

PRODUCTION OF EXTRACELLULAR KERATINASE BY *CHRYSOSPORIUM TROPICUM* AND *TRICHOPHYTON AJELLOI*

Jaroslava Kačínová^{*1}, Dana Tančinová¹, Juraj Medo¹

Address(es): Ing. Jaroslava Kačínová,

¹Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, +421 37 641 4433

*Corresponding author: kacinova.jaroslava@gmail.com

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ABSTRACT

Keratinous wastes constitute a troublesome environmental contaminant that is produced in large quantities in companies processing of poultry and their further use has ecological significance. We can use for degradation of keratinous wastes enzymes or strains, which produce these enzymes. The aim of this study was isolation of keratinophilic fungi from the soil samples and optimization of culture conditions of keratinase producing strains *in vitro*. For the isolation of our strains, we used hair - baiting method. From the all isolated strains, we used for other screening *Chrysosporium tropicum* (JK39) and *Trichophyton ajelloi* (JK82). Production of keratinase we monitored with different time of cultivation (7th, 14th, 21th days), sources of carbon (glucose, fructose, mannitol, sucrose), concentration of carbon sources (1%, 2%) and cultivation temperature (20, 25, 30, 37°C). Keratinase production was studied in a liquid medium containing chicken feathers as a source of keratin. We recorded the maximum production of keratinase (10.51 KU/ml) by *Chrysosporium tropicum* on 21th day of incubation with 1% glucose at 25°C.

Keywords: Keratinase, *Chrysosporium tropicum*, *Trichophyton ajelloi*, feather, keratin

INTRODUCTION

Keratin is a fibrous and insoluble structural protein extensively cross linked with hydrogen, disulphide and hydrophobic bonds (Harde *et al.*, 2011). Keratins, which are among the hardest-to-degrade animal proteins, are the major component proteins in poultry feathers and are characterized by a tightly packed form in α -helixes and β sheets with a high degree of disulfide bonds (Yasushi *et al.*, 2009). Only some bacteria, actinomycetes and keratinophilic fungi and, of macro-organisms, larvae of the common clothes moth (*Tineola bisselliella* Hummel) are capable of using keratin as the sole source of C, N, S and energy (Kornilowicz-Kowalska and Bohacz, 2011). Keratinophilic fungi including keratinolytic fungi are a group of fungi that degrades keratin, and they include several of the most important dermatophytes and saprophytic species. Most of the keratinophilic fungi are soil inhabitants (Javoreková *et al.* 2012). Kushwaha (2000) has studied the degradation of peacock feathers, using the keratinases of 20 different fungi and have reported that some dermatophytes were most active. Keratinophilic fungi are important ecologically and recently have attracted attention throughout the world (Sharma and Rajak, 2003; Marchisio, 2000).

Keratinases are the key enzymes elaborated by keratinophilic fungi for the degradation of keratin (Tawfik *et al.*, 2001; Vidal *et al.*, 2000). Keratinase producing microorganisms have ability to degrade chicken feathers, hair, nails, wool etc. (Gradišar *et al.*, 2005). Keratinases belong to the group of serine hydrolases that are capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds. The keratin chain of hair (hard keratin) is similar to that of the epidermis (soft keratin) as it is highly packed as an α -helix but differs from the latter in that it contains several fold higher amounts of cysteine. In feathers, the polypeptide chain assumes a β -confirmation, which is more readily hydrolysed than α -keratin (Ramnani and Gupta, 2004).

The hair from humans and animals and feather from birds which come to the soil either as dropped off or dead are affected by microbial decomposition. In the past few decades some studies on the decomposition of keratin in submerged cultures appeared biodegradation of keratin by using *Chrysosporium* and other related fungi in submerged culture is reviewed (Kushwaha, 1998) and scattered reports are available in literature. The process of keratin decomposition has also been found to be very fast in soil and it plays a very important role in energy transformation and nutrient cycling in soil (Kushwaha, 2000). Long term studies on the biology of *Chrysosporium*, and other scattered reports, revealed that its

wide distribution is due to its antagonistic potential and ability to produce enzymes and other extracellular metabolites (Kushwaha, 2000).

Chrysosporium tropicum produce amylase, urease, pectinase, keratinase, esterase lipase, leucine aryl amidase, cystine arylamidase, alpha galactosidase, alpha glucosidase, beta glucosidase, N acetyl glucosaminidase, alpha mannosidase (Calvo and Calvo, 1991).

The most promising application of keratinase is in the production of nutritious, cost effective and environmentally benign feather meal (Gupta and Ramnani, 2006). Microbial keratinase can be used in the textile, cosmetic and leather industries and in medicine (Farag and Hassan, 2004; Gupta and Ramnani, 2006; Brandelli *et al.*, 2010).

