Paper B35

Evaluation of increase at the production of keratinolytics enzymes

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Introduction

The beamhouse area in leather technology has a notable influence on the gaseous and liquid effluent because they have to satisfies discharge standards. The organic matter content and the presence of suspended solids in the liquid effluent greatly depend on the unhairing process used: the conventional hair-burning unhairing, lime and sodium sulphide based, give the maximum values for beamhouse pollution.

Nowadays, conventional unhairing process employing lime and sulphide contribute 50-60% of total pollution load in terms of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) besides a high alkaline effluent of 100% toxicity. Hydrogen sulphide emanating from the unhairing process is proven to be fatal even in concentrations as low as 200 ppm. The extensive use of hazardous sulphide not only leads to unfavorable consequences for the environment but also undermines the efficacy of the effluent treatment plants. The pressure has increased and it has become common occurrence that tanneries are forced to close down not only in developed countries but also developing countries like China (Wang R et al. 2009). As compared with the classical sulphide unhairing method, a 50% reduction of sulphide was observed in the wastewater, as well as a 40%reduction in the suspended solid level. The decreased in sulphide reduced odours and made it possible, by simple oxidation, to convert all the sulphide to sulphate giving the concentrations well below environmental specifications (Crispim et al. 2003).
The interest in the enzyme unhairing process trying to solve the difficulties that appear when intend to unhair only with enzymes without sodium sulphide. Dificulties are originated to the proteolysis, that can not be controlle and thus the extension of enzyme activity towards the desired reticular structure of the dermis can not be avoided, notably impairing the properties of the elaborated leather.
Unlike the above difficulties present, attractive aspects are maintaining into the activities of research and development of enzyme unhairing, and not only confine their use in the soaking and bating process (Cantera et al 2000). In recent years enzyme applications has been extended to degreasing and wet-blue bating.

In special, enzymes for proteolytic depilation or keratin degradation are called keratinases (Riffel et al 2003). These enzymes act as proteases but differ from the non-keratinolytic proteases for their ability to degrade insoluble keratin (El Refai et al. 2005). They have a broad specificity proteolytic activity involving proteases of the endopeptidase type.

They have been purified and characterized from pancreas and different microorganisms such as some fungi, actinomycetes and bacteria genera. Some fungi, especially that belonging to Dermatophytes genera (Kunert, 2000; Simpanya, 2000) and others from fungi imperfecti (*Chrysosporium, Aspergillus, Alternaria, Trichuris, Curvularia, Cladosporium, Fusarium, Geomyces, Gleomastix, Paecilomyces, Scopulariopsis, Penicillium and Doratomyces* have been reported as a good producers of keratinolytic enzymes (Friedrich et al 1999).

Keratinases would be taking part in the socalled “sulphitolysis”process. During this mechanism the disulphide bond of the keratin breaks providing the sulphite ion that would act on the same kind of bond to generate thiosulphuric esters like S-sulfocysteine and thiols:

$$RS\text{-}SR + SO_3^{-2} _____ RS^- + RS\text{-}SO^{3-}$$

Disulphide bridges of cystine — thiosulphuric esters

The sulphite ion reacts selectively with the S-S bond but it does not react with other amino acids residues except for the tryptophan (Yu et al. 1981).

As the culture medium lacks the sulphite ion, its synthesis must take place before sulphitolysis occurs. Little is known about the mechanism by wich sulphites are formed in the early stages of keratinolysis. Proteolytic digestion and sulphitolysis are simultaneous and complementary phenomena wich are used by microorganisms to transform keratin into nutritive material (Galarza et al 2004; Ruffin et al 1976).

Yates reported that the only enzymatic activity that may be related with depilation is a proteolytic one wich involves proteases of the endopeptidase types (Yates 1968).

With regard to a casein substrate, a protein that was used in many research works as a depilatory activity marker it's appropriate to refer to Yates's work where the author finds that caseinolytic activity may be used to predict depilatory activity (Cantera et al 2004). In the present work casein substrate is replaced by azocasein, a diazotized aryl amine that produce a chromophoric derivative.

Objectives

In the present work the enzimatic yields and proteic concentrations from crude extract of *Trichophyton ajelloi* growth in solid state cultures and submerged cultivation in the presence of reducing agents were determined.

