ENZYMATIC REMOVAL OF DUNG FROM CATTLE HIDES

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This thesis is submitted for the degree of Doctor of Philosophy

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September, 2000

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ABSTRACT

One of the major problems facing the meat and leather industries is dung cladding on hides. Dung dried onto hides is difficult to remove during the early stages of processing, resulting in the necessity for prolonging the soaking step, with the consequence of risking bacterial damage to the hides.

Microscopic examination of dung on hide reveals that adhesion is between the dung and the hair alone, with no sticking of the dung to the epidermis. This accounts for the difficulty in removing dry dung; the matrix of the hair within the dung creates a strong composite material.

Analysis shows that major components of the composition of dung are lignocellulosic materials (lignin, hemicellulose and cellulose). This project concerns solubilising the dung faster, to reduce soaking time by targeting these components with enzyme technology, using hemicellulases, cellulases, and laccases from commercial products and a range of fungal organisms (*Coriolus versicolor* and *Aspergillus niger*).

Removal of dung from hide pieces is affected by the action of cellulase, xylanase and laccase separately over 20-24 hours. Mixture of these enzymes removes dung in 6-8 hours demonstrating that synergistic activity is more effective than separate enzyme treatments.

When commercial enzyme mixture or *Coriolus versicolor* or *Aspergillus niger* cultural broths were used to effect dung removal in tannery drums in a larger scale experiment, in some cases the removal of dung was observed within 1 hour by the help of the mechanical action and nonionic surfactant. The enzyme treatment did not affect the components of hide, such as collagen, hyaluronic acid and dermatan sulphate and it was found that enzyme treated leathers and control samples showed no differences in terms of their physical characteristics.

Investigations in the mechanism of dung removal by spectrophotometry, chromatography and chemical techniques show that the enzyme treatments remove the dung from hides without changing the fundamental structure of the substrate. The enzymes probably attack the structural parts in lignocellulose, which are most susceptible to solubilising with enzymes: these attacks make small changes in the carbohydrate and lignin contents, but these changes are powerful enough for effective dung removal.

This thesis is dedicated to my beloved parents,

Nevzat and Gűlşen

ACKNOWLEDGEMENT

I should like to take this opportunity to express my gratitude to all those who played a role in the execution and presentation of this work. I should like to thank the following:

My supervisors, Professor Anthony Covington, from BSLT and Professor Christine Evans, from the Fungal Biotechnology Group, University of Westminister, for their interest, guidance and support. I would like to thank Turkish Ministry of Education, Department of Higher Education, for their financial support throughout this period. I would like to thank Dr. R. Law, Department of Chemistry, Imperial College, for his help in solid-state NMR experiments. I would like to give my thanks to Professor G.E. Attenburrow and Mr. E.M. Pickles for useful discussions on physical testing aspects of my project. I would like to thank staff from BSLT, especially Mrs. Pat Potter and Mrs. Sue Maloney for their assistance and patience during my practical work. I should like to extend thanks to the staff of the Fungal Biotechnology Group, University of Westminister, for their assistance. I would like to thank all my colleagues in BSLT Research Group, especially Mr. M. Syed and Mrs. S. Jeyapalina for their friendship and support. I should like to thank Mrs. S. Bobuş for her continuous encouragement, moral and patience during these years. Thanks to my closest friends, Dr. S. Cukur, Mr. R. Tekeli, Mr. C. Altan, Mr. U. Cihaner and Mr. S. Savranoğlu.

Finally, I would like to thank my parents, Nevzat and Gűlşen, my sisters, Emine and Esin, my brother Hakan and my little nephew Ege for their support, moral and encouragement. Thank you all.

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ABBREVIATIONS

Å: Armstrong

BLC: British Leather Confederation

BSA: Bovine Serum Albumin

BSLT: British School of Leather Technology

^{13}C CP/MAS NMR: Carbon-13 Cross Polarisation-Magic Angle Spinning Nuclear Magnetic Spectroscopy

CBH: Cellobiohydrolase

CMC: Carboxylmethycellulose

COD: Chemical Oxygen Demand

DMAB: Dimethylaminobenzaldehyde

DMSO: Dimethysulphoxide

DNS: Dinitrosalicylic acid

EDTA: Ethylenediaminetetraacetic acid

EPR: Electron Paramagnetic Resonance

ESR: Electron Spin Resonance

FT/IR: Fourier Transform Infrared Spectroscopy

GAG: Glycosaminoglycans

GC/MS: Gas Chromatography- Mass Spectroscopy

GPC: Gel Permeation Chromatography

HACCP: Hazard Analysis and Critical Control Point

IR: Infrared Spectroscopy

IUP: International Union of Physical Testing

kD: kilo Dalton

LiP: Lignin Peroxidase

MHS: Meat Hygiene Service

OVC: Official Veterinary Surgeon

SD: Standard Deviation

U/ml: Enzyme unit per millilitre

µl: micro litre

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND INFORMATION ABOUT LEATHER MANUFACTURING

The leather industry is one of the oldest industries in the world. It may be considered as a bridge between production of the hide as a by-product of the meat industry and its manufacture into shoes and wearing apparel.

The practice of leather manufacture varies considerably from tannery to tannery and from country to country, so that there is no single universally applied process. The main sequences of operations and their aims are the same, however, there are wide differences in detailed techniques (O'Flaherty *et al.*, 1956).

The principal types of raw hides and skins used for leather industry are cattle (ox and cow), calf, sheep and goat. After hides and skins are removed from the animal, they undergo the curing operation in which they receive either long term or short term protection against bacteria. The most common method of preservation of hides and skins is curing with common salt, sodium chloride (Sharphouse, 1971; Tuck, 1981).

Leather manufacture commences with the soaking of cured stock in water to rehydrate and to cleanse it. The hides are restored as far as possible to their freshly flayed condition, with fibre structure fully hydrated and separated. The rehydration process can be aided by chemicals (to adjust the pH), surface active agents, bactericides and enzymes (O'Flaherty *et al.*, 1956; Heidemann, 1993).

In most cases the removal of the hair and wool and epidermis layer is necessary in leather production. It can be achieved in various ways. The most common systems employ the use of sodium sulphide, an alkaline reducing agent, and lime but both enzymes and oxidising agents have been developed for this purpose to avoid the presence of sulphides in the effluent. Moreover, the physical and chemical modifications of the fibre structure take place in the liming-unhairing operation (Thorstensen. 1993).

After the liming process, the removal of alkali from limed pelts is carried out by the use of acidic materials, mainly ammonium salts (ammonium sulphate and ammonium chloride). This is followed by the bating process, during which the stock is subjected to the action of proteolytic enzymes. Finally, the pelt is pickled in solution of acids and salts, before being chrome tanned. The reaction of chromium salts with carboxylic groups of the hide protein, collagen, leads to high hydrothermal stability and stability against microbial action of the hides. After chrome tanning, hides and skins are called wet-blue (Heidemann, 1993; Covington, 1997).

Most leather requires retannage by using a variety of tanning materials. In general, the retannage gives specific properties to the leather such as tightness and fullness. The process of fatliquoring involves the treatment of leather with oil emulsion to lubricate the fibre structure and to control or adjust the physical properties of the finished leather. Leathers are mainly dyed with either acid or direct dyestuffs; the former is used when a penetrated dyeing is required and the latter when surface dyeing is required (Tuck, 1981).

After post tanning operations, the leather undergoes drying and mechanical operations. The final operation in leather manufacturing is finishing. The aim of finishing is to produce fashionable, attractive leather with a standardised and uniform

appearance. At the same time, the finish protects the leather surface and assists in its care and maintenance (Sharphouse, 1971).

Having discussed very briefly the leather manufacturing stages, in the next section, the dung problem in the leather and meat industries and its implications will be discussed.

1.2. DUNG PROBLEM IN LEATHER INDUSTRY

Contamination of hide with dung is a common problem in many parts of the world. It occurs mainly with beef cattle; dairy cattle are kept cleaner for the prevention of mastitis that can affect milk production (Pearson, 1994). The dung problem happens mainly in the winter period of the year when cattle are housed (Pearson, 1995). There is usually only a limited amount of space in the housing and so after the animal has defecated it cannot move away and at some stage will either sit or lie in the faeces that stick to the hide and are carried around by the animal (Pearson, 1994). Figures 1.1 and 1.2 illustrate heavily dung contaminated cattle and sheep. Figure 1.3 shows a dung contaminated hide (the amount of dung on this particular hide is over 20 kg).

The British Leather Confederation (BLC) have been conducting a dung survey since 1994 in order to provide quantitative evidence of the levels of contamination of animals and illustrate the seriousness of the problem. Information collected was based on four criteria: where the hides originated; the weight category; how much dung was on the hide and the date the assessment was made. The summary of cattle hide dung contamination from 1994 to 1998 is given in Table 1.1. Regional variations in dung contamination can be seen in Table 1.2. Also, the effects of hide weight and seasonal variations on dung contamination are illustrated in Table 1.3 and Figure 1.4 respectively.

Figure 1.1: Heavily dung contaminated cattle (from MHS operation manual, 1997)

Figure 1.2: Heavily dung contaminated sheep (from MHS operations manual, 1997)

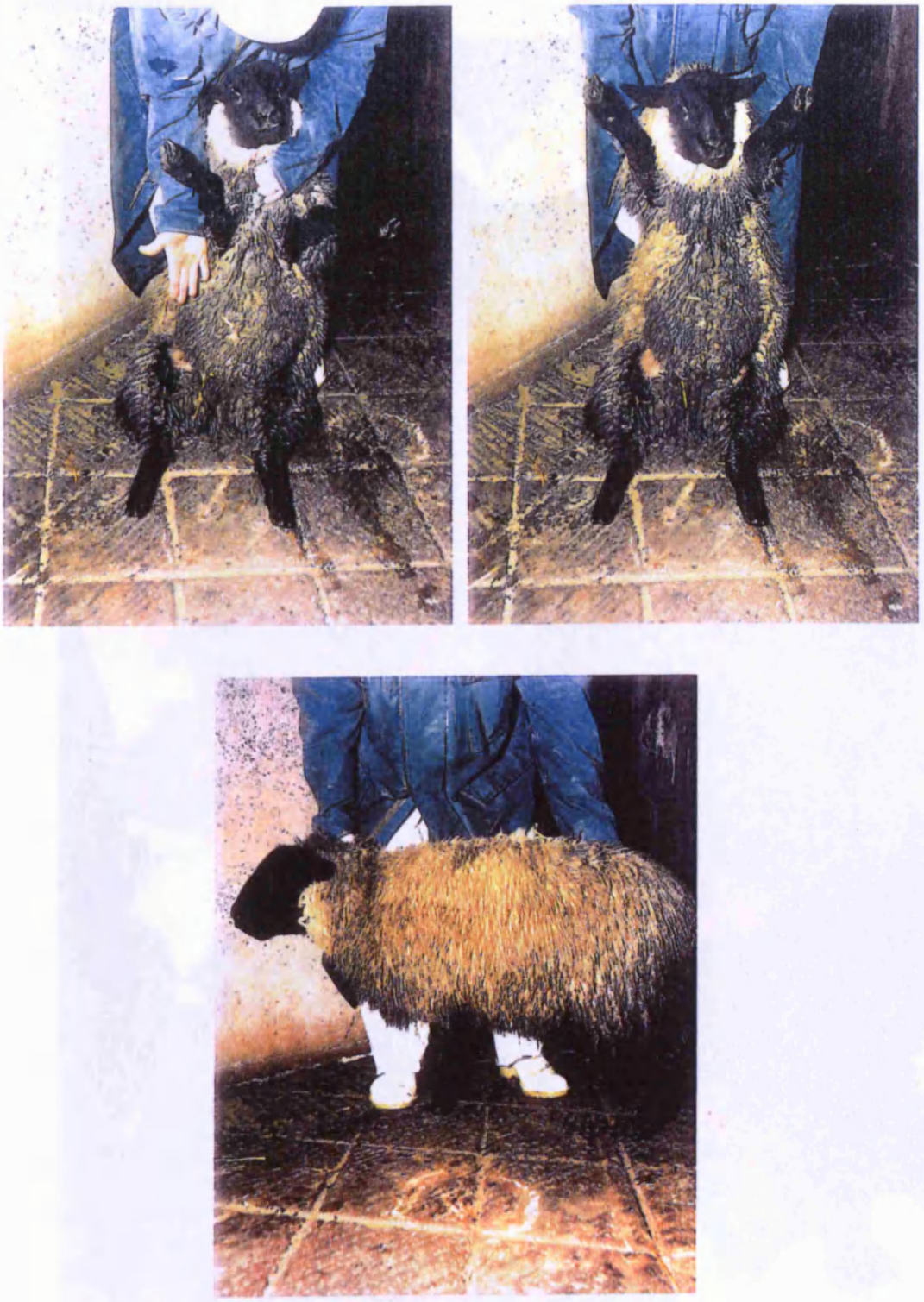


Figure 1.3: Heavily dung clad hide containing over 20 kg dung (from British Leather Confederation)

The survey results indicate that there is a real problem over the winter months, with the situation improved in May. The dirtiest hides that have been reported so far can carry up to 20 kg of dung on them. Although the results indicate that hides from England are in better condition than those from the other sources, quite a number of English hides are still contaminated. Overall there appears to have been an improvement in the levels of contaminated animals going through the UK slaughtering process. However, there are still significant numbers of heavily contaminated animals originating from Northern and Southern Ireland. This apparent improvement may be partly due to increased awareness of hygiene problems or to the mild weather and reduced housing period.

Table 1.1: The summary of cattle hide dung contamination results from 1994 to 1998 (from BLC- Dung Survey, 1998).

	1994		**1995**		**1996**		**1997**		**1998**	
	Ox	**Cow**	**Ox**	**Cow**	**Ox**	**Cow**	**Ox**	**Cow**	**Ox**	**Cow**
Hides inspected	8263	2222	3282	745	15628	-	4021	3915	18872	4132
Amount of dung/hide	2.5	0.3	1.8	0.5	3.7	-	1.4	0.3	1.3	0.3
% of hides with dung	53	27	33	17	73	-	47	15	49.2	19.2
% of hides with over 4.5 kg dung	22	2	17	3	36	-	11	1	8.6	0.7

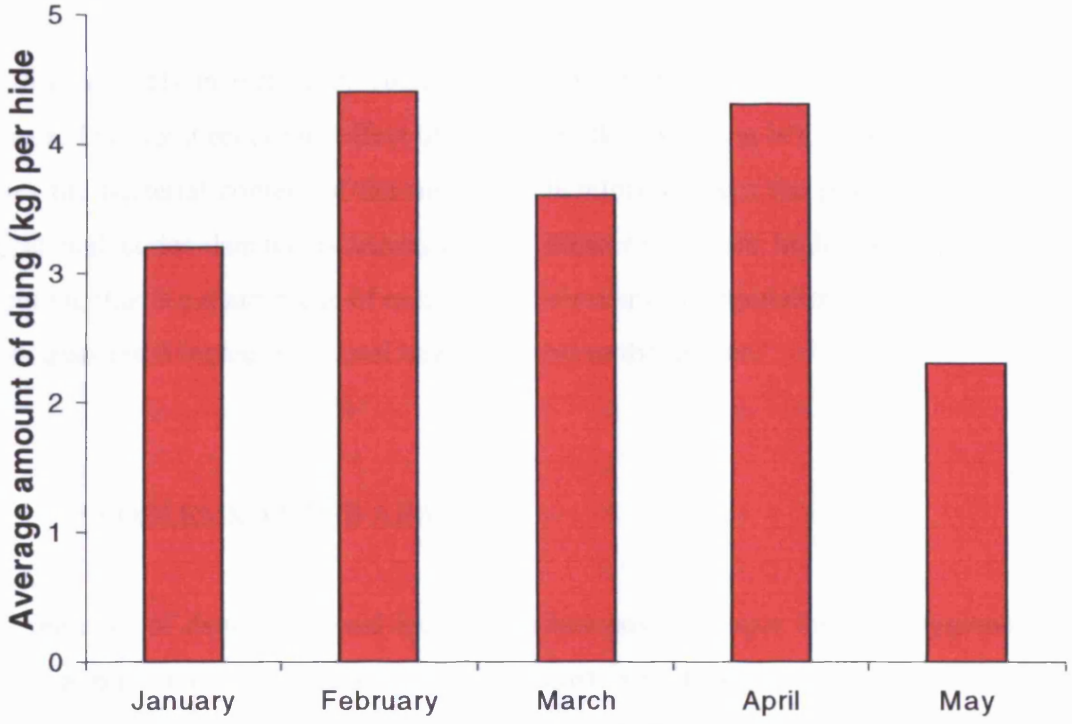
Table 1.2: Yearly comparison of average amount of dung per ox hide/(kg) by country (from BLC- Dung Survey, 1998)

Country	1994	1995	1996	1997	1998
England	1.2	1.6	2.4	0.9	0.33
Northern Ireland	5.3	2.4	4.3	2.82	1.08
Scotland	2.2	1.6	1.9	1.5	0.23
Southern Ireland	2.4	3.0	5.6	1.36	2.04

Table 1.3: Hide weight variations in ox/heifer hides affected by dung (Stosic, 1998)

	Hides Affected by Dung (%)		
Weight Category	**1994**	**1995**	**1996**
22-22.5 kg	-	8	44
26-30.5 kg	34	14	54
31-35.5 kg	41	7	74
36 kg upwards	64	59	80

Figure 1.4: Average amount of dung (kg) per hide on a monthly basis in 1996 (Stosic, 1996) .



A report from the Federal Meat Research Institute at Kulmbach, Germany, shows that every second bull and every third cow in Germany are excessively dung covered in the winter period (Schroer, 1995). Moreover, it is evident that dung contamination of hides is a very common problem in USA and Australia, which are the major hide producers in the world.

Contamination of animals with dung and dirt can cause a number of problems at the farm, the abattoir and the tannery. Excessive amounts of dung on animals affects the quality of milk, meat and the subsequent leather.

1.2.1 PROBLEMS AT THE FARM

Dung and dirt accumulate on cattle when they are held in enclosed or confined conditions. Prolonged contact with dung results in severe irritation of the skin and can result in secondary bacterial infections of bites, stretches and wounds etc.

Holding animals in wet, dirty conditions can promote the spread of parasites and viruses. The worst economic effect of dung in milk production is that lack of hygiene raises the bacterial content of the milk and therefore reduces the price. The risk of nipple and udder damage is increased and these organs are highly susceptible to infection, this is a main cause of mastitis and consequent amputation. There will also be an associated increased animal treatment cost to the farmer.

1.2.2 PROBLEMS AT THE ABATTOIR

The presence of dung on animal hides and skins poses a major threat to hygiene in the abattoir. The hide represents the most significant source of bacterial contamination for red meat carcasses (Newton *et al.*, 1978). Manure, whether wet or dry, hard or soft, large or small, adheres in clumps to the hide of the legs, belly and side of the animal, so careless removal can result in the transfer of bacteria to the carcass surface (Donkersgoed *et al.*, 1997). There is also visually evaluated evidence that excessive dunginess of the hide leads to increased microbial surface contamination of the carcass (Ridell and Korkeala, 1993). Moreover it was indicated that there is a direct correlation between the microbial loads on a cattle hide and on the carcass surface (Newton *et al.*, 1978). Table 1.4 shows the mean aerobic plate counts (APC) 37°C and *Escherichia coli* numbers for fresh cattle dung and faecally contaminated hide sites. Table 1.5 illustrates the number of sampling sites

contaminated with *Enterobacteriaceae* on carcasses derived from sheep with different scours of fleece cleanliness.

During the flaying procedure, the slaughterman must cut through the dung to effect removal of the hide or skin from the animal. Even if good hygiene practices are maintained in the abattoir, microbial contamination can be passed to the carcass from direct contact with the surface of the hide; also, sterile knives, the hands, arms and apron of slaughterman are likely to become contaminated with faecal organisms, with consequent infection of the carcass. The outcome is a risk of passing on the organisms to the consumers of the meat. While there is a general danger of gastro-intestinal problems from *Escherichia coli* infection, there is mortal danger from *E.coli* O157. The appearance of this bacterium in contaminated meat can cause infectious epidemics which can be fatal to the weaker members of society, the young and old. In 1996, the largest *E. coli* 0157 outbreaks were witnessed in the UK. In Scotland, there were over 400 cases and 18 elderly adults died due to this infection (Eley, 1997). *E. coli* 0157 is able to survive during normal cooking procedures (Phillips and Roscoe, 1996), and in frozen condition (Kudva, *et al.*, 1998) and therefore maintain the potential for human pathogenicity.