The aim of this study was optimization of production of extracellular keratinase from *Chrysosporium tropicum* and *Trichophyton ajelloi* isolated from the soil sample. Optimization of cultivation is one of the most important criteria for developing of any new microbial process.

MATERIAL AND METHODS

Isolation of keratinophilic fungi from soil

From the all isolated strains from 10 soil samples, we used for other screening *Chrysosporium tropicum* (JK39) and *Trichophyton ajelloi* (JK82). *Chrysosporium tropicum* and *Trichophyton ajelloi* were isolated by the hair-baiting method (Vanbreuseghem, 1952) from the soil samples from Slovakia. For the isolation of *Chrysosporium tropicum*, only the superficial layer 5 cm of the humus horizon was used. Soil samples were poured into Petri dish (up to 5 per sample), i.e. 1 sample=5 subsamples. Based on soil moisture, we applied 10 mL cycloheximide (actidione). 500 mg/L + 50 mg/L chloramphenicol solutions. Five fragments of sterilized horsehair on a Petri dish were applied as bait. Cultivation was carried out at 25°C for 2–3 months (every week check if any growth does occur on the fragments).

Isolation of the fungi from colonized hair fragments

Potato Dextrose Agar (PDA) was prepared and 100 μ L of the antibiotic solution (chlortetracycline/chloramphenicol) was applied on the surface and evenly spread. Solution (chlortetracycline/chloramphenicol) was prepared: 100 mg/L + 100 mg/L distilled water sterilized at 120°C for 15 min. The mycelium was

transferred from a fragment colonized hair into the PDA plate with antibiotic solution. We made three/four lines on the plate, because we wanted prevent possible contamination by other fungi. The cultivation temperature for PDA plates was 25°C, darkness, 4–6 days, until colonies appeared. Cultures were transferred to Sabouraud's dextrose agar medium (dextrose 40 g; peptone 10 g; agar 20 g; distilled water 1000 ml) for identification. These plates were incubated at 25°C for 7 days and then were used for identification. The identification of the experimental keratinophilic fungi was based on their phenotypic characteristics according to Van Oorschot (1980), and De Hoog et al. (2000).

Isolation and maintenance of microorganisms and screening of keratinolytic enzymes

From Slovak soil samples the fungi *Chrysosporium tropicum* (JK39) and *Trichophyton ajelloi* (JK82) were isolated. The fungi on Sabouraud's dextrose agar medium were maintained. Liquid media according Wawrzkiwicz et al. (1991) was used for crude keratinase enzyme production. Chicken feathers were used as a source of keratin. 500mg of keratin source was added to the mineral salt medium: K₂HPO₄, 1.5 g/L; MgSO₄·7H₂O, 0.05 g/L; CaCl₂, 0.025 g/L; FeSO₄·7H₂O, 0.015 g/L; ZnSO₄·7H₂O, 0.005 g/L. Erlenmeyer flasks containing 50 ml of mineral salt medium with keratin source were sterilized at 121°C for 15 min. Each flask was inoculated with agar plug (also with mycelium). Four carbon sources were tested, namely glucose, fructose, sucrose, mannitol. The carbon sources were sterilized separately and were aseptically added to the sterilized medium at final concentration of 1% respectively 2% (w/v). For studying the effect of temperature, fungi were grown for 3 weeks in a mineral salt medium at different temperatures, 20, 25, 30, 37°C, respectively. All variants were prepared in 3 replications. The cultures were harvested after every week of incubation up to 3 weeks. Aliquots of the culture medium were taken from the flask, filtered, and then centrifuged at 4000 rpm for 5 min. The supernatant was used as the crude enzyme (enzyme source).

Determination of Keratinolytic activity

Keratinase activity was assayed by the modified method of Yu et al. (1968). In brief, 20 mg of chicken feathers were suspended in 3.8 ml of 100 mM Tris-HCl buffer (pH 7.8) to which 200 µl of the culture filtrate (enzyme source) was added. The reaction mixture was incubated at 37°C for 1 h. After incubation, the assay mixture was dipped into ice-cold water for 10 min and the remaining feathers were filtered out. Then the absorbance of the clear mixture was measured at 280 nm (Genova, MK3 UV-vis spectrophotometer). The keratinase activity was expressed as one unit of the enzyme corresponding to an increase in the absorbance value 0.1 (1 KU=0.100 corrected absorbance), 1 KU=keratinase unit.

Statistical analysis

Keratinase activity was analysed with multifactor analysis of variance, followed by Tukey test in statistical package Statgraphics XV.

