ISOLATION AND CHARACTERIZATION OF FUNGAL AND BACTERIAL PROTEOLYTIC STRAINS FROM CHROME SHAVINGS

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The chrome shavings waste obtained as a result of the leather finishing process accumulates in a large volume in tanneries and represent a major problem for the environment. This waste are particularly resistant to attack of microorganisms, due to the significant concentration of chromium and are thus difficult to degrade. In this study, chrome shavings were analyzed microbiologically by determining the total number of germs and the number of yeasts and molds on specific culture media. Several bacterial and fungal strains were isolated from the cultures in Petri dishes, after the growth of the colonies. These strains were characterized in terms of the production of proteolytic enzymes, by a method of screening on the media with casein, which allows the determination of proteolytic indices of microorganisms. As a result of the tests performed, five bacterial strains probably belonging to the genus *Bacillus* and two fungal strains from the genera *Penicillium* and *Cladosporium* were selected.

Keywords: chrome shavings, proteolytic bacteria and fungi

INTRODUCTION

Industries of leather-manufacturing or tanneries are highly polluting and generate significant solid and liquid wastes and obnoxious smell due to the degradation of proteinaceous components of waste (Pillai and Archana, 2012). In addition, these activities release gases like H₂S, NH₃ and CO₂ (Thanikaivelan *et al.*, 2004).

The main form of solid tanned waste, constituting about 10% of the total weight of raw materials processed, is chromium and protein in the form of “chrome shavings” (CRS), which are small, thin pieces of leather generated during the hide shaving operation and represent high value protein-based waste (John Sundar *et al.*, 2011). Chromium is added to raw hide during leather manufacture to produce chromium tanned leather. This is done to prevent microbial degradation and produce a more durable product.

The traditional practice of disposal of chrome shavings is by landfilling, but lately there are restrictions on the disposal of chromium bearing waste in many parts of the world. The existence of valuable proteins in this waste necessitates alternative waste management strategies (John Sundar *et al.*, 2011). In the environment, the chromium exists in many oxidation forms, ranging from Cr²⁺ to Cr⁶⁺, but the most stable and common states are trivalent, Cr(III) and hexavalent, Cr(VI) species (Fendorf, 1995; Dhala *et al.*, 2013). Cr(III) has a high potential for environmental contamination, especially of aquifers and surface water, but it has a lower biological toxicity than Cr(VI). Hexavalent chromium has a high toxicity, because of its easy diffusion across the cell membrane in prokaryotic and eukaryotic organisms and subsequent Cr(VI) reduction in cells, which gives free radicals that may directly cause DNA alterations as well as toxic effects (Arslan *et al.*, 1987; Kadiiska *et al.*, 1994; Liu *et al.*, 1995).

In the treatment of waste from tanning industries various physico-chemical (Lofrano *et al.*, 2013) and biological systems have been used (Durai and Rajasimman, 2011; Fernandez *et al.*, 2019), but the biological methods have low operational cost and high efficiency in the removal of contaminants (Delgado, 2009).

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The biotechnology and green chemistry, represent sustainable activities to remediate pollutants by eco-friendly approaches through bioremediation, waste reuse and/or by-product recovery (Rao *et al.*, 2002). Microbiological and enzymatic treatment of CRS has been used to recover chromium and protein from CRS. Alkaline proteases like alcalase and combination of trypsin and esterase are used in the preparation of soluble collagen hydrolysate from CRS called chrome cake (Kupec *et al.*, 2002). Along with enzymes, the microbial management of industrial or other wastes for the removal of hazardous compounds can be achieved through degradation, biosorption or bioconversion to less toxic forms (Zouboulis *et al.*, 2003; Feng *et al.*, 2011).

An attractive and less expensive alternative for efficient disposal of leather industry waste, especially protein waste is the biological treatment with enzyme-producing strains of bacteria and fungi (Pillai and Archana, 2012). The used microbial populations should have the ability to grow in highly concentrated chromium containing environment (Thacker *et al.*, 2006). Although there are not many reports on microbial degradation of CRS, some studies have shown the possibility of selecting and using hydrolytic microorganisms. For example, Katsifas *et al.* (2004) obtained a fungal strain of *Aspergillus carbonarius*, that is able to degrade CRS in SSF (solid state fermentation) process and is tolerant to the presence of chromium. This fungal strain should be used in the treatment of waste from tanning industries for the disposal of CRS.

