Anson's method using control as a blank test. The preparation of control was identical to the preparation of the proper test but the TCA solution was added before the incubation. The results were determined using the calibration curve for tyrosine and were given in mcg of released tyrosine in 1 cm^3 of after-culture filtrate after 3 hours.

The keratinase in after-culture filtrates was determined with the method of Y u et al. (1968) modified by Takiuchi (T a k i u c h i et al., 1982), applying the crumbled feathers instead of guinea-pig hair as substrate. Non-condensed after-culture filtrates and 20-times condensed ones, as well as non-dialysed ones were used for the analysis. The dialysis of condensed after-culture filtrates was carried out for 24 hours at 4°C (dialysing tubes "Visking" type 30/32) in phosphate buffer solution (3000 cm^3 0.28 M of phosphate buffer with pH 7.8). In each case, 1 cm^3 of enzymatic material mixed with 50 mg of feathers, soaked previously in phosphate buffer and rinsed 4 times in redistilled water and then dried in the stream of cool air was used for the determination. The enzymatic reaction was carried out in 5 cm^3 of 0.028 M phosphate buffer (+ 1 mM $MgSO_4$) with pH 7.8 for 16 hours at 37°C applying shaking. In order to inhabit the development of bacterial contamination 0.01 % solution of mertiolat was applied. After completing the incubation the mixture was cooled for 5 minutes at of 0°C and centrifuged for 10 minutes at the speed of 3000 rpm. Protein was determined in clear supernatant liquid at the wave length of 280 nm, by means of spectrophotometer Specord M-40. The ezymatic material was boiled for 30 minutes in the control test. The determinations were carried out in the presence of clear mineral nutrient medium (II) as a blank test.

It was, assumed after (Y u et al., 1968; T a k i u c h i et al., 1982) that 0.100 absorbance at 280 nm (A_{280}) corresponds to 1 unit of keratinase (1 KU = 0.100). Therefore the obtained values were changed into the number of enzymatic units (KU) in 1 cm^3 of after-culture filtrates. The specific keratinase activity was expressed as the number of enzyme units per 1 mg of protein.

RESULTS AND DISCUSSION

The avaiable literature dealing with fungi keratinolysis shows that the degree of keratin substrate utilisation is one of the indices of keratinolytic activity of fungi determined on the basis of hair weight loss C h e s t e r s, M a t h i n s o n (1963), P a g e, S t o c k (1974), E v a n s, H o s e (1975), D e s h m u k h, A g r a w a l (1982, 1985). This was also confirmed in the present study research carried out with the use of chicken feathers as the source of C, N and S. It was manifested in the gradual loss of substrate mass from fungi culture reaching 60 % after 3 weeks

(Fig. 1). When keratin constitutes at least 90 % of feather weight (N o v a l, N i c k e r s o n, 1959), the rate of solubility of this keratin material reflects the dynamics of keratinolytic activity of the examined fungi.

It was also demonstrated that the increase in solubility of keratin is accompanied by the accumulation of peptide substances in the growth medium. The dynamics of liberation of these compounds is convergent with the dynamics of substrate decomposition (Figs. 1 and 2 A). An especially quick formation of peptide substances took place in the first 3 weeks of the fungi growth on the feathers (Fig. 2 A). Subsequently the process was slowed down with the rate of substrate decomposition. Maximum net values (after eliminating the by control) of peptide substances were noted at the end of the research. They corresponded to 95-98 % of solubilization of native feathers (Fig. 1). Because the liberation of the peptide compounds was associated with substrate decomposition it also reflected the keratinolytic activity of the investigated microorganisms. Similar observations regarding the fungi hair keratinolysis were made by E v a n s and H o s e (1975) and D e s h m u k h and A g r a w a l (1982, 1985).