RESULTS AND DISCUSSION

Taxonomic classification of fungi

Keratinolytic fungi are represented by two species, *Chrysosporium tropicum* (taxonomic classification: *Onygenaceae*, *Onygenales*, *Eurotiomycetidae*, *Eurotiomycetes*, *Pezizomycotina*, *Ascomycota*, *Fungi*) and *Trichophyton ajelloi*

(taxonomic classification: *Arthrodermataceae*, *Onygenales*, *Eurotiomycetidae*, *Eurotiomycetes*, *Pezizomycotina*, *Ascomycota*, *Fungi*) (<http://www.indexfungorum.org/>).

Determination of keratinolytic activity

This study demonstrated that the keratinase produced by *Trichophyton ajelloi* and *Chrysosporium tropicum* was able to degrade the chicken feather as substrate. These fungi were able to produce a significant amount of keratinase enzyme, which is responsible for keratin hydrolysis in nature. On the base of our previous screening study, *Trichophyton ajelloi* and *Chrysosporium tropicum* were selected as potential candidates to for analysis of the change of the culture medium, time of incubation and temperature.

All factors affected production of extracellular keratinases highly significant (species $p < 0.0001$, carbon source $p < 0.0001$; concentration $p = 0.0005$; duration of cultivation $p < 0.0001$; and temperature $p < 0.0001$). Keratinase production rose significantly from 7th to 14th day but further growth was not statistically confirmed. In both species, lower concentration of carbon sources seems to be better for keratinase production as it was significantly lower in higher concentration.

Among all the factors, maximum keratinase production was in species *Chrysosporium tropicum* (10.51 KU/ml) on the 21th day of incubation with using of 1% glucose as carbon source at 25°C. Results are shown in the figure 1.

Maruthi et al. (2011) presented a study where keratinolytic activity was 8.56 KU/ml on the 40th day in other study Venkatesan et al. (2010) detected recorded higher keratinase activity from *Microsporum gypsum* (77.65 KU/ml) and from *Microsporum canis* (76.20 KU/ml) on the 20th day. Awasthi and Kushwaha (2011) keratinase activity detected *Chrysosporium tropicum* (24.69 KU/ml) isolated from dropped feather.

In our study among all used carbon source the keratinase activity increased in case of glucose (figure 1a). The enzyme secretion was suppressed when the level of glucose concentration was increased above 1% (figure 1b). The higher concentrations (2%) of glucose inhibited their activity. Fructose, mannitol and sucrose were a equally efficient in terms of keratinase activity only glucose support fungi to produce significantly more enzyme. The keratinase was inducible by the keratin substrate and its production was stimulated by glucose (Malviya et al., 1992; El-Naghy et al., 1998). El-Naghy et al. (1998) had also reported that a higher concentration of glucose was more favourable for mycelial growth, but it decreased keratinase activity. Kushwaha (2000) indicates that glucose supported maximum mycelial growth of *Chrysosporium tropicum* and fructose was assimilated slower than mannose among the monosaccharides.

The effects temperature on keratinase activity are shown in figure 1d. There were observed some significant interactions between factors. Definitely, optimal temperature for keratinase production in species *Trichophyton* is significantly higher than in species *Chrysosporium*. Muhsin and Aubaid (2000) determined optimal temperature (50°C) for the highest keratinase activity from *Trichophyton mentagrophytes*. Anbu et al. (2006) determined from preliminary studies an optimum temperature of 30°C.

Among different temperatures tested, the highest enzyme production was registered of *Trichophyton ajelloi* at 30°C (6.27 KU/ml) and *Chrysosporium tropicum* at 25°C (10.51 KU/ml). Anbu et al. (2008) in the study of extracellular keratinase from *Trichophyton* sp. detected the highest enzyme production was registered at 35°C (5.0 KU/ml). In other study Anbu et al. (2006) detected the highest keratinase activity by *Scopulariopsis brevicaulis* (3.2 KU/mL) and *Trichophyton mentagrophytes* (2.7 KU/mL) in the culture medium with chicken feathers during 5 week of cultivation.