Table 1.4: Mean aerobic plate counts (APC) 37 °C and *Escherichia coli* numbers for fresh cattle dung and faecally contaminated sites (Bell, 1997).

Sample site	**Microbial population (log10 cfu)**	
	APC ± S.D.	***E. coli* ± S.D.**
Fresh dung (g^{-1})	6.64 ± 0.36	4.67 ± 0.77
Fresh dung (cm^{-2})	5.13 ± 0.58	3.90 ± 0.635
Faecally contaminated hide (cm^{-2})	5.71 ± 0.81	3.10 ± 1.02

Table 1.5: The number of sampling sites contaminated with Enterobacteriaceae on carcasses derived from sheep with different scores of fleece cleanliness (Hadley *et al.*, 1997).

Fleece cleanliness score*	**Proportion of sites contaminated with Enterobacteriaceae (%)**	
	Shoulder	**Abdomen**
1	35	30
2	20	5
3	50	55
4	85	70
5	100	100

* Score 1: clean, dry fleece with only a small amount of attached straw.
Score 5: extensive soiling of the complete underside and all four limbs with wet, dripping faecal material.

1.2.3 PROBLEMS AT THE TANNERY

Heavily contaminated hides are of concern to the tanner in three areas of the leather making process: raw material and final leather quality, production losses, increased effluent and transport costs (Stosic, 1996).

The irritation caused to the skin of the animal by contact with dung and urine for long periods has the following consequences for leather quality:

• Coarsening of and damage to the grain surface of hide
• Staining of the surface
• Affecting dye and other chemical uptake
• There is danger of dermatitis (Koeppen, 1991)

The presence of dung has the following consequences for leather production:

• Manure is ideal nutrients for bacterial growth during soaking and it promotes microbial attack in this process (Goetz, 1956)
• When hides are covered with manure, the fleshing blades must be backed off, resulting in poor fleshing. If it is not done, the hide might be cut by the fleshing blades (Bailey, 1994)
• Handling dungy hides is unpleasant and difficult

Hides are purchased on a weight basis and it is part of the dealing mechanism that an estimate of the weight of dung is made, so that the price can be adjusted accordingly. However, it is undoubtedly the case that tanners are buying some dung at the hide prices.

Presence of dung matter presents a false and variable hide substance weight in the determination of amounts of materials required for subsequent processes, therefore processing chemicals can be wasted (Goetz, 1956). Expected production yields of limed and wet-blue material from hides with heavy dung cladding can be affected due to the increased dung to hide ratio (Stosic, 1996).

Corning (1982) and Daniels (1995) discussed the contribution of dungy hides to tannery effluent charges. These investigations were based on comparing the soaking

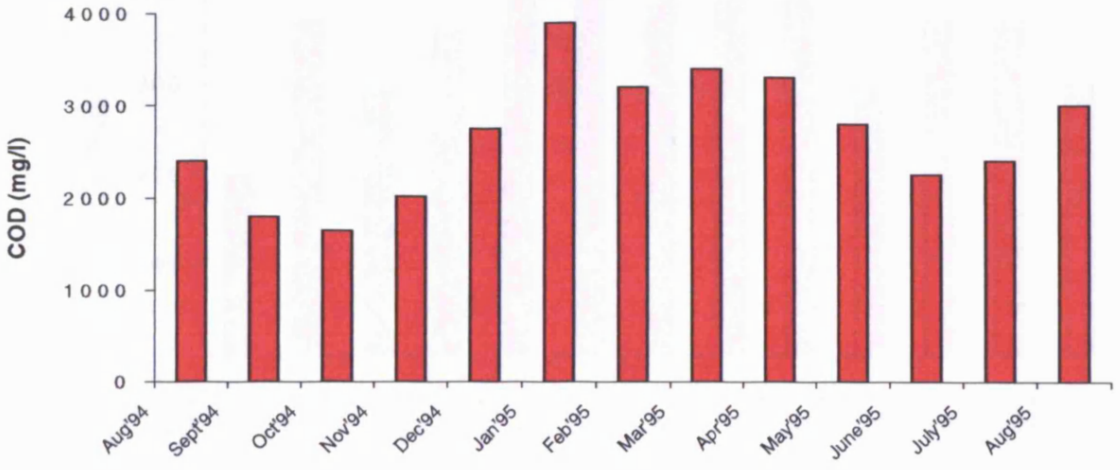
and liming/unhairing of clean hide parts with contaminated areas. It was found that each kilogram of dung contributed:

Chemical oxygen demand between 60-100 g
Biological oxygen demand between 20-50 g
Total Kjeldahl nitrogen approximately 10 g

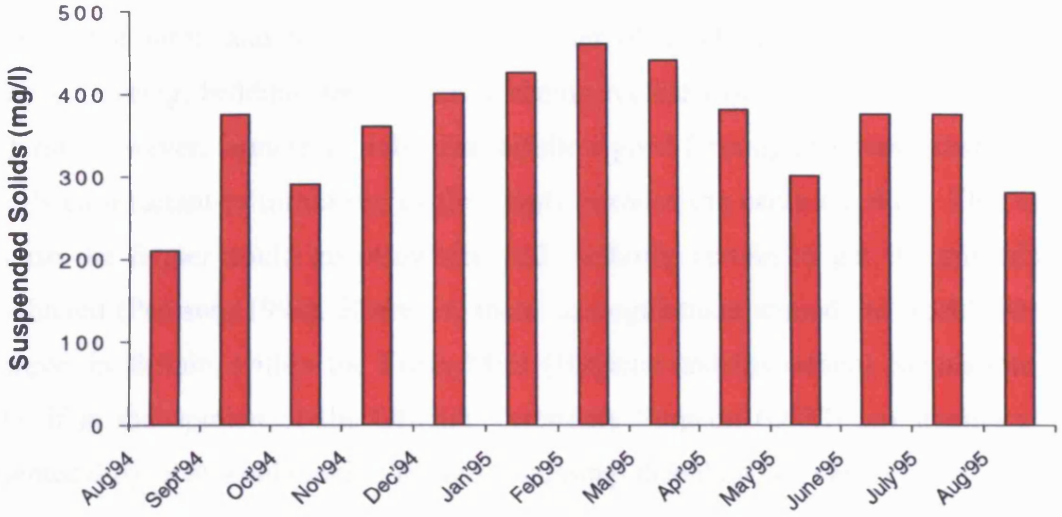
In the investigation of Corning (1982), the processing was said to be conventional and most of the dung was removed in the soak. However, Daniels (1995) argued that if the dung is dry, more will be carried into the liming process, especially if soaking times are short. He claimed that increases in both suspended solids and COD from dung breakdown in liming could be expected. It was further demonstrated that dung contamination contributed to the suspended solids, COD and solid waste of one tannery in the winter period (see Figure 1.5).

Figure 1.5: The effect of dung on effluent from a wet-blue tannery, following full primary treatment (Daniels, 1995).

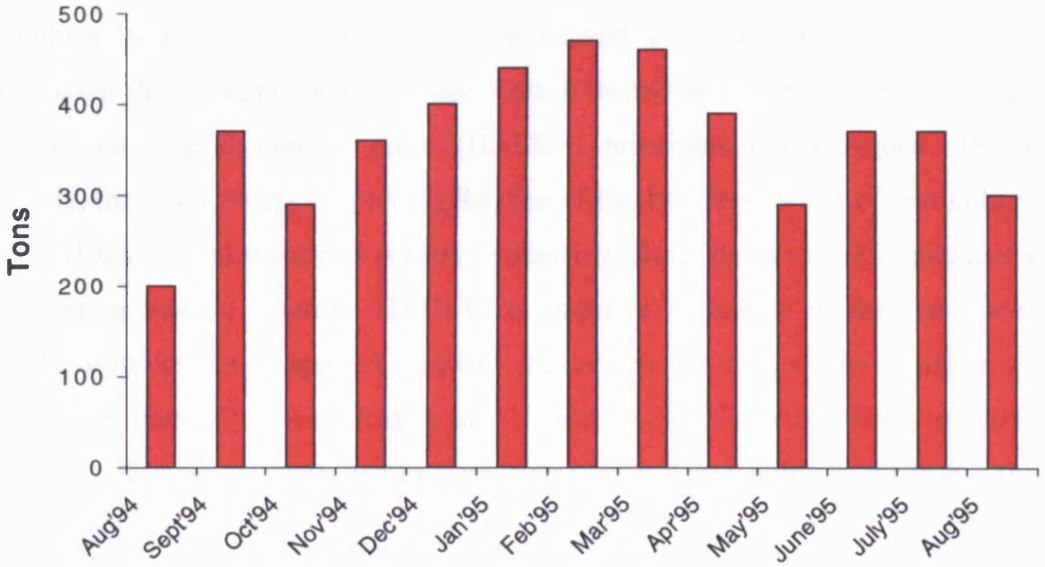
a. Chemical Oxygen Demand



b. Suspended solids



c. Solid waste



1.3 SOLUTIONS TO THE DUNG PROBLEM

It is commonly thought that if the good farming conditions are undertaken, the dung contamination problem can be eliminated in the first place. Animals need to be kept clean on the farm, and this is mainly a matter of good husbandry, appropriate housing, flooring, bedding, feeding and cleaning regime over the winter period on the farm. However, farmers are reluctant to follow good farming practices. Abattoirs have been reluctant to turn away cattle simply because the cattle are dirty. This is because the farmer could go elsewhere and be fairly certain to get the animals slaughtered (Pearson, 1994). However, there are regulations around the world. For example, in Britain, within the Fresh Meat (Hygiene and Inspection) Regulations 1995, if in the opinion of the Official Veterinary Surgeon (OVC) any animal is presented dirty, that would inhibit hygienic dressing, then the OVC can:

- prohibit slaughter of the animal
- request detention of an animal in the lairage
- request the occupier of the slaughterhouse to clean the animal prior to re-presenting the animal for ante-mortem inspection (MHS- Clean Technology Policy, 1997)

Agriculture and Agri-Food Canada have created a Food Safety Enhancement Program which is designed to encourage abattoir inspection systems based on hazard analysis and critical control point (HACCP) principles (Donkersgoed, 1997). Similarly, the Food Safety Inspection Service of the US Department of Agriculture has mandated that all meat and poultry processing plants develop and implement a system of preventive controls (HACCP) to improve the safety of their products. Finnish abattoirs have applied special regulations to reduce the number of "excessively dungy" cattle (Ridell and Korkeala, 1993). According to these regulations, farmers are requested to clean excessively dungy animals; otherwise the

animals undergo casualty slaughter or are taken separately after clean animals and slaughtered with greater care, with the extra costs for the special treatment incurred by the farmer.

Attempting to clean a dirty animal is not only extremely difficult and stressful to the animal, it is likely to cause damage to the hide which would mar and devalue the leather (IHATIS, 1975). These attempts range from warm showers to scrubbing with stiff brushes; the former is ineffective, except for fresh dung on dairy cattle, and the latter is both ineffective and scratches the skin, devaluing the hide. Washing of cattle shortly before slaughter is not only ineffective for dung removal but also leads to an increased potential putrefactive damage to the hide and risk of contaminating the carcass is further increased due to splashing of dirty water from the hide surface onto the meat. In addition, it was shown that the microbiological population of a dry hide may increase by five to ten times with the addition of sufficient moisture (Hadley *et al.*, 1997).

Although all the regulations and actions are in place to prevent and clean the dung contamination of animals, there is still a large number of winter slaughtered dungy hides arriving at tanneries. Therefore, the leather industry has to resolve this problem by itself.

Tanners are faced with the problem of dissolving adhering dung in the earliest step in the wet processing, soaking; as might be expected, this becomes increasingly difficult as the dung becomes drier. Dissolution can be achieved by the action of warm water, but the process may take several hours, helped somewhat by the addition of anionic surfactant. Enzymes have been used to assist in soaking, including proteases, amylases and lipases in conjunction with detergents (Christner *et al.*, 1991), typically with limited success in removing dung.

In order to remove dung from hides successfully, components of dung have to be targeted by enzymes. So what are the components of the dung? One may think that it is composed of digestive waste of animal food which is mainly grass, seed, some protein additives. Brown (1972) analysed dung in order to investigate its digestion by dung beetles. Results from these analyses are given in Table 1.6. It can be seen from the table that about 74% of the dung is made of lignocellulosic components, mainly cellulose, hemicellulose and lignin. The remaining components are some soluble matter and protein. Rosenberg (1979) and Rosenberg and Wilke (1980) reported the ethyl alcohol production from cattle manure by using the thermotolerant fungus *Chrysosporium pruinosum*. In that study, dung was analysed and 74 % lignocellulosic materials and 10% crude protein was found in the dung samples. These findings suggest that the main components of the dung are lignocellulosic materials and for successful enzymatic removal of dung, these lignocellulosic components should be addressed.

Therefore, in order to understand the chemistry of dung degradation, the chemistry and biodegradation of these lignocellulosic components should be evaluated.

Table 1.6: Composition of fresh dung (modified from Brown (1972))

Components	**% by weight of dried dung**
Soluble matters	13.4
Hemicellulose (contains mostly xylan)	22.4
Cellulose	31.9
Lignin	19.9
Total nitrogen	6.5

1.4 CHEMISTRY OF LIGNOCELLULOSIC MATERIALS AND THEIR BIODEGRADATION

1.4.1 CHEMISTRY OF CELLULOSE AND ITS BIODEGRADATION

Cellulose is the most abundant naturally occurring organic substance, being found as the principal cell wall constituent of higher plants in which it provides the main structural elements (Gascoigne and Gascoigne, 1960). The amount of cellulose in the plants varies according to source (see Table 1.7) and is usually associated with such materials as lignin, hemicellulose, water, pectin, proteins and mineral substances. It is also found as a constituent of various algae, and synthesised by a small number of bacteria, e.g. *Acetobacter xylinum* (Aspinall, 1970).

In terms of chemical structure, cellulose is among the simplest of the natural polymers in that it consists of a single repeating unit, D-glucose, linked through carbons 1 and 4 by β linkages. This means that the ring substituents other than hydrogen, including the bonds linking the glucose together, are all equatorial. Each glucose residue is rotated 180^{o} relative to its neighbours, so that the basic repeating unit is in fact cellobiose. When cellulose is subjected to acetolysis by treatment with a mixture of acetic anhydride and concentrated sulphuric acid, cellobiose octa-acetate is formed. Thus the structure of cellulose is based on the cellobiose unit (see Figure 1.6). Cellobiose is known to be the disaccharide, 4-O-β-D-glucopyranosyl-D-glucopyranose. Acetolysis of cellulose produces a cellotriose, a cellotetraose and a cellopentaose and in each of these all the 1,4-links have been shown to be β-links. The degree of polymerisation of native cellulose is probably 4000- 5000.

Table 1.7: Cellulose Content of Natural Sources (from Tsao and Chiang, 1983)

Raw Material	Cellulose Content (%)
Hardwoods	40-45
Softwoods	45-50
Grasses-straws	25-40
Corn/Wheat	3-4
Cotton	89
Cornstover	38
Tall fescue	34
Leaves	15-20
Birch	44.9
Spruce	46.1

Three hydroxyl groups per anhydroglucose unit as well as the configuration of pyran rings permit many intramolecular and intermolecular hydrogen bonds in cellulose polymer. These bonds together with the van der Waal's attractions hold the conformation of the chains in different degrees of lateral order which range from a perfect or nearly perfect geometric packing of the crystal lattice to random arrangement. As a result, cellulose is not thermoplastic but decomposes before melting. X-ray diffraction indicates that the length of the unit cell along the chain axis is about 10.3 Å corresponding to that of a cellobiose unit. The special properties of cellulose result from the association of bundles of microfibrils of individual molecules held together in a highly ordered structure. Estimates of the width of these microfibrils, based on electron microscopy, vary for different samples of cellulose and generally fall within the range of 50-100 Å but may be low as 35 Å.

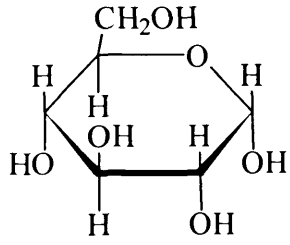
Despite its large number of hydroxyl groups, cellulose is insoluble in water and other common solvents, although it will dissolve in certain solvent mixtures. Solutions of metal complexes such as copper (II)-ammonia or copper (II)-diamine dissolve cellulose, presumably by forming complexes with the hydroxyl groups of the polymer.

1.4.1.1 CELLULOSE DEGRADING ENZYMES

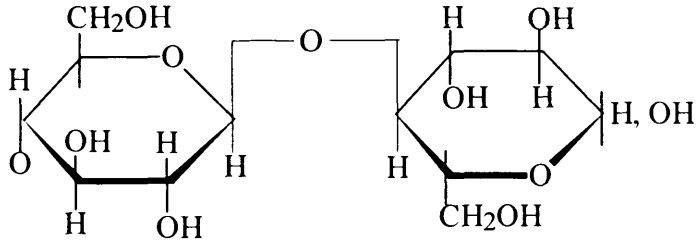
Cellulolytic enzymes are synthesised by a large number of microorganisms which include fungi and bacteria. However, only the fungi appear to excrete large amounts of cellulase enzymes in active form into culture media (Wood, 1985). *Trichoderma reesei* mutants are the most powerful producers of such enzymes. Other fungi such as *Aspergillus niger*, *Trichoderma koningii*, *Fusarium solani*, *Penicillum funiculosum*, *Sporotrichum pulverulentum* and the bacterium *Clostridium thermocellum* are also being considered for commercial exploitation, *Thermomonospara ssp.*, *Talaromyces emersonii* and others show promise in this regard (Mandels, 1982 and Coughlan, 1985).

Cellulase is not a single enzyme, but a unique system in which several enzymes act together to carry out the hydrolysis of cellulose that no one of them acting alone can achieve (Mandels, 1982). There are three types of enzyme that have been traditionally assigned to the cellulase system: endo-glucanases (endo-1,4-β-glucanases or 1,4-β-D-glucan 4-glucanhydrolases, EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-glucanases or 1,4-β-D-glucan cellobiohydrolases, EC 3.2.1.91), and cellobiases (β-glucosidases or β-D-glucoside glucohydrolases, EC 3.2.1.21) (Mandels, 1982; Wood and McCrea, 1979; Wood, 1985, 1992 and Eveleigh, 1987).

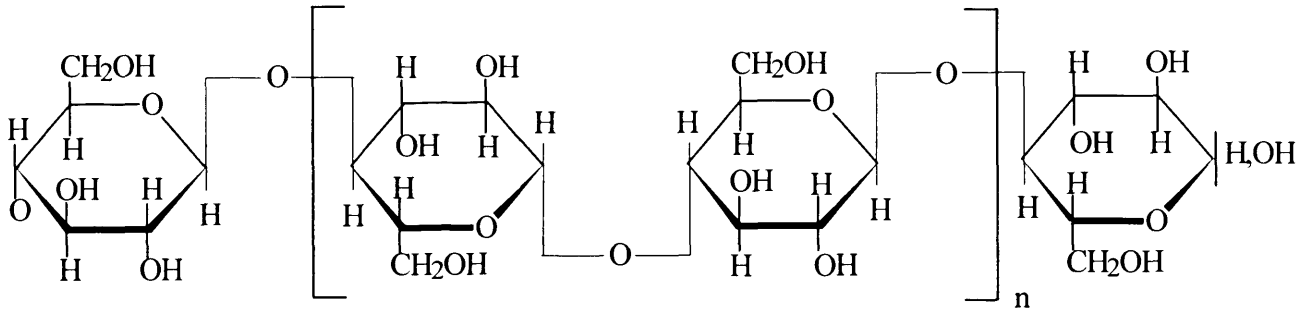
Figure 1.6: Cellulose linear structure and its repeating units.



D- GLUCOSE



CELLOBIOSE



CELLULOSE

Endoglucanases, often called CM-cellulases or C_x, can be found commonly in filtrates from cellulolytic fungi and bacteria. Most of the filtrates lack exo-β-glucanase so they have only limited action on crystalline cellulose, however, cellobiase (β-glucosidases) is produced with endo-β-glucanase by most organisms (Mandels, 1982). Endoglucanases have little ability to hydrolyse crystalline cellulose, however, CM-cellulose or ortho phosphoric acid-swollen cellulose, barley β-glucan or soluble cello-oligosaccharides are readily hydrolysed in a random fashion, leading to a rapid fall in the degree of polymerisation (Wood, 1985, 1992; Walker and Wilson, 1991). The products of endoglucanase reaction are glucose and cellobiose.