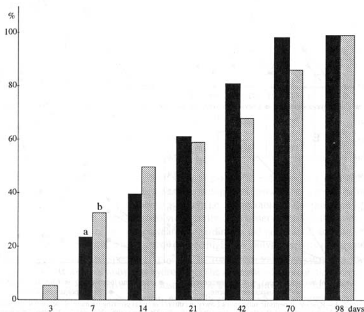

Fig. 1. Percentage of the substrate digestion in cultures of fungi growing on medium with the chicken feathers as sole C and N source
a – *Chrysosporium tropicum*, b – *Ctenomyces serratus*

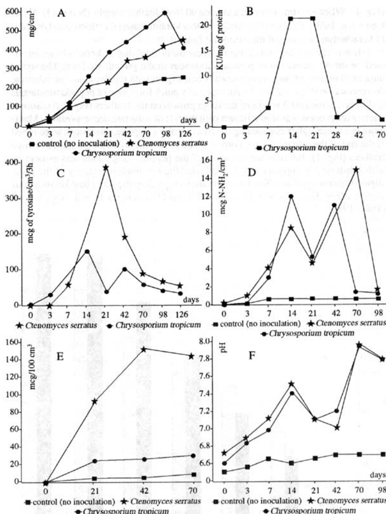

Fig. 2. A – Release of peptidic substances Lowry in cultures of fungi growing on mineral medium with chicken feathers. Lowry – positive substances expressed as mcg/cm^3 bovine serum albumin; B – Keratinolytic activity of the culture filtrates of *Ch. tropicum* against native keratin (20 x condensed and dialysed); C – Proteolytic activity of the culture filtrates of fungi growing on mineral medium with chicken feathers; D – Release of ninhydrin reactive compounds in cultures of fungi decomposing chicken feathers; E – Content of cystein in the culture fluid of fungi decomposing chicken feathers; F – Changes in the of pH cultures of fungi growing on mineral medium with chicken feathers

T a k i u c h i et al. (1982) reported that a specific protease, called keratinase is responsible for liberating peptides from hair keratine in Microsporum canis cultures. This enzyme activity is considered by many authors (Y u et al., 1968, 1969; T a k i u c h i et al., 1982; K u s h w a b a, 1983) as the basic measure of keratinolytic activity of fungi and is determined according to guinea pig hair. However, preliminary research indicated very low or none keratinase values in fungi cultures after applying this substrate. The presence of keratinolytic enzymes was revealed only by the application of native feathers as a substrate. The number of obtained enzymatic units was comparatively low, even in 20 times condensed after-culture filtrates (Table 1). Its increase was noted only after dialysing off low molecular compounds (Table 1, Fig. 2 B). Low keratinase values in the cultures of some dermatophytes decomposing feathers were also noted by K u s h w a b a (1983). In the present investigations the increase in keratinase activity corresponded to the rate of substrate hydrolysis and the liberation of peptides only during the first 3 weeks of decomposition of feathers. Subsequently the dynamics of keratinase activity and substrate solubility were not convergent. The interpretation of the obtained results are difficult and it demands additional research exceeding beyond the question of the method of choice.

Table 1

Keratinase activity in the culture filtrates of *Chrysosporium tropicum* (in KU/cm^3)

Filtrates	Days					
	7	14	21	28	42	70
not-condensed	0.18	0.32	0.15	0.10	0.34	0.02
20-times condensed not-dialysed	0.18	0.62	0.43	0.04	0.36	–
20-times condensed dialysed	3.60	6.40	5.80	3.00	6.40	0.40

The results of some studies (M e e v o o t i s o n, N i e d e r p r u e m, 1979) indicated that the after-culture filtrate of dermatophytes growing in keratin salts broth is characterized by a proteolytic activity (i.e. measurable in the presence of ordinary proteins) apart from keratinolytic activity. The observations of the above authors were the basis of my own research regarding to the proteolytic activity of fungi decomposing keratin. The research proved high caseinolytic activity of culture filtrate of both examined species (Fig. 2 C). Its maximum values occurred in the second and third week of substrate hydrolysis. The decrease of proteolytic activity in the later period might have been caused by the accumulation of products of the enzymatic hydrolysis of feather keratin, especially amino acids, the accumulation of which is indicated by the level of amine residues in the medium (Fig. 2 D). This assumption may be confirmed by the observations of M e e v o o t i s o n and N i e d e r p e u e m (1979). They proved the inhibition of keratinolytic proteases production of fungi under the influence of amino acids.

