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RECOVERY & REUSE OF Cr (III) USING FUNGAL BIOMASS & LEATHER SCRAPPINGS; APPLICATION OF FUNGAL PROTEASES IN LEATHER HYDROLYSIS

A PROJECT REPORT

Submitted by

ADITHYA B

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in

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KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

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APRIL 2006

BONAFIDE CERTIFICATE

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This is to certify that the project work entitled "**Biosorption and Recovery of chromium from tannery waste water using waste leather scrapings and fungal biomass and application of Fungal Protease for leather hydrolysis**" is a record of original work done by **Mr. B. Adithya.** This is submitted to Kumaraguru College of Technology, affiliated to Anna University, Coimbatore in partial fulfillment of requirements for the degree of B.Tech in Industrial Biotechnology, done in the Microbiology Laboratory, Central Leather Research Institute, Adyar, Chennai 600020 under my supervision. The project report has not been previously formed the basis for the award of any degree.

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ABSTRACT

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Biosorptive studies were carried on using two biomasses; 1.Fungal Biomass 2.Protein biomass (leather scrapings). The fungal biomass was prepared using the fungus isolated from the soil near the tanneries. The fungus was isolated and optimized. This fungal strain was used in the production of enzyme (alkaline proteases) by both the solid and liquid state fermentation. The substrate used for solid-state fermentation was wheat bran. The protease enzyme secreted by the fungus was used for the hydrolysis of leather waste and the biomass from the liquid state fermentation was used as the fungal biomass for biosorptive studies. Both the biomasses were used for the recovery and reuse of Cr (III). Both showed optimum removal of Cr in the solution and favorable conditions were optimized. The kinetic experiments data fit the well for the pseudo second order reaction and Freundlich Isotherm was found to be favorable. The biosorption of Cr (III) was studied with FT-IR for analysis of the functional groups in adsorption. Desorption studies were done with varying acid strength for recovery of Cr (III) from the biosorbent. The Cr was desorbed and was recycled for tanning. The proteins from leather hydrolysis by the protease were also used for other applications.

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INTRODUCTION

1. INTRODUCTION

1.1 GENERAL:

Leather industry is an age-old industry and has been serving the society as an important consumer industry. It provides a wide range of consumer goods such as shoes, garments, bags etc. However the tanning industry has been categorized as one of the most highly polluting industries and there are concerns that leather making activity can have adverse impacts on the environment. The global production of leather is about 24 billion sq. meters by the year 2005; this presents a considerable challenge to the industry considering the harmful nature of some of the chemicals used in leather processing. The tannery effluents are characterized by high contents of dissolved; suspended organic and inorganic solids giving rise to high oxygen demand and potentially toxic metal salts and chromium metal ions. The disagreeable odour emanating from the decomposition of protein waste material and the presence of sulphide, ammonia and other volatile organic compounds are also associated with tanning activities.

The last two decades have witnessed substantial relocation of leather making activity from industrialized countries to the developing countries on account of high cost of effluent treatment installations and treatment methods, however in the recent times, leather industries in the developing countries are also facing threat of closure due to orders based on public interest litigations. An increased public awareness leading to stricter enforcement of pollution control regulations has forced the leather industries in these countries to adopt cleaner leather processing methods in the recent times.

1.2 LEATHER INDUSTRY:

Current status:

The raw materials for leather industry are the rawhide or skin. It is subjected to a wide range of pH alterations and it involves the use of copious amounts of water. Environmental challenges from leather processing arise from the nature and quantum of chemicals used as well as the amount of wastes generated and discharged.

The salt used for preserving the skin/hide discharge huge amount of pollution load in terms of Total Dissolved Solids (TDS) and chlorides. Other major polluting chemicals used in tanning industry that cause pollution are lime, sodium, sulphide, ammonium salts, sulphuric acid, chromium salts and vegetable tanning materials. Typical emission factors expressed as wastes generated for every tons of leather processed at various stages of processing are presented in Table 1.1 and Table 1.2.

Environmental Issues:

Water pollution: Currently about 6.5 million tons of wet salted hides and skins are processed world wide annually. Based on this figure, it is estimated that about 3.5 million tons of various chemicals are used for leather processing. A considerable part of this amount is not taken up by the hide/skin during the process and is discharged into the effluent. At an average consumption of 45-50 cubic meters of the waste liquor per ton of rawhide, over 300 million cubic meters of waste liquor containing thousands of tons of chemicals and solid waste are discharged by the leather industry. They are to be treated adequately and disposed scientifically complying with the statutory requirements. The composition of solid wastes generated in tanneries worldwide is presented in Table 1.3.

The characteristics of the tannery wastewater are complex. They vary in strength from tannery to tannery and region to region. The principles involved in these processes are same at every center. In addition, the intensity of pollution is related to the quality of raw materials including the hides and skins and other ancillary chemicals with reference to geographical location. It is observed that tannery wastes are stronger than sewage and far different in constituent pollutants. For example, chromium, sulphides and total dissolved solids are unique to tannery wastewater.

Soil pollution: The untreated wastewaters from tanneries have been applied on land merely to contain them at one place. The long-term use of land for disposal of wastes has resulted in contamination of soil. The soils holding it directly and irrigated with contaminated groundwater have been reported to lose productivity (Mariappan 1997).

Atmospheric pollution: The tanneries are proverbially known generating malodor. The hydration of salted hides and skins generally emit odour of volatile fatty and amino acids evolved in the course of biological decomposition in the presence of water. In addition, the stench of hydrogen sulphide along with acids, fats, carbohydrates etc in liming, deliming and tanning processes is predominant within the tanneries. The venting out of malodorous substances to ambient air and subsequent transports to further distance are responsible for atmospheric pollution. Hydrogen sulphide at 20 ppm ($30mg/m^3$) in ambient air is lethal to human kind.

Solid wastes: Several researchers are carrying our study in solid wastes for the reduction of pollution problem in leather processing. The tannery generates huge amount of fleshing as a solid waste. The Table 1.2 shows environmental balance of leather production.

Thus, out of 1000Kg of raw hides, nearly 850Kg of the raw materials is generated as solid wastes in leather processing. Only 150 Kg of the raw material is converted into leather. The solid wastes in the leather processing constitute 80% from beam house, 19% from tanning and 1% from finishing. The solid wastes can be hydrolyzed and used as a useful by product in many ways. Table 1.3 shows the percentage of various solid wastes generated in the leather industry.

Several technologies have been explored for the utilization of solid wastes generated in leather industry. Among the solid wastes, keratin plays an important role because of the difficulty in hydrolyzing it. The disulfide bond plays an important role in safe guarding the keratin. It is highly resistant towards enzymes and most of the chemicals. It is rich in cysteine containing amino acids. The chrome shavings and fleshing also play a major source of proteins commercially utilizable if processed properly.

Tannery Liquid Effluents: The tanning industry discharges different types of waste into the environment, primarily in the form of liquid effluents containing organic matters, chromium, sulphides, ammonium and other salts. Pollution becomes acute when tanneries are concentrated in clusters in arid areas. Out of this chromium has been the most under study due to its harmful effects.

1.3 CHROMIUM (Cr):

Chromium (Cr) is listed as one among the 129 top pollutants by the EPA. Chromium is considered one of the 14 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 at priority superfund sites.

General Hazard and Toxicity: The EPA regards all chromium compounds as toxic. Hexavalent chromium causes cellular damage via its role as a strong oxidizing agent, whereas trivalent chromium can inhibit various enzyme systems or react with organic molecules.

Carcinogenicity: Chromium in general is listed by EPA as a Class A human carcinogen. Some salts of chromium are carcinogenic and humans exposed to chromium fumes have an increased risk for lung cancer.

Developmental, Reproductive, Endocrine, and Genotoxicity Hazards: Hexavalent chromium is associated with cancer risk and kidney damage, and may cause damage to DNA and many other tissue structures.

1.4 BIOSORPTION:

The search for new technologies involving the removal of toxic metals from wastewaters has directed attention to biosorption, based on metal binding capacities of various biological materials. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico-chemical pathways of adsorption (Gadd 1992). Algae, bacteria and fungi and yeasts have proved to be potential metal biosorbents (Volesky, 1986). The major advantages of biosorption over conventional treatment methods include (Kratochvil and Volesky, 1998)

- Low cost
- High efficiency
- Minimization of chemical and or biological sludge
- No additional nutrient requirement
- Regeneration of biosorbent and
- Possibility of metal recovery

The biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, metal ions). Due to higher affinity of the sorbent for the sorbate species, the latter is attracted and bound there by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phases.

Biosorbent material: Strong biosorbent behavior of certain microorganisms towards metallic ions is a function of the chemical make-up of the microbial cells. This type of biosorbent consists of dead and metabolically inactive cells. Some types of biosorbents would be broad range, binding and collecting the majority of heavy metals with no specific activity, while others are specific for certain metals. Some laboratories have used easily available biomass whereas others have isolated specific strains of microorganisms and some have also processed the existing raw biomass to a certain degree to improve their biosorption properties. Recent biosorption experiments have focused attention on waste materials, which are by products or the waste materials from large-scale industrial operations.

Biosorption mechanisms: The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell. The biosorption mechanisms are various and are not fully understood. They may be classified according to various criteria.

According to the dependence on the cell's metabolism, biosorption mechanisms can be divided into:

- Metabolism dependent

According to the location where the metal removed from solution is found, biosorption can be classified as

- Extra cellular accumulation or precipitation
- Cell surface sorption or precipitation
- Intracellular accumulation.

Transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of biosorption may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal.

During non-metabolism dependent biosorption, metal absorbed is by physico-chemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cell's metabolism. Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups. This type of biosorption, i.e., non-metabolism dependent is relatively rapid and can be reversible (Kuyucak and Volesky, 1988).

In the case of precipitation, the metal absorbed may take place both in the solution and on the cell surface (Ercole *et al.*, 1994). Further, it may be dependent on the cell's metabolism if, in the presence of toxic metals, the microorganism produces compounds that favor the precipitation process. Precipitation may not be dependent on the cell's metabolism if it occurs after a chemical interaction between the metal and cell surface.

Physical adsorption: In this category, physical adsorption takes place with the help of Van der Waals' forces. Kuyucak and Volesky in 1988

by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chiarella vulgaris* for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger*.

Ion Exchange: Cell walls of microorganisms contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of K^{-}, Na^{+}, Ca^{2+}, and Mg^{2+}. These ions can exchange with counter ions such as CO^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} resulting in the biosorptive uptake of heavy metals (Kuyucak and Volesky 1988). The biosorption of copper by fungi *Ganoderma lucidium* (Muraleedharan and Venkobachr, 1990) and *Aspergillus niger* was also up taken by ion exchange mechanism.

Precipitation: Precipitation may be either dependent on the cellular metabolism or independent of it. In the former case, the metal removal from solution is often associated with active defense system of the microorganisms. They react in the presence of toxic metal producing compounds, which favor the precipitation process. In the case of precipitation not dependent on the cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface. The various biosorption mechanisms mentioned above can take place simultaneously.

1.5 FACTORS AFFECTING BIOSORPTION:

The investigation of the efficacy of the metal adsorption by the microbial biomass is essential for the industrial application of biosorption, as it gives information about the equilibrium of the process that is necessary for the design of the equipment.

The metal adsorption is usually measured by the parameter "q" which indicates the milligrams of metal accumulated per gram of biosorbent material and "q_h" is reported as a function of metal accumulated, sorbent material used and operating conditions.

The following factors affect the biosorption process:

1. Temperature seems not to influence the biosorption performances in the range of 20-35 ^{0}C (Friis *et al.*, 1998).

2. pH seems to be the most important parameter in the biosorptive process; it affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of metallic ions (Friis and Myers-Keith, 1986; Galun *et al.*, 1987) .

3. Biomass concentration in solution seems to influence the specific adsorption; for lower values of biomass concentrations there is an increase in the specific adsorption (Fourest and Roux, 1992; Gadd *et al.*, 1988) they suggested that an increase in biomass concentration leads to interference between the binding sites. Fourest and Roux in 1992 invalidated this hypothesis attributing the responsibility of the specific adsorption decrease to metal concentration shortage in solution. Hence this factor needs to be taken into consideration in any application of microbial biomass as biosorbent.

4. Biosorption is mainly used to treat wastewater where more than one type of metal ions would be present; the removal of one metal ion may be influenced by the presence of other metal ions. For example: Uranium

the presence of manganese, cobalt, copper, cadmium, mercury and lead in solution (Sakaguchi and Nakajima, 1991). In contrast, the presence of Fe^{2+} and Zn^{2+} was found to influence uranium adsorption by *Rhizopus arrhizus* (Tsezos and Volesky, 1982) and cobalt adsorption by different microorganisms seemed to be completely inhibited by the presence of uranium, lead, mercury and copper (Sakaguchi and Nakajima, 1991).

1.6 BIOSORPTION EQUILIBRIUM MODELS:

Assessment of sorption performance: Examination and preliminary testing of solid liquid sorption system are based on two types of investigations; equilibrium batch sorption tests and dynamic continuous flow sorption studies.

The equilibrium of the biosorption process is often described by fitting the experimental points with models (Gadd, *et al.,* 1988) usually used for the representation of isotherm adsorption equilibrium. The two widely accepted and linearized equilibrium adsorption isotherm models for single solute system are given by the following:

Where q is milligrams of metal accumulated per gram of the biosorbent material; C_{eq} is the metal residual concentration in solution; q_{max} is the maximum specific adsorption corresponding to the site saturation and b is the ratio of adsorption and desorption rates. This is a theoretical model for monolayer adsorption.

These models can be applied at a constant pH. These models are used in literature for modeling of biosorption equilibrium in the presence of one metal. These values are plotted in a 2D line where the specific adsorption q is reported as a function of the metal concentration C_{eq}.

But the above said adsorption isotherms may exhibit an irregular pattern due to the complex nature of both the sorbent material and its

some metallic compounds (Volesky and Holan, 1995). Evaluation of equilibrium sorption performance needs to be supplemented by process-oriented studies of its kinetics and eventually by dynamic continuous flow tests.

1.7 DESORPTION:

If the biosorption process were to be used as an alternative to the wastewater treatment scheme, the regeneration of the biosorbent may be crucially important for keeping the process costs down and in opening the possibility of recovering the metals extracted from the liquid phase. For this purpose it is desirable to desorb the sorbed metals and to regenerate the biosorbent material for another cycle of application.

The desorption process should:

- Yield the metals in a concentrated form
- Restore the biosorbent close to the original condition for effective reuse with undiminished metal adsorbed
- No physical changes or damage to the biosorbent

While the regeneration of the biosorbent may be accomplished by washing the metal laden biosorbent with an appropriate solution, the type and strength of this solution would depend on the extent of binding of the deposited metal.

1.8 PROTEASES:

Proteases from a large group of enzymes, ubiquitous in nature are found in are wide variety of microorganism. They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins. These enzymes are impotent in a number of diverse and crucial biological processes; for example, they are

modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao *et al.*, 1998).