Cellobiohydrolases (Wood and McCrae, 1972; Wood, 1985, 1992 and Eveleigh, 1987) degrade cellulose by splitting off cellobiose units from the non-reducing end of the chain. Substituted celluloses (CM-cellulose) are not attacked, but swollen partially degraded amorphous celluloses and soluble cello-oligosaccharides, triose to hexose, are readily degraded. Cotton fibre, which is highly hydrogen bonded, with a degree of polymerisation of approximately 10,000, has few chain ends accessible to the enzyme and is apparently not degraded rapidly. Avicel (microcrystalline cellulose) in having an average degree of polymerisation of only 200 has more chain ends than cotton and it is more easily degraded. β-Glucosidases hydrolyse cellobiose and soluble cello-oligosaccharides to glucose, but cellulose is not degraded.

The classical action of cellulase is envisioned as an initial attack by endoglucanases at multiple internal sites in amorphous zones of the substrate, thereby creating new sites for subsequent attack by cellobiohydrolase. This enzyme initiates its attack at non-reducing termini and then sequentially cleaves cellobiose residues from the glucan chain and final hydrolysis of the small oligosaccharides to glucose by cellobiase (Figure 1.7).

Fractionation studies have demonstrated that each of the types of enzyme comprising the cellulase system consists of a multiplicity of forms with apparent duplication of function. The nature and origin of these isocomponents has been subject of much discussion (Wood, 1985). This discussion has addressed the possibility that the multiplicity of components could be genetically determined or be caused by partial proteolysis or by differential glycosylation of a common polypeptide chain. A typical *T. reesei* commercial cellulase contains six endoglucanases, three cellobiohydrolases and one cellobiase (Beldman *et al.*, 1985). A typical cellulase found in cultures of *T. koningii* has been found to contain ten cellulase components. These included two cellobiohydrolases, two β-glucosidases and six endoglucanases (Wood and McCrae, 1978). Cellulases of *P. funiculosum* (Wood *et al.*, 1980) and *T. emersonii* (McHale and Coughlan, 1981) have been reported to be equally heterogeneous.

Synergism occurs between cellulose components during the action of enzymes towards substrate. The synergism is most significant when crystalline cellulose is the substrate, but it is low or non-existent with amorphous highly hydrated celluloses and absent with soluble derivatives of cellulose (CM-cellulose) (Wood, 1992). Synergism is a phenomenon resulting in a mutual increase of the efficiency of action of two or more components of a system when they act together in, comparison with their additive action when they act separately (Klyosov, 1990). Three types of synergistic activity operate in cellulases (Wood, 1992):

- between endoglucanase and cellobiohydrolase
- between two homogenetically unrelated cellobiohydrolases
- between β-glucosidase and the other two type of enzymes

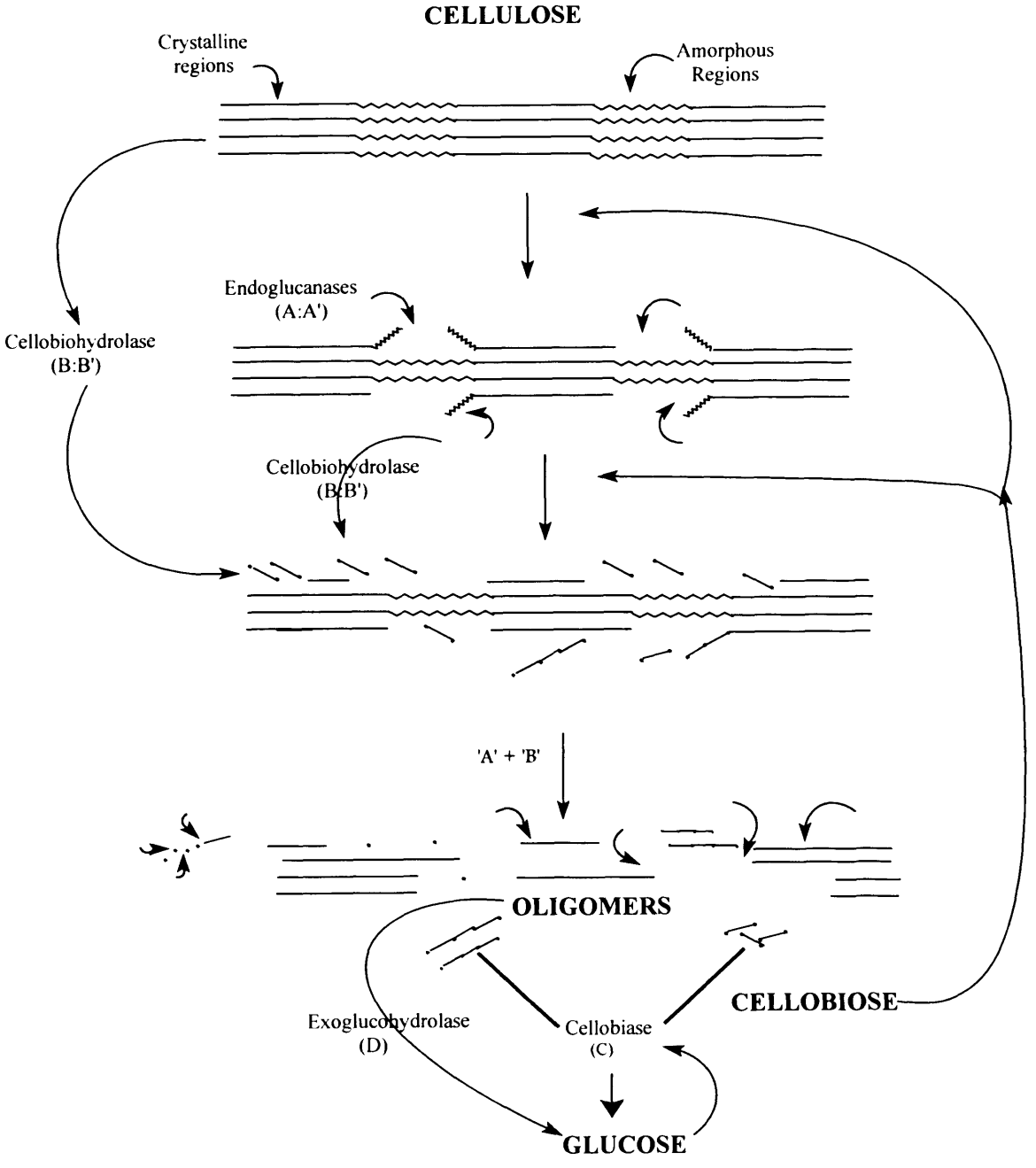
Synergism between endoglucanases and cellobiohydrolases is traditionally interpreted by a sequential mechanism of enzyme action (Wood *et al.*, 1979 and Beldman *et al.*, 1988). The endoglucanases initially attacking the amorphous regions of the cellulose, provide new non reducing polyglucose chain ends for the subsequent action of the cellobiohydrolases.

Another mechanistic model for endo-exo synergism is based on the results of Ryu *et al.* (1984) and Kyriacou *et al.* (1989) suggesting that competition for adsorption sites on cellulose and a well co-ordinated adsorption and desorption of individual enzyme components lead to an enhanced cellulase turnover on the cellulose surface and consequently to an increased rate of substrate degradation.

For the synergistic action of two cellobiohydrolases, Wood (1985) tried to explain synergism between two homogenetically distinct cellobiohydrolases from *Penicillium pinophilium*. Cellobiohydrolases I and II are two enzymes exhibiting a different substrate stereospecificity that have been synthesised to attack the two different non-reducing end groups that can be found in cellulose (see Figure 1.7). The action of one cellobiohydrolase, by removing cellobiose units successively from one type of non-reducing chain end, might expose a non-reducing end group on another chain with the correct configuration for attack by the other stereospecific cellobiohydrolase.

A third type of synergism exists between cellulases and β-glucosidases (Walker and Wilson, 1991 and Woodward, 1991). It is assumed that the role of β-glucosidase is to hydrolyse cellobiose, which is the principal reaction product of the major *T. reesei* cellulases and which strongly inhibits cellobiohydrolases, for example CBH I from *T. reesei*.

Figure 1.7: General scheme for cellulolysis (Eveleigh, 1987)



1.4.2 CHEMISTRY OF HEMICELLULOSE AND ITS BIODEGRADATION

Hemicellulose is a collective term for a group of chemically heterogeneous carbohydrates found in the cell walls of plants, in association with lignin and cellulose (Wilkie, 1983; Betts *et al.*, 1991). Definitions of hemicellulose are made ambiguously and have included "any structural polysaccharide which is not cellulose" and "those plant cell wall polysaccharides soluble in alkali" (Betts *et al.*, 1991).

Hemicelluloses are usually extracted from plant material which has first been sequentially extracted with ethanol-benzene or chloroform-methanol to remove lipid material; cold and hot water to remove proteins and water soluble carbohydrates including polysaccharides; ammonium oxalate-oxalic acid solution or EDTA to remove the pectin substances; and acid sodium chlorite to remove lignin material yielding a holocellulose (made essentially of hemicellulose and cellulose) which is then extracted with alkali (Dekker, 1980). The sugar residues in these polysaccharides include D-xylose, L-arabinose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-galactose, L-galactose, D-mannose, L-rhamnose and L-fructose (Betts *et al.*, 1991).

Unlike cellulose which is a linear polysaccharide with the same basic structure in all species, hemicelluloses are heteropolysaccharides which may be highly branched and have widely differing structures from species to species. Structures and diversities of wood and grass hemicelluloses have been discussed in the reviews from Timell (1964 and 1965) and Wilkie (1979). Hemicelluloses are considerably smaller than cellulose with a degree of polymerisation usually less than 200 and they are generally non-crystalline.

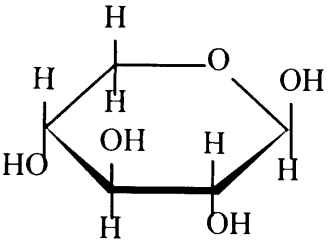
Hemicelluloses can be divided into three predominant types according to their chemical structure: xylans, glucans and mannans. In the cell walls of land plants, xylan is the most common and abundant hemicellulosic polysaccharide, representing more than 30% of dry weight (Joseleau *et al.*, 1992). For this reason, the chemical properties of xylan and its biodegradation by enzymes will be focused on in more detail than other hemicellulosic polysaccharides.

1.4.2.1 XYLANS

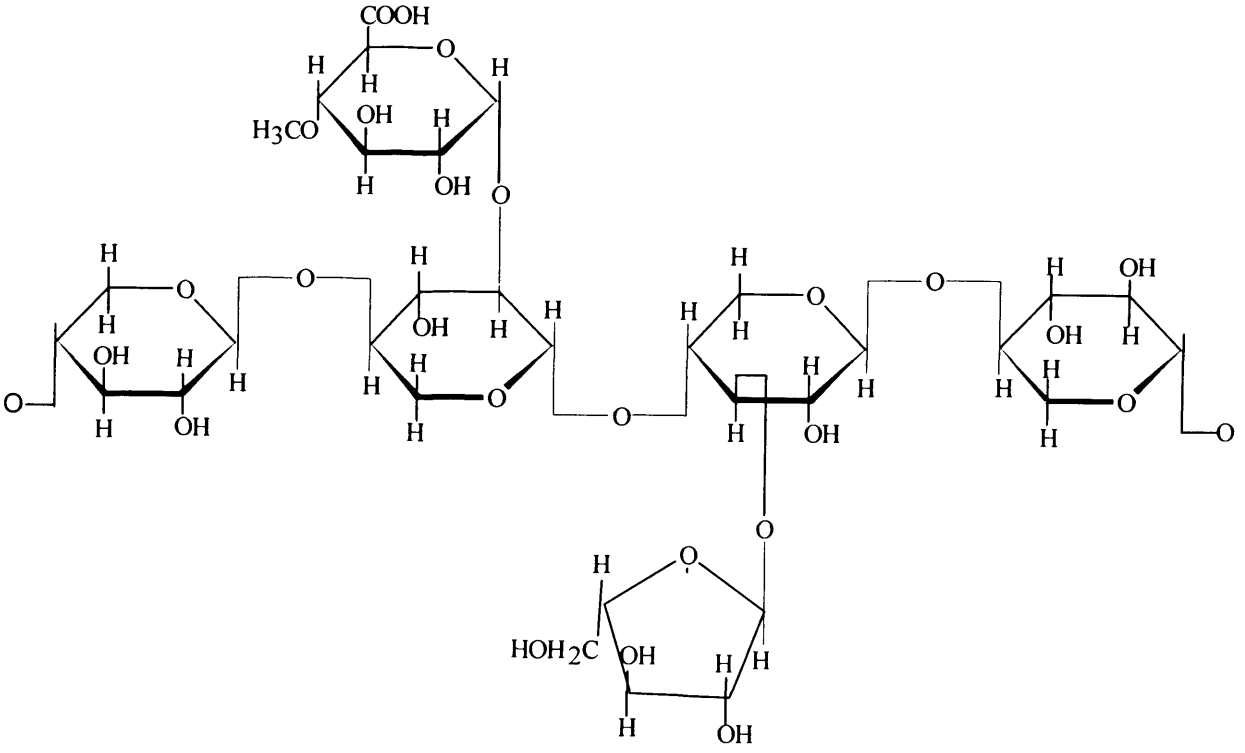
Xylans are composed of 1,4- linked β-D-xylopyranosyl residues (Whistler and Richards, 1970). Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain and in the side chain (Biely, 1985). The common substituents found on the backbone of xylan are acetyl, arabinosyl and glucuronosyl residues (Whistler and Richards, 1970). However, homoxylans consist exclusively of xylosyl residues. This type of xylan is not widespread in nature and has been isolated from such as esparto grass (Chanda *et al.*, 1950). A structural representation of xylan polymer is illustrated in Figure 1.8.

The xylans in grasses are generally arabinoxylans and some xylans have branches containing various combinations of arabinosyl, galactosyl, glucuronosyl, and xylosyl residues (Wilkie, 1979). The xylan of hardwood is O-acetyl-4-O-methylglucuronoxylan (Timell, 1964). Every tenth xylose residue carries a 4-O-methylglucuronic acid attached to the 2 position of xylose. Softwoods are composed of arabino-4-O-methylglucuronoxylans. The 4-O-methyglucuronic acid residues are attached to the C-2 position. Softwood xylans are not acetylated and instead of acetyl groups they have α-L-arabinofuranase units linked by α-1,3 glycosidic bonds to the C-3 position of the xylose (Sunna and Antranikian, 1997).

Figure 1.8: Structural representation of xylan



D-XYLOSE



XYLAN

Xylan appears to be a major interface between lignin and other carbohydrates in plant cell walls (Wong *et al.*, 1988). It is a major carbohydrate component in many isolated phenolic carbohydrate complexes and may be covalently linked to phenolic residues via its arabinosyl and glucuronosyl residues. Therefore, it is suggested that due to the high phenolic content in these complexes, xylan is linked to the lignin structure. Xylan tends to adsorb onto cellulose and aggregate with other hemicellulosic components. This is likely to be the result of hydrogen bonding interactions (Wong *et al.*, 1988). Both these covalent and non covalent interactions can contribute to the structural integrity of lignocellulose.

1.4.2.2 XYLAN DEGRADING ENZYMES

The occurrence of both 1,3 and 1,4-β-D-xylanases in marine and terrestrial bacteria, marine algae, fungi, rumen and ruminant bacteria, snails and a variety of invertebrate animals has been reported by Dekker and Richards (1976). However, the most intensively studied xylanases are from fungi.

Due to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system. This is usually composed of endo-1,4-β-D-xylanases (EC 3.2.1.8), exo-1,4-β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucoronidase (EC 3.2.1) and acetylxylan esterase (EC 3.1.1.6) (Reilly, 1981; Woodward, 1984; Wong *et al.*, 1988; Bajpai, 1997; Sunna and Antranikian, 1997).

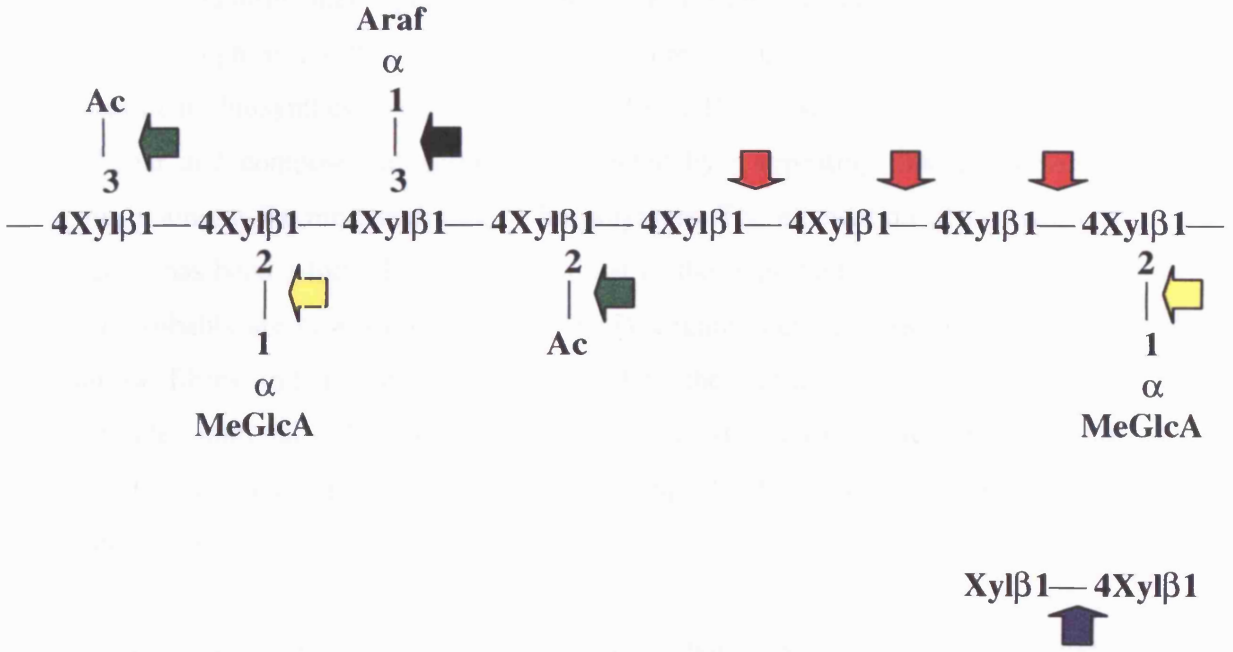
β-1,4-endoxylanase cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased degree of polymerisation of the substrate. During the early course of hydrolysis of xylan, the main products formed are xylooligosaccharides. As the hydrolysis proceeds, these oligosaccharides will be

further hydrolysed to xylotriose, xylobiose and xylose (Sunna and Antranikian, 1997). There are two main types of endo-xylanases: those are capable of hydrolysing the 1,3-α-L-arabinofuranosyl branch points of arabinoxylans and those that do not cleave the branch points of arabinoxylan (Dekker and Richards, 1976). Many organisms are able to produce both debranching and non debranching xylanases, resulting in the maximum efficiency of xylan hydrolysis (Wong *et al.*, 1988).

β-D-xylosidases are exoglycosidases that hydrolyse short xylooligosaccharides and xylobiose from the non reducing end to liberate xylose (Wong *et al.*, 1988). The affinity of the enzyme towards xylooligosaccharides degreases with increasing chain length.

The other enzymes such as acetyl xylan esterase, α-glucoronidase and α-L-arabinofuranosidase are responsible for cleavage of the side chains of heteroxylan polymer. The action of xylanolytic enzymes towards to xylan is illustrated in Figure 1.9.

Figure 1.9: The actions of xylanolytic enzyme on xylan (from Bajpai, 1997)

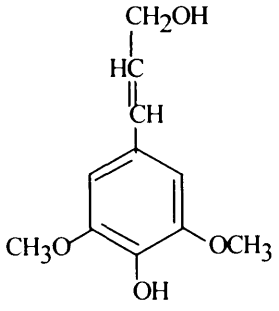


1.4.3 CHEMISTRY OF LIGNIN AND ITS BIODEGRADATION

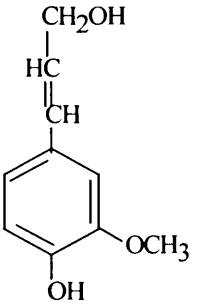
It has been stated that the plant polymer lignin is the second most abundant organic compound on Earth, and that cellulose is the only other organic substance present in the larger quantities than lignin (Crawford, 1981). Lignin content of plant biomass may be as high as 20-25%. The chemical nature of lignin is known largely from studies of its biosynthesis (Sarkanen and Ludwig, 1971). Most biological polymers are linear and composed of subunits connected by a repeating linkage; however, lignin is among the most complex of biopolymers. The elucidation of its chemical structure has been a formidable task but most of the important structural features of lignin probably are now known (Adler, 1977). Lignin is closely associated with the cellulose fibres and it is chemically bonded to the hemicellulose. In this natural composite material, the cellulose fibrils provide tensile strength and the hemicellulose and lignin provide cross-linking, binding the structure together (Evans, 1991).

