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Original Research Article

Enzymatic Hydrolyses of Chrome Shaving Waste from Tannery through *Aspergillus tamerii*

Paramasivam Senthil Kumar[1*], Lesley Sounderraj[2], Kandaswamy Sivakumar[3] and Suseela Rajakumar[4]

[1] National Environmental Engineering Research Institute (NEERI), Chzl, CSIR-Complex, Taramani, Chennai- 600 117, INDIA
[2] P.G. Department of Zoology, Voorhees College, Vellore 632 001, INDIA
[3] Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Madhuranthagam, 603308. Tamilnadu, INDIA
[4] Microbiology Laboratory, Central Leather Research Institute (CLRI), Chennai 600 020, INDIA

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ABSTRACT

The isolated fungi were optimized and used in the production of enzyme (alkaline Proteases) by the Solid state fermentation. The enzyme extracted from the fungi was used in the hydrolysis of leather protein present in the chrome shaving waste and the recovered chromium was recycled and used in the tanning process. To completely hydrolyze 1 gram of leather wastes 6 unites of the enzyme protease is required. In the optimization, the fungi maximum production shows at 72 hrs, the nitrogenous source are the milk extract and the optimum temperature to hydrolyse is 55ºC and the pH is 9.5.

Key Words: Alkaline Proteases, A*spergillus tamerii,* chromium, Leather waste hydrolysis

INTRODUCTION

The current scenario to remove the highly toxic chromium from the tannery waste is by chemical and biological techniques. Biological techniques is recommended worldwide, because, in the chemical method, recovery of chromium is difficult. Among the biological methods, enzymatic hydrolyses is one of the excellent method to recover the chromium from the tannery wastes (Gupta et al 2005, Zayed and Terry 2003). Chromium (Cr) is listed one among the 129 top pollutants and is considered as the 14th most noxious heavy metals. Chromium is also listed among the 25 hazardous substances and is thought to pose the most significant potential threat to human health in priority superfund sites by the EPA (EU 1996, UNEP/IEO 1994). Chromium (III) used in the tannery process is converted into the chromium (VI) in the final waste due to the oxidation. But it can be recycled by the same process. Chromium (III) is essential for the living things for their metabolism (Shaili Srivastava and Indu Shekhar Thakur 2006, Kuber and Stanislaus 1999, Scheuer 1990).

Enzymes present in the microbes have a wide range of application in the industrial biotechnology, dye industrial, medical and basic research laboratory. (Ravindran et al 2011, Elsayed and Saba, 2009) Among the enzyme group, alkaline proteases plays the important role in the tannery industries. Fungal alkaline proteases produced by the *Aspergillus sp.* is used in the removal of the chromium from the tannery effluent and soil (Berla Thangam and Suseela Rajkumar 2000, Ganesh Kumar and Hiroshi Takagi 1999), in the dehairing of goat skins in the tannery, collagen hydrolysates in retanning the good quality of leather, and also in detergent industry and peptide synthesis (Santosh Kumar Yadav et al 2011, Kanagaraj et al 2006).

In the present study, the isolated *Aspergillus tamerii* were optimized and grown in the SSF and the enzyme is partially purified and hydrolyzed by the enzyme protein from the tannery waste (chrome shaving)

MATERIALS AND METHODS

Isolation and identification of Proteolytic fungus

Isolated fungal *Aspergillus tamarii* were discussed in the pervious paper (Dayanandan 2003).

Assay for Protease

The enzyme activity assay and standard prepared by the referred (Dayanandan, 2003).

* Corresponding author: microsenthil@gmail.com

Optimization of parameters for enzyme production

To achieve maximum growth of the organism for the maximal production of the enzyme various parameters such as age of inoculum, size of inoculum and the incubation time plays a vital role. In the case of solid-state fermentation, the percentage of moisture content is also essential. Taking all these factors into account, the parameters were optimized after performing various combinations of experiments. The investigations on the production of enzyme is based upon the effect of incubation period, effect of protein sources, effect of inoculum size and effect of age of inoculum.

