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CONCENTRATION AND IMMOBILIZATION OF A PROTEOLYTIC COMPLEX

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Abstract

Proteases are very important industrial enzymes, accounting for more than a half of the total enzymes used in the world. For leather industry they have an important technological role as well as for waste degradation.This paper reports the researches done for obtaining immobilized proteolytic complexes for using in biodegradation purposes. Three bacterial strains were cultivated in minimal medium with 0.6% sheep fur as carbon source. The concentration of the culture medium was performed by rotaevaporator, at 35 C, followed by lyophilisation at 0.04 mbar, for 18 hours. The concentrated complex was immobilized on clay and adabline, which are inert and cheap supports that are technically convenient because we used the adsorption process for immobilization. Collagenase and keratinase activity was assayed for estimation of the immobilization efficiency. The best results were obtained when the immobilization support was clay, using a 2:1(ml enzyme/g immobilization support) ratio.

Keywords*: collagenase, concentration, keratinase, immobilization, lyophilization.*

INTRODUCTION

Leather industry is among the major pollution causing industries and has a high environmental impact. A lot of chemicals mainly responsible for pollution are used in tanning processes, therefore using proteases for processing tanneries materials and wastes has been recognized as a reliable alternative (Sivasubramanian et al., 2008).

Proteases are industrially important due to their wide applications in leather processing, food industries, textile industry etc (Deng et al., 2010). Proteases are obtained from plants, animal organs and microorganisms, but currently a large proportion of commercially available proteases are derived from bacteria and fungi (Raj, 2012).

The keratinase productions by various microorganisms were reported by a number of authors. It was found that keratinase produced by fungi, *Streptomyces* and bacteria were produced at alkaline pH and almost thermophilic temperatures (Allpress, 2002).

Although microbial collagenases have been found in a wide variety of mesophilic bacterial strains, the industrial-scale application of known bacterial collagenase for collagen peptide production has been disturbed because of their insufficient stability (Tsuruoka, 2003). Maintenance of their structural stability during any biochemical reaction is highly challenging. Enzyme immobilization provides an excellent base for increasing their stability and repetitive use. Attaching enzymes to a solid support results also in an improved availability to the substrate, so that several natural and synthetic supports have been assessed for their efficiency (Datta, 2013).

This paper reports the researches done for obtaining immobilized proteolytic complexes for using in biodegradation purposes. Three bacterial strains were cultivated in minimal culture medium with 0.6% sheep fur as carbon source. The concentrated complex was immobilized on some inert and cheap supports as clay and adabline, which are technically convenient because the adsorption process may be used. Collagenase and keratinase activity was assayed for estimation of the immobilization efficiency. Enzymes activity

and protein content were determined using spectrophotometrical methods.

MATERIALS AND METHODS

Microorganisms and growth conditions Three bacterial strains were used from the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest: 7.2 and BN7 strains were recently isolated, while Bl strain is a collection strain (*Bacillus licheniformis* ATCC 14580).

The inoculation was done in a minimal salt medium supplemented with 0.6% sheep fur as carbon source. Proteolitic enzymes were produced in 500 ml flasks, kept at 27^{o}C, agitated at 150 rpm, for 15 days. The culture medium was centrifuged at 6000 rpm, at 4^{o}C, for 20 minutes.

Concentration of the culture medium. The concentration of the culture medium was performed by rotaevaporator, at 35^{o}C, followed by lyophilisation at 0.04 mbar, for 18 hours.

Testing of some immobilization supports. The concentrated complex was immobilized on clay and adabline. They are inert and cheap supports that are technically convenient because the adsorption process for immobilization was used. Different immobilization ratio was used (1:2; 1:1; 2:1 ml enzyme/g immobilization support).

Obtaining of the immobilized enzymatic complex. For this purpose two methods were tested: drying at 40^{o}C in air stream for 24 hours and vacuum drying.