From a chemical point of view, it is an amorphous, three-dimensional aromatic polymer, formed at the sites of lignification in plants by enzyme mediated polymerisation of three substituted cinnamyl alcohols: p-coumaryl, coniferyl and sinapyl alcohols (4-hydroxy-, 4-hydroxy-3-methoxy- and 4-hydroxy-3,5-dimethyoxycinnamyl alcohol, respectively) (Crawford, 1981; Kirk, 1983). The chemical structure of these cinnamyl alcohols are represented in the Figure 1.10. Lignin molecules vary widely in molecular weight, but it is likely that *in vivo* natural lignins have molecular weights in the range of 105 kD or higher (Sarkanen and Ludwig, 1971).

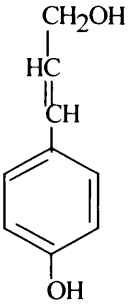
Figure 1.10: Structures of three lignin precursors



sinapyl alcohol



coniferyl alcohol



p-coumaryl alcohol

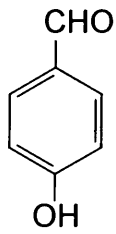
Higuchi *et al.* (1977) and Chen (1988) classified lignin in three major groups:

i- Guaiacyl lignins; also called softwood (gymnosperm) lignins, composed mainly of coniferyl alcohol units with only small amounts of sinapyl and p-coumaryl alcohol. It was reported that the relative proportions of coniferyl, coumaryl and sinapyl alcohol derived units in guaiacyl lignin were 80:14:6 respectively (Crawford, 1981); however, Erikson and Miksche (1974) reported somewhat lower quantities for the amounts of coumaryl and sinapyl units in guaiacyl lignin (94:5:1). Vanillin is the major oxidation product of guaiacyl lignin (see Figure 1.11).

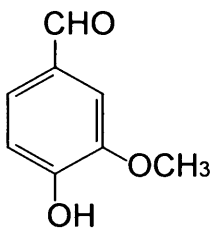
ii- Guaiacyl-syringyl lignins; also called hardwood (angiosperms) lignins, contain monomeric units derived from approximately equal amounts of coniferyl alcohol and sinapyl alcohol with only minor amounts of coumaryl alcohol units. The estimated ratio of coniferyl:coumaryl:sinapyl is about 49:5:46 respectively (Freudenberg, 1968). Their oxidation products are mainly vanillin and syringaldehyde (see Figure 1.11).

iii- Guaiacyl-syringyl-p-hydroxyphenyl lignins, also called grass lignin, is thought to be composed of approximately equal amounts of all three cinnamyl alcohols. Their oxidation produces 4-hydroxybenzaldehyde, vanillin and syringaldehyde (see Figure 1.11).

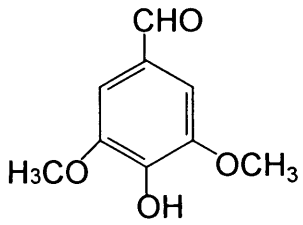
Figure 1.11: Major oxidation products from lignins



p-hydroxybenzaldehyde



Vanillin



Syringaldehyde

Lignin is synthesised by plants from carbon dioxide by way of the shikimic acid pathway (Higuchi *et al.*, 1977). The aromatic amino acids phenylalanine and to a lesser extent tyrosine are responsible for the formation of hydroxycinnamyl alcohol derivatives. Both of these amino acids are synthesised by the shikimic acid pathway as well. Figure 1.12 shows the pathways in biosynthesis of lignin precursors from carbon dioxide. The lignin polymerisation is initiated by specific cell wall peroxidases that catalyse the one-electron oxidation of lignin precursors, producing phenoxy radicals, which can form a number of resonance structures each with a short half life (Betts *et al.*, 1991) (Figure 1.13).

A wide range of different bond types is formed in the lignin structure during the synthesis. Major types are illustrated in Figure 1.14.

Little research has been conducted on grass lignin, hence detailed structures are not available. However, it is thought that wood and grass lignins are closely related (Betts *et al.*, 1991).

The structure analysis of lignin represents a fundamental problem because lignin cannot be isolated in its native state. A number of methods have been developed in order to establish the chemical structure of lignin (Adler 1977). These methods rely on extensive depolymerisation of lignin by using mild chemical procedures that do not cause serious side reactions. After mild chemical treatment, monomeric to tetrameric phenols derived from lignin can be isolated. Adler (1977) isolated lignin by such a treatment and his structural representation of lignin is reported in Figure 1.15.

Figure 1.12: Lignin biosynthesis in plants via the shikimic acid pathway (Higuchi *et al.*, 1977)

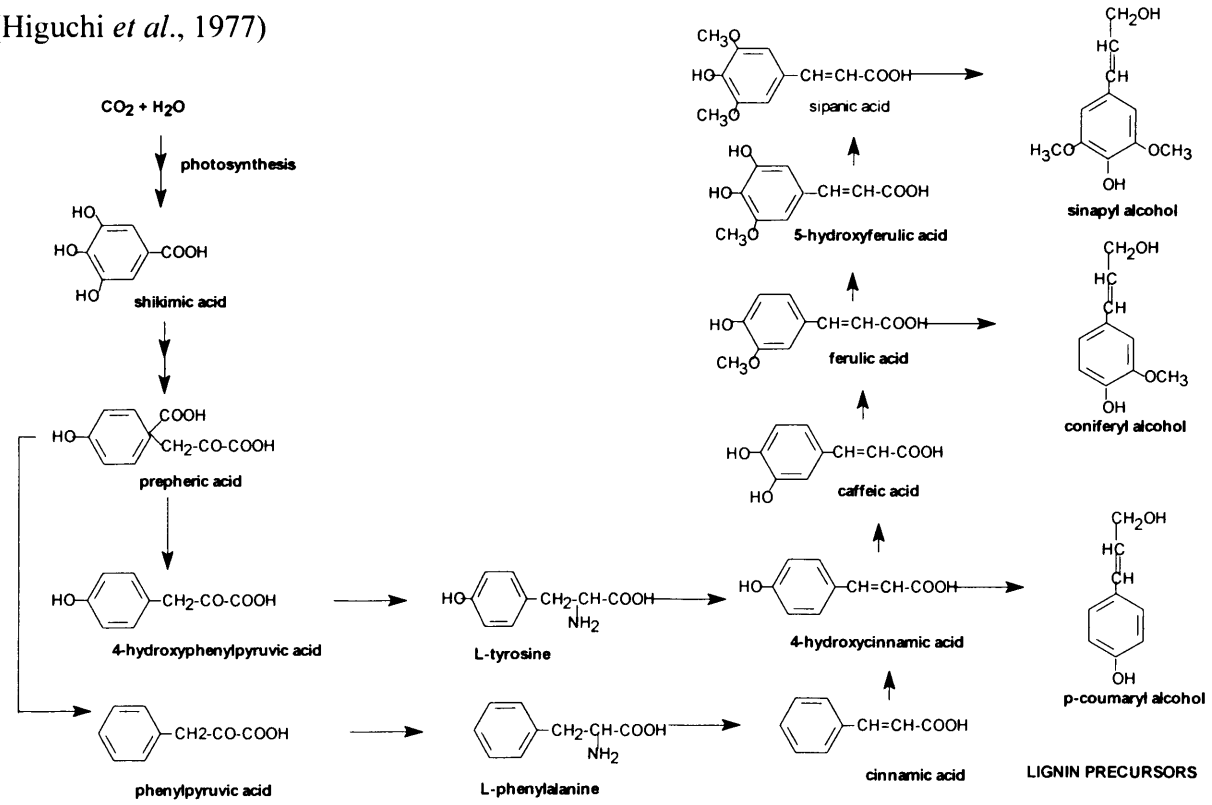


Figure 1.13: Radical coupling of coniferyl alcohol during biosynthesis (Betts *et al.*, 1991)

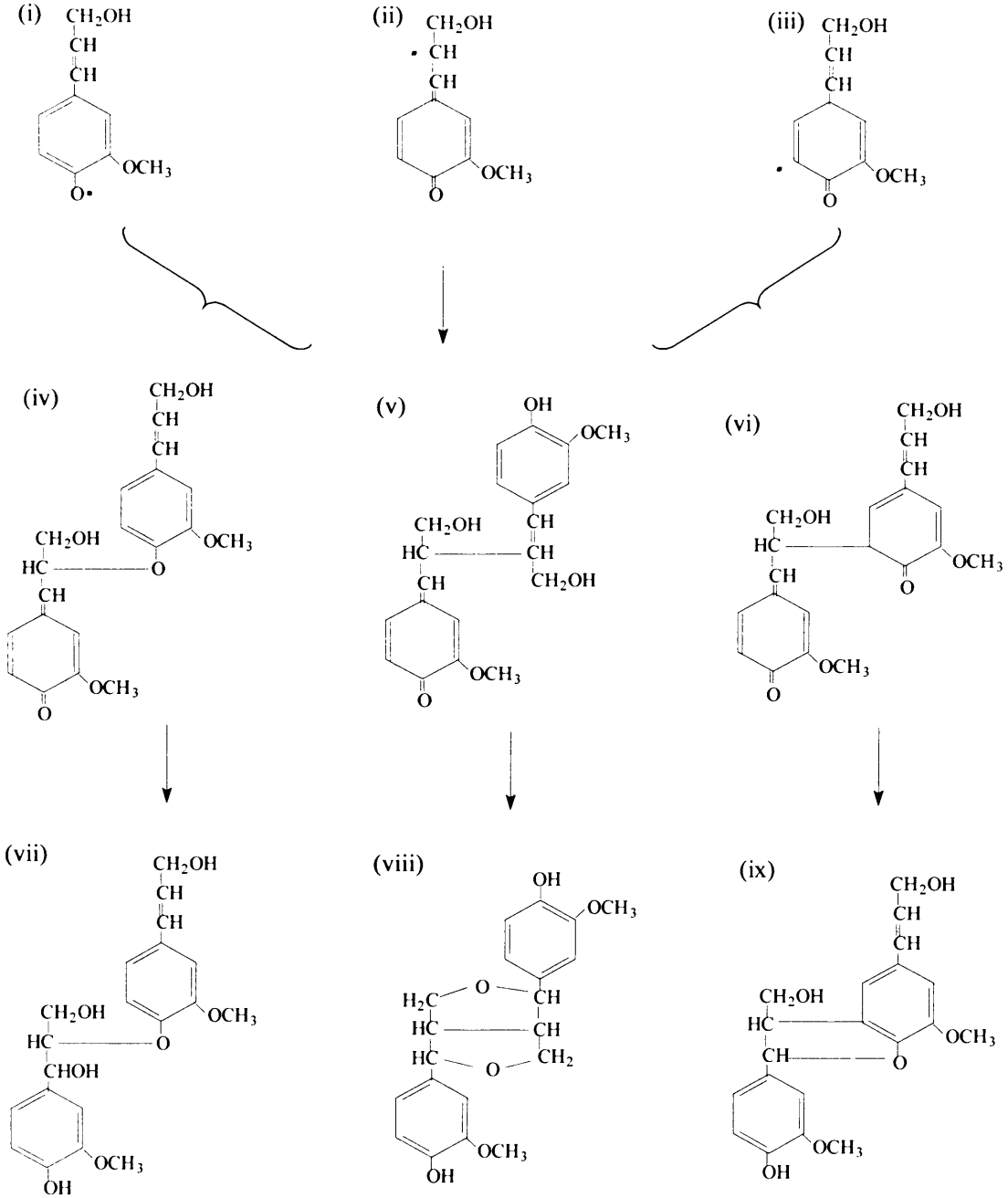
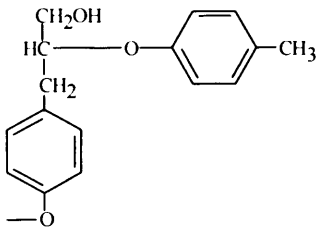
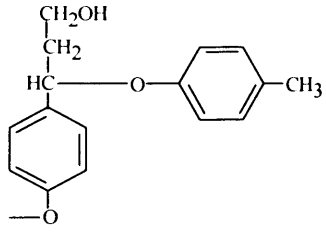


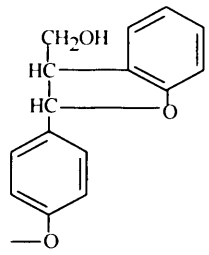
Figure 1.14: Proportions of some major types of bonds connecting phenylpropanoid units in spruce (*Picea abies*) lignin (Adler, 1977)



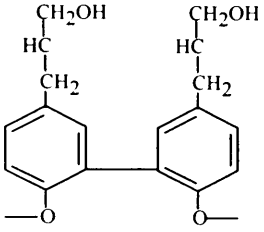
arylglycerol-β-aryl ether
48%



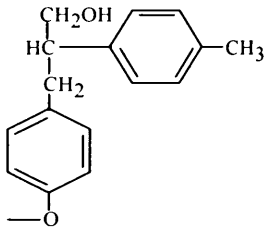
arylgycerol-α-aryl ether
6%-8%



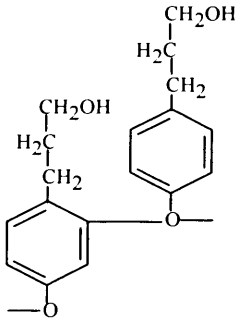
phenylcoumaran
9%-12%



biphenyl
9.5%-11%

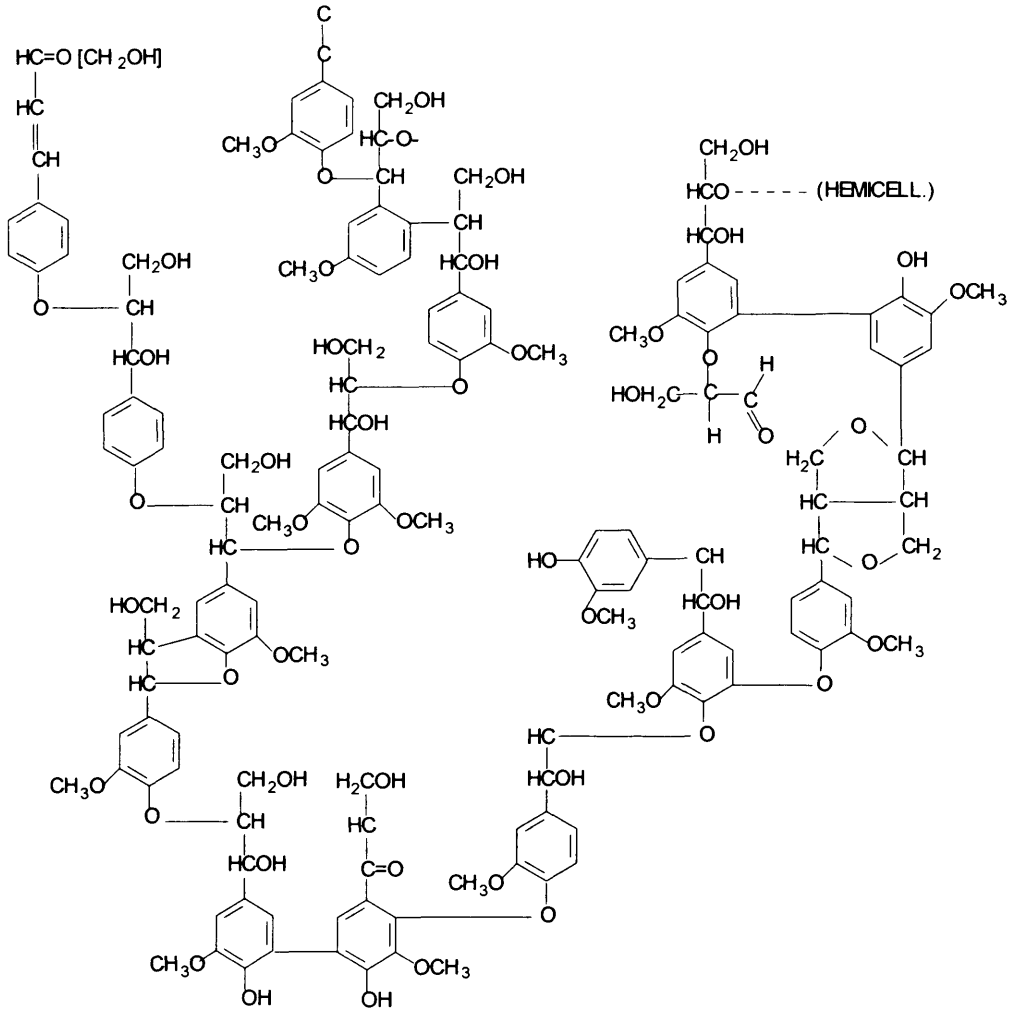


1,2-diarylpropane
7%



diphenyl ether
3.5%-4%

Figure 1.15: Schematic structural formula of an average conifer lignin structure (Adler, 1977)



1.4.3.1 LIGNIN DEGRADING MICROORGANISMS

Fungi are the only organisms known to extensively degrade lignin polymer (Kirk, 1987). The white-rot fungi, the most effective lignin degraders, are *Basidiomycetes* (Crawford, 1981). They are able to extensively decompose all the important structural components of the cell wall to carbon dioxide and water. These fungi characteristically produce extracellular enzymes involved in the process of lignin degradation and deplete lignin and carbohydrate components at about the same proportional rates (Crawford, 1981). Amongst these fungi *Phanerochaete chrysosporium* (Kirk, 1987), *Sporotrichum pulverulentum* (Ander and Eriksson, 1976), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1997) and *Coriolus versicolor* (Evans, 1985a) are the most widely studied organisms.

Brown-rot fungi, in contrast to white-rot fungi, depolymerise and remove the polysaccharides from wood while leaving the lignin as a brown residue. The lignin in brown rotted wood has been demethylated, and partially oxidised and depolymerised but not completely degraded (Reid, 1995). Brown-rot fungi are *Basidiomycetes*, and are classified in the same families as white-rot genera.

The third type of wood decay is soft-rot, caused by members of *Fungi Imperfeeti* and *Ascomycetes*. Soft-rot fungi attack wood under conditions of high humidity, causing softening of surfaces of the tissue, accompanied by weight loss. These decays involve lignin degradation, although many soft-rot fungi studied have been shown to attack the polysaccharides preferentially (Buswell and Odier, 1987).

1.4.3.2 LIGNIN DEGRADING ENZYMES

1.4.3.2.1 LIGNIN PEROXIDASE

In 1983, Tien and Kirk (1983) and Glenn *et al.* (1983) announced the discovery of an extracellular H_2O_2-requiring enzyme activity that they called ligninase (Lignin peroxidase) in *Phanerochaete chrysosporium.* Ligninase (LiP) (diarylpropane: oxygen, hydrogen-peroxide oxidoreductase, EC 1.11.1.14) is a haem containing peroxidase with an unusually high redox potential and low optimum pH (Tien, 1987; Umezawa and Higuchi, 1991; Gold and Alic, 1993).

LiP purified from *P. chrysosporium* is a mixture of 15 isoenzymes (Tien and Kirk, 1983; Glenn *et al.*, 1983) with isoelectric point varying between 3.2 and 4.9. However, the differences between isomers may be attributed to the level of glycosylation of the protein moiety rather than any major changes in amino acid number or composition (Evans, 1991).

LiP shows little substrate specificity and even degrades unrelated molecules, however, all LiP isomers are able to oxidise veratryl alcohol to verataldehyde in the presence of hydrogen peroxide. The enzyme can be assayed by the oxidation of veratryl alcohol to veratraldehyde, the formation of which is monitored by its absorbance at 310 nm (Evans, 1991). Most of the studies for understanding the biochemistry of lignin degradation have concentrated on the use of lignin model compounds (Tien, 1987; Kirk, 1987; Higuchi, 1993). Two of these models, represent the β-O-4 and β-1 structures of lignin are the most commonly used compounds. Like the lignin polymer, these structural models are also degraded. The C_α-C_β cleavage and aromatic ring opening reactions shown to occur during the reaction with these models are analogous to those shown to occur in the reaction with lignin polymer.

Apart from this, ligninase catalyses the oxidation of a wide range of substrates to yield multiple products (Kirk, 1987 and Tien 1987). Figure 1.16 shows the reactions catalysed by the ligninase. The reactions can be divided into five categories: (1) benzylic alcohol oxidation (A), (2) carbon-carbon bond cleavage (B), (3) hydroxylation (C), (4) phenol dimerisation (or polymerisation) (D), and (5) demethylation (E).