Effect of period of incubation

In order to enhance the production of the enzyme, the inoculated fermentation medium was incubated at various time periods and the enzyme activity was determined at regular intervals. The results are presented

Estimation of protein content

The amount of protein present in the culture filtrate was assayed by the method of Lowry et al. 1ml of sample was mixed with 5ml of freshly prepared alkaline copper sulphate and incubated at room temperature for 10 minutes. To this 0.5ml of Folin- Ciocalteus`s reagent was added and incubated at room temperature for 20 minutes and the absorbance was measured at 660nm. The blank was prepared using sterile water instead of sample. The protein content was assayed by calibration with the standard graph.

Effect of size of inoculum

The size of initial inoculum to be used in solid –state fermentation was also optimized by performing the experiments with different inoculum size such as 1%, 2%, 3%, 4% and their enzyme activity was tabulated respectively.

Effect of age of inoculum

The effect of the age of the culture influence enzyme production. The optimal production of enzyme depends mainly on the phase of growth of the microorganisms. Inoculum can be prepared within 2 days, 3 days, 4 days and 5days and the old culture and the age of inoculum at which there is maximal production of enzyme was determined. The results were noted.

Enzyme production

Taking all the optimal parameters into consideration, the fungus was grown in large scale for further studies on leather waste hydrolysis. The fermentation was carried out in Hoffkin`flask (3000, ml Borosil). 100mg of wheat bran was inoculated with 10% inoculum and moistened with 60ml of 48 basal medium to obtain 60% moisture content and mixed well and sterilized at 121^0C for 15 minutes. To this substrate, 10ml of 7 days old culture of *Aspergillus sp.* was inoculated and flask was carefully shaken. This was incubated at 28^0 c for 3 days, and then enzyme was extracted by shaking with 1000ml of cold 20mM borate buffer pH 8.0 having 0.2mM $CaCl_2$ for 30minutes in an orbital shaker. Initially, the extracts is filtered at 4^0c using cheesecloth and then by using Whatman No.1 filter paper. Further the crude enzyme was centrifuged at 10,000rpm for 20minutes at 4^0c. The supernatant was harvested and enzyme activity was assayed. By this the protein content in the sample was found out (Gupta et al 2005).

Concentration of enzymes

Ammonium Sulphate Precipitation

Precipitation is a basic reaction by which the proteins present in a solution are made to precipitate using solvents or salts. The salt used for precipitation was Ammonium Sulphate. Ammonium Sulphate was added to the filtrate with constant stirring at 4^0c up to 80% to attain saturation. The precipitate obtained at this stage was removed by centrifugation at 10,000rpm for 15 minutes. The pellet obtained was dissolved in minimum quantity 25ml of 20mM Tris HCl pH 8.0 containing 2mM $CaCL_{2.}$

Dialysis

The sample was dialyzed for 48 hrs against the same buffer. The buffer was changed every 24hrs during dialysis. The enzyme activity and the protein content were assayed. Further the sample was lyophilized in a stored at 4^0 C.

Analyses of Chrome shaving

For the pH moisture content, nitrogen and protein were done by the std. method.

Leather Waste hydrolysis

Recovery of hydrolyzed protein is concerned with the recovery of protein by enzymatic decomposition of leather waste. The leather wastes obtained after liming, which have the alkalinity that corresponds to the pH of the enzyme used, have been used in the work. The process was conducted at a constant temperature for this enzyme. Before the tests, the waste contained 15% of water and the pH was maintained 7.0 to 7.5. The known amount of 100g of leather waste was taken in 1-liter conical flask and it was pretreated with agitation at 60-70^0c in water bath for two hours. This pretreated step is necessary to obtain the pH that will be optimal for the enzymatic digestion. Thus the sample was pretreated with 0.4% alkaline solution for 1 hour.

After the pretreatment, the enzyme 0.372ml (6units/g) was added and the samples were kept at 55ºC for a period of 2 to 4 hours. The solution was filtered through Whatman No.1 filter paper and the hydrolyzed protein solutions were stored at 40c. The chrome cake was air-dried.

The unhydrolzsed waste material was air dried for estimation of percentage of hydrolysis. The total protein of hydrolyzed protein solution was determined via total nitrogen. The result obtained in determining total nitrogen concentration (g/l) was multiplied by 6.25 to give the total protein concentration. The same experiment was conducted in various temperatures, pH and enzyme concentration to find out the percentage of solubility of protenicity of enzymatic digestion.