Recently, several proteolytic bacteria of the genus *Bacillus* have been tested for waste treatment in the leather industry, due to their properties for the synthesis of hydrolytic enzymes, especially collagenases and keratinases, as well as resistance to environmental factors. Pillai *et al.* isolated a hot spring bacterium, called *Bacillus subtilis* P13, that is able to effectively degrade and grow using chrome shavings (Pillai and Archana, 2012). Due to the high resistance of some compounds to biodegradation, bioaugmentation strategies have been studied, (Chen *et al.*, 2017; Dahiya and Venkata Mohan, 2016; Nzila *et al.*, 2016). Bioaugmentation involves the addition of microorganisms that have the ability to biodegrade recalcitrant molecules in a polluted environment (Nzila *et al.*, 2016; Fernandez *et al.*, 2019).

Circular economy (CE) model has been tested as a new way of raw materials, water and energy consumption reduction in the leather industry. This model was suggested for reducing, reusing, recycling and recovering of the tannery effluents in leather processing to the different operation processes (Kanagaraj *et al.*, 2015).

MATERIALS AND METHODS

The chrome shavings waste comes from the process of hide shaving from the tannery SC PIELOREX SA Jilava, Ilfov county.

The process of shaving is performed to obtain a uniform thickness of the skin, and due to its low specific weight, a large volume of waste accumulates in tanneries.

The chrom shavings were analyzed for microbial load. The total number of mesophilic aerobic germs and the number of yeasts and molds were determined by cultural methods. In order to determine the total number of germs, the sterilized Plate Count Agar culture medium was used and poured into Petri dishes containing 0.1 – 0.2 grams of sample and, after homogenization, the samples were incubated at 30°C. The colony counting was performed after 48-72.

The number of yeasts and molds was performed using Potato Dextrose Agar culture medium supplemented with chloramphenicol. The dishes were incubated at 25°C, and the colonies were counted after 5-7 days.

The results represent the average of 3 determinations.

Several species were isolated from the colonies using an inoculation needle, and the microorganisms were grown in test tubes on Nutrient Agar for bacteria and Potato Dextrose Agar for fungi. The cultures were stored at 4°C until use.

The proteolytic activity of isolated microorganisms was determined by a semi-quantitative screening method in Petri dishes, on an agar medium containing 0.25% casein as sole carbon source. Due to the hydrolysis of casein (which causes opacification of the agar medium), a transparent area appears around the colonies producing proteolytic enzymes.

After the development of the colonies (3 days for bacteria and 5 days for molds) the diameters of the colonies and of hydrolysis zone were measured. The proteolytic index was determined as the ratio between the 2 diameters:

$$I_p = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}} \quad (1)$$

RESULTS AND DISCUSSION

The results obtained showed that on this type of waste there is a significant number of microorganisms, probably most in sporulated form, such as sporogenic bacteria of the genus *Bacillus* and several fungi. These microorganisms were able to withstand the presence of chromium in CRS and to germinate and grow in Petri dishes with autoclaved CRS samples (for 15 minutes at 121°C). The appearance of Petri dishes after the growth of microorganisms is shown in figure 1.

Figure 1. Determination of a) total germs number and b) fungi number in Petri dishes

The average value of number of total germs was 3×10^3 CFU·g⁻¹, while the number of fungi was smaller, 5.5×10^2 CFU·g⁻¹. On Petri dishes, we can see bacterial white colonies with a diameter of a few millimeters, shiny or rough, with irregular margins, flat profile, and odorless.

In Petri dishes containing Potato Dextrose Agar as a culture medium for fungi, three major types of colonies were observed. The most numerous were the green-blue colonies, with white edges, fluffy appearance, and characteristic odor. The microscopic preparations show thin hyphae, hyaline and reproductive structures specific to the genus *Penicillium* (figure 2c). Other, less numerous, gray-beige colonies belong to the genus *Cladosporium*, as can be seen under a microscope (figure 2b). The third type of colonies, large, gray, with a pasty appearance, belong to the coenocytic molds of the genus *Mucor*. They appear on the microscope slides as being made up of thicker hyphae, some of them with black sporangia with sporangiospores (figure 2a).