Proteases are difficult to characterize because of their diversity of action and structure. Originally proteases were classified based on molecular size, charge or substrate specificity. However, with the advent of molecular biology, proteases are now grouped into families based on the following; chemical nature of the catalytic or active sites, mechanism(s) of action, and the evolutionary relationship of their three-dimensional structure (Rao *et al.*, 1998).

Proteases are broadly divided into either exopeptidases or endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzymes cleave peptide bonds distant from the termini of a substrate, they are classified as endopeptidases.

Proteases are one of industrially most important enzymes. The main sources of these enzymes are animals (e.g. calf stomach) and plants (e.g. pineapple, fig, papaya). Due to the irregular production associated with plant sources and large number of moral and ethical issues related to animal sources, microbial source have occupied an important place in the production of all the three major types of proteases acidic, neutral, and alkaline microbial proteases account for approximately 40% of the total worldwide enzyme sales. For this reason, different studies are currently being carried out to determine new sources of production or to increase the production of known sources.

A wide range of microorganisms including bacteria, moulds, yeast and also mammalian tissues produces alkaline proteases. Various species of *Bacillus*, *Pseudomonas*, *Vibrio*, *Aspergillus* and *Streptomyces*, have

proteases. *Trichoderma, Rhizopus,* and *Penicillium* represent some other alkaline protease producing genera among fungi.

Bacteria are active producers of extra cellular alkaline proteases. It is well established that extra cellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH temperature, dissolved oxygen and incubation time.

Proteolytic microorganisms can grow only in media containing peptones (protein hydrolysates). Because of their rich source of amino acid and peptides, peptones have become one of the most important constituents of culture media and are widely used today for the production of biomass and a large variety of metabolites, including enzymes. At present, commercial peptones come mainly from casein, soy, gelatin, meat, fish peptones have only been used to a minor extent.

30% to 40% of the production cost of industrial enzymes is estimated to be accounted for by the cost of the growth medium considering this fact, the use of cost-effective growth medium for the production of alkaline proteases from an alkalophilic *Bacillus sp* is especially important, because these enzymes account for approximately 25% of world wide enzyme consumption. For this purpose, soybean meal was recognized as a potentially useful and cost effective medium ingredient, because it is largely produced as a by-product during oil extraction. Furthermore, chemical analysis showed that it is composed of approximately 40% protein and is rich in other organic and inorganic components suggesting it as a good candidate for culture media. Wastes or agro-industrial substrates, such as defatted soybean cake, gram bran, wheat bran, rice bran, and banana waste etc. for enzyme production.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 LEATHER INDUSTRY:

Keratin wastes:

Using strong alkali such as sodium hydroxide and acid like hydrochloric acid, keratin is hydrolyzed. The hydrolysate prepared from keratinous material has been employed in chrome tanning and rechroming at various levels and the exhaustion is studied. The study shows that the hydrolysates help us to improve the chrome exhaustion as tanning bath and rechroming bath. The physical strength properties are also not altered (Ramamoorthy *et al.*, 1989).

Fleshing wastes:

Modified fleshing hydrolysate has been prepared from fleshing and acrylic acid. The resultant product has been used as an aid in chrome tanning and rechroming. Modified fleshing hydrolysates at the level of 2% showed improved adsorption of chromium in chrome tanning and rechroming. The physical strength properties and organo-leptic properties of leather are better than conventionally produced leather (Kanagaraj *et al.*, 2002.)

Chrome shaving wastes:

Chrome tanned leather; splits and trimmings have also been found to be a useful by product. These solid wastes have been useful in obtaining glue, gelatin, protein flavor and reconstituted collagen; the above products can be made by treating with hydrogen peroxide to bring about important degree of maceration and then grinding and extraction to yield the by-product in the order of 95% (Colen *et al.*, 1998).

Chrome shaving has also been hydrolyzed by many ways. Various alkalinity-inducing agents, such as magnesium oxide, alone or in combination with calcium hydroxide or sodium carbonate have been employed to maintain the optimal environment for enzyme hydrolysis. It has been shown that magnesium oxide alone in combination with calcium hydroxide, sodium hydroxide or sodium carbonate will increase the efficiency of the solubilization and at the same time reduce the amount of enzyme needed and thus making the treatment more cost effective (Taylor *et al.*, 1990).

Gels, adhesives and films of high molecular weight gelable protein fraction have been obtained from chrome shavings under mild alkaline conditions, however the characteristics of the products depends on the composition of the original chromium waste product depends on the composition of the original chromium waste product and on the specific treatment conditions (Brown *et al.*, 1992).

The interaction of gelatin and polyphenols has been studied (He Xianqi *et al.*, 1994). The relation between molecular structure of polyphenols and their hydrophobicity has been investigated. The results showed that galloyl and polyphenols had hydrophobic sites, then polyphenol-amino acid and polyphenol-gelatin interaction showed that hydrophobic association between predominant patterns of polyphenol-gelatin interaction.

Gelable and hydrolyzed protein products from chromium containing leather waste have been obtained by using various alkali inducing agents. Magnesium oxide (MgO), carbonates and hydroxides have been used to obtain the product compared to the use of MgO alone; the use of MgO in combination with sodium or potassium hydroxide increases the chromium content of the hydrolyzed protein products.

Carbonates and hydroxides have a detrimental effect whereas MgO has the most significant effect (Taylor *et al.*, 1994).

The use of hydrolysates from chrome-tanned wastes for producing biodegradable plastic particularly applicable in agriculture for the breakdown of synthetic polymers has been studied. It has been confirmed that protein hydrolysates from chrome shavings markedly increases the biodegradation of material and also exerts a positive influence on mechanical properties (Kresalkova *et al.*, 2002).

Films of high quality have been made from commercial gelatins; commercial gelatins have been enzymatically treated with a microbial transglutaminase mixed with glycerol as plasticizer. The results showed that the amount of glycerol added affected the mechanical properties, particularly the maximum strain. Increasing the concentration of cross linking agent gave products with higher tensile strength and improved water adsorption properties (Taylor *et al.*, 2002).

Chrome shavings have been used for making chrome cakes. Characterizations of the chrome cakes and the protein generated by these methods have been studied. The unwashed chrome cakes showed moisture content of 83.9%, ash 40.57%, Total Kjeldahl Nitrogen (TKN), 6.07%, Chromium content (Cr_2O_3), 4.2% (Taylor *et al.*, 1992).

Chrome shavings have been used to obtain gelatin. These shavings have been treated with enzyme solution, pepsin A (3900 U/mg), trypsin (10,400 U/mg) for 6-24 hours at the optimum pH 8 and gelatin has been extracted at 70^0C. The results showed that pepsin behaves as mild-enzymes with a controllable effect on leather wastes and trypsin gave a better yield (Cabeza 1997).

Solid wastes such as lime fleshing and chrome shavings have been hydrolyzed by *Paecilomuces litacinus*. The effectiveness of the

showed that the rate of fleshing hydrolysis is 93% in comparison with conventionally hydrolyzed fleshing of 87% (Chakraborty and Sarkar 1998).

Utilization of chrome shavings and improved penetration as well as adequate exhaustion of chrome liquors in chrome tanning process has been studied. Shavings containing about 2% Cr_2O_3 has been hydrolyzed with sulphuric acid and the resultant hydrolysates has been added in different amounts to commercial chrome tanning salts for carrying out chrome tanning process (Manzo *et al.*,1993).

Chrome shavings have been treated with pepsin-trypsin and pepsin alkaline protease and various products such as gelatin; chrome cake and hydrolysates have been prepared. The shavings have been pretreated with pepsin at room temperature and gelatin has been extracted with water at 70^0C. The chrome sludge recovered after centrifugation has been treated with trysin or alkaline protease and hydrolysates has been extracted at 70^0C. The chemical properties showed that high quality of gelatin is produced by these methods. (Cabeza *et al.*, 1998).

Alkaline hydrolysis assisted by proteolytic enzyme at moderate temperature of 55^0C for the chrome-tanned leather had resulted in collagen hydrolysates, chrome cake and Cr(III) tanning salts. Application of collagen hydrolysates in retanning gave good quality of leather (Cantera *et al.*, 1997; Cantera *et al.*, 2003).

Pilot plant trials of a process to treat chrome shavings to isolate protein products and purified chromium have been studied. The chemical and physical properties showed that the extracts of gelatin with alkali gave a better molecular weight fraction (Cabeza *et al.*, 1999).

Enzymatic hydrolysis of chrome-tanned leather waste, have been studied that yielded hydrolysates of 50-60%. The same study indicated

low ash content (2.4-3.3%) and low content of chromic compounds (13.6-54.5 mg per kg) for the dry weight of the hydrolysates (Langmaier 1999).

Collagen hydrolysates have been obtained by treatment of chrome shavings with enzyme. This hydrolysate has been used as a retanning agent and the performance has been studied (Cantera *et al.*, 2000).

Chrome collagen residues have been used to obtain gelatin using conventional methods. A large part of this type of residue has been used for the production of agglomerates known as regenerated leather, mixing with latex in batch piles and various articles have been manufactured (Colen *et al.*, 1998).

Chrome shavings have been hydrolyzed and the resulted hydrolysates,acrylic copolymer of the hydrolysates has been directly used as a retanning agent in post tanning process of leather making. The procedures help for avoiding cost of disposal of shavings (Cantera 2003).

Two approaches for the utilization of chrome shavings have been developed. In one approach cross-linking with glutaradehyde has followed an acidic protease-pepsin. The dried material has been used for leather board. The second approach is by using an alkali protease with completely digested the wet blue shavings into a smaller material. The dried material has been used for casein formulations in leather finishing (Crispim and Mota 2003).

A comparative study of alkali and enzymatic conditioning of "twinned" hide from calves, young and aged animals have been carried out. Some unique properties of gel strength and isoelectric point have been obtained. The results obtained from polyacrylamide gel electrophoresis and gel permeation chromatography of the gelatin yielded results showed that it has been possible to suggest a peptide-type collagen cross linking developed with animal's age (Cole and McGill 1988).

The hydrolysates of chrome tanned leather waste from tanning manufacture react with glutaraldehyde to produce thermo-reversible and thermo irreversible gels. Thermo reversible gels have been employed as glues and thermo irreversible gels have been applicable in currently widespread encapsulation techniques (Langmaier *et al.*, 1999).

2.2 BIOSORPTION:

Biosorption of Cr(III) by *Sargassum* biomass: Eneida Sala Cossich *et al.*, used brown marine alga *Sargassum sp* from Brazilian coast as the biomass to absorb Cr(III). The purpose of this experiment was the determination of the contact time required to reach the equilibrium between dissolved and solid-bound sorbate (*i.e.* ions). The experiments were made with two biomass sizes: biomass in its natural size (with leafs and thallus) and milled biomass.

Biosorption experiments: Experiments to determine the contact time required for equilibrium sorption experiments were performed in Erlenmeyer flasks, using 1l of metal solution and approximately 1g of biomass (dry matter). The flasks were maintained at 30°C under constant agitation in a rotary shaker. Samples were removed at different time intervals, membrane filtered and analyzed for chromium by Atomic Adsorption Spectroscopy (AAS) (Eneida Sala Cossich *et al.*,2002).

Batch equilibrium sorption experiments were carried out Erlenmeyer flasks for 6 hours in a rotary shaker. These experiments were done at pH 2.0, 3.0 and 4.0 and temperatures at 20°C, 30°C and 40°C. After the sorption equilibrium was reached (6 hours), the solution was separated from the biomass by membrane filtration. The initial and equilibrium chromium concentrations in each flask were determined by AAS (Eneida Sala Cossich *et al.*,2002) .

Data evaluation: The chromium biosorption coefficient (q) the construction of sorption isotherms was calculated from the initial concentration (C_i) and the final or equilibrium concentration (C_f) in every flask, as follows:

$$q = \frac{V(C_i - C_f)}{M}$$

Where, V is the volume of the chromium solution in the flask and M is the dry mass of biosorbent.

Two models were used to fit the experimental data: Langmuir model and Freundlich model (Eneida Sala Cossich *et al.*,2002).

Chromium biosorption kinetics: Equilibrium time is a function of many factors, such as type of biomass (number and kind of biosorption sites), size and form of biomass, physiological state of biomass (active or inactive, free or immobilized), as well as the metal involved in the biosorption system. Reported values for equilibrium time are in the range from 15 minutes to ten days.

2.3 EFFECT OF CONCENTRATION:

Results indicate that sorption can be divided into two stages: one in which the sorption rate is very high (60% of biomass saturation capacity was reached in a contact time of 10 minutes), followed by a second stage with a much lower sorption rate. (Crist *et al.*, 1988 and Crist *et al.*, 1990), observed that proton adsorbed by algal cells consists of two processes, a fast surface reaction and a slow diffusion of protons into the cells.

Influence of biosorbent size on chromium biosorption: The influence of biosorbent size on chromium biosorption can be evaluated. The experimental results indicate that the biosorbent size did not influence the capacity and rate of chromium biosorption. Although this is contrary to

to point out that the two sizes of biomass are actually of the same thickness (dimension which determines the diffusion distance). This is so because size grading of ground biomass particle by standard sieves works on the length and width dimensions. Then, the influence of biosorbent size on metal adsorbed seems to be a function of both the type of biomass and the metal ion.

2.4 EFFECT OF pH:

It is now well established that heavy metals are taken up from water predominantly by ion exchange. Carboxyl and sulphate groups have been identified as the main metal-sequestering sites in seaweed and as these groups are acidic its availability is pH dependent. At pH in the range 3.5-5.5 these groups generate a negatively charged surface and electrostatic interactions between cationic species and this surface can be responsible for metal biosorption. The chromium biosorption capacity was at all temperatures; higher at pH 4.0 (at pH 5.0 a chromium precipitate was observed). This pH dependence suggests a competition of metallic ions and protons by the same binding sites, since in this pH range chromium ion is present as a cation. The decrease of pH can be attributed to the chemical features of the Cr (III) solution rather than to the sorption mechanism (Kuyucak and Volesky, 1989; Kratochvil *et al.*, 1998). Cr (III) in water can undergo hydrolysis and/or complexation reactions. The extension of these reactions depends primarily on the total Cr (III) concentration, pH and the type of anions in the solution. The hydrolysis reaction generates divalent cations $Cr(OH)^{2+}$ and protons which contribute to the increase of acidity of the Cr(III) solutions (Kratochvil *et al.*, 1998).

2.5 EFFECT OF TEMPERATURE:

Tsezos and Volesky, 1981; Kuyucak and Volesky, 1989; reported a slight increase in cation adsorbed by seaweed in the range of 4°C to 55°C. The effect of temperature on chromium biosorption by *Sargassum sp.*, was not as pronounced as the effect of pH.