Materials and methods

Isolation of fungus strain

The strain selected for this study *Trichophyton ajelloi*, was isolated from the soil of a Yak yard from La Plata Zoo using "Vanbreuseghem's hair baiting technique"(Vanbreuseghem 1952). This technique consists of incubating portions of nonsterile soil in Petri dish using keratin protein substrate upon wich fungal growth is observed after two weeks of incubation in humid atmosphere. After isolation, the selected strain was kept in Sabouraud's agar at 28ºC enriched with chloranphenicol used as antibacterial agent and cycloheximide to avoid the growth of environmental fungal growth. Reports indicated that this species presents no pathogenesis for men or animals.

Culture conditions

Solid state culture

Assays were performed in Petri dishes containing 3 g of "hair waste" from "hair saving unhairing process" cleaned, autoclaved at 121ºC for 15 min and ground (Galarza et al. 2007).

Innoculum preparation

The innoculum was prepared as follows: cultures of seven days in Sabouraud's Agar de *Trichophyton ajelloi* in 250 ml Erlenmeyers was resuspended whit stir agitation in 10 ml water destilated aditionated with Tween 80. Then, 90 ml minimal mineral medium (MMM) with buffer NaH_2PO_4-K_2HPO_4 pH 7,2, chloranphenicol 0,5 g/l and trace amounts Cl_3Fe, Cl_2Zn and Cl_2Ca was added to suspension.

Then dishes were inoculated with 10 ml conidia suspension (fungal spores) whose viable-count was an average of 10^5 ufc/ml. In each experiment system the follows reducing agents were aditionated in

final concentration 5 mM. 1) L-cistein hydrochloride monohydrate 2) sodium thioglycolate 3) sodium sulphite. In parallel, experiment system without reducing agent was made to compare the effect of reducing agent.

Incubation was done at 28ºC in oven with humid atmosphere until 28 days. Two dishes were withdrawn every three days and stirred with 30 ml NaCl 0,5 N solution for 15 min to extract the enzymes adsorbed on solid substrates. The "crude extract" obtained was vacuum filtered through 0,45 µm cellulose membrane in an ice bath and kept at -20ºC until used for protein and activity determination.

Submerged culture

Submerged cultures were carried out in 500 ml Erlenmeyers flasks using 200 ml of MMM with 1,0% (w/v) of "hair waste" treated as it described before. Reducer agent was added (duplicate) to flask in concentration 5mM, autoclaved for 15 min at 121ºC, inoculated with 10 ml of inoculums. Flasks were incubated at 28ºC, 200 rpm for 28 days, withdrawing 5 ml of the culture every two days to obtain crude extracts. The samples were centrifugates at 3000xg for 15 min. The different extracts wer fractionates and kept at – 20ºC until used.

Assessment of protein content and proteolytic activity

Protein concentration was determined by Bradford's method (Bradford).

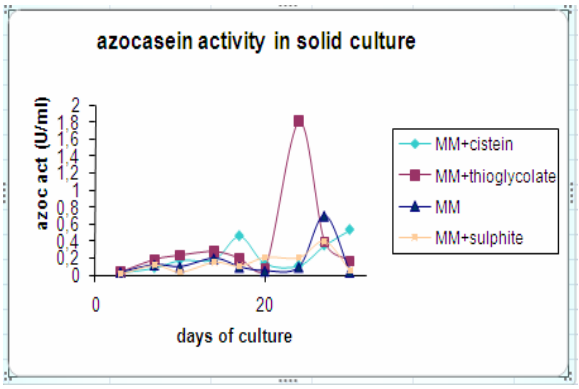
Assay of proteolytic activity

Enzyme activity was determined using azocasein (sulphamide azocasein) as substrate from Sigma Chem.Co., St. Louis, MO. Reaction mixture containing 100 µl of crude extract and 250 µl of the 1% (w/v) substrate solution in 0,1 M Tris-HCl buffer pH 9 was incubated for 30 min a 37ºC and then stopped by the addition of 1 ml of tricloroacetic acid (TCA) 10% (w/v) and centrifuged (3000xg, 15 min). One ml of NaOH was added to 0,9 ml of the supernatant, agitated and measured at 440 nm (triplicate). Blank was performed using enzyme solution inactivated for heating at 100ºC 5 min.