The variety of reactions catalysed suggests that radical chemistry might be involved (Kersten *et al.*, 1985; Schoemaker and Leisola, 1990). Electron spin resonance (ESR) study showed that aryl cation radicals are generated during LiP catalysis of 1,4-dimethoxybenzene, 1,2,3,4-tetramethoxybenzene and 1,2,4,5-tetramethoxybenzene in the presence of hydrogen peroxide (Kersten *et al.*, 1985). The demethoxylation mechanism involves one-electron oxidation of the substrate, and subsequent addition of water to the radical cation, followed by methoxyl elimination as methanol (Figure 1.17).

The involvement of a cation radical during the enzyme reaction is an important phenomenon because radicals exhibit several properties in lignin biodegradation (Camaioni and Franz, 1984; Palmer *et al.*, 1987). Radicals can act as one electron oxidants which are able to oxidise an appropriate donor and become reduced themselves to the ground state. Radicals also can undergo side-chain reactions and this explains the C_α-C_β bond cleavage observed in lignin and lignin-model compounds.

Having discussed the mechanism of lignin degradation with ligninase, it should be mentioned that lignin peroxidases have not only been isolated from *P. chrysosporium* but also from several other white-rot fungi such as *C. versicolor* and *Phlebia radiata* (Dodson *et al.*, 1987 and Kantelinen *et al.*, 1989). The same

characteristics might be observed from these proteins, however, slight modifications of culture conditions are required to obtain equivalent yields (Evans, 1991).

Figure 1.16: Wide range of reactions catalysed by ligninase (Tien, 1987)

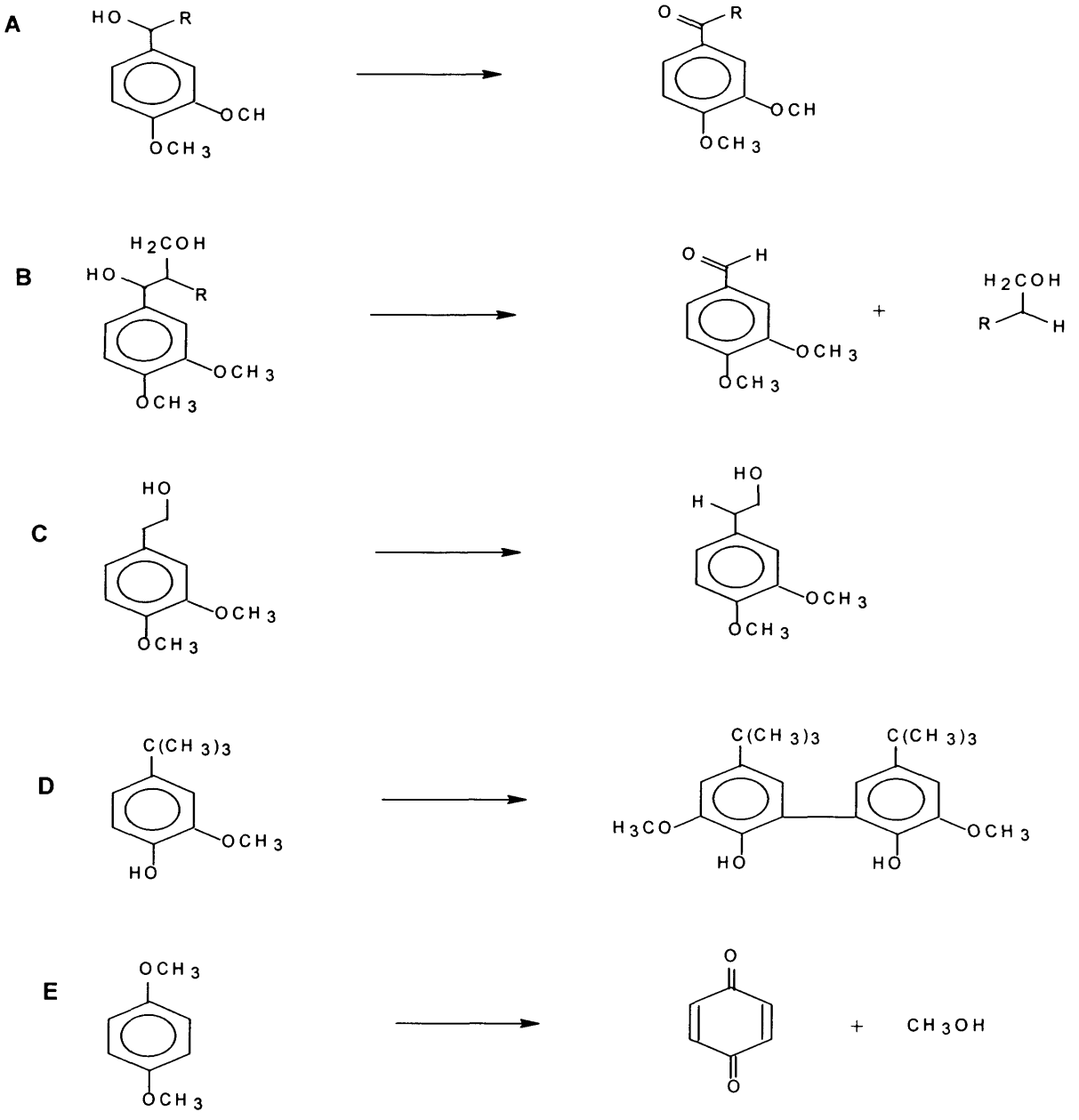
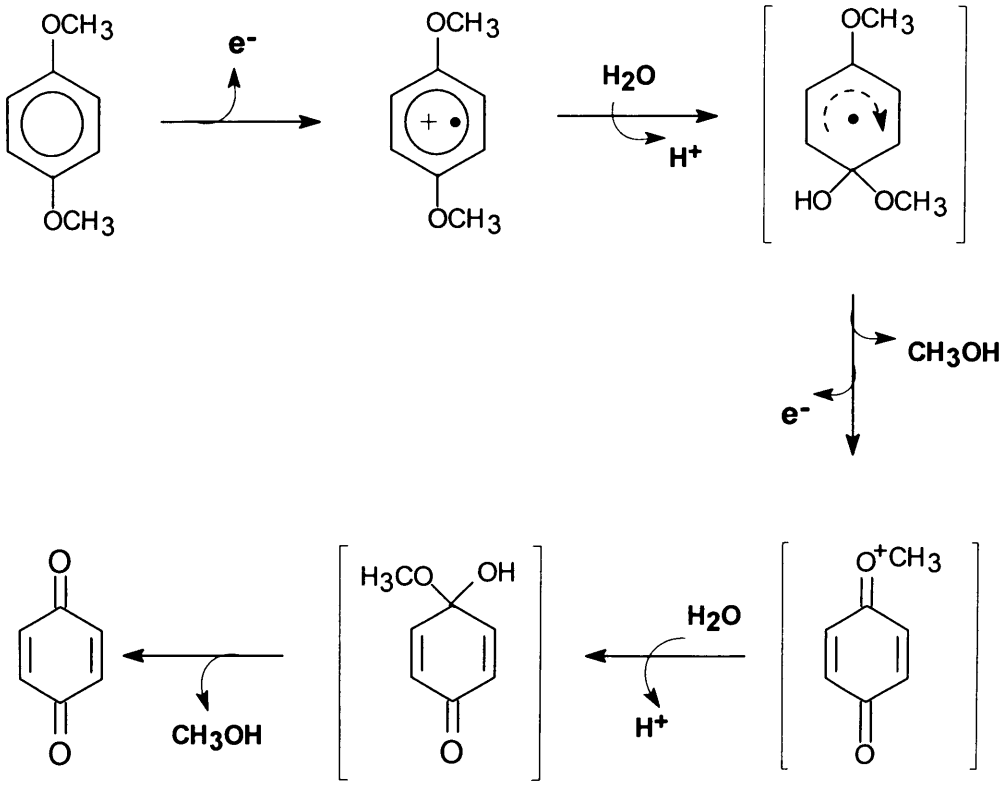


Figure 1.17: Scheme showing two sequential one-electron oxidation of 1,4-dimethoxybenzene by ligninase and addition of water and loss of methanol (Kersten *et al.*, 1985).



1.4.3.2.2 LACCASE

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is the most widely distributed of the large blue copper-containing phenol oxidases, being found in higher plants and many fungi (Thurston, 1994). Laccase differs from peroxidases in that it does not require hydrogen peroxide to oxidise substrate. Laccase is dependent on four copper ions distributed among three different binding sites for its function and each copper ion appears to play an important role in the catalytic mechanism. Copper may be distinguished using UV/visible and EPR spectroscopy (Leontievsky *et al.*, 1997a). Type I copper gives a blue colour to the protein from an absorbance at about 600 nm and is EPR detectable. Type II copper confers no colour, but it is EPR detectable and Type III copper is a pair of copper atoms that give a weak absorbance in the near UV and have no EPR signal. The copper can be removed quite readily from all laccases with loss of activity. For their involvement in lignin biodegradation, the wood rotting fungi are the best documented. Laccase is present in the white-rot fungi and absent from soft-rot fungi (Haider and Trojanowski, 1975; Blaich and Esser, 1975).

Laccase oxidises a wide range of substrates including simple diphenols, catechol, guaniacol, 2,6-dimethoxyphenol, p-phenylene diamine, syringaldizine (Thurston, 1994). Substrate oxidation by laccase is a one-electron reaction generating a free radical like peroxidases. With 1,2,4,5-tetramethoxybenzene as substrate the initial product is the carbon-centred cation radical formed by removing one electron from the aromatic nucleus (Kersten *et al.*, 1990). The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation (converting phenol to quinone with many substrates), may undergo non-enzymatic reaction such as hydration and may take part in a polymerisation reaction giving an amorphous insoluble melanin-like product.

For several years, the role of laccase in the process of lignin degradation has been investigated: some researchers conclude that laccase plays an essential role in lignin biodegradation, whereas others conclude that laccase is involved in the synthesis of lignin rather than its biodegradation.

Morohoshi (1991) showed that laccase III from *C. versicolor* catalyses β-O-4 cleavage and Cα-Cβ cleavage and alkyl-phenyl cleavage, which are the main bonds in the lignin polymer, by using model compounds such as guaiacylglycerol-β-guaiacyl ether (GOG), guaiacylglycerol-β-syringyl ether (GOS), syringlyglycerol-β-guaiacyl ether (SOG) and syringlyglycerol-β-syringyl ether (SOS) (Figure 1.18).

It is known that the methoxyl content of lignin in decayed lignocellulose is generally lower than in intact lignocellulose. Ishihara (1980) demonstrated that laccase can catalyse demethoxylation of several lignin model compounds. Kawai *et al.* (1988) found that 4, 6-di-t–butylguaiacol is converted to a ring-opening product by the laccase of *C. versicolor*. They proposed the pathway for ring opening of 4, 6- di- t-buthylguaiacol by laccase (see Figure 1.19).

Although all these studies on the side chain cleavage and ring opening of lignin model compounds showed that laccase is involved in degradation of lignin, there are some observation that suggest otherwise. Evans (1985a) demonstrated that degradation of milled-wood lignin with laccase from *C. versicolor* was unaffected when laccase activity was inhibited by addition of a laccase specific antibody and partial depolymerisation of milled-wood lignin was observed. However, later Dodson *et al.* (1987) isolated an extracellular peroxidase from the same fungus, which was similar to the lignin peroxidase isolated from *P. chrysosporium*. Therefore, the presence of lignin peroxidase or manganese peroxidase might contribute to the lignin degradation.

One of the problems in the study of the role of laccase in lignin degradation is that the redox potential of laccase is not sufficiently high to remove electrons from non-phenolic aromatic substances that must be oxidised during lignin degradation.

However, Bourbonnais and Paice (1990) demonstrated that two artificial laccase substrates, ABTS (2, 2'-azinobis-[3-ethylbenzthiazoline-6-sulfonate] and Remazol Blue, could act as redox-mediators that enable laccase to oxidise non-phenolic lignin model compounds. In addition to that Eggert *et al.* (1996) isolated laccase from *Pycnoporus cinnabarinus* which secretes neither lignin peroxidase nor manganese peroxidase. Laccase oxidised five dimeric lignin model compounds. In presence of a redox mediator, 3-hydroxy anthranilic acid (3-HAA), laccase degraded a non phenolic lignin model dimer.

Leontievsky *et al.* (1997a, b) isolated yellow and blue copper containing laccase from solid-state culture on wheat straw containing lignin and submerged cultures of *Panus tigrinus*. Yellow laccase isolated from solid-state culture showed unusual properties in that it oxidised a non-phenolic dimeric lignin model compound without any addition of redox mediators unlike blue laccase.

From these finding it can be concluded that laccase can play an important role in lignin degradation. For this reason, in this research we have used laccase for the effective removal of dung from hides.

Figure 1.18: Proposed degradation pathway of syringlyglycerol-β-guaiacyl ether by laccase from *C. versicolor* (Morohoshi, 1991)

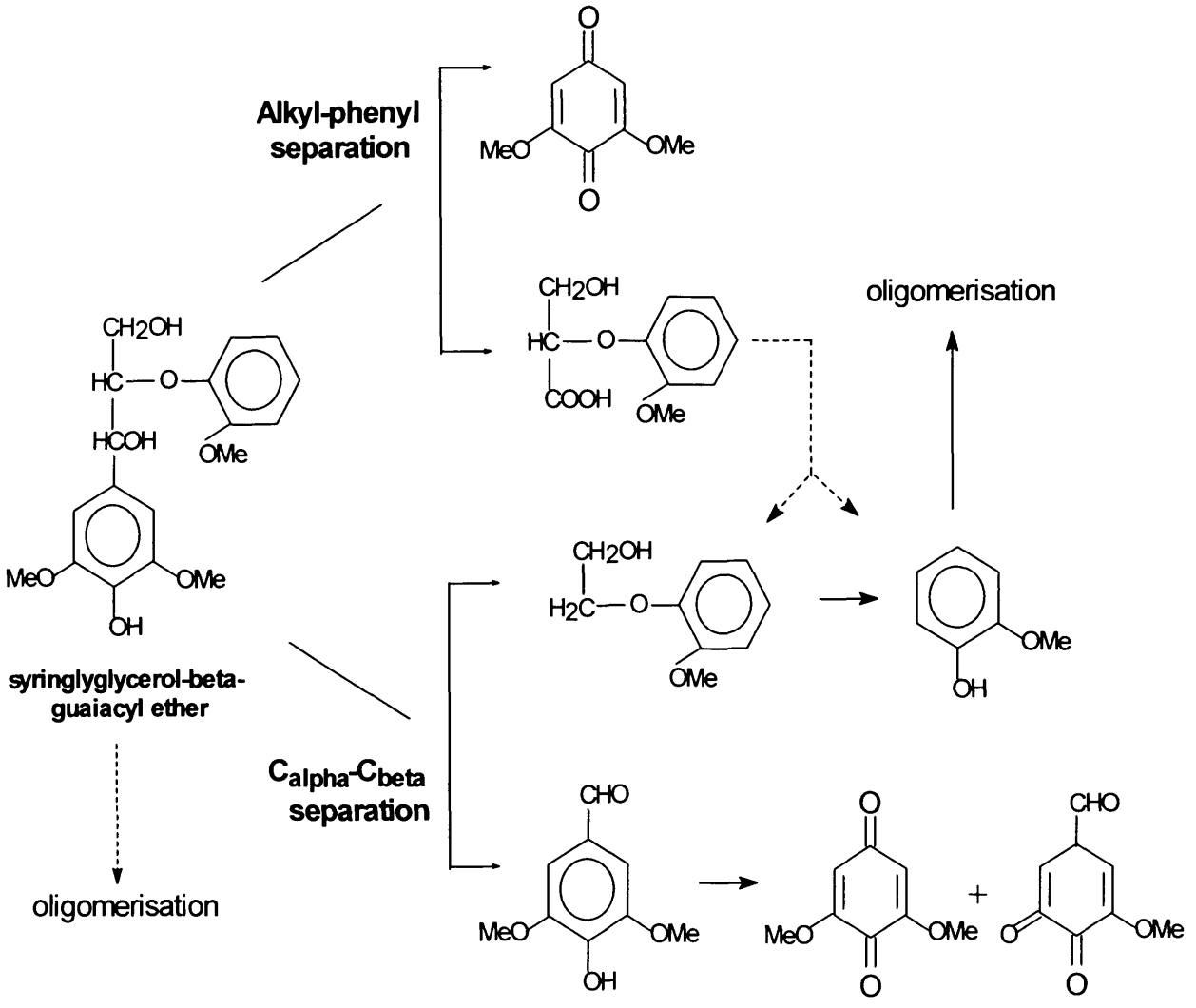
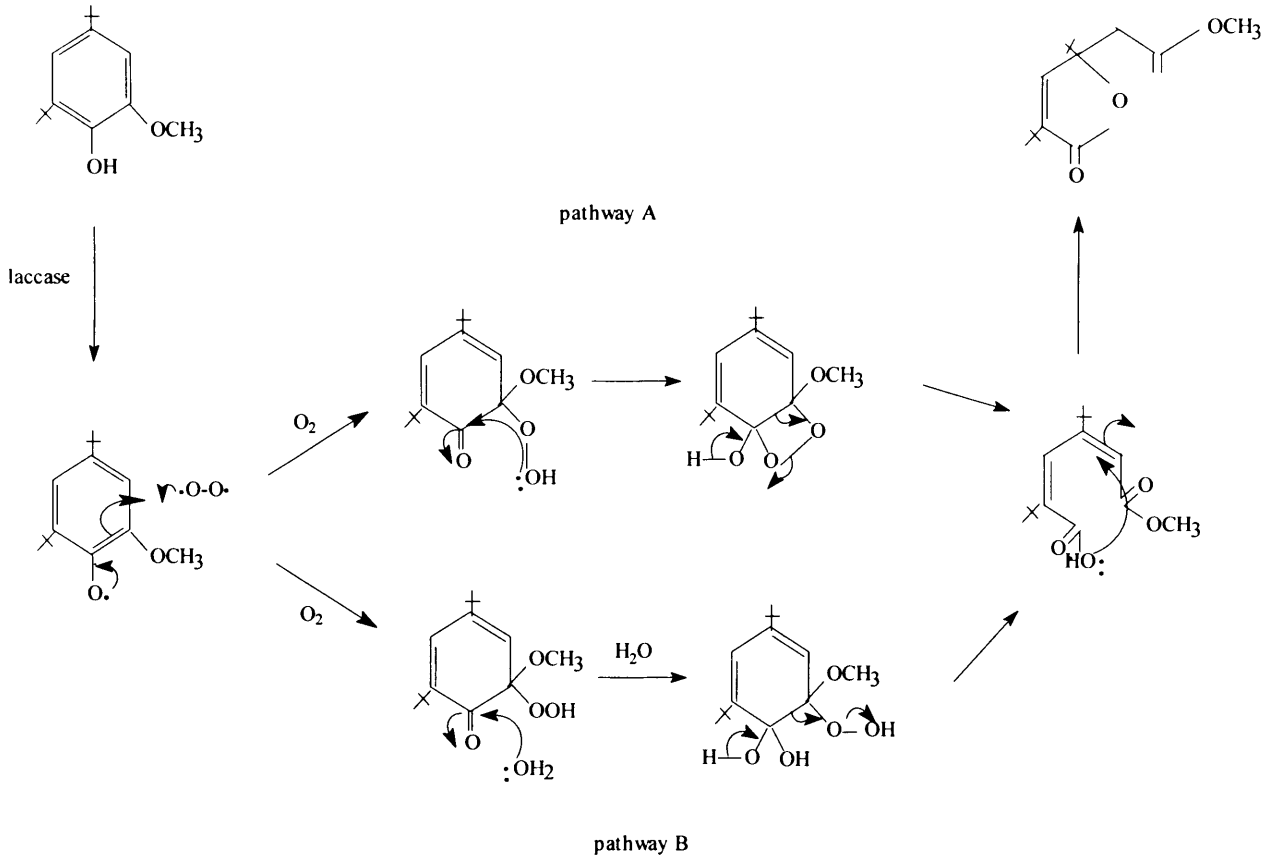


Figure 1.19: Mechanism for degradation of 4,6-di-t-butylguaiacol by laccase of *C. versicolor* (Kawai *et al.*, 1988)



1.5 ENZYMOLOGY IN LEATHER INDUSTRY

Before introducing dung removing enzymes into the beamhouse process, it is useful to review the roles of enzymes in the early stages of leathermaking, in order to understand the reactions currently exploited and to see how new enzymes might react with this component.