RESULTS AND DISCUSSION

Isolation and identification of Fungi

The fungus stains isolated from tanning soil were found to give high yield of protease, when compared with bacterial proteolytic strain. The fungus isolated in microbiology lab, CLRI, Chennai was identified as *Aspergillus tamarii*. The tyrosine standard was made for the enzyme assay and Bovine Serum Albumin standard for the protein estimation (Dayanandan 2003) in Figure 1.

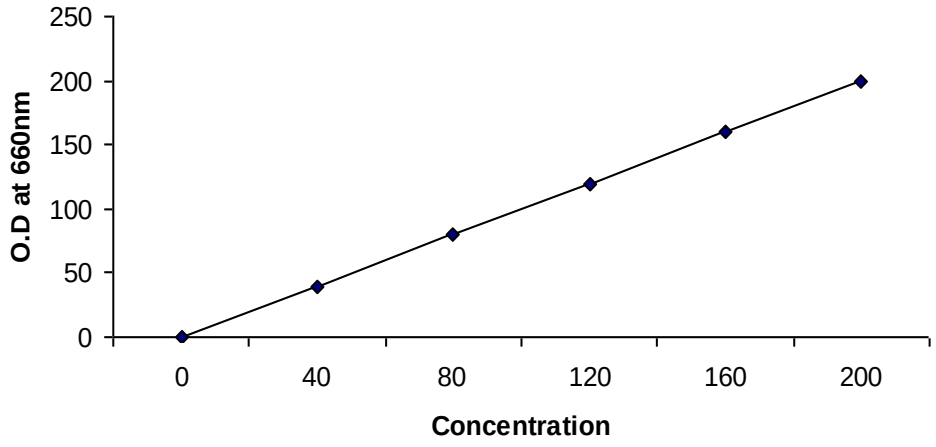

Figure 1. Estimation of protein content.

Optimization of environmental conditions of maximal production of protease

Effect of incubation period of enzyme production was recorded at regular intervals. The results obtained are presented in the Figure 2. Enzyme production was higher in the 3rd day of incubation and maximum production was observed in 72 hours. Effects of protein on medium composition on protease production by *Aspergillus tamarii* were studied and results obtained were represented graphically in Figure 3.

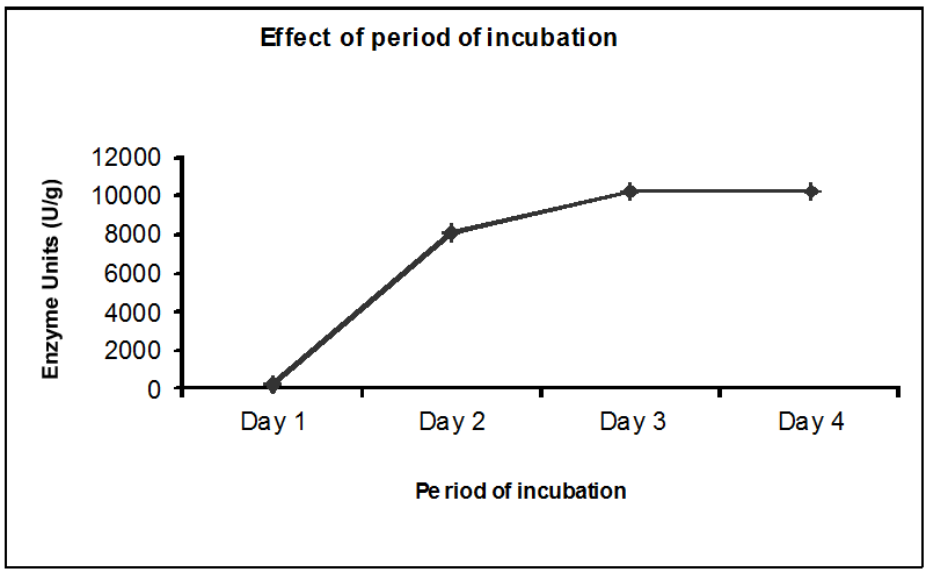

Figure 2. Effect of period of incubation.