Enzymatic assay of collagenase, based on some classical refferences (Moore and Stein, 1948; Mandl et al., 1953), was made as following: after collagenase promotes hydrolysis of collagen, the degree of proteolysis is measured by color development with ninhydrin (absorbance was measured at 570 nm). The enzymatic activity of collagenase was measured in U/ml (*unit definition*: one unit liberates peptides from collagen equivalent in ninhydrin colour to 1 μmol of leucine in 2 hours at pH 7.4 and at 37 ^{O}C).

Enzymatic assay of keratinase was performed using 4 mg keratin azure as substrate. One unit of keratinase activity was defined as the amount that caused an increase in absorbance of 0.01 at 595 nm within 60 min reaction at 60°C.

The protein concentration was determined by Lowry method, which is based on the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Lowry et al., 1951).

RESULTS AND DISCUSSIONS

Selection of the most efficient method for concentration of culture medium

The bacterial collagenases registered the highest values of the enzymatic activity in the lyophilized preparates compared to the ones concentrated by rotary evaporation for all the tested strains (Figure 1).

Remarcable among these was Bl strain, which reached initial the highest collagenase specific activity (18.21 U/mg protein); after lyophilization the activity of collagenase produced by this strain increased 1.3 times. The collagenase activity of Bl strain decreased 1.56 times after concentration by rotary evaporation. Also the BN7 strain manifested initial a good specific activity of collagenase (11.27 U/mg protein), which increased 1.87 times by lyophilization. The rotary evaporation leads to a decreasing of collagenasic activity also for this strain.

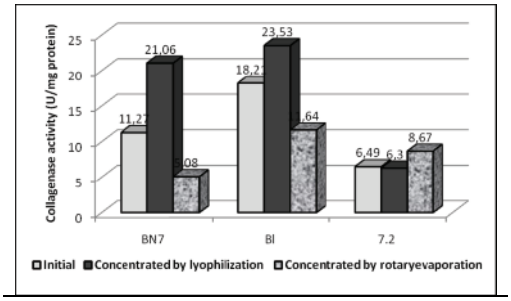

Figure 1. Activity of colagenase in concentrated enzymatic complex

Regarding the specific activity of keratinase, the obtained results indicated also the lyophilization as the most efficient method of

concentration (Figure 2). Initial, the highest keratinase activity was registered by the Bl strain (5.67 U/mg protein); a slight increasing of keratinase activity was observed after lyophilization (6.19 U/mg protein).

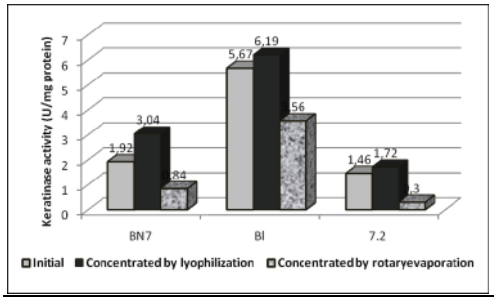

Figure 2. Activity of keratinase in concentrated enzymatic complex

Generally, the values of collagenase and keratinase activities were significant lower after rotary evaporation than after lyophilization for all the tested strains. Considering the fact that rotary evaporation was performed at 40° C, probable the enzymatic activities of collagenase and keratinase were partly inhibated by this temperature value.

In conclusion, among the tested methods of concentration, the best results were obtained by lyophilization for all the used strains, such that the further experiments were performed on lyophilized enzymatic complex.

Selection of the most efficient immobilization support

The concentrated complex was immobilized on clay and adabline by drying at 40°C in air stream for 24 hours. The obtained results indicated a good conservation of the enzymatic activities in the immobilized preparates with differences depending on the support type. One can observe that the proteolytic complex manifested a higher enzymatic activity on clay as immobilization support than on adabline for all the tested bacterial strains (Figure 3 and 4).

For example, the BN7 strain, which generally proved a good colagenase activity, registered a value of 6.96 U/ml on clay support and only 3.67 U/ml on adabline (Figure 3).