2.6 COPPER BIOSORPTION BY BROWN SEAWEED:

Antonio Carlos A. Da Costa *et al.*, (1997) conducted a basic investigation into the removal of copper ions from aqueous solutions by *Sargassum sp.*, in batch conditions. The influence of different experimental parameters such as initial pH, shaking rate, sorption time, temperature, equilibrium conditions and initial concentrations of copper ions on copper adsorption was evaluated. Results indicated that for shaking rates higher than 100 rpm no significant changes in copper accumulation were observed, as well as for pH values between 3.0 and 5.0. No marked effect on the biosorption of copper was detected for temperatures between 298K and 328K. The Langmuir model better represented the sorption process, in comparison to the model of Freundlich. The process followed second-order kinetics and its calculated activation energy was 5.2 kcal/mol. Due to its outstanding copper adsorption capacity (1.48 mmol/g biomass) *Sargassum sp.*, proved to be an excellent biomaterial for accumulating and recovering copper from industrial solutions.

Also studies by Chen, Jiaping and Yiacoumi, Sotira show that the surface charge increases with decreasing pH. The biosorption of copper strongly depends on solution pH; the metal ion binding increases from 0 to 90 percent in pH ranging from 1.5 to 5.0. In addition, a decrease in ionic strength results in an increase of copper ion removal. Kinetic studies

Furthermore, a fixed-bed biosorption experiment shows that calcium alginate has a significant capacity for copper ion removal. The two-pK Basic Stem model successfully represents the surface charge and equilibrium biosorption experimental data. The calculation results demonstrate that the copper removal may result from the binding of free copper and its hydroxide with surface functional groups of the biosorbents.

2.7 BIOSORPTION CHARACTERISTICS:

Neurospora crassa (Fungal biomass)

The removal of Cr(VI) from aqueous solutions by live and pretreated *Neurospora crassa* fungal biomass was investigated in the batch mode (Fica *et al.*, 1994). The influences of solution pH, contact time and initial metal ion concentration as well as pretreatment of biomass on the biosorption efficiency were studied. All pretreatment methods were found to increase the biosorption capacity of biomass in comparison with the live biomass and biosorption capacity of acetic acid pretreated biomass was found to be 15.85 ± 0.94 mg/g biomass under optimum conditions. The adsorption constants were found from the Freundlich isotherm model at 25 °C. The biosorbent was regenerated using 10 mM NaOH solution with up to 95% recovery and reused five times in biosorption–desorption cycles successively. The biosorption mechanism of biosorbent was also evaluated by chemical and instrumental analysis including infrared spectroscopy, scanning electron microscopy and X-ray energy dispersion analysis.

2.8 DESORPTION STUDIES:

Dilute solutions of mineral acids like hydrochloric acid, sulphuric

biomass (Rome and Gadd, 1987, Zhou and Kiff, 1991, Luef *et al.*, 1991, Holan *et al.*, 1993; Pagnanelli *et al.*, 2002; Baiand Abraham, 2003).

A few experiments were conducted to desorb the metal ions from the loaded waste fungal biomass of *Aspergillus sp.*, (Fedele G *et al.*, 1993) as a function of HCl concentration in the case of iron, calcium and nickel. The results revealed that with increase in HCl concentrations, the desorption of the metal ions increased and at 5M HCl, complete removal of calcium and iron would be achieved while about 78% Nickel would be desorbed.

2.9 PROTEASE:

Microbes have been exploited in the last few decades for the production of many industrial enzymes for e.g. protease, amylase, lipase and diastases their industrial process have also been developed and reached commercial levels.

Proteolytic enzymes are leading the world's enzyme market as it accounts for nearly 60% of the total enzymes used;because of the high and ever increasing demand of these enzymes, there is a need for further research from different sources and with specific properties.

Production of proteases:

Microbial sources

Bacterial Proteases: Hiroshi Matsuzan, Masara Hamaoki, Takashia Ohta, (1982), showed production of extra cellular thermophilic protease by *Thermus aquaticus,* thermophilic bacteria living in hot springs. The progress of growth of organisms and production of casein hydrolytic enzyme in the culture supernatant were followed and found that their cell produced two types of extra cellular protease; first type was aqualysin-I, which has activity at 70-80 °C, the other which was called as aqualysin-II

Fungal Proteases: Berla Thangam and Suseela Rajakumar, did the characterization of extra cellular alkaline protease from *Alcaligens fecalis* in 2000 showed the characteristic feature of serine protease.

Attawat Impoolsup, Timothy W. Feigal, A. Marret, (1981), isolated an alkaline protease and neutral protease from *Aspergillus flavus*, which was purified using ammonium sulphate, precipitation, DEAE sephadex G-50 and higher purification was done using PAGE.

Banerjee R., Bhattacharyya B.C., (1992), showed the production of proteases from *Aspergillus niger* cultures, studied the fungal morphology and inoculum effects.

Yeast Proteases: Christen, P., Auria, R., Vega, C., Villegas, E. and Revah, S (1993), showed that proteinases from *Candida sp* with a precursor of 44 Da protein, at pH 6.0.

2.10 FERMENTATION:

Proteases produced by SSF processes have greater economic feasibility. In recent years, there have been increasing attempts to produce different types of proteases (acid, neutral, alkaline) through SSF route, using agro-industrial residues. It is interesting to note that although a number of subtracts have been employed for cultivating different microorganisms, wheat bran has been the preferred choice in most of the studies. Koji (1957), worked on isolation of alkaline and neutral proteases from *Aspergillus flagus* , *Aspergillus columnaris*, a Soy Sauce Koji Mold.

Narahara H *et al.*, (1982) studied the relationship of growth conditions and enzyme production using solid-state cultures of *Aspergillus oryzae*. Results showed a direct relationship between pressure drop, production of $C0_2$, and temperature increase. Acid protease

related with the fungus metabolic activity as represented by the total $C0_2$ evolved. They made stepwise changes in the gas environment and temperature during SSF process to mimic those changes, which arose during SSF due to mass and heat transfer limitations. It was observed that a decrease of 0_2 concentrations from 21% to 0.5% did not alter protease production.

Aunstrup K. (1974) showed the detailed co-relation between fungal growth and soil-state fermentation. The hyphal mode of fungal growth and their good tolerance to low water activity (A_w) and high osmotic pressure conditions make fungi efficient and competitive in nature micro flora for bioconversation of solid substrates. The hyphal mode of growth of filamentous fungi has a major advantage over unicellular microorganisms in the colonization of solids substrates and for the utilization of available nutrients and also the power to penetrate into the solid substrates. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of LSF what makes the action of hydrolytic enzymes very efficient and allows penetration into solid substrates. Penetration increases the accessibility of all available nutrients within particles. The fungal mycelium synthesizes and excretes high quantities of hydrolytic exoenzymes.

H.H.Wang (1987) studied effect of moisture, content, growth period and growth temperature for enzyme production. The microbial adhesion on solid substrate for proper fermentation depends upon the water holding capacity (WHC), water activity (A_w) inoculum density,

Aunstrup K. (1974) studied the culture conditions of *Aspergillus oryzae* for the production of protease enzyme. Optimization of fermentation conditions revealed that the maximum protease production was obtained at 28^0C and 200 rpm, for 48 h with a medium containing sucrose (3%); ammonium nitrate,(0.38%); tryptone, (2%); potassium dihydrogen phosphate, (0.1%); casein,(2%); zinc Sulphate, (0.001%); and calcium chloride, (0.0001%). The initial pH was 7.0-7.5; using 12% (v/v) preinduced inoculum grown in the same medium under similar conditions for 24h. In order to assess the suitability of the enzyme for use in detergents, its stability over a range of alkaline pH and temperature and its compatibility with commercial detergents was determined. The enzyme was stable between pH 6.0-8.5 and at temperature up to 40^0C.

Malathi and Chakraborty (1990) evaluated a number of carbon sources (brans) for alkaline protease production and reported wheat bran to be the best for cultivation of *A.flavus* IMI 327634.

2.11 PROTEIN ESTIMATION:

Lowry O.H. *et al.*, (1951) described protein measurement with the Folin Phenol Reagent. The method involved the reaction of protein with alkaline copper sulphate and the reduction of phosphomolybdate and phosphotungstate of Folin Ciocalteaus reagent by tyrosine and tryptophan residue of protein, which imparts blue colour to the solution, which has a maximum adsorption at 660 nm. It is highly sensitive method and quantities as low as 20μg of proteins can be measured. Several components like EDTA, Tris phenol, ammonia might interfere with colour development.

2.12 PROTEASE ENZYME ACTIVITY:

Anson M.L., (1938) formulated the caseinolytic method for studying proteolytic enzyme activity using casein as substrate.

2.13 FOURIER TRANSFORM – INFRA RED SPECTROSCOPY (FT-IR):

Samples of FT-IR studies were made by grinding the concern sample with potassium bromide followed by irradiation of UV light for 30 minutes and then sample was made into pellet using pellet maker. Then the pellet was kept under IR irradiation for 5 scans by using Perkin Elmer spectrum RX1 FT-IR instrument (Bajza *et al.*, 1997).

3. OBJECTIVES OF THE STUDY

Main objectives of this study are:

- To reduce the environmental pollution caused due to the discharge of tannery effluents by sorption of Chromium onto fungal biomass and waste leather protein biomass.
- To standardize the method used for the pre-treatment of the biosorbents.
- To hydrolyze the waste leather protein biomass using the enzyme (protease) produced by the fungal strain, which can be used commercially.
- Desorption studies of Cr using different acid strengths.
- To recover Cr from the leather protein biomass onto which Cr was adsorbed.
- To reuse the recovered Cr for Basic Chrome Solution (BCS) preparation, used as the tanning Agent.
- To analyze Langmuir, Freundlich adsorption isotherms and kinetic study to find the Best-Fit Model

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 ISOLATION OF PROTEOLYTIC FUNGUS:

Fungal cultures were obtained with the collection of various samples from tannery soil and fish market soil. The soil samples collected were serially diluted and plated on Czapeck dox medium.

4.2 IDENTIFICATION OF ORGANISMS:

The different fungal strains were isolated and the strain that secreted more protease enzyme was isolated by observing the zone of clearance and that strain was used for rest of the studies .

4.3 MAINTENANCE OF CULTURE:

The fungal strain was routinely sub cultured and maintained in Czapeck dox agar slants and was stored at $4^{o}C$.

4.4 PREPARATION OF INOCULUM:

Inoculum was prepared by harvesting the conidiophores of *Aspergillus* from the 7 day old slant culture and suspending in sterile water with Tween 80 as surfactant prepared inoculum.

4.5 CULTURE MEDIUM:

Wheat bran was used as solid substrate for the production of protease enzyme. It was moistened upto 60% and sterilized at $121^{o}C$ for 15 minutes.

4.6 BIOMASS PRODUCTION FROM LIQUID STATE FERMENTATION:

Medium Preparation (1500ml):

COMPONENTS	QUANTITY
Glucose	3g
Sodium Nitrate ($NaNO_3$)	0.75g
$K_2 HPO_4$	1.50g
KCl	0.75g
$MgSO_4$	0.75g
$FeSO_4$	0.015g
$CaCl_2$	2.5g/50ml of distilled water

- Glucose was taken as 6g/30 ml water in a separate conical flask and was sterilized.
- Similarly $CaCl_2$ was taken as 2.5g/50ml water and was sterilized.
- The remaining components of the media were dissolved in distilled water (1500ml) and were sterilized.
- After sterilization in autoclave, the media is inoculated using the inoculum prepared. 6 ml of the inoculum is added to 1500 ml of the media prepared.(Figure 4.1)
- The inoculated media is kept in shaker for 3 days.
- After 3 days, biomass is filtered, dried in oven and stored for further experiments.

Yield of 10 g obtained for 1000 ml of media (Figure 4.2,4.3 and 4.4)

4.7 EXTRACTION OF PROTEASE FROM FERMENTED SUBSTRATE:

Solid –state fermentation was carried out aseptically at 28^0C. After 3 days, the enzyme was extracted by shaking for 30 minutes with 50ml of 20mM borate buffer of pH-8, containing 2mM $CaCl_2$. Solids were separated by filtration; this is followed by centrifugation at 10,000 rpm for 20 minutes at 4^0C. The supernatant was used as crude enzyme and proteolytic activity was measured.

4.8 ASSAY FOR PROTEASE:

Alkaline protease activity was assayed by the modified method of Anson. The extra cellular fraction (supernatant of 10,000 rpm for 10 minutes at 4^0C) was dissolved in 1% casein as the substrate and it is also dissolved in 20mM borate buffer pH 8.0 containing 2mM $CaCl_2$. The proteolytic activity of the mixture containing 2ml casein (1% w/v) and 0.5 ml suitably diluted enzyme was carried out at 45^0C for 30 minutes. The enzymatic reaction was ceased with 2.5 ml of 10% TCA and then the mixture was allowed to settle for about 30 minutes. The mixture is then filtered using Whatman No.1 filter paper. To 1ml of the filtrate 5ml of 0.5mM Sodium bicarbonate solution and 0.5ml of diluted Folin-Ciocalteau reagent were added. The reaction mixture was incubated for 30 minutes in room temperature, the absorbance was measured at 660nm using the Spectrophotometer. Simultaneously a blank was prepared using the same steps; instead 10% TCA was added in prior to the addition of enzyme. The amounts of alkaline protease activity were determined using a standard graph prepared form tyrosine. From the standard graph, enzyme unit was calculated.

Results are expressed in amount of Alkaline Protease units (APU/ml) of enzyme extract /unit time.

$$\frac{\text{Concentration} \times \text{Unknown} \times \text{Dilution Factor} \times \text{Total Volume of Enzyme}}{\text{OD} \times \text{Enzyme for assay} \times \text{Total weight of wheat bran}}$$

4.9 PREPARATION OF TYROSINE STANDARD:

A stock solution containing 10mg of tyrosine and 50ml water was made so that the concentration is 0.2mg/ml. Various concentrations of the standard were made and were made up to 1 ml using sterile distilled water. The tubes were mixed with 5ml of 0.55mM of 1:3 diluted Folin-Ciocalteus`s reagent was added. The absorbance was measured at 660nm after 30 minutes of incubation at room temperature and plotted in a graph. Tyrosine standard graph was used in calculation of protease activity.

One alkaline protease Unit (APU) is defined as the amount of enzyme required for producing 1μg of tyrosine/ml of enzyme in 30 minutes at 45^0C. From the standard graph (Figure 1) the enzyme unit (U) was calculated.

4.10 ESTIMATION OF PROTEIN CONTENT:

The amount of protein present in the culture filtrate was assayed by the Lowry's method. One ml of sample was mixed with 5ml of freshly prepared alkaline copper sulphate solution and incubated at room temperature for 10 minutes. To the mixture 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.

4.11 STANDARD GRAPH:

A standard graph was done by using BSA as a known sample. A stock solution was prepared using 10mg in 50 ml of 0.5M Sodium hydroxide, so that the concentration is 0.2 mg/ml. Increasing concentration of the substrate was made up to 1 ml using sterile distilled water. To this 5ml of freshly prepared alkaline copper sulphate solution was mixed and incubated at room temperature for 10 minutes. To this mixture 0.5ml 1:1 diluted Folin-Ciocalteus's reagent was added and incubated at room temperature for 20 minutes and absorbance was measured at 640nm.A graph is plotted against the O.D values with the protein concentration of the known samples.