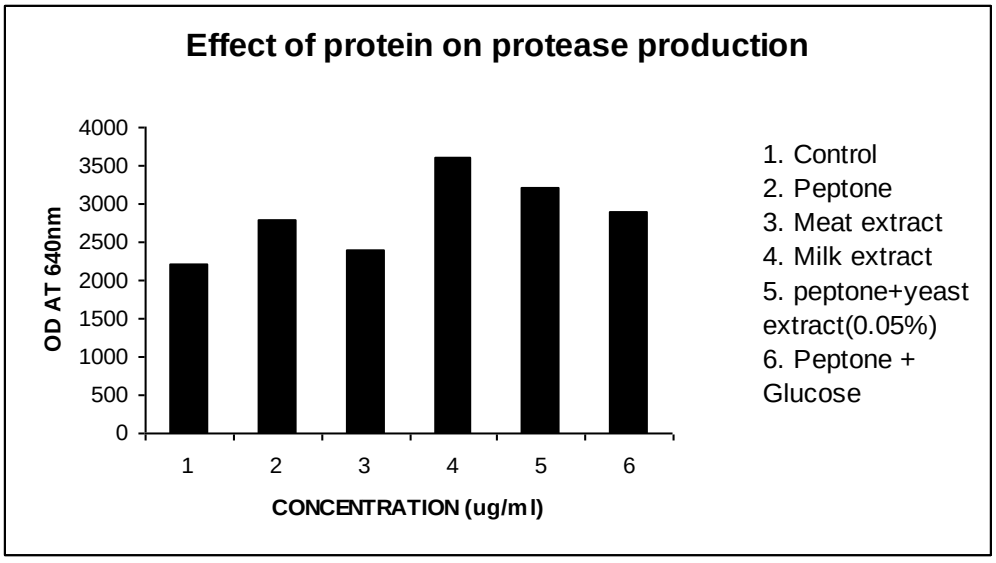

Figure 3. Effect of protein on protease production.

It is clear from the graph that the protease production by *Aspergillus tamarii* was influenced by the composition of the medium. Protease production was found to be high in wheat bran enriched with a mixture of basal medium and milk powder. Though protease activity was high in milk powder enriched basal medium, basal medium can only be used for later works.

The effect of age of the inoculum was found to play an important role in the production of enzyme and maximal activity was found when the medium was inoculated with 3 days old culture which is been explained in Figure 4.

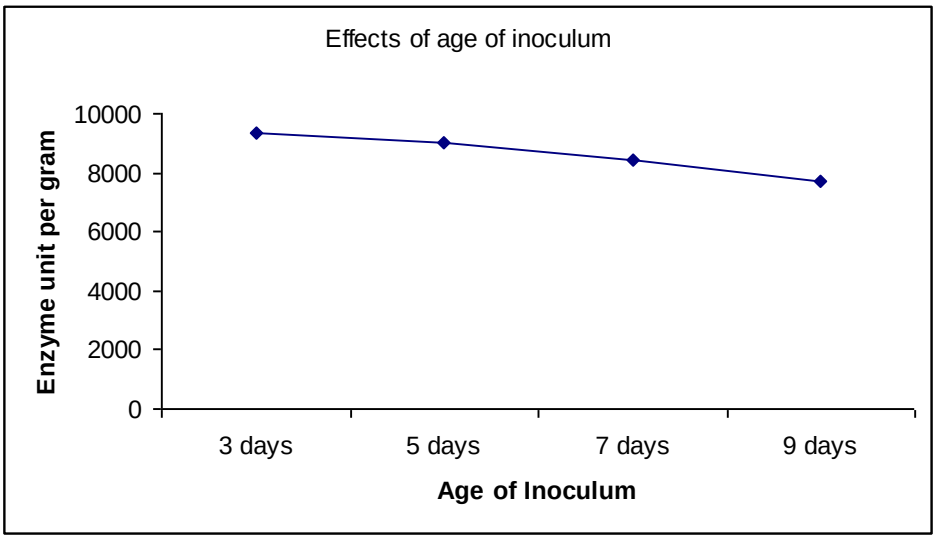

Figure 4. Effects of age of inoculum.