Also regarding the keratinase activity the same tendency can be noticed (fig. 4): higher values were determined on clay support (1.92 U/ml for Bl strain and 2.12 U/ml for BN7 strain) compare to the ones measured on adabline support (1.02 U/ml for Bl strain and 1.01 U/ml for BN7 strain).

The rate of residual enzymatic activity after immobilization was calculated for Bl strain, which manifested the highest proteolytic activity. So, if the activity of collagenase synthetized by Bl strain measured initial 18.02 U/ml, the value found after immobilization on clay support was 7.38 U/ml, which indicated 40.62% preservation of collagenase activity. On adabline support only 24% of collagenase activity was maintained.

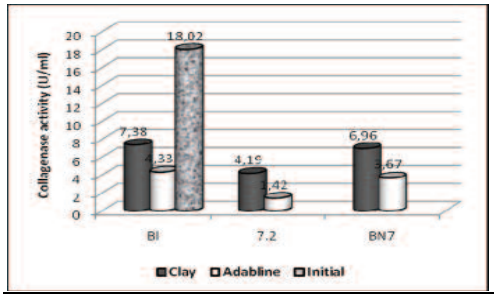

Figure 3. Activity of colagenase in immobilized enzymatic complex

The keratinase activity of Bl strain initial registered a value of 4.11 U/ml. Subsequent, a preservation of 46.71% of keratinase activity was noticed on clay support and only 24.81% when adabline was used as immobilization support.

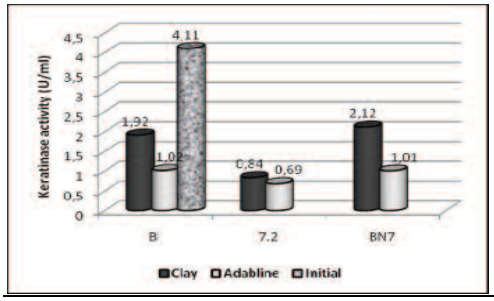

Figure 4. Activity of keratinase in immobilized enzymatic complex

Although clay was established as the most efficient immobilization support, the loss of enzymatic activity after immobilization was high (more than 50%), probably because of a partial inhibating of proteolytic activity at 40°C, the temperature value used by this immobilization method.

Selection of the most efficient method for enzyme immobilization

Further researches were performed in order to find an efficient immobilization method, which can preserve most of enzymatic activity of the proteolytic complex. For this purpose drying under vacuum at room temperature was used as method of immobilization on clay support.

Under these circumstances, the measured collagenase activity for Bl strain was initial 17.81 U/ml and 16.09 U/ml after immobilization, therefore 90.34% of enzymatic activity was preserved (Figure 5).

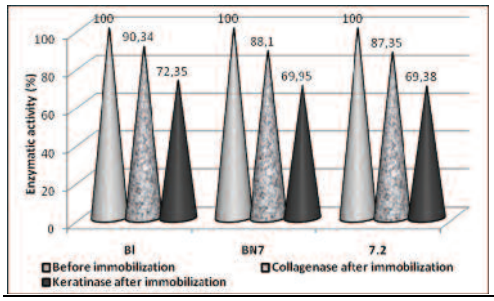

Figure 5. Preservation capacity of proteolytic activity after immobilization

Regarding the keratinase activity, also the Bl strain initial registered the highest value (4.16 U/ml), from which 72.35 % was preserved in the immobilized complex.

Analyzing these results, one can observe a great efficiency of this immobilization method, considering the high preservation capacity of colagenase activity (87–90%). Instead, keratinase proved less resistance to immobilization, taking account of lower remain activity measured after immobilization (70–73%).

CONCLUSIONS

Among the tested methods of concentration, the best results were obtained by lyophilization of cultivation medium.

The proteolytic complex manifested a higher enzymatic activity on clay as immobilization support than on adabline for all the tested bacterial strains.

Drying under vacuum at room temperature was proved as the most efficient method of immobilization.

Immobilization on clay by drying under vacuum leaded to the highest preservation of the enzymatic activity of the immobilized proteolytic complex: 87–90% of collagenase activity and 70–73% of keratinase activity.

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