The liberation of ninhydrin-positive substances is accepted as a criterion of the keratinolytic activity of fungi (Y u, H a r m o n, B l a n k, 1969; Y u et al., 1972). The present study showed that the formation of these compounds during the decomposition of feathers by fungi undergoes large oscillations (Fig. 2 D). They were manifested in the double stimulation of $N\text{-}NH_2$ content: in the initial and subsequent phase of feathers decomposition and their decrease in the third week of the study. The decrease in N-amine amount 3-week old cultures might have been caused by its assimilation by fungi. In this period the maximum increase of spawn biomass was noted (unpublished data). Thus, the assumption that the content of soluble amine compounds may reflect the keratinolytic ability of fungi demands taking the dynamics of liberation of these compounds into consideration.

When considering the high content of cystin in the keratin of feathers (W o l s k i, 1985) it would be advisable to determine the amount of liberated cystein in the cultures of fungi decomposing the substrate. K u n e r t's research (1973, 1976) showed that cystein is formed during the so-called sulphitolysis, i.e. reducible breaking of S-S bridges in keratin (hair keratin). The analysis of amino acids chromatograms of low molecular fraction of culture filtrates of the examined fungi indicated the increase in the content of this amino acid together with the intensification of solubilization of feather keratin (Fig. 2 E). The above effect was strongly marked in *C. serratus* cultures, indicating larger affinity of this fungi to the substrate.

The present investigations proved that the degradation of native feathers by fungi was accompanied by medium alkalization (Fig. 2 F). This effect was noted during the whole period of the experiment in spite of slight decrease of pH in the final phase of substrate decomposition. The alkalization of medium during fungi growth on the hair native keratin was observed by many authors (C h e s t e r s, M a t h i n s o n, 1963; E v a n s, H o s e, 1975; S a f r a n e k, G o o s, 1982; D e s h m u k h, A g r a w a l, 1982, 1985). Undoubtedly, the main cause of this phenomenon is the liberation of residues amine and ammonia. The results of studies which have not been published so far indicated that keratinolytic fungi change at least 50 % of inorganic N in feathers into ammonia N. The application of the changes of medium pH as a simple index of kerationlytic activity of fungi would demand, however, the exclusion of similar types of changes in the cultures of non-keratinophilic fungi decomposing non-keratinic compounds in native keratin substrates.

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Metody określania aktywności keratynolitycznej saprofitycznych micromycetes

S t r e s z c z e n i e

Uzyskane wyniki upoważniają do wykorzystania pomiaru ubytku masy substratu oraz uwalniania substancji peptydowych i aminokwasów siarkowych jako wskaźników aktywności keratynolitycznej (pióra

kurcząt). Znaczne wahania zawartości reszt aminowych w podłożu wskazywałyby natomiast na konieczność uwzględnienia w ocenie aktywności keratynolitycznej tych organizmów, dynamiki zmian zawartości tego składnika. Niska aktywność keratynolityczna wobec natywnego substratu, proteaz pozakomórkowych oraz silny spadek ich aktywności wobec zwykłych białek (kazeina) – w czasie nasilenia keratynolizy również nie odzwierciedlają w pełni właściwości keratynolitycznych micromycetes. Natomiast alkalizacja podłoża może być prostym wskaźnikiem aktywności keratynolitycznej, pod warunkiem wykluczenia podobnego typu zmian w hodowlach grzybów nie-keratynolitycznych na surowych odpadach keratynowych.