1.5.1 SOAKING ENZYMES

It is worthwhile briefly mentioning the preservation process before starting to discuss typical soaking and enzymatic soaking processes because these two processes are related to each other.

When the animal is slaughtered, the body's defence fails. Hides and skins begin to putrefy immediately after removing them from the animal carcasses. The putrefaction process is accomplished in two ways: autolysis (self-digestion) and bacterial action. The autolysis can be explained in the following way; the metabolic processes are changed dramatically due to the death of animal, i.e., the supply of oxygen and nutritional components are stopped. This results in destruction of the enzyme controlled processes because of inactivation by inhibition of some coenzymes. Due to the permeability change in the lysosomal membrane of the cell, some enzymes such as catepsins are freed and start to decompose proteins. Lysosomes not only contain protein digestive enzymes but also contain enzymes which can digest sugars, lipids and other components of the skin. The putrefaction process due to bacterial action can occur in the following way; bacteria may live on the skin of the animal or inside it, such as in scratches, insect bites, etc. or the body openings and hair pockets may be their route to the inside. In the living organisms,

bacterial action, growth and propagation are prevented because of immunological barriers. Once the animal is dead, these barriers collapse and bacteria are able to penetrate into the organism.

The object of preserving hides and skins is to make them non-putrescible so that they can be transported and stored until the tanner is ready to put them into work. Practically there are three ways to prevent hides and skins from putrefaction: reducing the moisture content (salting, drying), reducing the temperature (such as chilling, freezing) and the use of bacteriostatics and bactericides. Curing with common salt (NaCl) is still the most widely practised preserving method. Preserving by drying is performed in some developing countries. Other methods are not commonly used for hide preservation.

Soaking is the first process step in leather manufacture. It serves to remove the preserving salt and to free the hide from dung, blood and dirt. At the same time, hide is rehydrated to its original state, non fibrillar proteins (albumins, globulins) and some proteoglycans are eliminated, in other words, the hide structure is started to be opened up.

Many enzymatic products have been used in soaking of hides and the advantages of an enzymatic soak have been known to the tanner for many years. The enzymes are mainly proteases, lipases and carbohydrases. Before the days of using these enzyme products, it was customary to use a standing bath for several packs. This soaking involved much dirt and bacteria which had a proteolytic activity and accelerated the soaking process. However, this type of soaking was always in danger of damaging the hide substance due to uncontrolled bacterial action. Recently, with advances in biotechnology, enzymes have been employed in the process in isolated forms and measured quantities (Trabitzsch, 1966).

Several literature reports concerning the advantages of enzymes in the soaking process have been published (Trabitzsch, 1966; Dhar, 1974; Taeger, 1985; Taylor *et al.*, 1987; Alexander, 1988; Pfleiderer and Reiner, 1988; Christner, 1995). The claimed advantages of soaking might be summarised in the following way:

- Enzymes accelerate the soaking process, thus decrease the process time
- Removal of non fibrillar proteins and glycoproteins is increased
- Better and uniform liming can be achieved
- Better degreasing effect is obtained when compared to non enzymatic soaking
- Reduction of the wrinkles in the grain and loosening of the scud.

Particular suggestions were made for the use of proteases for soaking dried or dry salted hides and skins due to their difficulties in soaking back (Trabitzsch, 1966 and Dhar, 1974). Proteolytic enzymes are able to disintegrate the non collagenic proteins and in the course of this reaction these proteins are decomposed to smaller peptides and are made partly water soluble by an increase in hydrophilic end groups.

There are several literature reports and patents concerning the use of enzymes in soaking. A summary of the literature is given in Table 1.8. It can be seen from the summary table that most of the literature concerns the practical aspects of the enzymatic soaking; there are few reports concerning the mechanisms and actions of the enzymatic soaking materials. Therefore it is worthwhile mentioning these few references.

Alexander (1988) reviewed the enzymatic beamhouse process; particular attention was paid to the role of enzymes in proteoglycan removal. Lambskins tend to produce

firmer leathers due to their unusually high levels of endogenous non collagenous protein and proteoglycan. When proteolytic enzymes are used during the soak/scour process, removal of the non-collagenous protein and dermatan sulphate from skin is increased dramatically (see Table 1.9). Opening up is improved and the double faced shearlings produced are softer and lighter in substance.

In 1992, Zugno used biotinylated trypsin (trypsin labelled with hexahydro-2-oxo-1 H-thieno [3, 4-d]-imidazolo-4-pentanoic acid) to observe the penetration of trypsin into hide. It was found that trypsin penetrates through the epidermis and digests components in the epidermal-dermal junction, which facilitates the removal of the epidermis and increases the permeability of the hide. Also trypsin was found inside and around the blood vessels and arteries, sebaceous glands, fat cells and muscles, suggesting that trypsin digests the cytoplasm of the cells and these changes may result in leather with different properties (better fat removal and more elasticity).

Table 1.8: Summary of use of enzymes in soaking processes

Enzyme used	Claims	Reference
Proteases and Carbohydrases from *A. paraciticus*, *A. falvus*, *A. oryzae* and *B. subtilis*	Leathers were full, supple and showed no loose grain	Grimm (1966)
Carbohydrase from *Aspergillus avamori*	Improved wetting of hides, fewer flaws, 1% increase in the area and reduction of the time of treatment	Rokhvarger and Zubin (1971)
Aspergillus oryzae	Shortening the time to 4-5 hours, elimination of degreasing and increased skin whiteness	Toshev and Esaulenko (1972)
Pancreatin papain	Soft and finely grained leathers	Bayerlein *et al.* (1973)
Polygalacturonase	Increased surface yield and improved quality of fur	Dianova *et al.* (1977)
Bacillus subtilis	Long lasting enzyme activity and this activity was not affected by anionic surfactants	Cristescu and Bratulescu (1979)
Streptomyces rimosus fermentation broths	This inexpensive treatment produces a uniform hide swelling, which facilitates unhairing and does not adversely effect hide elasticity	Oertel *et al.* (1989)

Table 1.9: Removal of interfibrillary protein from lambskin during soaking (Alexander, 1988)

Process conditions (pH 10, 35 °C, 4 h)	**Removal (%)**		**Organoleptic assessment of skins (0 bad-10 good)**	
	Non collagenous protein	Dermatan Sulphate	softness	Lightness of substance
No enzyme (control)	9	13	5.0	4.6
Enzyme	16	38	7.0	5.9

1.5.2 LIMING ENZYMES

Depilation by lime alone is the oldest method known, having been used from ancient times, and it was still practised generally until about the middle of the 19th century. The operation was carried out in wooden vats which were distinguished by the names "dead", "weak" and "live" vats, corresponding to the old, mellow and new limes. Duration of liming today is about 18-24 hours, however, the distinguishing feature of all the old methods of liming was the extraordinarily long duration of the process. In the 18th century, nine or ten months was common. It is certain that bacterial action played an important part in the process of unhairing when hides and skins were limed for long periods.

Procter (1903) stated that very old limes, especially in hot weather, often contain large numbers of active bacteria, which may be seen under the microscope. Wood

(1912) also found a large number of bacteria in an old lime in which pelts had been worked for 3 to 4 weeks. A question arises from the old liming process as to whether bacteria can play any great part in the liming process. It is likely to be the case in the old liming process before the use of sulphide. With the cutting down of the time of liming, however, there seems little time available for bacteria to develop and play any major part in the unhairing and loosening process. Thus the liming process today brings about unhairing and loosening by virtue of the facts that lime is an alkaline buffer and that sodium sulphide acts on the hair protein.

1.5.2.1 CHEMICAL ASSISTED ENZYMATIC UNHAIRING/LIMING PROCESS

There is still no commercial process available in the leather industry to achieve unhairing by using only enzymes apart from traditional sweating process. In this process, sheepskins are left in dark, humid rooms in a warm condition of 21-27^{o}C until the wool is loose. Under these conditions, bacteria thrive and attack the soft keratin, resulting in hair slip. However, the sweating process for dewoolling sheepskins is essentially an uncontrolled enzyme process (Sharphouse, 1971). Putrefaction might occur and there might be serious weakening of the grain and in the overall strength of skin, if certain precautions are not taken.

Therefore, there is a need in the leather industry for enzymatic unhairing systems which eliminate the use of lime and sulphides, which are difficult and expensive to remove from effluent; such systems should be controllable, and at the same time should not have any side effects on leather.

The mechanism of enzymatic unhairing system is still unclear. Yates (1968) stated that "there is no doubt that the full potential of an enzyme system will not be

realised until the mechanism of the depilation process is understood and an enzyme system can be designed specially for the job".

In 1950s and 1960s, some researchers thought that removal of some hide components with enzymes would be a contributor to the unhairing. Burton, Reed and Flint (1953) considered that when mucoid materials, which are in close association with fibrous protein structure and are present in the spaces between the corium fibres especially at the epidermal-corium junction and around hair follicles, are removed, the cohesion of the protein fibres is reduced and the entire structure is loosened. They stated that mucoid material may be removed by two methods; treatment with alkalis and treatment with mucolytic enzymes. For this reason, mucolytic enzymes were used for unhairing, to attack the mucolytic materials at the epidermal-corium junction. Owing to the fact that those enzymes have no proteolytic activity, the protein structure should not be degraded. The skins were treated with enzymes at room temperature for 3 days. The epidermis was detached in the form of continuous sheets, bringing the hairs (including the short ones) with it. The hair and grain surface appeared to be undamaged.

Gillespie (1953) replied to the Burton *et al.* (1953) paper. He criticised the lack of precautions taken against bacterial depilation during the three days enzyme unhairing using mucolytic enzymes. Removing of the epidermis in the form of continuous sheets might be due to bacterial attack since similar attack is often to be seen during the bacterial sweating of sheepskins.

In the same paper Gillespie (1953) tested a number of fungal preparations for their unhairing actions. *A. parasiticus*, four strains of *A. flavus-oryzae*, two of *A. orchraceus* and one of *A. wentii* depilated the skin in 30 hours, whilst *A. terricola* and *A. niger* produced only partial depilation in 40 hours. In all instances when

moulds were capable of depilating skin, they also produced proteases which attacked gelatine. Under comparable culture conditions certain strains of *A. oryzae* produced a much more active depilatory enzyme than did *A. parasiticus* or any of the other species tested. Unlike the *A. parasiticus* culture, *A. oryzae* appeared to damage pelt, probably because they are richer in proteases. Gillespie (1953) also investigated some bacterial and animal enzymes for their unhairing action. Most strains of *Proteus vulgaris*, *Flavobacterium esteroaromaticum* and *Bacillus subtilis* produced active filtrates. All filtrates capable of sweating sheepskin in pure culture also produced depilatory enzyme and for depilatory activity they all required the addition of a compound which splits disulphide bonds.

Bose *et al.* (1955) prepared collagen, reticulin, elastin, keratin, albumin, globulin and glycoprotein (mucoids) from skins and the actions of protease isolated from the latex of modar plants (*Calotropis gigantea*) and amylase isolated from a cheap cereal 'ragi' (*Eleusive Coracona*) were studied under controlled conditions on the individual components. All the non-fibrous proteins viz., albumins, globulins and mucoids were quite readily hydrolysed by the protease whereas among the fibrous proteins, elastin and reticulin were found to be only slightly hydrolysed and collagen and keratin were found to be unaffected at all by the enzyme. It was indicated that the process of unhairing skins and hides by the protease depends in general on the hydrolysis and removal of the globular proteins. However, it was unclear whether the enzymatic unhairing was due to the hydrolysis of only one type of globular protein or all the three proteins together or any two proteins in combination. Amylolytic enzymes were equally capable of unhairing skins and hides as proteases. However, no protein components were hydrolysed by amylases. Glycoproteins treated with amylases showed the positive Molisch reaction that indicates the presence of a carbohydrate. Therefore it was concluded that the process of enzymatic unhairing by

means of proteolytic or amylolytic enzyme depends essentially on the hydrolysis and removal of mucoid materials.

Cordon (1955) used an amylase preparation with several antiseptics and pretreatment. When the fresh hide was treated with the amylase, there was little or no hair loosening. However, when hides were pretreated with NaCl or urea, unhairing was more effective than without any treatment. It was concluded that the function of pretreatment substances is not clear but it appears that they dissolve some constituent of the hide that prevents the action of the enzyme. Moreover, it was stated that which enzymes are responsible for unhairing is not known because commercial amylases used in the research were certainly not single enzymes, and it may be well be that on entirely unsuspected enzyme is responsible for the depilatory action.

Lindroth (1961) used raw pancreas which had been defatted, activated and desiccated; it contained trypsin and amylase in addition to being rich in lipase, carboxypeptidase and other pancreatic enzymes. A comparison of the cross-sections of both lime-sulphide and enzyme unhaired stock showed that the epidermal tissue surrounding the hair bulb was modified so that the hair was loosened and could be removed mechanically. The lime-sulphide stock showed slight alteration of the elastic tissue in the epidermal area. The enzyme-unhaired stock had no elastic tissue remaining in the epidermal area. The removal of the elastic tissue indicated that the enzyme system had good activity in the epidermal and lower corium areas, but its activity in the centre corium was less, as the elastic tissue in the arteries in the centre corium was still demonstrable. In the enzyme unhairing stock the activity of the enzyme system was not confined only to the elastic tissue but also had an effect upon the collagen, particularly at the junction of the epidermal-corium areas.

It is very desirable from many points of view if the depilatory activity of the enzyme preparations can be correlated with their activity against a simple substrate. This would considerably facilitate the assessment of new depilatory systems and undoubtedly shed some light on the mechanism. For this reason, Cordon *et al.* (1961) and Jones *et al.* (1961) attempted to find an assay procedure that would correlate with the depilatory action of the enzymes. There was no correlation found between hair loosening activity of enzymes with action on casein, starch and elastin. In addition to that, enzymes of plant, animal and microbial origin were assayed by three different procedures (formol titration, degree in viscosity and liquefaction) using gelatine as a substrate and no correlation was found.

Yates (1968) used a number of commercially available enzymes and for comparing the enzymatic unhairing results of the enzymes, the enzyme offer was based on an equivalent protein nitrogen rather than a weight basis, equivalent amount of activity basis and cost basis. It was thought that this is as close as possible to a valid criterion for comparison. All the enzymes with the exception of pielinase in the case of dry skin and pangestin in the case of green skin, were better than the water control. Pangestin, which is a highly active proteolytic preparation, has a very poor depilatory activity, while crystalline trypsin is only slightly superior. It was concluded from this study that depilation is not a single enzyme process. It was interesting that pronase, which is known to break down proteins completely to amino acids, had a strong depilatory activity. It was also very significant that pronase has a high mucolytic activity. The Novo bacterial amylase preparation had a reasonably high depilatory activity but this was not surprising when it was realised that it had an active proteolytic component too. It was interesting that the depilatory activity of this preparation was significantly increased by the addition of trypsin and it was important that the addition of hyaluronidase did not have a very marked effect. The pure α-amylase did not have any depilatory activity under any of the conditions.

Yates (1972) used a number of crystalline enzyme preparations, namely; pronase, alcalase, nagarase, bacterial proteinase, pangestin, trypsin and α-chymotrypsin. He investigated the correlation of hyaluronidase, chondroitinase, and some miscellaneous enzyme activities with depilation activity. However, it was found that there was no correlation with depilatory activity except activity against casein. He considered the problem of the mechanism of enzyme depilation under three following sections:

- what type of bonds are required to be broken by the enzyme system?
- Within these types do specific bonds have to be broken?
- Can the required bonds be broken by a single homogenous enzyme species or is a multiple enzyme system necessary?

The involvement of peptide bond breakage was further investigated by the same author, with regard to the peptide bonds which need to be broken. The high correlation between the caseinolytic activity and depilatory activity suggests that a broad specificity of peptide bond breakage may be the prime requirement. Furthermore, no attempt was made to verify the homogeneity of the crystalline enzymes used. The involvement of peptide bond breakage in the depilation mechanism is well established. However, the precise nature of the peptide bonds which need to be broken is not known, nor is it clearly established if peptide bond breakage is the only type of cleavage required for depilation to occur. Oxidised insulin and casein were used in these experiments as substrates for the crystalline enzyme preparation, to measure the rate and extent of the appearance of free amino groups as measured by the ninhydrin method.

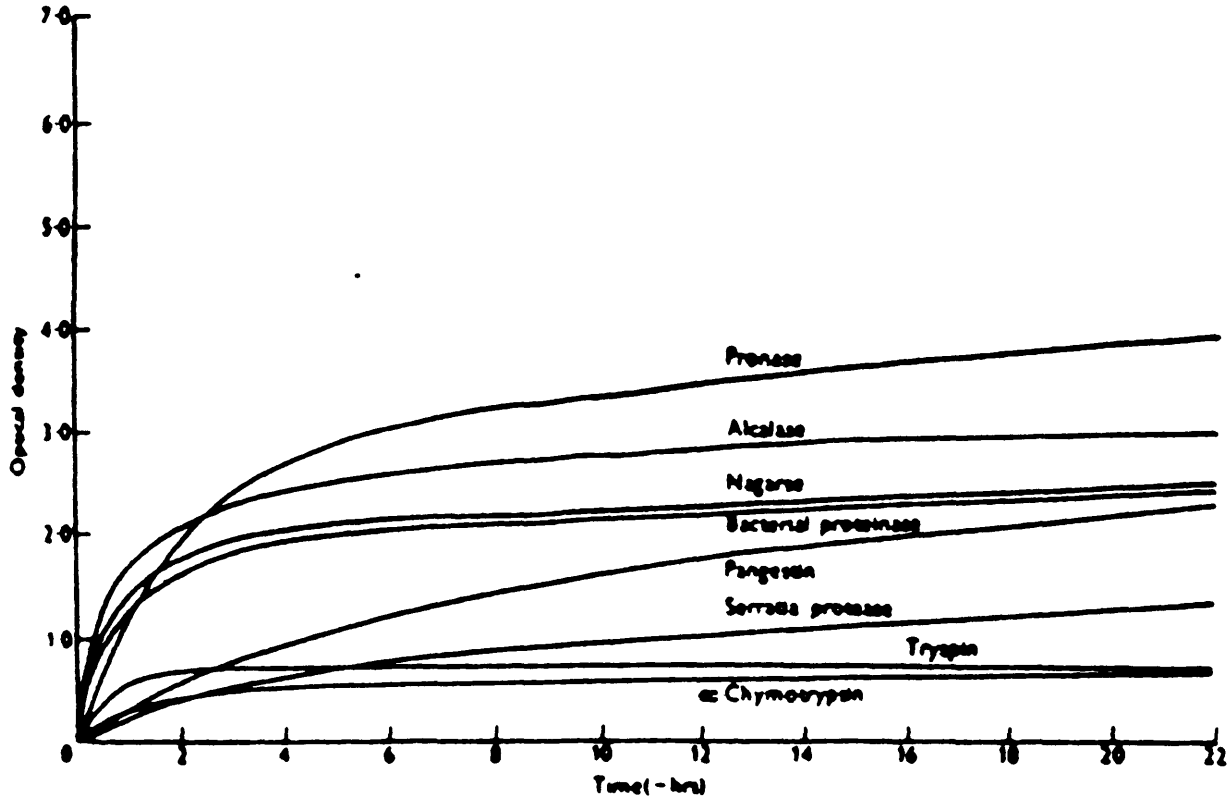
Figure 1.20 shows the rate and extent of appearance of free amino groups from the oxidised insulin experiment. The picture with casein was very similar. It can be seen that the enzymes divide themselves into two clear groups, the top four curves representing the good depilation agents and lower four the poorer ones. Pronase has been shown to have a very broad specificity and to break a large number of peptide bonds. It is, of course, a system containing a number of different enzymes, with both endo and exo-peptidase activity. Alkali proteases (Alcalase, bacterial proteinase and nagarase) hydrolyse peptide bonds containing the carboxyl groups of such amino acids as L-tyrosine, L-phenyl alanine and L-leucine and show a broad specificity, hydrolysing about 18% of the bonds in the oxidised B chain of insulin.

It can be seen from Figure 1.20 that the curves of pronase, pangestin and possibly serratia do not level off like the curves of alcalase, bacterial proteinase and nagarase that are theoretically single enzyme systems. Therefore, it was suggested that the pronase, pangestin and serratia preparations may contain exopeptidases which continue to split off single amino acid residues after the endopeptidases have split the main protein chain. Pronase is known to possess both amino and carboxypeptidase activity and pangestin also contains exopeptidase activity. In the case of pangestin, the main type of proteolytic activity is of the exopeptidase, and this accounts for the slow but continuous rise in the number of free amino groups. However, it was suggested that for adequate depilation to occur some endopeptidase activity is required to break the large peptide chain into smaller units; and the suggested balance of protease activity in pangestin with its predominantly exopeptidase nature, could account for the poor depilatory activity of this preparation.