Effect of size of inoculums is evident from the table that the production of enzyme was found in the entire medium containing different concentration of inoculum, maximum enzyme production was found in all the initial inoculum size 10%, which was the minimum concentration in the experiment and results were represent in Figure 5.

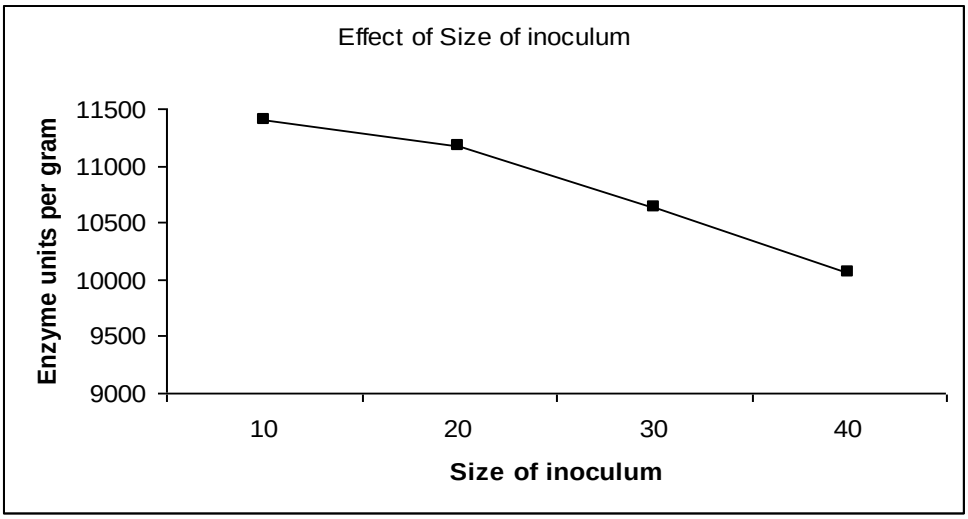

Figure 5. Effects of size of inoculum.

Analysis of leather scrapings

The parameters and the key factors of the leather scrapings used in the experiments are standardized and shown in the Table 1.

Table 1. Analysis of Chrome Shavings

S.No	PARAMETERS	% Standardized values
1	pH	3.5
2	Moisture (Room Temperature)	15
3	Total Solids	79.5
4	Total Nitrogen	14.4
5	Total Protein	90
6	Total Chromium as Cr (VI)	5.25

Leather waste hydrolysis

The concentration of alkaline protease ranges from 1 to 10 units /g and the percentage of hydrolysis of leather waste. Enzyme hydrolysis of leather waste at constant enzyme concentration with varying temperatures and pH are shown in Figure 6 and 7. Thus we can analyze the influence of alkaline protease concentration on solubility of leather waste. The solubility is increased by increasing the enzyme concentration in the range of 6 to 25 µ/g. During the hydrolysis process, enzyme concentration also influences the proportion of total nitrogen. At lower enzyme concentrations (1 to 6) during hydrolysis, there are no large changes in proportion of total nitrogen. On using high enzyme concentration, high values of nitrogen proportions are reached were shown in Figure 7. Thus, a temperature of 55^0C and pH adjusted to 9.5 was used for the entire digestion of leather waste by alkaline protease.