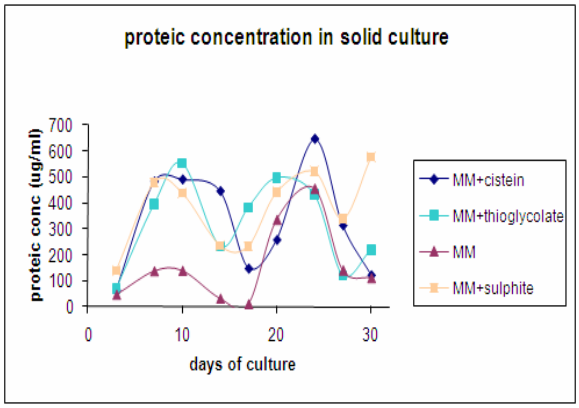
Azocasein activity unit (U_c) is defined as the amount of enzyme that, under the test conditions causes an increase of 0,1 Abs_{440} per minute.

Results and discussion

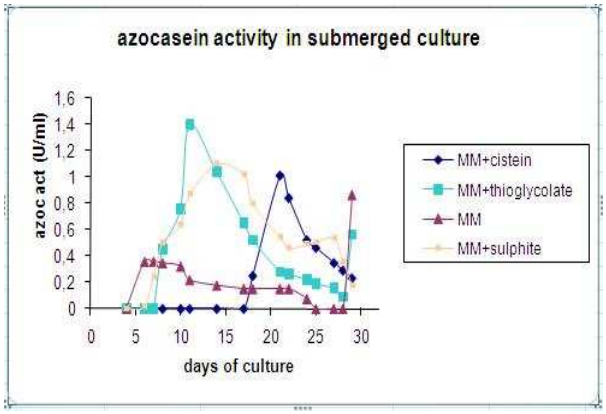
The follows graphics represents: I and III: azocaseinolytic activity as a function of day of culture in both, solid and submerged medium; II and IV: proteic concentration as function of day of culture in both, solid and submerged medium.



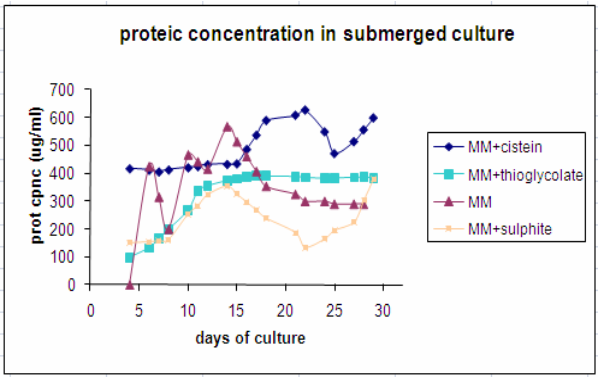
Graphic I



Graphic II



Graphic III



Graphic IV

It can be observed in graphics of proteic concentration vs days of culture, in both, solid and submerged cultura the highest values were after cystein addition. For azocaseinolytc activity, sodium thioglycolate in submerged culture produced a maximum on day 11. This value exceded in 7 times those produced by mineral medium addition for the same day. In solid culture the highest point of activity was on 24 day for sodium thioglycolate. This is the same rise than the previous (submerged culture) in comparison with mineral medium without aggregates.

The absolute values of the points of azocaseinolytic activity for solid culture were always higher than submerged culture while the highest proteic concentrations values were similar in both types of cultures.

Conclusions

It is possible to conclude that the aggregate of reducing agents to culture medium, in special sodium thioglycolate, increases the production of keratinolytic enzymes.
This finding can be explained taking account the sulphitolysis process.
In special case of *Trichophyton ajelloi*, sodium thioglycolate was used as reducing agent and as sole carbon energy source while cystein is besides incorporated as nitrogen source.
This action show a potential use of keratinases in tannery process as: soaking, unhairing, bating and deliming and adds value to the tannery solid waste as hair waste in process related with the bioconversion into feed and fertilizers**.**

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