Yates (1972) concludes that depilatory activity is uniquely related to the capacity of an enzyme preparation to break peptide bonds. Further, with certain qualifications the more peptide bonds that can be broken, the greater the depilatory activity of the

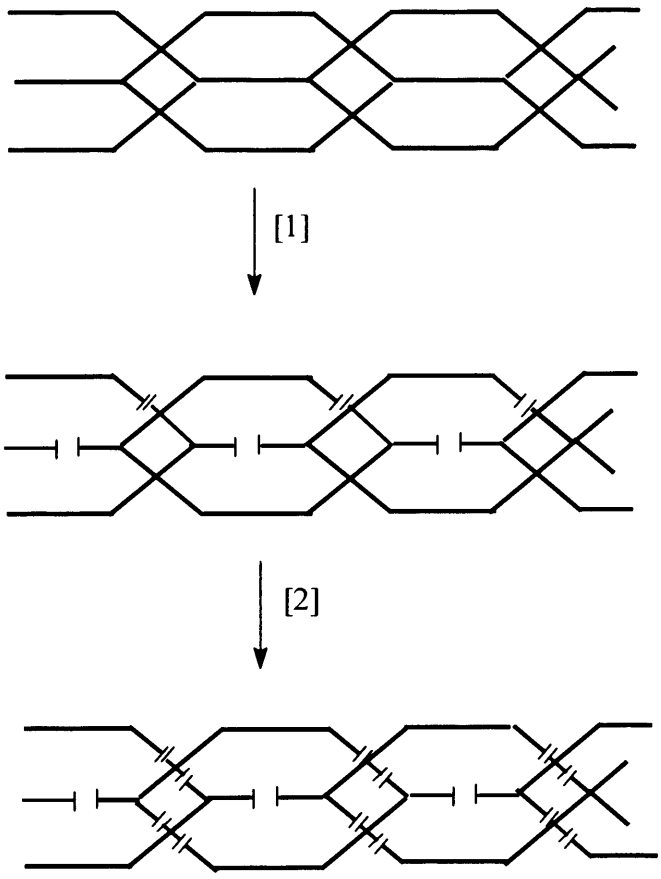
preparation. However, whether or not satisfactory leather can be produced from hides and skins depilated with strongly proteolytic preparations is another question.

Figure 1.20: Appearance of free $-NH_2$ groups with time from the action of the enzymes on oxidised insulin, measured by the ninhydrin technique (from Yates 1972)



Brady *et al.* (1990) supported the involvement of peptide bond hydrolysis and requirement for broad-specificity proteolytic activity of endopeptidase type enzymes. They discussed the involvement of the basement membrane at the dermal-epidermal junction, which is the primary site of depilatory action. The basement membranes consist of a network of globular glycoproteins, proteoglycans and non-fibrous type of collagen. As the basement membrane binds the epidermis to the dermis, its disruption would lead to the simultaneous removal of the epidermis and associated hair or wool. Unless the integrity of the basement membrane is disrupted by the strongly alkaline conditions of liming for instance, very broad specificity of enzymatic cleavage will be required, otherwise, it would require fewer enzymatic cleavages to disrupt the integrity of the protein network. Moreover, it is likely that denaturation would result in the exposure of more peptide bonds previously hidden in the hydrophobic centres of domains, making it more likely that a single protease with a narrower range of bond specificities could disrupt the integrity of the protein. This might allow depilation by proteases with a narrow range of specificity than might be required in proteolytic degradation of the intact, native protein. Brady *et al.* (1990) applied a model that is given in Figure 1.21. The model predicts that a narrow specific protease would be able, with increasing concentration or time, to cause a decrease in the depilation load, but up to a certain intermediate point. At this stage it would have degraded all the components of the basement membrane that it was capable of degrading. The number of complementary enzymes required would decrease if several of the intermolecular bonds were disrupted or more cleavage sites were exposed by hydrolysis or denaturation during liming.

Figure 1.21: Schematic representation of the degradation of the basement membrane. (1) The network remains intact. However addition of proteases of complementary specificities (2) is likely to lead to increased disruption of the network (from Brady *et al.*, 1990)



1.5.2.2 ENZYME ASSISTED CHEMICAL UNHAIRING PROCESS

Enzyme assisted chemical unhairing has been used in the leather industry for several years. Mostly proteolytic enzymes are used in order to facilitate the liming process and reduce the amount of chemical use. The opening up of collagen fibre structure of hide or skin during liming is associated with the removal of dermatan sulphate (Alexander *et al.*, 1986; Alexander, 1988). They found that under the practical tannery conditions over 50% of the endogenous dermatan sulphate was removed from bovine hide during an 18 hours sulphide unhairing/liming step. Essentially none was extracted during extensive soaking and this was attributed to the strong binding of the proteogylcan to the collagen. Proteoglycan can also be degraded by proteolytic enzymes as well as by alkali. Addition of an alkali stable enzyme to the lime/sulphide liquor accelerated the removal of dermatan sulphate and the opening up of the fibre structure (see Table 1.10).

Alexander (1988) summarised the advantages of enzyme assisted chemical unhairing in the following way:

- shortening of liming time
- increased opening-up of the fibre structure and production of softer leather
- improved pulping and production of cleaner leather
- reduction of sulphide requirement
- increased area yield

Table 1.10: Enzymatic degradation of dermatan sulphate proteogylcan during liming of bovine hide (from Alexander *et al.*, 1986)

Liming conditions	**Removal of dermatan sulphate (%)**
None	0
6 h	22
6 h + 0.01% NUE*	47
6 h + 0.02% NUE*	53
18 h (control)	50

*NUE: Novo Unhairing Enzyme

1.5.3 BATING ENZYMES

Bating is one of the most curious and also one of the most important procedures in manufacture of leather. It is the only step in leather processing where chemical methods cannot be substituted for enzymatic processes (Pfannmuller, 1956). The mechanism and application of the bating process have been reviewed by Green (1956), Pfannmuller (1956), Wilson (1956) and Alexander (1988).

The purpose of bating is to obtain certain desired characteristics in the final leather. The highly swollen state of unhaired skin prior to bating makes it unsuitable for most tanning processes. Therefore, previously tanners realised the need for further treatment of limed skin. For this reason, they treated skins with animal dung whose action was mainly bacterial. Fowl dung was used when a relatively weak action was desired, for stronger action, dog dung (Pfannmuller, 1956). These processes were

very difficult to control and required expert judgement in order to avoid putrefaction and damage to the skin. Wood (1912) discovered that the dung bates owed their primary activity to the action of bacterial proteases. In 1908, Otto Röhm patented the first standardised bate, Oropon, based on pancreatic enzymes.

The main enzyme system responsible for the bating action is always proteolytic. The enzyme systems most extensively used for commercial bating purpose are still those derived from animal pancreas. The bate manufacturer utilises as the source of raw material the pancreas glands of pigs, sheep, cattle and other mammals (Pfannmuller, 1956). However, bating enzymes can be prepared from bacterial and fungal origins such as *Streptomyces ssp.* (Padmavathi *et al.*, 1995), *Aspergillus flavus* (Malathi *et al.*, 1991), *Bacillus subtilis* (Hameed *et al.*, 1996a and 1996b).

Actions of bating on skin components have received a lot of attention. According to Heidemann (1993) the main purposes of bating are removal of predigested or prehydrolysed noncollagenous proteins and denatured collagen as well as removal of scud (composed of epidermal matter, hair roots, fine hairs, pigments, fats, sebaceous and sweat glands).

It has been argued whether the bating process has any effect on elastin structure (Green, 1956 and Pfannmuller, 1956). If elastolytic enzymes are present in the bating preparation, it may be expected that a change in the elastic tissue of the skin during bating is likely. Alexander *et al.* (1991) developed assay techniques for characterising the relative activities of enzymes against the different components of skin (e.g. elastin, keratin, albumin, etc). When elastolytic activity rich protease preparations are used in bating (in this case bacterial proteases), some special effects were obtained. The breakdown of elastin in bating resulted in a reduction in grain thickness and overall increase in leather strength and area yield. Moreover, it was

suggested to use elastase active bates in production of soft, thin, strong bovine clothing leathers, upholstery leathers, for reducing growthiness in heavy hides and reducing mottle in sheepskins.

It is also believed that in soaking and liming operations, the majority of the interfibrillary substances, which are albumins, globulins and mucoids, are removed. However, some still remaining after these operations are removed in the bating process by the action of proteolytic enzymes.

1.6 INTRODUCTION TO THIS STUDY

Contamination of hides with dung leads to great problems in the leather industry. Avoiding this problem seems to be simple, however, farmers are mostly reluctant to apply any action against this problem. Therefore, this problem has to be resolved by tanners. It has been shown that attempts so far have not been successful due to lack of scientific background. Effective removal of dung from hides in the soaking process can lead to reduced process time, chemical additions and potential damages in subsequent mechanical operations. In addition to that, currently hides are split into the layers either at the limed stage or wet-blue stage. Due to the presence of dirt on soaked hides, green splitting is always avoided. Therefore, there is potential to split hides in the soaked stage if clean and dung free hides are produced. This operation will produce uncontaminated flesh splits which can be used in food and cosmetic industries and make a higher value byproduct. Moreover, effective dung removal can have potential application for live animals, either on the farm or at the abattoir to address the problems of hygiene and associated dangers of *E. coli* O157.

In order to target the dung with potential enzymes, the composition of cattle dung has to be established. It has already been shown in section 1.3 that lignocellulosic

components are likely to be involved in the constitution of dung. Therefore, more attention will be paid to lignin, cellulose and hemicellulose chemistry.

Experiments can be designed to establish the background strategies for the enzymology of dung removal by using commercial enzyme preparations. The kinetics of dung removal and solubility can be assayed by using a number of techniques.

Coriolus versicolor, *Aspergillus niger* and *Trichoderma reesei* fungal cultures are effective lignocellulosic enzyme producers. Their growing conditions can be modified to produce enzymes which are responsible for dung removal from hides.

The mechanism of dung removal should also be addressed such as the effects of enzymatic treatment on the components of dung. This can be done by using spectrophotometry, chromatography and chemical techniques.

After establishing the basic principles of dung removal, tannery trial experiments can be conducted in order to evaluate effects of commercial enzymes and fungal cultures on dung removal on the larger scale. Moreover, leather produced by these treatments can be evaluated in terms of their chemical and physical properties.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS AND REAGENTS

Unless otherwise stated all reagents used were supplied by either Sigma Chemicals Ltd., Poole, Dorset or by BDH Chemicals Ltd., Poole, Dorset.

2.1.2 EQUIPMENT

Unless otherwise stated the equipment used throughout this work was:

Spectrophotometer: Spectronic 501- Milton Roy Company
Rotary shaker water bath: Grant OLS 200
Incubator: Orbital Incubator SI 50- Stuart Scientific
Centrifuge: MSE Centaur 2
Microscope: Leica WILDM10 (light microscope)
S2500 Hitachi Scanning Electron Microscope

2.2 ANALYSIS OF PLANT MATERIALS

The difficulty in analysing plant material arises because one is not always dealing with chemical substances that can be clearly defined. This applies especially with cellulose, hemicellulose and lignin. As Jermyn (1955) wrote,

"It cannot be too strongly emphasised that 'cellulose' and 'hemicellulose' are normally determined as the resultants of certain sets of operations rather than as chemically defined species."

The result of this is that each method of analysing these substances may give different results. For this reason, it is necessary to give the exact conditions under which analysis was carried out. Proximate analysis was carried out because the complexity of the composition of plant materials makes complete analysis extremely difficult. When proximate analysis is used the percentages of the different fractions cannot be expected to add up to 100 %. Dung samples used throughout in this section were collected from heavily dung clad hides which originated from UK domestic cattle slaughtered in Midland Meat Packers Ltd., Crick, UK in December, 1996. Dung samples used for other experiments were collected from the same source at different times. Before analysis the pellets of dung were broken up, dried in air and cleaned from residual hairs.

2.2.1 ETHER SOLUBLE FRACTION

Five grams of air-dried dung were extracted with ether for 15 hours in a Soxhlet apparatus. The solvent was evaporated and the residue dried for 12 hours at 100°C and then weighed (Brown, 1972).

2.2.2 COLD WATER EXTRACT

The material left after ether extraction was transferred to a beaker and mixed with 150 ml of distilled water. After 24 hours the aqueous extract was filtered through a small, previously dried and weighed filter paper. The plant residue was washed with

2 or 3 lots of distilled water. The filtrate and washings were evaporated on a water bath. The residue was dried at 100 °C to constant weight (Brown, 1972).

2.2.3 HOT WATER EXTRACT

The same material used in section 2.2.2 was returned to a beaker. About 150 ml of distilled water was added. The beaker was placed on a boiling water bath for 3 hours. The material was filtered and the extract was treated in the same way as the cold water extract (Brown, 1972).

2.2.4 ALCOHOL SOLUBLE PORTION

150 ml of 95% alcohol were added to the plant material, then the mixture was placed on a boiling water bath for 2 hours. The remaining plant material was washed with hot alcohol. The filtrate and washings were evaporated on a water bath and dried at 100 °C to constant weight (Brown, 1972).

2.2.5 HOLOCELLULOSE

In this work holocellulose was isolated from dung using the method given by Jermyn (1955). The dung was first treated by breaking it up and boiling it for 30 minutes in 85% ethanol. The alcohol was filtered off and the residue washed with alcohol and ether and allowed to dry in air.

The weighed material (up to 5 g) was suspended in 160 ml of water in a 250 ml conical flask. The flask was immersed up to the neck in a water bath at 75 °C in a fume cupboard. 10 drops of glacial acetic acid and 1.5 g of sodium chlorite were added to the flask. A 25 ml conical flask was inverted in the top of the large flask to

form a loose stopper. After one hour and two hours the same amounts of acetic acid and sodium chlorite were added. After 3 hours the contents of the flask were filtered through a sintered glass funnel and washed ten times with ice cold water. These operations were carried out in the fume cupboard because chloride dioxide is given off. The residue was then washed with acetone and ether, and air dried.

2.2.6 CELLULOSE

The method used to isolate cellulose from the holocellulose was based on that of Corbett (1963).

Sodium hydroxide solution was prepared using carbon dioxide free water. (This solution contained 17.5 ± 0.1 g of sodium hydroxide per 100 g of solution). A weighed sample of about 3.0 g of air-dried holocellulose was used. 35 ml of alkali at 20 °C were added to this in a beaker. The mixture was allowed to stand for five minutes, and then macerated with a glass rod with a flattened end for 10 minutes. During the maceration 40 ml of alkali were added in 10 ml portions. The beaker and its contents were kept at 20 °C for a further 30 minutes. 75 ml of distilled water at 20 °C was added with thorough stirring. The mixture was filtered through a fritted glass funnel. The residue was washed with 750 ml of distilled water by means of gentle suction. It was then soaked for five minutes in 40 ml of 10% acetic acid at 20 °C. The residue was finally washed until free of acid, treated with ethanol and ether and dried to constant weight at 100-105 °C.

2.2.7 HEMICELLULOSE

The method used to isolate hemicelluloses was based on that described by Whistler and Faether (1965).

A 10% solution of sodium hydroxide was made with carbon dioxide free water. The solution was deoxygenated by passing nitrogen through it for at least two hours. The nitrogen was first passed through Fieser's solution* to remove traces of oxygen. Elimination of the oxygen minimises the oxidative degradation of the hemicellulose. (*Fieser's solution: This was prepared by dissolving 20 g potassium hydroxide in 100 ml of water. 2.0 g of anthraquinone-2-sulfonic acid (sodium 2-anthraquinone-sulphonate) was added to the warm solution. The red solution was used at room temperature and retained its efficiency in absorbing oxygen until it turned dull red or brown.)

About 2 g of holocellulose were weighed and placed in a 100 ml flask. 100 ml of deoxygenated alkali solution was added. Oxygen free nitrogen was bubbled through the solution for 30 minutes. The flask was then tightly stoppered and shaken gently for 20 hours at 20 ^{o}C. The contents of the flask were filtered through a sintered glass funnel. The residue was washed with 25 ml of the alkali solution followed by 75 ml of distilled water. The filtrate and washings were transferred to a beaker and cooled in an ice bath. The solution was acidified to pH 5 with 50% acetic acid. This causes part of the hemicellulose (hemicellulose A) to precipitate. The precipitate was removed by centrifugation and dehydrated by washing with 95% alcohol, absolute alcohol, acetone and ether. The remainder of the hemicellulose (hemicellulose B) was precipitated out of the solution by adding 3 volumes of acetone. The precipitate was centrifuged off and dehydrated by washing with ether. The two types of hemicellulose were combined and dried for 24 hours at 40^{o}C. They were placed in a dessicator and brought to room temperature before being weighed.

2.2.8 LIGNIN

The method described by Adams (1965) was used to isolate lignin from dung or other samples.

Approximately 1 g of the sample was weighed and extracted in a Soxhlet extractor for 4 hours with 95% ethanol. The extraction process was continued for a further 4 hours using 1:2 v/v ethanol and toluene. The residue was washed with 50 ml of ethanol to remove toluene. It was heated on a water bath for 3 hours with 400 ml of hot water. At the end of this time the sample was filtered off, washed with 100 ml of hot water and then with 50 ml of ethanol. The residue was dried in air.

The cellulose was removed from the sample by placing it in a small beaker and adding 15 ml of cold sulphuric acid (72%) slowly and with constant stirring for one minute. The beaker was covered with a watch glass and placed in a water bath at 20 ^{o}C for 2 hours. The acid was stirred frequently. The sample was transferred to a 1 litre flask and 560 ml of distilled water were added to dilute the acid to 3%. The flask was boiled for 4 hours and the volume was kept approximately constant by the addition of distilled water as necessary.

The lignin was allowed to settle and filtered in a fritted glass filter that had been dried to constant weight. The lignin was washed with 500 ml of hot water to remove the acid. The filter and contents were dried at 105 ^{o}C to constant weight.

2.2.9 PROTEIN

The protein fraction of the dung was determined by the standard Kjeldahl method. According to this method, 1 g of ground dung was transferred to a dry 800 cm^3

Kjeldahl flask. Nine grams of catalyst mixture (1 part $CuSO_4$ and 10 parts K_2SO_4, by weight) and 20 cm^3 of concentrated sulphuric acid were added. The flask was heated in a fume cupboard until the mixture went clear, and then for one hour longer. A blank containing only catalyst and acid was set up at the same time. The solution was cooled and 250 cm^3 of distilled water was added. And also some anti-bumping granules and some anti-foaming agent were added to the solution and then the flask was transferred on the distillation apparatus. 100 cm^3 of saturated boric acid were placed in a 500 cm^3 of conical flask and then some screened methyl red was added. This flask was placed under the outlet of the condenser. 85 cm^3 of 30% sodium hydroxide was placed in the tap funnel. After alkali was run into the Kjeldahl flask, the tap was closed. When the volume in the receiving flask was 300 cm^3, the tap in the funnel was opened and the heat was turned off. Finally, the distillate was titrated with 0.2 M HCl back to the purple colour. The percentage protein in the sample was calculated by applying the factor of 6.25 to convert the nitrogen value (Rosenberg, 1979).

2.3 MICROSCOPIC EXAMINATION OF DUNG CLAD HIDE

The physical interaction between dung and hide was examined using conventional light microscopy. Samples were taken from the heavily dung clad hide by using a surgical scalpel. Ten fold magnification was used in this investigation.

2.4 ENZYMES, CULTURAL TECHNIQUES AND ASSAY PROCEDURES

2.4.1 ENZYMES

All of the commercial enzymes used were obtained from Sigma Chemical Co., Poole, UK, except for enzymes used for the tannery trial, which were obtained from Biocatalyst, UK. In order to compare enzyme activities with fungal cultural enzymes and different producers, the enzymes used in this project were assayed with the procedures outlined in this chapter. However, some commercial enzymes used for initial experiments (laminarinase, β-D-galactosidase, protease, α-amylase and β-D-mannosidase) were employed according to their activities determined by the manufacturers.

2.4.2 FUNGAL ORGANISMS

Coriolus versicolor, *Aspergillus niger* and *Trichoderma reesei* were supplied by Professor Christine Evans, Fungal Biotechnology Group, University of Westminster, London, UK.

2.4.3 CULTURE MAINTENANCE

Cultures were maintained on a solid medium consisting of 2% (w/v) nutrient agar, supplemented with 3% (w/v) malt extract on Petri dishes. Stock cultures were sub-cultured at regular intervals routinely after 15 days incubation at 25°C.