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Figure 2. Microscopic aspect of a) *Mucor* sp., b) *Cladosporium* sp., c) *Penicillium* sp.

Microorganisms Producing Proteolytic Enzymes

The selection of the colonies that produce proteolytic enzymes was performed by observing the hydrolysis of casein in the culture medium and measuring the diameter of transparent lysis area. The proteolytic colonies were transferred in tubes and kept at 4 °C until use. The aspect of proteolytic colonies in Petri dishes are shown in figure 3 and 4. This test method is a semi-quantitative one, and the proteolytic activity will have to be analyzed after this selection, by more accurate methods. The action of microorganisms could be tested directly, by cultivating selected microorganisms in culture media containing chrome shavings or other materials as the sole source of nutrients.

Figure 3. Proteolytic bacterial colonies grown on casein agar medium

Figure 4. Proteolytic *Penicillium* (a) and *Cladosporium* (b) colonies on casein agar medium

The values of the proteolytic indices are shown in table 1. With the exception of the *Mucor* colonies, all other selected species had considerable proteolytic activity, with indices between 1.6 and 3.3, and were isolated, cultivated and preserved for future research.

Table 1. Proteolytic indices of selected bacteria and fungi

Microorganism	Bacteria/Fungi	Time, hours	Diameter of hydrolysis zone, cm	Proteolytic index
B1	Bacteria	72	3	3.3
B2	Bacteria	72	3	1.6

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Microorganism	Bacteria/Fungi	Time, hours	Diameter of hydrolysis zone, cm	Proteolytic index
B3	Bacteria	72	3.4	2.2
B4	Bacteria	72	3	3.3
B5	Bacteria	72	3.5	2.3
<i>Penicillium</i> sp.	Fungi	96	2.5	2.5
<i>Cladosporium</i> sp.	Fungi	96	3.6	1.6
<i>Mucor</i> sp.	Fungi	72	0	No proteolytic activity

Numerous other studies have been conducted on this subject, which attest to the importance of microorganisms in the biodegradation process of CRS. Various microbial species producing proteolytic enzymes have been isolated, able to survive in the presence of chromium ions and transforming the protein substrate into peptides and amino acids (Chen *et al.*, 2012; Desta *et al.*, 2014; Liang *et al.*, 2016; Ma *et al.*, 2018). The research has resulted in significant results on the importance of using single microorganisms or in microbial communities, and also the effect of bioaugmentation on prokaryotic microbial community structure (Sul *et al.*, 2016). It has been shown that the microbial community of wastewater treatment systems is affected by various factors such as influent composition, operational parameters, and environmental conditions (Chen *et al.*, 2017; Gao *et al.*, 2016; Niu *et al.*, 2016).

CONCLUSION

The use of chromium-resistant proteolytic microorganisms represents an alternative of interest for the degradation process of chromed leather waste. The selection of microorganisms is the first step in degrading this waste into peptides and amino acids that can be used for other purposes, such as biogas production by codigestion.

The waste represented by chrome shavings was analyzed microbiologically in terms of the total number of germs and the number of yeasts and molds. The obtained results showed that the samples contained 3×10^3 CFU·g⁻¹ total aerobic microorganisms and 550 CFU·g⁻¹ fungi, probably in sporulated form.

Of these microorganisms that resisted in the presence of chromium, several randomly selected colonies were isolated, belonging to the group of bacteria and fungi.

The selected bacteria and fungi were tested for the production of proteolytic enzymes by a semi-quantitative method, on a casein culture medium. These microorganisms showed proteolytic indices between 0 and 3.3, and will be tested later for enzyme production and hydrolysis of chromed leather waste.

5 bacterial strains probably belonging to the genus *Bacillus* and two fungal strains of the genera *Penicillium* and *Cladosporium*, which had significant proteolytic activity, were kept for further testing.

The microorganisms are therefore a permanent source of biodegrading agents, by virtue of its tolerance to chromium and capability to utilize thermally processed collagen present in the chrome shavings as sole protein source.

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