4.12 OPTIMIZATION OF PARAMETERS FOR ENZYME PRODUCTION:

To achieve maximum growth of the organism with maximal production of the enzyme; various parameters such as age of inoculum, size of inoculum and incubation time play a vital role. In the case of solid-state fermentation, the percentage of moisture content is also critical. Taking all these factors into account, these parameters were optimized after performing various combinations of experiments.

Effect of proteins on enzyme:

Presence of substrate is the basic requisite for an organism to produce enzyme. The effect of enrichments in addition to the basal medium was studied. To compare protease production, five different substrates such as Peptone (1%), Yeast extract (1%), Milk powder (1%), with glucose (1%), were used as nutritional supplements at the above

supplements was inoculated with the fungal spores and solid-state fermentation was performed.

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 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.

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 were prepared from 2 days, 3 days, 4 days and 5 days old culture and the age of inoculum at which there is maximal production of enzyme was determined.

4.13 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 (3000ml Borosil). 100mg of wheat bran was inoculated with 10% inoculum and moistened with 60ml of 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. It was incubated at 28^0C for 3 days.

The enzyme was extracted by shaking with 1000ml of cold 20mM borate buffer of pH 8.0 having 0.2mM $CaCl_2$ for 30 minutes in an orbital shaker. The extracts were filtered at 4^0C initially 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. The protein content in the sample was calculated.

4.14 CONCENTRATION OF ENZYMES:

Ammonium sulphate Precipitation:

Precipitation is a basic reaction by which the proteins present in a solution are made to settle down 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, the mixture was allowed to attain saturation. The precipitate obtained at this stage was removed by centrifugation at 10,000rpm for 15 minutes in a refrigerated centrifuge. The pellet obtained was dissolved in 25ml of 20mM Tris HCl of pH 8.0 containing 2mM $CaCl_2$.

4.15 DIALYSIS:

The sample was dialyzed for 48 hrs against the 20mM Tris HCl 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.

4.16 DETERMINATION OF CHROME OXIDE CONTENT (WET OXIDATION METHOD):

Principle:

In this method, Cr(III) in leather is oxidized with an oxidizing mixture containing Perchloric acid, Nitric acid and Sulphuric acid to Cr(VI), which is determined colorimetrically by measuring the O.D at 372 nm after neutralizing with NaOH. As a result of addition of acids the Cr(III) is converted to Cr(VI) due to strong oxidizing action which results in production of white fumes. Further neutralized mixture is calorimetrically measured at 372nm.

Reagents required:

- Concentrated Sulphuric acid
- Concentrated Perchloric acid
- Concentrated Nitric acid
- Sodium Hydroxide solution (40%)

Procedure:

Acid Digestion:

0.1gm of solid sample was taken in an Erlenmeyer flask. Conc. Sulphuric acid, Conc. Perchloric acid, and Conc. Nitric acid were added in the ratio of 2.5: 5.0:11.5.

Few porcelain beads were added to avoid bumping and were kept for digestion with a funnel placed over its mouth. The digestion was performed till the color of the digest turns green to orange, which indicates the conversion of trivalent chromium to its hexavalent state. The digest was allowed to cool and 40% NaOH was added until the fizzling stops. The contents were made up to 50ml in a standard flask. The color of the diluted digest was read at 372nm.

Calculation:

Amount of Chromium as Cr (VI) = $\frac{\text{OD at 372 x Dilution Factor x 52}}{4830 \times 20}$

Amount of Chromium as Cr (III) = $\frac{\text{OD at 372 x Dilution Factor x 52 x 152}}{4830 \times 20}$

4.17 ESTIMATION OF NITROGEN CONTENT BY TOTAL BY TOTAL KJELDAHL METHOD:

Principle:

Nitrogen in the leather is converted to ammonium sulphate by digesting with conc. Sulphuric acid in the presence of catalyst mixture. The ammonia liberated by the addition of excess caustic soda is distilled in to an absorbent solution and estimated by titration. In the absence of added nitrogenous compounds, the ammonia liberated is proportional to the hide substances in the leather.

Reagents required:

- Accurately standardized 0.2N Sulphuric acid
- Pure conc. Sulphuric acid (sp gr 1.84) free from $(NH_4)_2 SO_4$ and nitrates.
- NaOH solution (40%) free from nitrates.
- Boric acid solution (4%)
- Catalyst mixture ($CuSO_4$: K_2SO_4 = 1:10)
- Mixed indicator (Methyl red: Bromo cresol green = 1:3)
- 0.1% solution in ethanol.

Procedure:

0.5 gm of leather is weighed accurately and transferred to a clean, dry Kjehldahl flask. 10ml of conc. H_2SO_4 is added and the contents are heated placing the flask in an inclined position. 5g-catalyst mixture is added and the leather is completely disintegrated, the temperature is varied and the contents boiled briskly for 60 min until the solution becomes quite clear and colorless. A small funnel is kept in the neck of acid during digestion. The content was allowed to cool.

After cooling, 200 ml water is added to dilute the contents. 1 or 2 porous chips, 1ml of 1% indicator solutions are added. An excess of 40% NaOH (nearly 80ml) are poured carefully into the flask through its sides. The flask is then connected to the distillation for a minimum period of 45 min.

The distillate is collected in 80 ml of 4% boric acid solution containing a few drops of mixed indicator. When the evolution of ammonia is completed, the receiver is removed and the distillate is titrated with 0.2 N HCl. The end point is the color change from green to faint red. The amount of Nitrogen present is calculated by multiplying the percentage of Nitrogen by 5.62 provided no other N2 other than the hide substance is present.

Preparation of Cr Stock Solution:

An aqueous stock solution (10,000mg/l) of Cr (VI) was prepared using $K_2Cr_2O_7$ salt using distilled water. The concentration of the stock solution is 10,000ppm. This is used as the stock solution in all the experiments carried out. The solution was prepared freshly for each experiment to ensure efficiency and reproducibility of the results.

4.18 PRETREATMENT OF THE BIOSORBENT MATERIALS:

Fungal Biomass: The fungal biomass left over after the production of the protease enzyme is used as the sorbent material. The biomass is dried well in the room temperature or in hot air oven at 50-60 °C overnight. After drying, fungal biomass is obtained as pellets. These pellets were powdered using mortar and pestle. This biomass in the powdered form was used as the sorbent material for all the experiments carried out.

Waste Leather Protein Biomass: The solid protein waste (Chrome Shavings) obtained from the leather industry was used as another sorbent material. The chrome shavings usually contained about 3-4% of Cr (III). Therefore the biomass is made devoid of Cr by soaking in 1N Sulphuric acid for 1hr and by keeping in shaker for constant agitation. Then the biomass is washed twice with water to ensure the utmost removal of chromium. The protein biomass was dried in hot air oven at 45-55 °C overnight. The treated biomass was used as the sorbent material in the experiments done.

4.19 BIOSORPTIVE STUDIES:

pH vs Adsorption of Chromium:

The effect of pH on chromium adsorption was studied. The pH of the samples were adjusted to 1.6,2.5, 3.5, 4, 5 by using 0.05 M Sulphuric acid and 0.1 M NaOH with 50ml of standard chrome solution prepared from the stock. The solution is made in contact with 500mg of biomass for a period of 3-4 h in a rotary shaker at room temperature. The chromium content was measured using the wet oxidation method.

Time vs Adsorption of Cr:

Experimental trials were carried out by varying the time of contact

(2000ppm) in a 100 ml conical flask using 500mg of biomass. They were agitated in a rotary mechanical shaker at room temperature. The quantity of Cr remaining in solution after the specified time interval was determined by wet oxidation method. Further, the time profile study was related to kinetics of adsorption. A plot of q_t vs t was plotted for the first order kinetics and a plot of t/q_t vs t *was* plotted for the kinetic studies.

Pseudo first order reaction:

$$\log (q_e - q_t) = \log q_e - (k_1/2.303)\, t$$

where k_1 is the first order rate constant

q_e is the amount of chromium adsorbed by the adsorbent.

Pseudo Second order reaction:

$$t/qt = 1/k_2\, q_e^{\,2} + 1/q_e\; t$$

where k_2 is the second order rate constant.

The regression coefficient values were calculated and the best-fit model was plotted.

Quantity of biomass vs Adsorption of Cr:

Experimental trials were carried out to study the adsorption of Cr by varying the amounts of biomass (0.25g, 0.5g, 1.0g, 1.5g). 50 ml of 250ppm Cr solution was made with contact with the biomass and was agitated in room temperature for 5 hours. Finally the Cr content in the supernatant was calculated using wet oxidation method.

4.20 EQUILIBRIUM STUDIES:

Adsorption isotherms were used to characterize the adsorption capacity of the biomass. Freundlich and Langmuir adsorption isotherms were tested for their validity of the chromium adsorptive behavior of both fungal and the protein biomass. Adsorption isotherms were determined using 1g biomass treated with 39, 78, 117, 156, 195, 234, 273, 312ppm

Cr remaining in solution after the specified time interval was determined by wet oxidation method.

Experiments with Chrome liquor from tannery:

Using the chrome liquor form the tannery biosorptive studies were performed using both fungal and protein biomass with contact time of 4 hrs in agitation at room temperature. The Cr adsorbed was calculated using wet oxidation method.

4.21 INFRARED SPECTRA ANALYSIS:

In order to determine the functional groups responsible for metal adsorption, an un-reacted biomass and a pre-treated biomass with 312mg/l chromium were analyzed using a Fourier Transform Infrared Spectrometer (FTIR). FTIR technique is used mainly to identify functional groups (like carboxyl, hydroxyl, etc.) that are capable of adsorbing metal ions.

4.22 UTILIZATION OF CHROMIUM TREATED BIOMASS:

Preparation of basic chromium sulfate tanning agent:

To 100g of sodium dichromate well dissolved in 160ml of distilled water, 120 g of sulphuric acid (98% purity) was added. The solution was mixed using a magnetic stirrer at $95 \pm 3\ ^{0}C$. Chromium adsorbed biomass was added to the stirred solution as reductant in the ratio 1:0.4 (dichromate: biomass – w/w). Stirring was continued until the reduction of Cr (VI) to Cr (III) was complete. The absence of Cr (VI) was also ensured using a standard diphenylcarbazide (DPC) method in which hexavalent chromium reacts with DPC under acidic conditions to form a pink/purple color. 1 ml of the sample was diluted 10 times, to which DPC was added. The non-appearance of a pink color confirms the absence of

(III) was about 2hours. The pH of the reaction mixture was finally adjusted to 2.6 – 2.8 by the addition of sodium carbonate.

4.23 PURIFICATION OF CHROME CAKE:

Chrome cake was dissolved in 98% Sulphuric acid until pH was is 1. pH is then raised slowly to 2 by adding 50% sodium hydroxide solution. The solution was heated for 30 minutes at 60° C and was allowed to incubate overnight at room temperature. The solution was then filtered through Buchner porcelain funnels with Whatman No: 1 filter paper. The retentive (protein) was discarded. The filtrate was adjusted to a pH between 8 and 9 with 50% w/v sodium hydroxide solution. The solution was heated at 70° C for 2 hours and allowed to settle for 2 to 3 hours. Solution was then filtered with Whatman filter paper and the filtrate containing Cr (III) was used for tanning process.

4.24 LEATHER WASTE HYDROLYSIS:

Recovery of hydrolyzed protein concerns on 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, is used. The process was conducted at a constant temperature for the 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 was pretreated with agitation at 60-70 ^{0}C in water bath for 2 hours. This pretreatment 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 (6U/g) was added and

was filtered through Whatman No.1 filter paper and the hydrolyzed protein solutions were stored at 4^0C. The chrome cake was air-dried and TKN is performed from which protein is estimated.

RESULTS AND DISCUSSIONS

5. RESULTS AND DISCUSSIONS

5.1 SCREENING OF MICROORGANISM

The fungus was isolated from tannery soil. It was found to give high yield of protease, when compared with bacterial proteolytic strain. The isolated fungus was found to be *Aspergillus sp.*, (Figure 5.19)

5.2 TYROSINE STANDARD:

Using tyrosine as standard, an enzyme assay was made and a standard graph was plotted. The results were tabulated and standard graph was plotted (Table 5.1 and Figure5.1).

5.3 PROTEIN STANDARD:

Results of standard protein values from Bovine Serum Albumin were tabulated and standard graph was plotted based on the results. (Table 5.2 and Figure 5.2)

5.4 OPTIMIZATION OF ENVIRONMENTAL CONDITIONS OF MAXIMAL PRODUCTION OF PROTEASE:

Effect of incubation period on protease production:

The enzyme production was recorded at regular intervals. The results obtained are presented in the Table 5.6. It is clear from the graph (Figure 5.3) that enzyme production was higher in the 3rd day of incubation and maximum production was observed in 72 hours. (Table 5.3 and Figure 5.3).

Effect of age of the inoculum on protease production:

The effect of 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 (Table 5.4 and Figure 5.4).

Effect of protein on protease production:

The effect of medium composition on protease production by *Aspergillus sp.*, was studied and results obtained were tabulated and represented graphically (Table 5.5 and Figure 5.5).

It is clear from the results that protease production by *Aspergillus sp.*, 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 was only used for later works.

Effect of size of inoculum:

It's evident from the results (Table 5.6 and Figure 5.6) 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.

5.5 BIOSORPTION STUDIES BY VARYING THE BIOMASS AND WITH CONSTANT Cr CONCENTRATION:

The quantity of the biomass (fungal and protein) was varied from 0.25 to 1.5 g with a constant Cr concentration of 250ppm. This

the solution, adsorbed by the biomass. The system attains equilibrium at the saturation level of the absorbent. Corresponding results are shown in Tables 5.7, 5.9 and Figure 5.7, 5.9).

5.6 BIOSORPTION STUDIES BY VARYING THE Cr CONCENTRATION WITH CONSTANT BIOMASS QUANTITY:

The Cr concentration was varied from 250ppm to 2000ppm and the biomass (fungal and protein) quantity was taken as 1g. At lower concentrations, all metal ions present in the solution would interact with the binding sites and thus facilitated maximum adsorption. At higher concentrations, more Cr ions are left unadsorbed in the solution due to the saturation of the binding sites. Corresponding results are shown in Tables 5.8, 5.10 and Figure5. 8.

5.7 EFFECT OF pH:

The removal of Cr with fungal and protein (leather waste) biomass is highly dependent on the pH value of the effluent. Here, the pH range was studied between 2.5 and 5.0, since at lower pH, the amount of Cr ions bound in biosorption was low and at higher pH, precipitation of Cr occurred. The Cr absorbed at different pH values is shown in Tables 5.11 and 5.12 for fungal and protein biomass respectively and in Figures 5.11 and 5.12 for protein and fungal biomass respectively. Maximum adsorption of 97.97% was obtained at a pH range of 3.5 for protein biomass and a maximum absorbed of 95.31% was obtained at a pH range of 3.5 for fungal biomass.