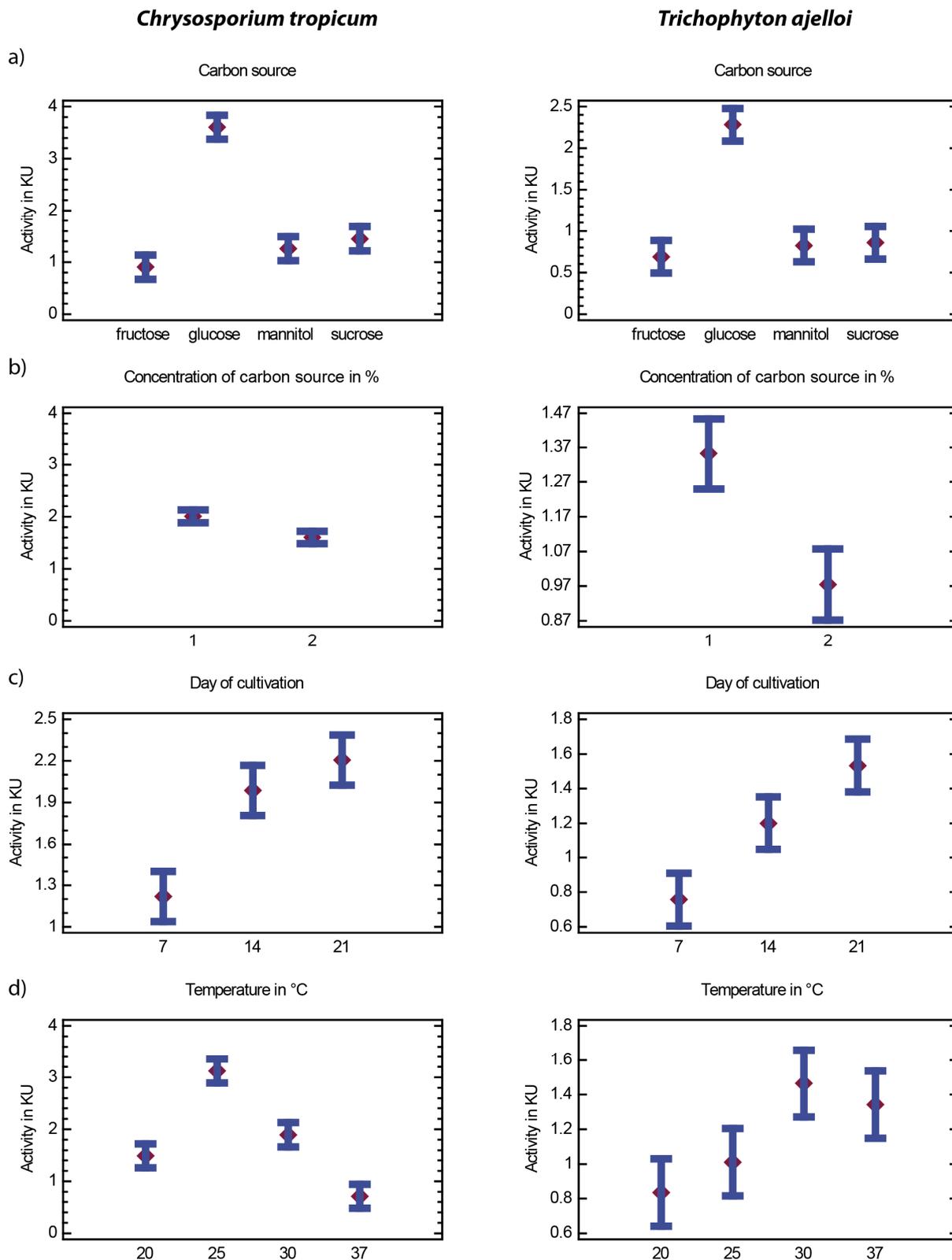


Figure 1 Effect of a) various carbon sources, b) concentration of carbon sources, c) cultivation period d) cultivation temperature to extracellular keratinase activity produced by *Chrysosporium tropicum* and *Trichophyton ajelloi*. Average values of enzyme activity with Tukey confidence interval.

Similarly, moderate keratinase productions were reported previously in *Aspergillus flavus* as 3 KU/ml (Muhsin and Hadi, 2001), in Antarctic actinomycetes as 0.2-0.3 KU/ mL (Gushterova et al., 2005). According to Gupta and Ramnani (2006), microbial keratinases have become biotechnologically important since their target the hydrolysis of highly rigid, strongly cross/linked structural polypeptide keratin recalcitrant to the commonly known proteolytic enzymes trypsin, pepsin and papain. The test strains were comparable in production keratinase with other published in the available literature.

CONCLUSION

Keratinolytic activity has an important role in degradation of feather in natural environment. According the results are evident that *Trichophyton ajelloi* and *Chrysosporium tropicum* were capable of producing keratinase enzyme and degradation feather. We recorded the maximum production of keratinase (10.51 KU/ml) by *Chrysosporium tropicum* on 21th day of incubation with 1% glucose at 25°C. Mostly *Chrysosporium* can be used for utilization and recycling of keratinous waste in soil and in water. Secretion of some their metabolites, particularly enzymes and antimicrobial compounds can be useful for

pharmaceutical industries. Mostly of these enzymes, arouse great interest for biotechnologies.

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