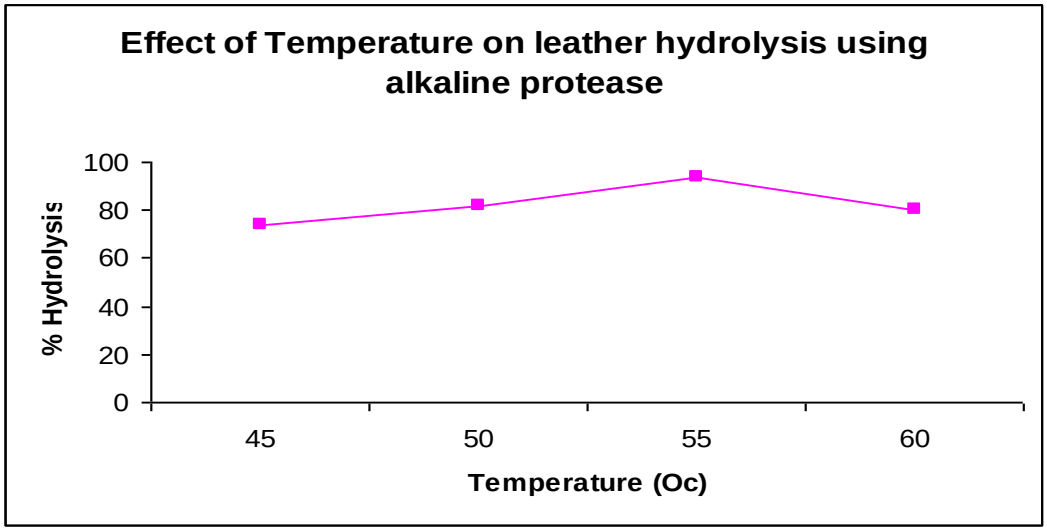

Figure 6. Effect of temperature on leather hydrolysis using alkaline protease.

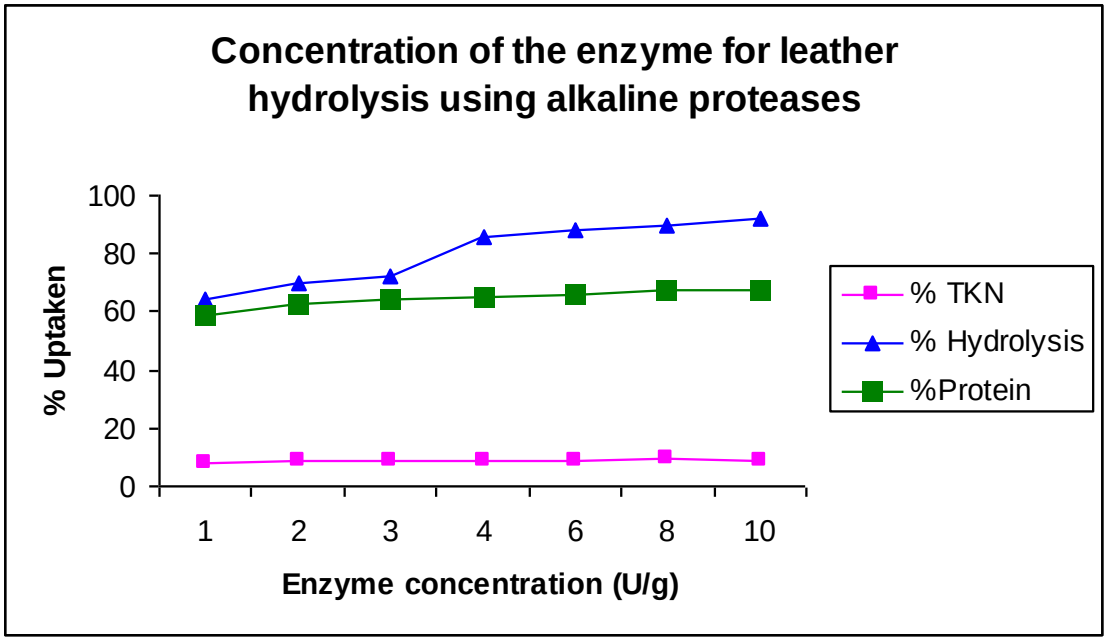

Figure 7. Concentration of the enzyme for leather hydrolysis using alkaline proteases.

CONCLUSIONS

Thus from the present studies it is concluded that the enzyme used for the protein hydrolysis is used in very small quantity i.e. 6 units/g. Hydrolysis process is so simple without any risk. This hydrolysis protein is purified further and is used for animal feeding, in pharmaceutical industries etc. Chrome cake is also re-used in tanning. Chromium is recycled and used in leather tanning. Application of enzymes to this protein hydrolysis gives processes and this uses less energy, produce less toxic waste, reduced process times, and also less expensive. In Paper and pulp industry, the protease enzyme is used as a biofilm removal De-linking, starch coating Pulp bleaching De-linking, Drainage bleaching pulp. It is also used for cleaning, in washing powder, drain cleaner and also for restoring colour for cotton. Further it is also used in bioprocessing technology, X-ray films for silver recovery. In future, for the high production of enzymes by the fermentation technology, protein engineering is required (Ganesh Kumar and Hiroshi Takagi 1999).

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