This increase in Cr adsorbtion at this pH could be due to the ionization of the carboxyl groups present in both the biomass. With further increase in pH to about 5.0, the Cr adsorbed by fungal and protein

increase of pH, the basicity of the Cr complexes also increases and at pH values close to and greater than 5.0, Cr starts precipitating in the liquor. The optimal pH removal of Cr is around 3.5, which is the pH of wastewater generated from the chrome-tanning stream. Thus, the pH 3.5 was maintained in subsequent experiments.

5.8 EQUILIBRIUM STUDIES:

To study the maximum absorbed capacity of the fungal and leather waste biomass, the concentration of the synthetic chromium was varied (Table 5.7, 5.8, 5.9, 5.10 and Figures 5.7, 5.8, 5.9). The maximum absorbed of fungal biomass was 49.25 mg/g and maximum absorbed of leather waste biomass was 53.42 mg/g at an initial chromium concentration of 39 to 312ppm.

The values obtained from the experiment were analyzed using Freundlich and Langmuir Isotherms.

Freundlich Isotherms:

The Freundlich constants n and k were obtained from the linear regression analysis of the equation:

$$\log q = \log k + 1/n \log C_e$$

Where q is the amount of chromium adsorbed by the adsorbent used (mg/g), C_e is the equilibrium concentration of chromium in solution (mg/dm^3). k and n are constants. (Adsorption capacity and Intensity of adsorption); plot of log q_e vs log C_e should give a straight line with a slope of 1/n and intercept of log k. A linear relationship was observed among the plotted parameters at different biomass quantity both protein (leather) and fungal biomass (Figure 5.14 and 5.15) which indicates the applicability of the Freundlich equation. The k and n values and the correlation coefficients for the adsorption of Cr from the synthetic

from the graph and are tabulated below. The value of k represents the adsorption capacity and the value of n represents the intensity of adsorption.

Constant values and regression coefficients obtained from the experiments conducted on biosorption using fungal biomass from Chrome liquor for Freundlich equation:

K	n	R^2
7.323	1.6077	0.97255

Constants values and regression coefficients obtained from the experiments conducted on biosorption using leather waste biomass from Chrome liquor for Freundlich equation:

K	n	R^2
1.3194	0.7509	0.97962

Langmuir Isotherms:

The Langmuir constants q_o and b can be determined from the linear plot of Ce/q vs Ce, which has the slope of the $1/q_o$ and intercept of $1/q_ob$. The linear form of the Langmuir plot is given as

$$\mathbf{Ce/q= 1/q_ob+ 1/q_oCe}$$

Where Ce is the concentration of the chromium in solution (mg/ dm^{-3}) at equilibrium. The constant signifies the adsorption capacity (mg g^{-1}) and b related to affinity of adsorption (dm^3 mg^{-1}). The constants q_o and b along with the correlation coefficient value (R^2) are given in Table below. The adsorption capacity (q_o) value was 63.29mg g^{-1}, when 1g of fungal biomass was used. The large value of b also implies the

characteristics of the isotherms can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, R. According to McKay *et al.*, (1982), R^2 values between 0 and 1 indicate favorable adsorption. According to Kadirvelu and Namasivayam (2000), the n values between 1 and 10 represent beneficial adsorption.

As the regression value R^2 was so low and weak, this system doesn't fit the Langmuir model.

Constant values and regression coefficients obtained from the experiments conducted on biosorption using fungal biomass from Chrome liquor for Langmuir equation:

Q_o	b	R^2
63.2911	0.1045	0.903

The Langmuir and Freundlich capacity or q_{max} gives the efficiencies of fungal and leather waste biomass. The magnitude of k and n values shows the easy separation of heavy metal ion from wastewater and high adsorption capacity. The value of n, which is related to the distribution of the bonded ions on the sorbent surface, found to be greater than the unity for fungal and protein biomass. Though the regression values of Freundlich and Langmuir of this system seem similar, Freundlich fits better than Langmuir on further calculations. This indicates that this system is a heterogeneous system.

5.9 BIOSORPTION KINETICS:

EFFECT OF CONTACT TIME ON BIOSORPTION USING FUNGAL AND PROTEIN BIOMASS:

The concentration of both the metal ions and the biosorbent is a significant factor to be considered for effective biosorption. It determines the sorbent/ sorbate equilibrium of the system. The rate of adsorption is a function of the initial concentration of the metal ions. The kinetic profiles of the chromium biosorption are shown in (Table 5.13, 5.14, 5.15, 5.16 and Figure 5.12, 5.13). A maximum adsorption of Cr using protein was attained at the 30^{th} minute (Figure 5.13); the time taken to attain equilibrium for 0.5g of fungal biomass was at 140^{th} minute (Figure 5. 12). This indicates the saturation of the binding sites at that course of time and some Cr ions are left unabsorbed. Similarly, the time taken to attain equilibrium for 0.5g of protein biomass was at the 120^{th} minute (Figure 5. 13).

Effect of contact time on biosorption using fungal biomass and protein biomass:

Biomass	K_1 values for Pseudo first order reaction	K_2 values for Pseudo second order reaction	R Values
Fungal	K_1=0.04863	K_2=0.006782	R_1=0.99113 R_2=0.99998
Protein	K_1=weak	K_2=0.01734	R_1=0.4054 R_2=0.9999

This indicates that the Pseudo second order reaction fits best for both the biomass and the rate of the reaction is optimum. The results are shown in Table 5.13, 5.14 and Figures 5.16, 5.17.

5.10 INFRA RED SPECTRAL ANALYSIS:

An untreated fungal biomass sample and treated fungal biomass with the Cr solution were analyzed using FTIR, and the percentage transmission for various wave numbers is presented in Table 5.22. The adsorption bands identified in the spectra and their assignment to the functional groups in the fungal biomass are listed in the below in Table 5.22 and Figure 5.21 for treated fungal biomass and in Figure 5.20 for untreated fungal biomass.

Ahalya *et al.*, have shown that the wave number of 3000 and 3750 cm^{-1} for Bengal gram husk indicates the presence of OH groups on the husk surface. The trough observed in their IR at 2918.8cm^{-1} and 893.25cm^{-1} indicates the presence of C-H groups.

Aravindhan have shown their IR spectra to substantiate the interaction of chromium with Turbinaria seaweed. After the contact with chromium solution, the see weed exhibits spectra with clear shifts of the carboxyl stretching bands to lower frequencies.

Similarly, the IR spectra from our experiments indicate the presence of several functional groups for binding Cr (III) ions on the fungal biomass and the main functional groups involved in chromium absorbed are carboxyl, sulfydryl and hydroxyl, mainly those from the polysaccharides which are the prime constituents of fungal biomass. The broad band obtained in the IR spectra of the treated fungal biomass is due to the moisture content.

5.11 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 5.18.

5.12 LEATHER WASTE HYDROLYSIS:

The concentration of alkaline protease in the range of 1U/g to 10 U/g and the percentage of hydrolysis of leather waste is shown in Table 5.19.

The results of enzyme hydrolysis of leather waste at constant enzyme concentration with varying temperatures and pH are shown in Tables 5.20 and 5.21.

Thus we can analyze the influence of alkaline protease concentration on solubility of leather waste. Increasing the enzyme concentration in the range of 6 to 25 U/g increases the solubility.

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.

Thus, a temperature of 55^0C and pH adjusted to 9.5 was used for the entire digestion of leather waste by alkaline protease.

5.13 DESORPTION STUDIES:

Different acid strengths of HCl were used to remove the adsorbed metal Cr (III) from the biomass. Thus the percentage of desorption is shown in Table 5.17. The desorbed chromium is again reused and recycled for leather tanning.

TABLE 1.1:

COMPOSITION OF LIQUID AND SOLID WASTES GENERATED IN TANNERIES WORLDWIDE

PARAMETERS	TONS
Chemical Oxygen Demand	1470
Biochemical Oxygen Demand	610
Suspended Solids	920
Chromium	30
Sulphides	60
Solids Wastes (Trimming, Fleshing, Shaving and bugging dust)	3000

TABLE 1.2:

ENVIRONMENTAL BALANCE OF LEATHER PRODUCTION

MATERIAL GENERATED	kg/TON OF RAW SKIN/HIDE
Leather	150
Solid waste (skin trimming, fleshing, buffing, chrome shaving wastes)	850
Fleshing	400

TABLE 1.3:

SOLID WASTES GENERATED IN LEATHER INDUSTRY

TYPES OF SOLIDS WASTES GENERATED	PERCENTAGE
Hair	2-5%
Fleshing	50-60%
Chrome shavings, chrome splits and buffing dust	35-40%
Skin trimmings	5-7%

TABLE 5.1:

TYROSINE STANDARD

CONCENTRATION (µg/mg)	OPTICAL DENSITY AT 660nm
200	0.29
400	0.59
600	0.863
800	1.138
1000	1.601

TABLE 5.2:

PROTEIN ESTIMATION

CONCENTRATION (mg/ml)	OPTICAL DENSITY AT 660 nm
0	0
40	0.106
80	0.713
120	0.287
160	0.389
200	0.478

TABLE 5.3:

EFFECT OF INCUBATION PERIOD

PERIOD OF INCUBATION	CONTROL	TEST	ENZYME UNITS U/g
Day 1	0.240	0.254	220
Day 2	0.410	1.306	8082
Day 3	0.193	1,329	10224
Day 4	0.328	1.462	10206

TABLE 5.4:

EFFECT OF AGE OF THE INOCULUM

AGE OF INOCULUM	CONTROL O.D	TEST O.D	(CONTROL – TEST) O.D	ENZYME (U/g)
3 days	0.221	1.257	1.036	9324
5 days	0.195	1.195	1.000	9000
7 days	0.149	1.085	0.936	8429
9 days	0.260	1.114	0.854	7686

TABLE 5.5:

EFFECT OF PROTEIN ON PROTEASE PRODUCTION

CONCENTRATION (μg/ml)	OPTICAL DENSITY AT 640nm
Control	0.22
Peptone	0.28
Meat Extract	0.24
Milk extract	0.36
Peptone + Yeast Extract (0.05%)	0.32
Peptone + Glucose	0.28

TABLE 5.6:

EFFECT OF SIZE OF INOCULUM

SIZE OF THE INOCULUM (%)	CONTROL O.D	TEST O.D	(CONTROL – TEST) O.D	ENZYME UNITS U/g
10	0.306	1.572	1.266	11394
20	0.284	1.526	1.242	11178
30	0.290	1.471	1.181	10629
40	0.294	1.142	0.848	10062

TABLE 5.7:

VARYING FUNGAL BIOMASS CONCENTRATION vs CONSTANT CHROMIUM CONCENTRATION

FUNGAL BIOMASS (g/l)	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (g/l)
0.25	250	89.06	222.66
0.5	250	96.81	242.03
1.0	250	97.52	243.80
1.5	250	97.91	244.73

TABLE 5.8:

CONSTANT FUNGAL BIOMASS CONCENTRATION vs VARYING CHROMIUM CONCENTRATION

FUNGAL BIOMASS (g/l)	Cr CONCENTRATION. (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (g/l)
1.0	250	99.45	248.65
1.0	500	98.54	494.68
1.0	1000	93.30	956.06
1.0	2000	89.09	1932.95

TABLE 5.9:

VARYING PROTEIN BIOMASS CONCENTRATION vs CONSTANT CHROMIUM CONCENTRATION

PROTEIN WASTES (g/l)	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (g/l)
0.25	250	87.15	217.88
0.5	250	90.10	225.25
1.0	250	94.92	237.30
1.5	250	95.88	239.70

TABLE 5.10:

CONSTANT PROTEIN BIOMASS CONCENTRATION vs VARYING CHROMIUM CONCENTRATION

PROTEIN WASTES (g/l)	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (mg/l)
1.0	500	99.58	496.95
1.0	1000	95.88	979.80
1.0	2000	90. 91	1961.40
1.0	4000	87.56	3924.20

TABLE 5.11:

EFFECT OF pH ON BIOSORPTION USING FUNGAL BIOMASS

FUNGAL BIOMASS (g/l)	pH	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (mg/l)
0.5	1.6	1000	92.52	925.20
0.5	2.5	1000	92.61	926.01
0.5	3.5	1000	95.31	952.80
0.5	4.0	1000	94.19	941.60

TABLE 5.12:

EFFECT OF pH ON BIOSORPTION USING PROTEIN BIOMASS

PROTEIN WASTES (g/l)	pH	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (mg/l)
0.5	1.6	1000	96.66	966.68
0.5	2.5	1000	96.95	969.60
0.5	3.5	1000	97.97	979.60
0.5	4.0	1000	97.66	976.80
0.5	5.0	1000	96.96	969.60

TABLE 5.13:

EFFECT OF CONTACT TIME ON BIOSORPTION USING FUNGAL BIOMASS

FUNGAL BIOMASS (g/l)	TIME (min)	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (mg/l)
0.5	20	2000	96.45	1929
0.5	40	2000	96.69	1933.80
0.5	60	2000	96.86	1937.20
0.5	80	2000	96.99	1939.80
0.5	100	2000	97.18	1943.60
0.5	120	2000	97.47	1949.40
0.5	140	2000	97.50	1950.00

TABLE 5.14:

EFFECT OF CONTACT TIME ON BIOSORPTION USING PROTEIN BIOMASS

PROTEIN WASTES (g/l)	TIME (min)	Cr CONCENTRATION (mg/l)	% ADSORBED	TOTAL Cr ADSORBED (mg/l)
0.5	20	2000	97.50	1950.0
0.5	40	2000	97.55	1951.2
0.5	60	2000	97.85	1957.0
0.5	80	2000	97.94	1958.8
0.5	100	2000	98.10	1962.0
0.5	120	2000	98.15	1963.0
0.5	140	2000	98.16	1963.2
0.5	160	2000	98.16	1963.2

TABLE 5.15:

BIOSORPTIVE STUDIES USING PROTEIN BIOMASS FROM CHROME LIQUOR

LEATHER BIOMASS (PROTEIN BIOMASS) (g)	Cr CONCENTRATION (mg/l)	Cr ADSORBED (mg/152 ml)	TOTAL Cr ADSORBED (mg/l)
1.0	39	0.5187	1.256
1.0	78	0.7331	2.669
1.0	117	0.9555	3.380
1.0	156	1.120	7.368
1.0	195	1.380	9.078
1.0	234	1.703	11.203
1.0	273	2.061	13.559
1.0	312	2.449	16.11

TABLE 5.16:

BIOSORPTIVE STUDIES USING FUNGAL BIOMASS FROM Cr LIQUOR

FUNGAL BIOMASS (g)	Cr CONCENTRATION (mg/l)	ADSORBED (mg/152 ml)	TOTAL Cr ADSORBED (mg/l)
1.0	39	0.1047	0.688
1.0	78	0.4712	3.1
1.0	117	0.6316	4.155
1.0	156	0.7838	5.156
1.0	195	0.9131	6.007
1.0	234	1.4870	9.782
1.0	273	2.552	16.789
1.0	312	3.256	21.421

TABLE 5.17:
DESORPTION STUDIES USING DIFFERENT ACID STRENGTH

ACID STRENGTH (N)	% OF Cr DESORBED
0.25	13.6
0.5	45.3
1.0	79.7
2.0	87.6

TABLE 5.18:

ANALYSIS OF CHROME SHAVINGS

PARAMETERS	STANDARDIZED VALUES
pH	3.5
Moisture (Room Temperature)	15
Total Solids	79.5
Total Nitrogen	14.4
Total Protein	90
Total Chromium as Cr (VI)	5.25

TABLE 5.19:

LEATHER HYDROLYSIS USING VARYING CONCENTRATIONS OF THE ENZYME

ENZYME CONCENTRATION (U/g)	% TKN	% HYDROLYSIS	PROTEIN (%)
1	8.0	64	58.82
2	8.5	70	62.5
3	8.7	72	63.97
4	8.8	86	64.70
6	9.0	88	66.18
8	9.2	90	67.64
10	8.7	92	67.64

TABLE 5.20:

LEATHER HYDROLYSIS AT VARYING TEMPERATURE

TEMPERATURE (^{O}C)	% HYDROLYSIS
45	74
50	82
55	94
60	80

TABLE 5.21:

EFFECT OF pH ON LEATHER HYDROLYSIS

pH	% OF HYDROLYSIS
7.5	72
8.0	76
8.5	80
9.0	84
9.5	90
10.0	80
10.5	71

TABLE 5.22:

FT-IR ADSORPTION BANDS AND CORRESPONDING POSSIBLE GROUPS

FREQUENCY (cm^{-1})	FUNCTIONAL GROUP
3430	-0H, -NH
2895	-CH
1640	-COO, -C=O
1055	-C-O, C-N
610	-CH

FIGURE 4.1:

LIQUID SUBSTRATE FERMENTATION OF FUNGAL BIOMASS

FIGURE 4.2:

FUNGAL BIOMASS RETRIVED FROM LIQUID FERMENTATION (WET BIOMASS)

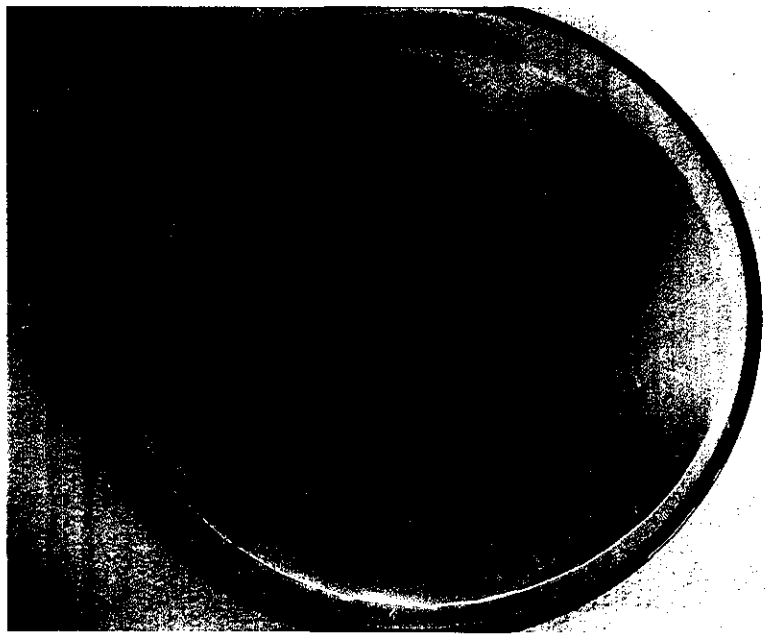


FIGURE 4.3:

DRIED FUNGAL BIOMASS

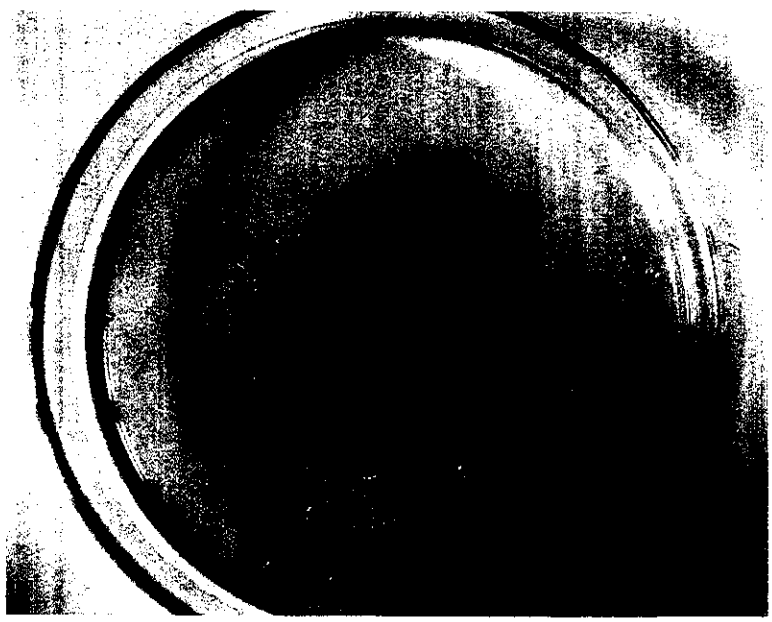


FIGURE 4.4:

DRY POWDERED FUNGAL BIOMASS

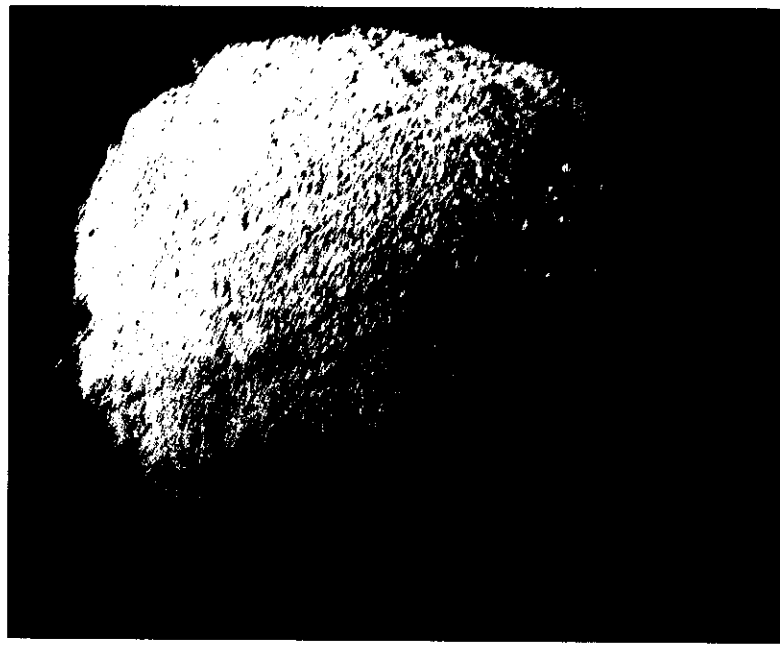


FIGURE 5.1:

TYROSINE STANDARD GRAPH

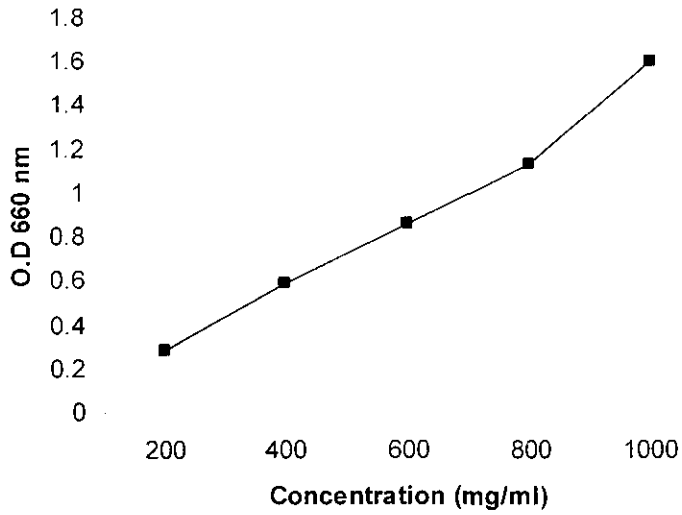


FIGURE 5.2:

PROTEIN STANDARD GRAPH

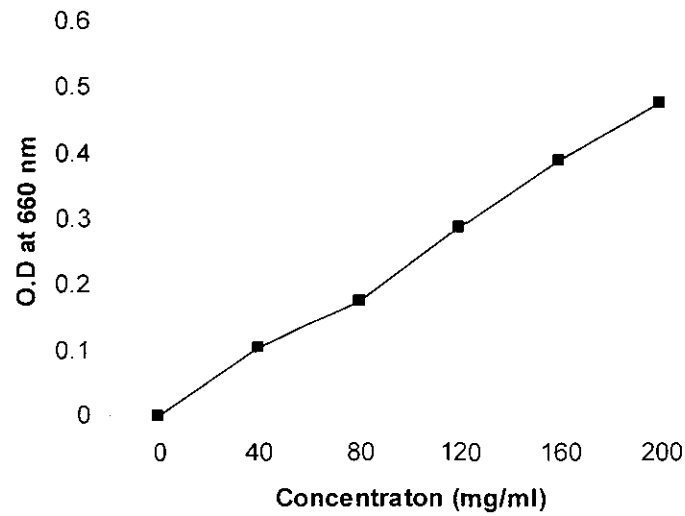


FIGURE 5.3:

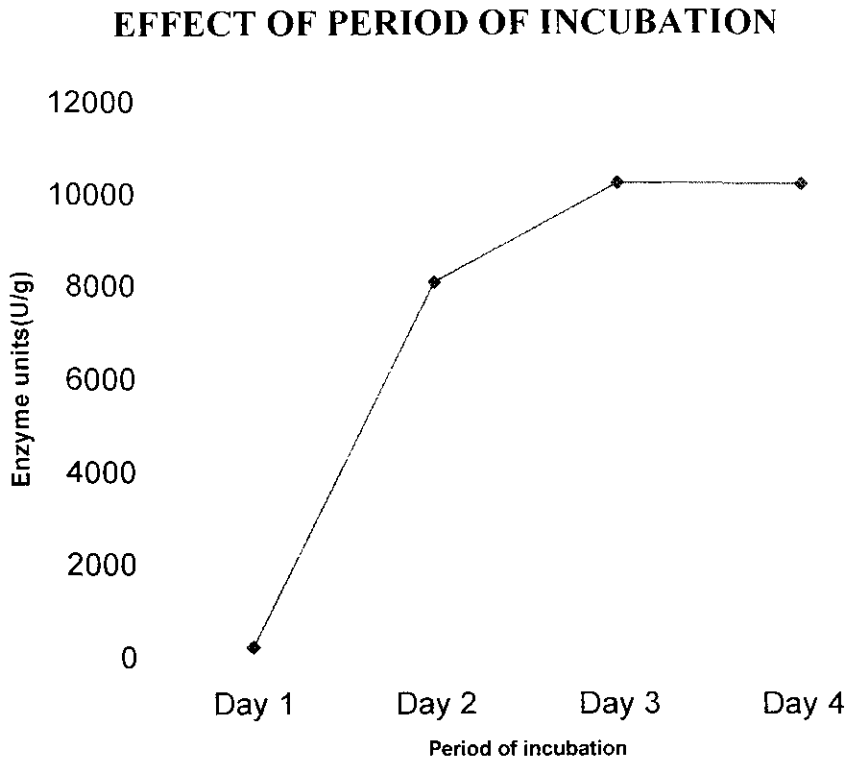


FIGURE 5.4:

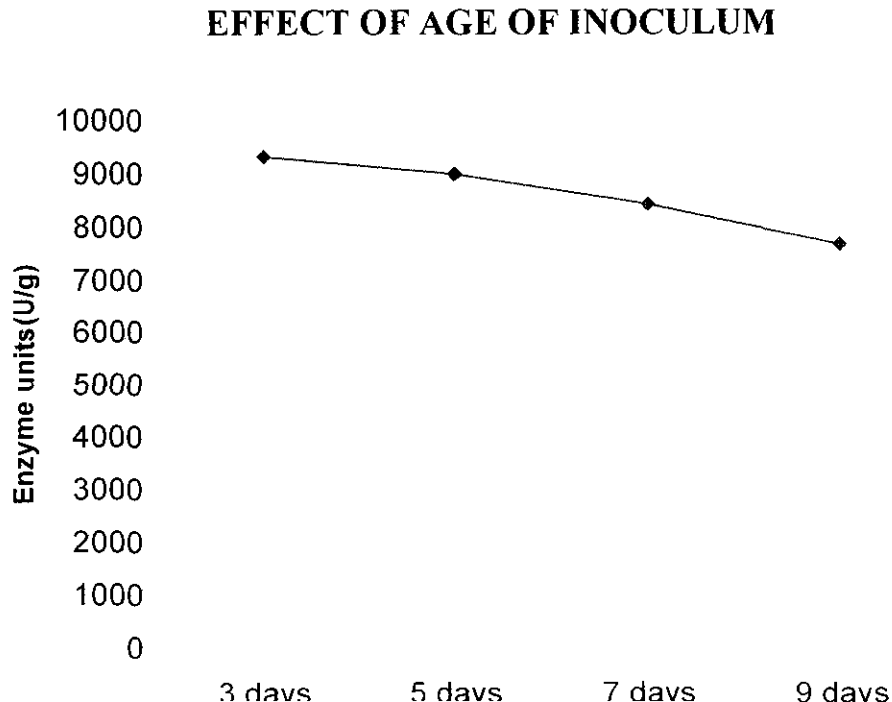
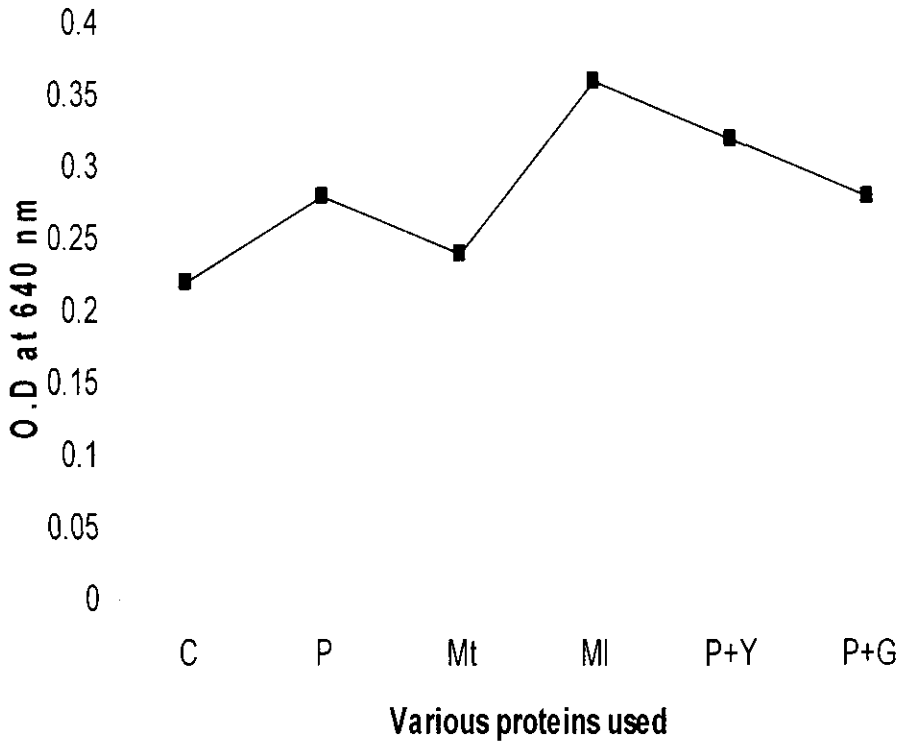


FIGURE 5.5:

EFFECT OF VARIOUS PROTEIN USED IN MEDIA ON PROTEASE PRODUCTION



LEGEND

C - Control
P – Peptone
Mt – Meat Extract
Ml – Milk Extract
P+Y – Peptone +Yeast Extract
P + G – Peptone + Glucose

FIGURE 5.6:

EFFECT OF THE SIZE OF THE INOCULUM

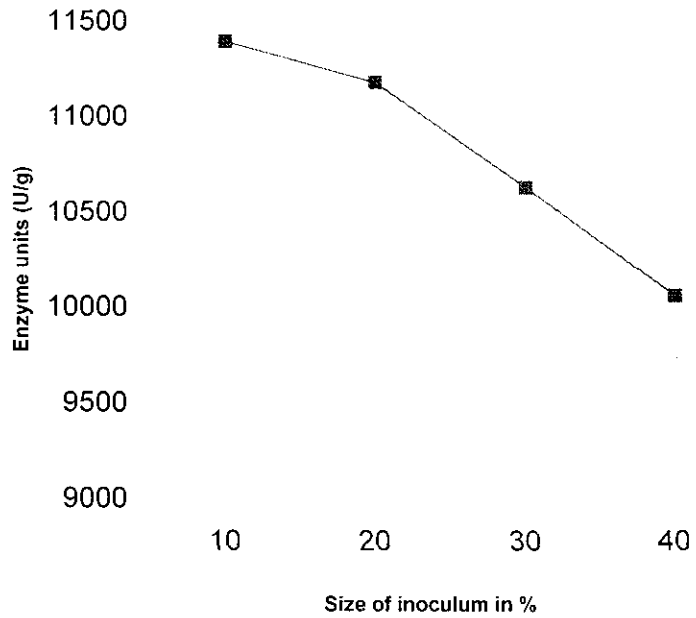


FIGURE 5.7:

VARYING FUNGAL BIOMASS CONCENTRATION vs CONSTANT CHROMIUM CONCENTRATION

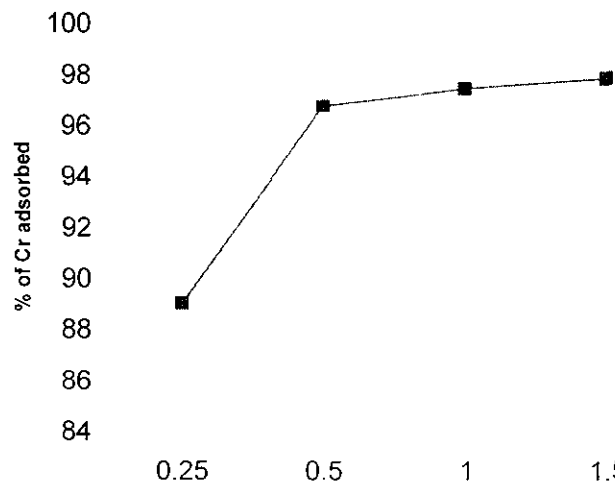


FIGURE 5.8:

VARYING CHROMIUM CONCENTRATION vs CONSTANT FUNGAL BIOMASS CONCENTRATION

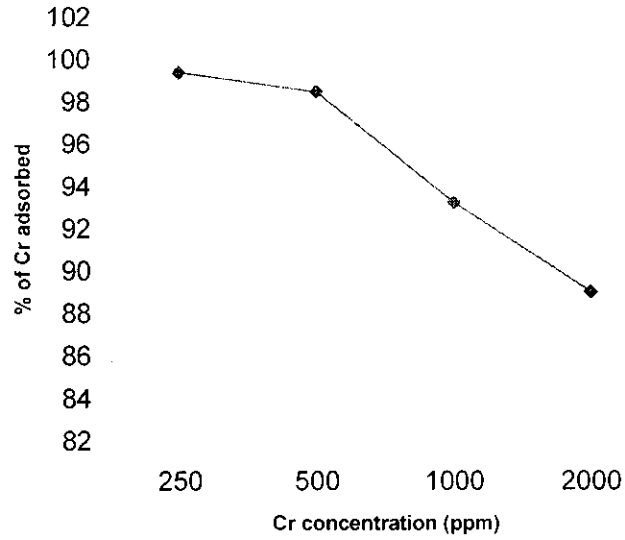


FIGURE 5.9:

VARYING PROTEIN BIOMASS CONCENTRATION vs CONSTANT CHROMIUM CONCENTRATION

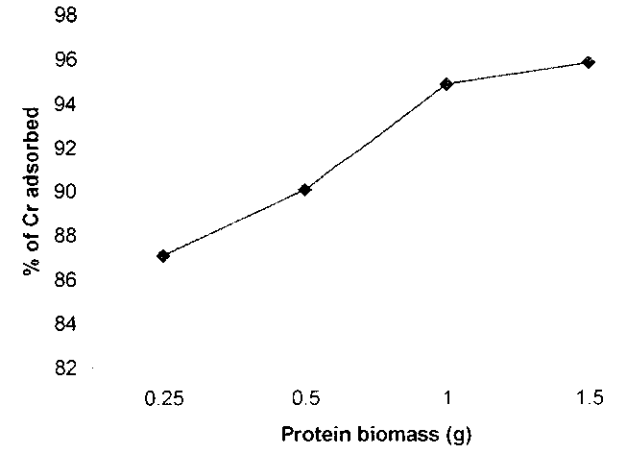


FIGURE 5.10:

EFFECT OF pH ON BIOSORPTION USING FUNGAL BIOMASS

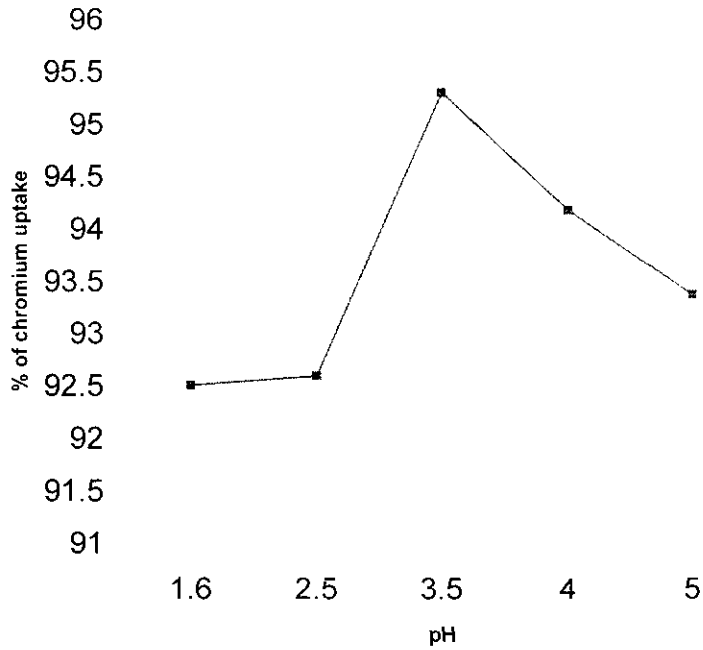


FIGURE 5.11:

EFFECT OF pH ON BIOSORPTION USING PROTEIN BIOMASS

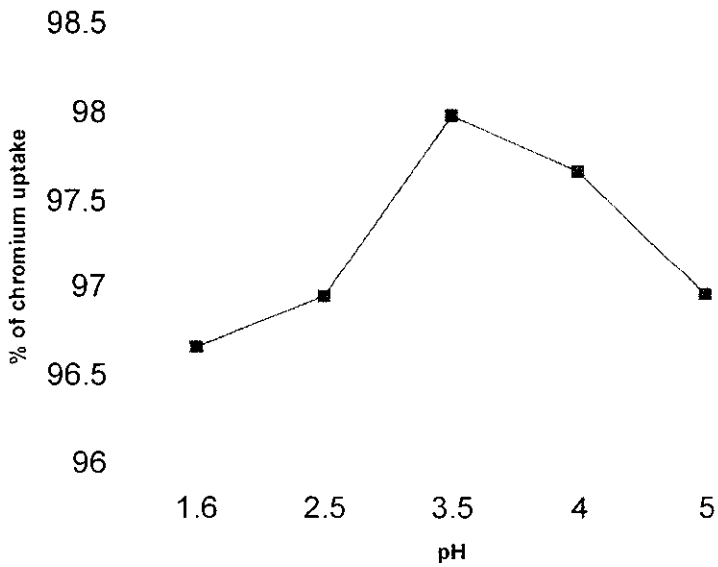


FIGURE 5.12:

EFFECT OF CONTACT TIME ON BIOSORPTION USING FUNGAL BIOMASS

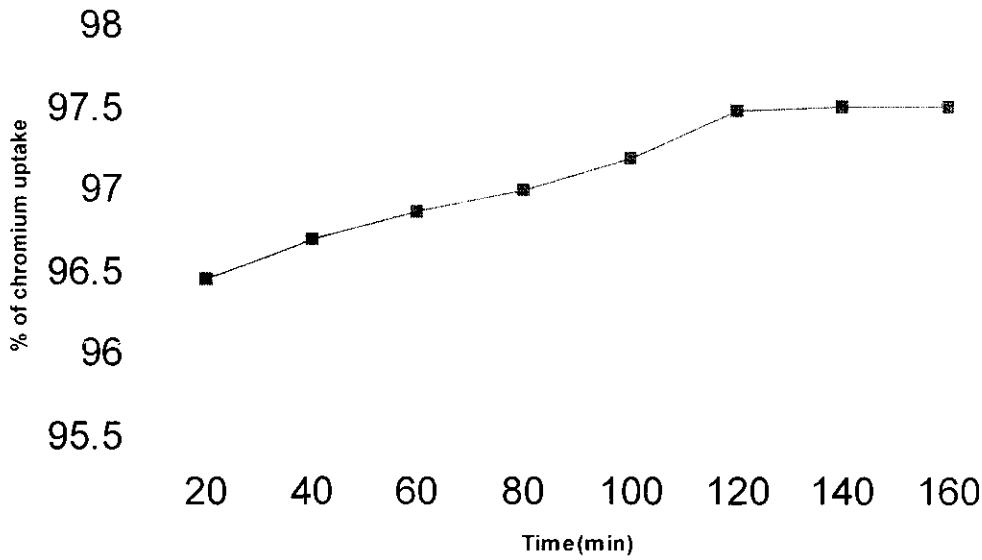


FIGURE 5.13:

EFFECT OF CONTACT TIME ON BIOSORPTION USING PROTEIN BIOMASS

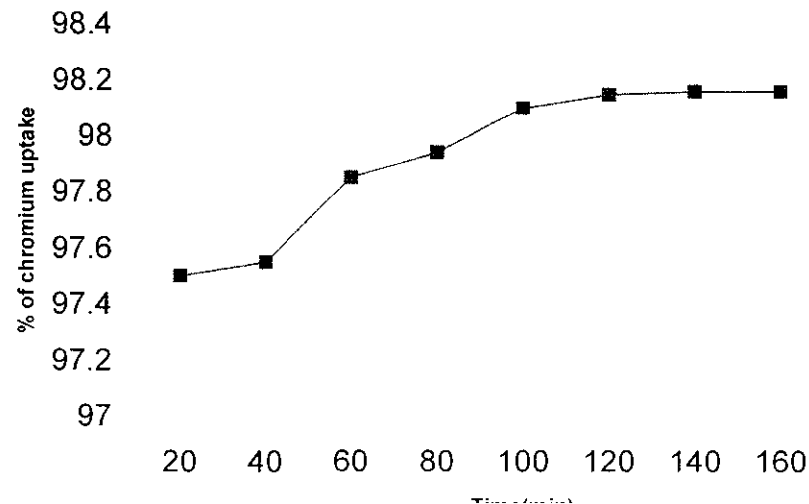


FIGURE 5.14:

BIOSORPTIVE STUDIES USING PROTEIN BIOMASS FROM Cr LIQUOR *(Freundlich Isotherm)*

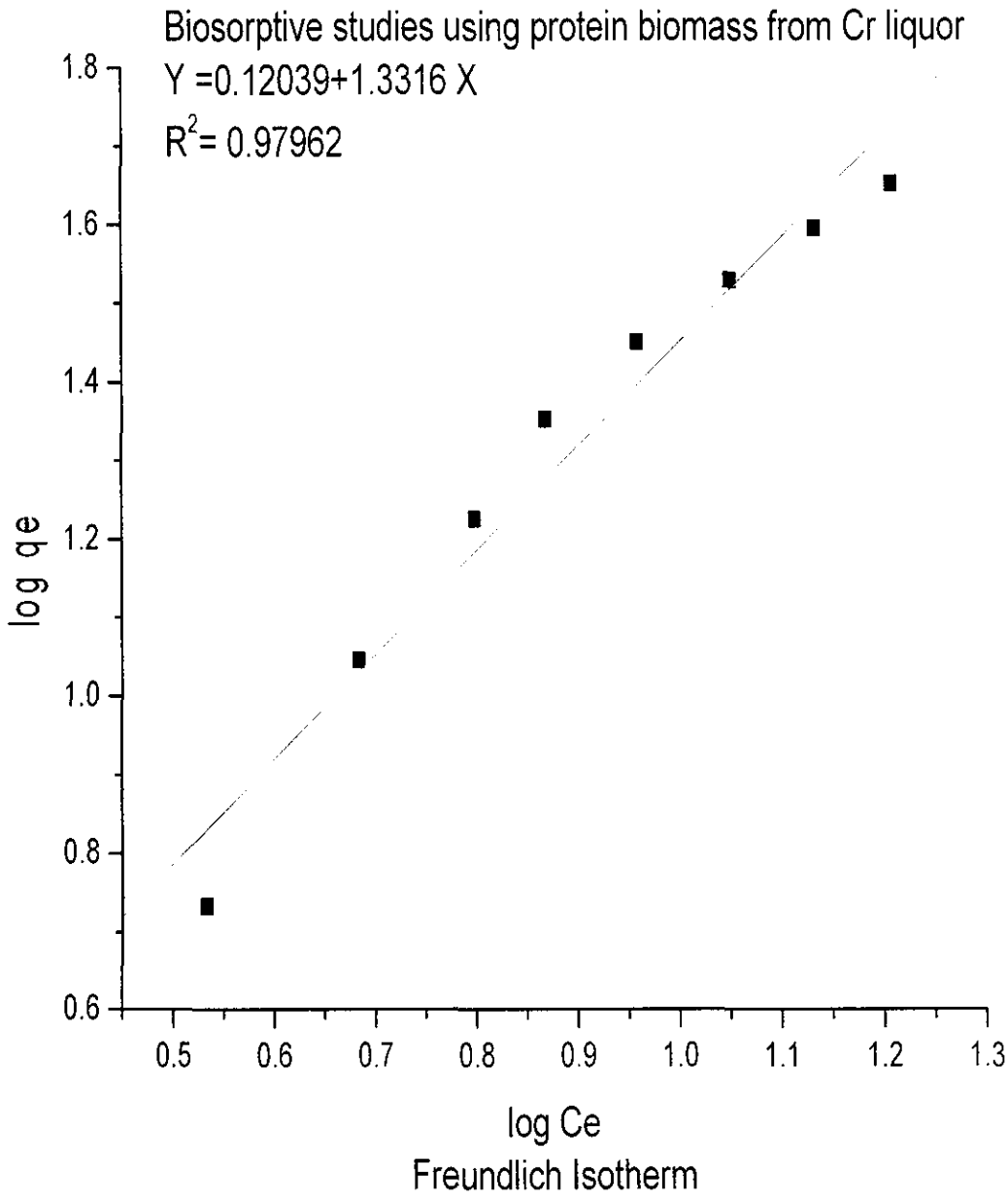


FIGURE 5.15:

BIOSORPTIVE STUDIES USING FUNGAL BIOMASS FROM Cr LIQUOR *(Freundlich Isotherm)*

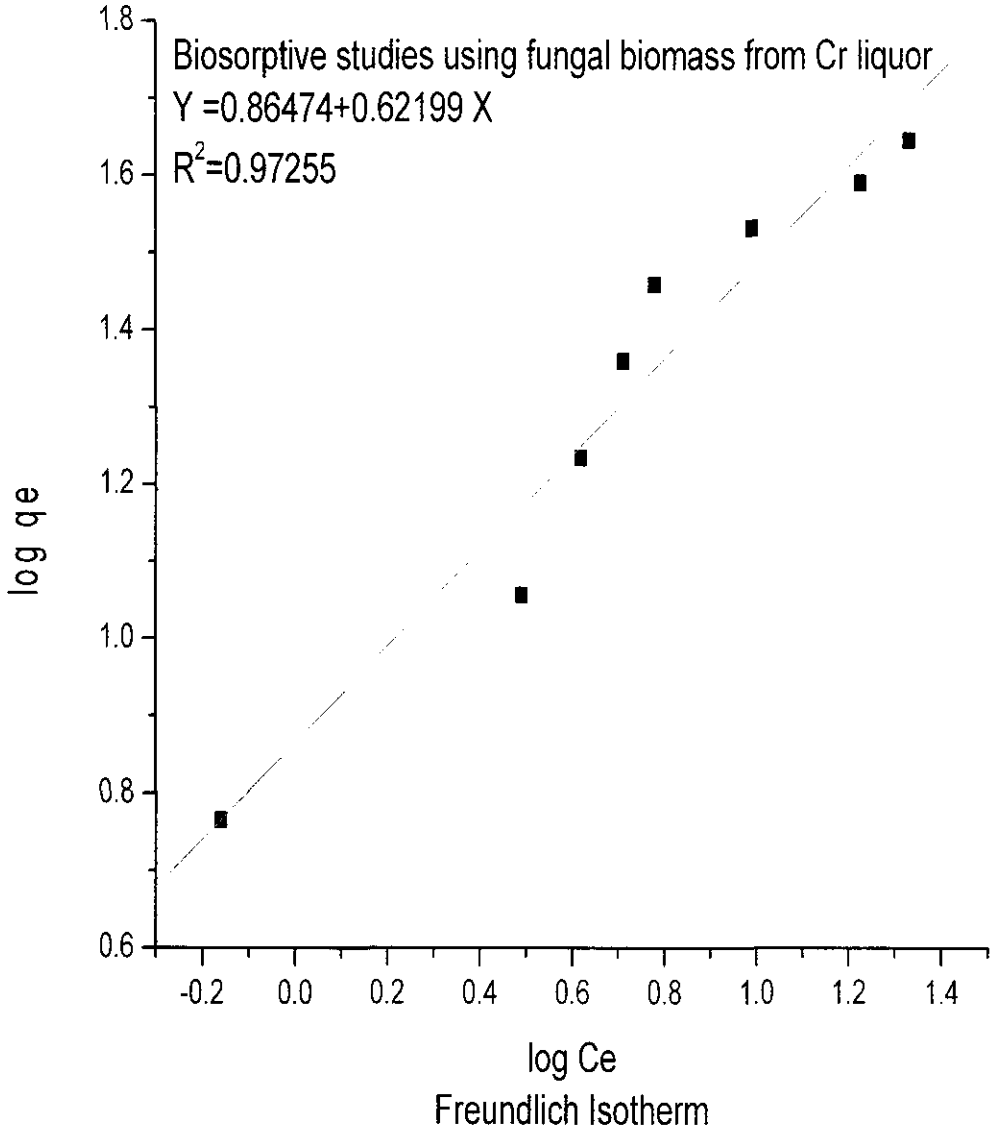


FIGURE 5.16:

EFFECT OF CONTACT TIME ON BIOSORPTION USING PROTEIN BIOMASS *(Pseudo second order)*

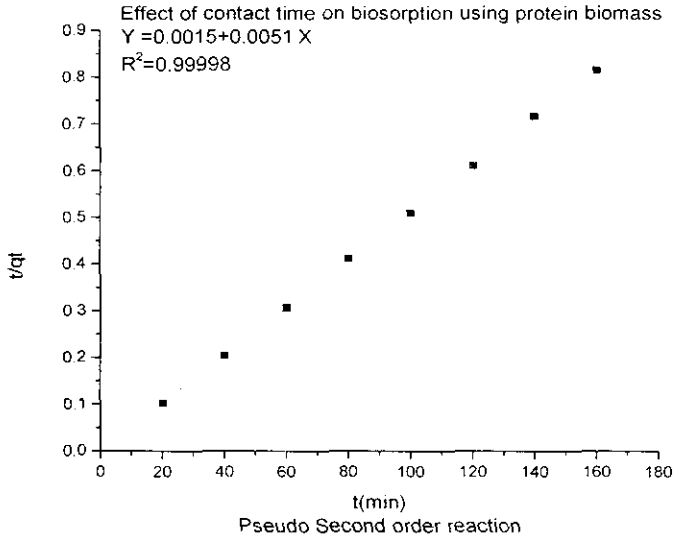


FIGURE 5.17:

EFFECT OF CONTACT TIME ON BIOSORPTION USING FUNGAL BIOMASS *(Pseudo second order)*

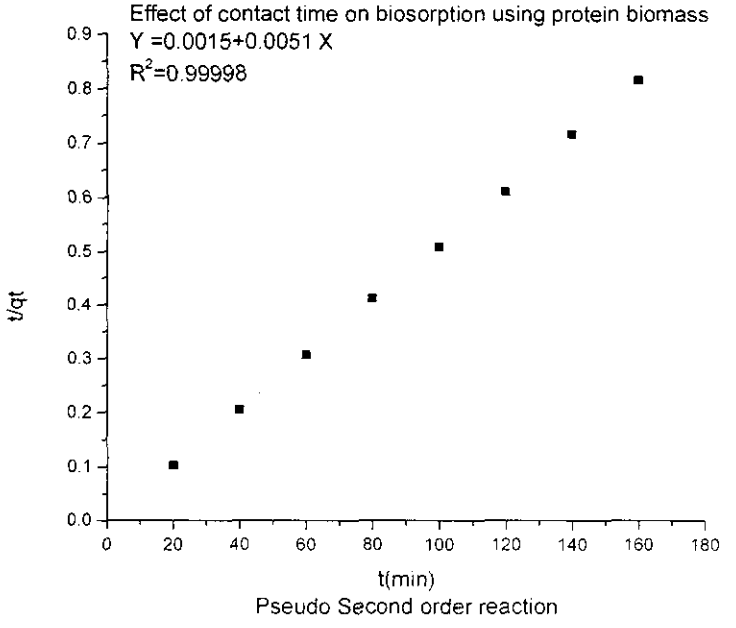


FIGURE 5.18:
DESORPTION STUDIES USING DIFFERENT ACID STRENGTH

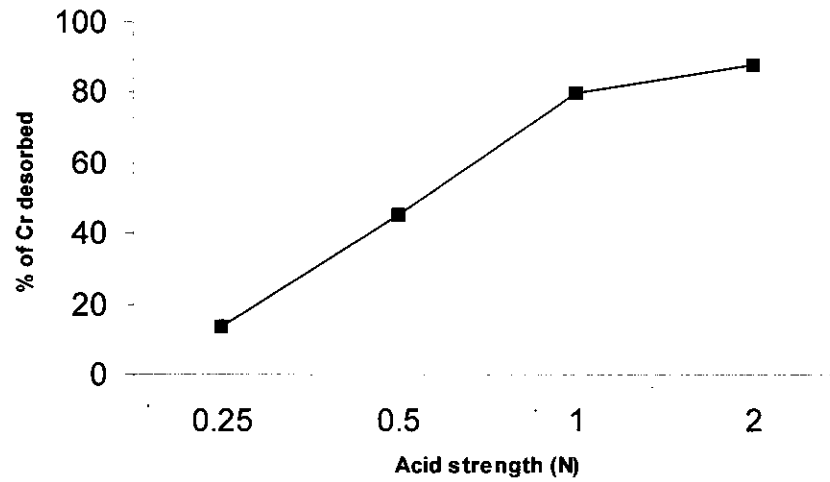


FIGURE 5.19:

ISOLATED FUNGAL CULTURE (*Aspergillus sp.,*)

FIGURE 5.20:

FT – IR ANALYSIS OF NON TREATED FUNGAL BIOMASS (CONTROL)

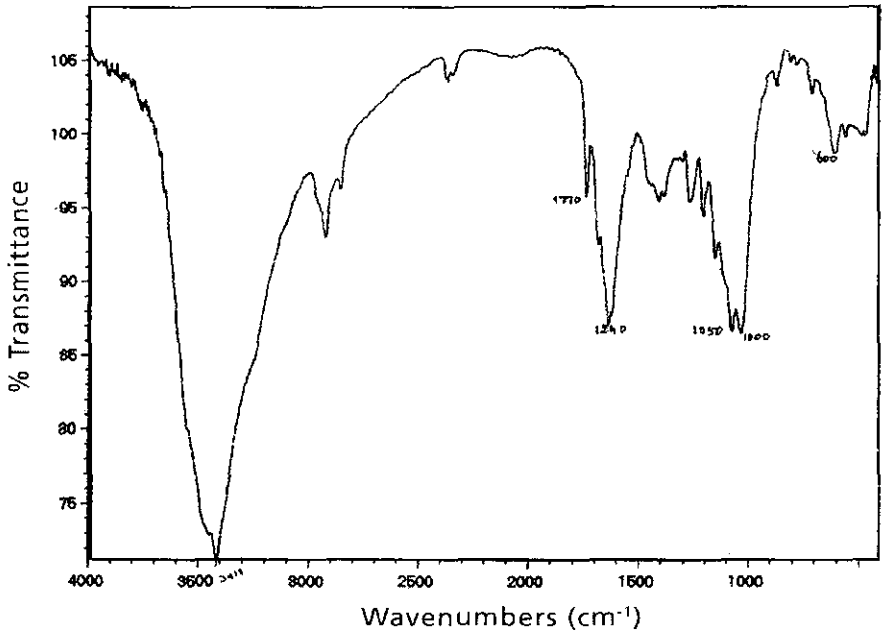
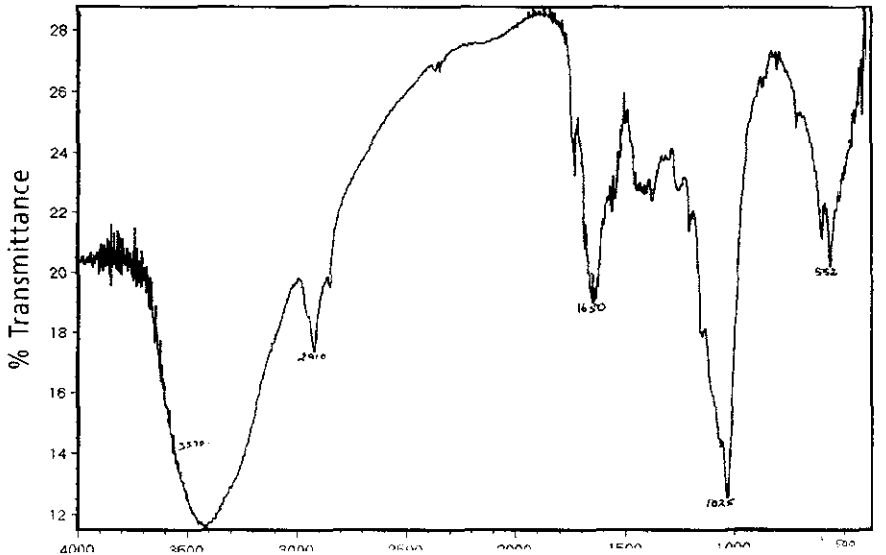


FIGURE 5.21:

FT – IR ANALYSIS OF CHROME TREATED FUNGAL BIOMASS (TEST)



6.0 CONCLUSION:

The present study gives substantiate results to use the fungal biomass and leather waste scrapings as suitable adsorbents for the removal and reuse of chromium from tannery effluent. It is so feasible and economic that the application of the biological means for the removal chromium from chrome bearing tannery wastewater not only gives a low cost treatment option but also recycles chromium for leather tanning. Leather scrapings i.e., the protein biomass can be very much preferred over the fungal because of the fact that they are very much cheaper and is a waste bi-product of leather tanning. Also, the enzymes used for leather hydrolysis is used in very small quantities 6 U/g. It ensures a complete break down of the leather waste. This hydrolysis process is so simple, economic and without any risk and the hydrolyzed protein would be purified and used as an animal feed, pharmaceutical industries etc. The chrome cake can be reused in tanning of leathers.

Thus the study proves to be an application oriented work with lot of potential towards the removal and reuse of Cr (VI) that is a potent carcinogen. It is also very much economical and industry focused.

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