2.4.3.1 SUB-CULTURE TECHNIQUES

Agar plugs were removed from plate cultures aseptically using a 10 mm cork borer

and were placed in the centre of an agar plate. All cultures were incubated for 7 days at 25 °C.

2.4.3.2 INOCULATION OF CULTURES FOR EXTRACELLULAR ENZYME PRODUCTION

Four agar plugs were removed aseptically from 15 days old plate culture, using a 10 mm cork borer. The four agar plugs were placed in a sterile universal bottle containing 7 ml of distilled water and 5 g of glass beads. The universal bottle was shaken vigorously to remove the culture from the agar surface. The suspended mycelia were then inoculated into a 500 ml flask containing liquid medium. *C. versicolor* was incubated stationary, however, *A. niger* and *T. reesei* were incubated on a shaker at 100-120 rpm for 7 days at 25 °C.

2.4.4 LIQUID MEDIA

2.4.4.1 CULTURE MEDIUM FOR LACCASE PRODUCTION BY *CORIOLUS VERSICOLOR*

The liquid culture medium used for the growth of *C. versicolor* was the medium used by Fähraeus and Reinhammer (1967).

Liquid Culture Medium

Glucose	20 g/l
L-aspargine	2.5 g/l
D,L-phenylalanine	0.15 g/l
Adenine	0.275 g/l

Thiamine Hydrochloride	50 μg/L
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g/l
Di-sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	0.1 g/l
Calcium chloride ($CaCl_2$)	0.01 g/l
Iron Sulphate ($FeSO_4.7H_2O$)	0.01 g/l
Manganese sulphate ($MnSO_4.4H_2O$)	0.001 g/l
Zinc sulphate ($ZnSO_4.7H_2O$)	0.001 g/l
Copper sulphate ($CuSO_4.5H_2O$)	0.002 g/l
Magnesium sulphate ($MgSO_4.7H_2O$)	0.50 g/l

The volume of medium was adjusted to 1 litre with distilled water and dispensed into flasks, stoppered with non-absorbent cotton wool. The flasks were autoclaved at 15 p.s.i./121 °C for 15 minutes. The pH of the medium was 5.0.

2.4.4.2 CARBOXYMETHYLCELLULOSE LIQUID MEDIUM FOR EXTRACELLULAR ENZYME PRODUCTION FROM *CORIOLUS VERSICOLOR*

The liquid culture medium used for the growth of *C. versicolor* was adapted from a medium used by Abrams (1948).

Carboxymethylcellulose (CMC) Liquid Culture Medium

Glucose	1%
Carboxymethylcellulose	5%
Ammonium tartrate	96 mg/l
Dimethylsuccinate	1.49 g/l
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g/l

Magnesium sulphate ($MgSO_4.7H_2O$)	0.05 g/l
Calcium chloride ($CaCl_2$)	0.01 g/l
Thiamine hydrochloride	0.00005 g/l
Adenine	0.0275 g/l
Iron sulphate ($FeSO_4.7H_2O$)	0.01 g/l
Manganese sulphate ($MnSO_4.4H_2O$)	0.001 g/l
Zinc sulphate ($ZnSO_4.7H_2O$)	0.001 g/l
Copper sulphate ($CuSO_4.5H_2O$)	0.002 g/l

The volume of the medium was adjusted to 1 litre with distilled water and dispensed into flasks, stoppered with non-absorbent cotton wool. The flasks were autoclaved at 15 p.s.i/121 ^{o}C for 15 minutes. The pH of the medium was 5.0.

2.4.4.3 CARBOXYMETHYLCELLULOSE LIQUID MEDIUM SUPPLEMENTED WITH DUNG FOR EXTRACELLULAR ENZYME PRODUCTION FROM *CORIOLUS VERSICOLOR*

The liquid culture medium used was the same as that used for production of extracellular enzyme from *C. versicolor*. In this medium, however, the cultural condition was modified by adding to the medium 3% ground cattle dung which was moisture free and had particle size less than 250 μm.

2.4.4.4 EXTRACELLULAR ENZYME PRODUCTION FROM *ASPERGILLUS NIGER* AND *TRICHODERMA REESEI*

The liquid culture medium used for the growth of *A. niger* and *T. reesei* was a modified medium of Abrams (1948).

Liquid Cultural Medium

Carboxymethylcellulose	15 g/l
Xylan	5 g/l
Ammonium nitrate (NH_4NO_3)	3 g/l
Potassium dihydrogen phosphate (KH_2PO_4)	4.5 g/l
Magnesium sulphate ($MgSO_4.7H_2O$)	2 g/l
Calcium chloride ($CaCl_2$)	0.1 g/l
Thiamine hydrochloride	0.001 g/l
Boric acid (H_3BO_3)	0.000570 g/l
Ferric chloride ($FeCl_3$)	0.000145 g/l
Cobalt chloride ($CoCl_2.6H_2O$)	0.0040 g/l
Copper sulphate ($CuSO_4.5H_2O$)	0.000060 g/l
Manganese chloride ($MnCl_2.4H_2O$)	0.000030 g/l
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	0.000020 g/l
Zinc sulphate, ($ZnSO_4.7H_2O$)	0.000310 g/l

The volume of the medium was adjusted to 1 litre with distilled water and dispensed into flasks, stoppered with non-absorbent cotton wool. The flasks were autoclaved at 15 p.s.i/121 ^{o}C for 15 minutes. The pH of the medium was 5.0.

2.4.5 INDUCTION OF LACCASE FROM *C. VERSICOLOR* with 2,5-DIMETHYLANILINE

On day six of growth in 200 ml of liquid medium in the flask (section 2.4.4.1), *C. versicolor* was induced to produce laccase by the addition of 2,5-dimethylaniline to the medium. 2-5-Dimethylaniline was dissolved in 50% ethanol to give a concentration in the growth medium of $2x10^{-4}$ M.

2.4.6 HARVEST OF EXTRACELLULAR LACCASE

After induction the extracellular laccase was harvested 24 hours later, by filtering the culture through glass wool and muslin on ice. The filtrate was assayed for enzymes activity by techniques described in section 2.4.8 and stored in sterilised bottles at 4 °C.

2.4.7 HARVEST OF EXTRACELLULAR ENZYME PRODUCED BY *C. VERSICOLOR*, *A. NIGER* AND *T. REESEI*

After incubation of the fungus in the culture medium that is described in section 2.4.4.2 and section 2.4.4.4 in a stationary and shaken culture respectively at 25 °C for 7 days, cultures were filtered through 2 layers of muslin and filtrates collected. *C. versicolor* which was grown in the CMC medium supplemented with cattle dung (section 2.4.4.3) was harvested by using the same method after incubation in stationary medium at 25 °C for 14 days.

The filtrate was assayed for enzymes activity by techniques described in section 2.4.8 and stored in sterilised bottles at 4 °C.

2.4.8 ASSAY PROCEDURES

2.4.8.1 PROTEIN ASSAY

The method of Lowry *et al.* (1951) was used for protein determination.

Reagents:

10% (w/v) deoxycholate

2 % (w/v) sodium carbonate in 0.1 N sodium hydroxide

1 % (w/v) copper sulphate

2 % (w/v) sodium/ potassium tartrate

Folin and Ciocalteau's Phenol Reagent

Assay Procedure:

10 - 100 µl of enzyme solution was placed in a test tube and the volume was made up to 100 µl with distilled water. The following solutions were then added:

0.4 ml of 10 % deoxycholate, 5.0 ml of 2 % sodium carbonate in 0.1 M sodium hydroxide, 50 µl of 1.0% copper sulphate and 50 µl of 2% sodium/ potassium tartrate. The reaction mixture was thoroughly mixed on a vortex mixer and allowed to stand for 10 minutes at 25 °C. While mixing, 0.5 ml of Folin and Ciocalteau's phenol solution (1 part Folin: 2 parts water) was added. The reaction mixture was allowed to stand for 20 - 30 minutes at 25 °C before the optical density at 590 nm was recorded.

Calibration:

A standard calibration curve was determined using Bovine Serum Albumin (BSA). A protein concentration range from 0 to 200 µg of BSA was used.

2.4.8.2 CATECHOL ASSAY

Polyphenol oxidase activity with catechol as a substrate was measured using the method given by Gallagher (1989).

The catechol assay is a general assay for polyphenol oxidases in which the development of the yellow coloured product is followed spectrophotometrically at 440 nm.

Reagents:

50 mM catechol dissolved in 50 mM sodium acetate buffer (pH 5.0).

Assay Procedure:

100 - 500 µl of culture medium was added to 3.0 ml of 50 mM catechol dissolved in 50 mM sodium acetate buffer at pH 5.0.

The increase in absorbance at 440 nm is proportional to polyphenol oxidase activity. 1 unit of polyphenol oxidase caused a change in absorbance of 1.0 per minute at 25 °C.

2.4.8.3 β-GLUCOSIDASE AND β-XYLOSIDASE ASSAY

β-Glucosidase and β-xylosidase activity of enzymes were assayed using the method given by Gallagher (1989).

Reagents:

10 mM p-nitrophenyl-β-D-glucopyranoside (β-glucosidase activity) and 10 mM p-nitrophenyl-β-D-xylopyranoside (β-xylosidase activity) in 50 mM sodium acetate buffer (pH 5.0).

1.0 M sodium bicarbonate

Assay Procedure:

10-100 µl of the enzyme solution was incubated with 1.0 ml of 10 mM p-nitrophenyl-β-D-glucopyranoside or 1.0 ml of 10 mM p-nitrophenyl-β-D-xylopyranoside in 50 mM sodium acetate buffer (pH 5.0) at 40 °C for 10 minutes. The reaction was stopped with addition of 2.0 ml of 1.0 M sodium bicarbonate. The absorbance of 4-nitrophenol at 410 nm was measured.

Under the reaction conditions, 1 unit of activity was defined as that amount of enzyme which catalysed the release of 1 µmole of 4-nitrophenol per minute.

2.4.8.4 ENDO-GLUCANASE AND ENDO-XYLANASE ASSAY

Endo-glucanase and endo-xylanase activities of enzymes were assayed by the method given by Evans (1996).

Reagents:

Carboxymethylcellulose

Birch xylan

50 mM sodium acetate buffer (pH 5.0)

DNS reagent (0.25 g 3,5-dinitrosalicylic acid, 75 g sodium potassium tartrate, dissolved in 50 ml 2.0 M NaOH, diluted to 250 ml with distilled water)

Assay Procedure:

10 mg of CMC (for endo-glucanase) or xylan (for endo-xylanase) was incubated in 3.0 ml of 50 mM sodium acetate buffer with 1.0 ml of culture medium for 1 hour at 37 °C. After that, 1 ml of DNS reagent was added to the incubated mixture. Then they were heated in a water bath at 100 °C for 12 minutes. After cooling, the absorbance at 570 nm was measured. The amount of reducing sugar produced per

ml of culture medium (as glucose or xylose equivalents) was calculated by reference to a calibration curve.

Under the reaction conditions, one unit of enzyme is defined as the amount of enzyme that will liberate 1 μg of reducing sugar, measured as glucose or xylose equivalents, from substrate per minute at 37 °C.

2.4.8.5 PROTEASE ASSAY

Protein activity with casein as a substrate was measured by the method of Kunitz (1947).

Reagents:

1% casein solution

0.3M Trichloroacetic acid

Assay Procedure:

One ml of culture medium was added to 1.0 ml of 1% casein solution and incubated for 30 min. at 30 °C. Protein was precipitated with 3.0 ml of 0.3 M trichloroacetic acid. After 30 minutes, the supernatant was separated by centrifugation at 10,000 x *g* for 30 minutes and absorbance at 260 nm determined. A blank was prepared in which 3.0 ml of trichloroacetic acid were added before incubation.

One unit of activity is defined as the amount of enzyme which releases 1 μmole of tyrosine under the assay conditions.

2.4.8.6 EFFECT OF SALT AND NON IONIC SURFACE ACTIVE AGENTS ON ENZYME ACTIVITY

Cellulase and xylanase activities of the commercial enzymes and culture filtrates

were measured in the presence of sodium chloride and nonionic fatty alcohol ethoxylate (Eusapon S- BASF) using the assaying methods described in section 2.4.8.4.

The cellulase and xylanase activities of the enzymes were measured with and without NaCl. Enzyme activity was measured in the presence of up to 3.0 M NaCl. Also the cellulase and xylanase activities were measured with and without nonionic fatty alcohol ethoxylate addition. In this case, enzyme activity was measured in the presence of 2% of nonionic surfactant.

2.5 ENZYME TREATMENT OF PIECES OF DUNG CLAD HIDE

Heavily dung clad hide pieces, each 5 cm^2 were treated with different commercial enzymes at pH 5.0 or 7.0 depending on their pH optima. 10 ml volumes of the enzymes, cellulase, xylanase, laminarinase, β-D-1,4-galactosidase, β-D-1,4-mannosidase, β-D-1,4-glucosidase, α-amylase and protease were used, containing approximately equal activities (50-100 units).

Samples of hide pieces and enzymes were agitated gently on a rotary shaker at ambient temperature for up to 24 hours. Dung removal was assessed by passing a spatula over the hair surface. Scores were given according the Table 2.1.

Table 2.1: Assessment of dung removal

Dung Removal	**Score**
No appreciable removal	0
Difficult and incomplete removal	*
Removal with moderate difficulty	**
Easy removal	***
Removal without any appreciable mechanical action	****

2.6 ASSAYING THE DIFFERENT ENZYME TREATMENT ON SOLUBILISATION RATE OF DUNG

In this experimental section, investigations were conducted to determine the effects of enzyme treatment on the solubilisation rate of dung. To determine the solubilisation rate of such a material is not an easy task due to the complexity of the substrate. For this reason, the experiments were conducted to show the overall solubilisation rate of the substrate (chemical oxygen demand) and the solubilisation of carbohydrates that is mainly cellulose and hemicellulose (reducing sugar). Not only was the solution derived from the enzyme reaction assayed, but also the substrate exposed to the enzyme action was analysed before and at end of the reaction period, to find out the effects of the enzymes on the dung components, i.e. cellulose, hemicellulose and lignin.

The cattle dung used in this experiment was prepared in the following way: it was left for 3 days in the fume cupboard in order to remove excessive moisture. The dried material was lightly ground in a coffee grinder. The dung powder was passed through a 1.00 mm screen and then a 0.25 mm screen. Therefore, the final particle size of the sample was between 0.25 mm - 1.00 mm. The fibrous powder was dried in an oven at 65 ^{o}C for at least 2 days prior to use, until constant weight was obtained.

2.6.1 ENZYME TREATMENT OF DUNG

5 g of dried dung sample were placed into 100 ml of 50 mM sodium acetate buffer (pH 5.0) in a 1 litre bottle. The types of enzyme applied, all at a total of 2 U/ml activity, are given in the following list:

Control (containing only buffer solution)

Cellulase

Xylanase

Laccase

Cellulase + Xylanase (50:50)

Cellulase + β-glucosidase (80:20)

Cellulase + xylanase + β-glucosidase (40:40:20)

Cellulase + xylanase + β-glucosidase + laccase (40:30:15:15)

10 ml aliquots from each bottle were taken at time intervals; 3, 8, 12, 18 and 24 hours. Then they were filtered through two layers of medium pore size filter paper and stored in the freezer until they were all collected. Each aliquot was subjected to freeze drying for 2 days in order to examine the samples by IR and solid state NMR techniques. Any substrate left after 24 hours of enzyme treatment was filtered again and then dried to constant weight at 65 ^{o}C.

2.6.2 CHEMICAL OXYGEN DEMAND OF SOLUTION

The standard I.U.C method was used in this experiment. 2 cm^3 of enzyme solution were digested with a mixture of 20% (w/v) mercuric sulphate, 0.042 M potassium dichromate and 2% (w/v) silver sulphate solutions for 2 hours. After that the solution was titrated with 0.1 M ferrous ammonium sulphate solution. The result was expressed as COD in mg per dm^3.

2.6.3 REDUCING SUGAR DETERMINATION

In this assay 1.0 ml of suitably diluted sample was mixed with 5.0 ml of a reagent containing 0.75 g anthrone in 1 litre of 72% (v/v) sulphuric acid. The mixture was

heated in a boiling water bath for 12 minutes, cooled to room temperature and the optical density at 620 nm was measured. Glucose was used as a standard. Anthrone reagent was made fresh daily (Rosenberg, 1979).

2.6.4. EFFECT OF ENZYMES ON THE INDIVIDUAL COMPONENTS OF DUNG

0.2 g of dry dung was mixed with 5.0 ml of 72% (v/v) sulphuric acid and allowed to stand for 3 hours with mixing every half hour for a few seconds. This was then diluted to 50 ml with distilled water, allowed to stand overnight and then vacuum filtered through tared 0.45 μm pore size 45 mm diameter membrane filters. Aliquots of the filtrate were retained for carbohydrate (reducing sugar) assays (see section 2.6.3).

The residue on the filter, consisting of lignin and insoluble inorganic material, was washed with distilled water until the filtrate pH reached 5.0 as measured with pH paper. The filter plus residue were dried overnight at 65 °C and weighed. The filters and samples were then transferred to tared crucibles and ashed overnight at 550 °C. Lignin is defined as the fraction of the sample (excluding the filter) which vaporised.

2.7 MECHANISM OF DUNG REMOVAL

2.7.1 PAPER CHROMATOGRAPHY FOR SEPARATION OF SUGARS

The method used to identify sugars was modified from Smith (1969).

Descending chromatography;
Solvent: n-Butanol / pyridine / water

Procedure:
60 ml of n-butanol were mixed with 40 ml pyridine and 30 ml water. A run typically took about 25 hours at room temperature. The chromatogram was dried in a fume cupboard for about 4 hours (until only a faint odour of butanol remained) before the use of detection reagents.

Detection reagents:
Alkaline silver nitrate
Two solutions were prepared:
[A]: 0.1 ml of a saturated solution of silver nitrate were added to 20 ml of acetone. If cloudiness appeared, a drop more water was added.

[B]: 2.0 g of sodium hydroxide were dissolved in a minimal volume of water (<1 ml) and made up to 100 ml with methanol.

The chromatogram was dipped in reagent A, dried again and then sprayed with reagent B.
This is a sensitive stain producing silver-brown spots with reducing carbohydrates at a sensitivity of about 1 μg.

2.7.2 GEL PERMEATION CHROMATOGRAPHY STUDIES

GPC analysis was carried out by using a gel permeation unit consisting of PL-DCU (Polymer Laboratories), LC1120 HPLC pump (Polymer Laboraties) and 132 RI detector (Gilson Ltd.). Half ml of suitably diluted sample was injected into the column. The samples were eluted by deionised water. The detection of the samples was at 100 millivolts, with the HPLC pump set at 4.4 MPa, and a flow rate of 1.00 ml/minute. Polysaccharides (Polymer Laboratories) were used as references.

2.7.3 INFRARED SPECTROSCOPY STUDIES

IR experiments were carried out on a Perkin Elmer 781 Infrared Spectrophotometer. KBr discs were prepared from both solid dung samples and freeze dried aliquots from enzyme-dung reaction solutions. One part of the sample and 200 parts of KBr were used for the preparation of discs.

2.7.4 SOLID STATE CROSS–POLARISATION MAGIC ANGLE SPINNING (CP/MAS) ^{13}C N.M.R STUDIES

CP/MAS N.M.R experiments were carried out on a Bruker DSX 200 wide bore spectrometer (Imperial College). The samples were put into a 7 mm zirconia rotor and rotors were spun at 5000 Hz. The contact time and recycle delay used were 1 ms and 5s respectively. The samples were externally referred to tetrakistrimethylsilylsilane.

2.8 PILOT SCALE TRIAL FOR DUNG REMOVAL

Before processing dung cladded UK domestic hides, they were sided. Left sides were exposed to enzyme treatment in soaking and right sides were used for the control experiment. Soaking processes were conducted in tannery drums according to the procedure given below:

Dirty Soak: 300% water at 20 °C 1 hour
Main Soak: 300% water at 20 °C
2.5 U/ml enzyme
0.5% non ionic detergent 18 hours

Control samples were soaked without enzyme addition in the same drum. In the first enzymatic soaking trial, a commercial enzyme mixture was used, except for the laccase preparation. The enzyme concentration used in this experiment was 2.5 U/ml which was made of cellulase, xylanase and laccase in the ratio 50: 30: 20. In the second, third and fourth trials, *C. versicolor* and *A. niger* culture broths were used. The enzyme concentration used was based on cellulase activity in the culture fluid, 2.5 cellulase unit/ml. After soaking, the hides were placed into the same tannery drum and the rest of the processes were carried out there, in order to eliminate any differences in processing effects on leather properties. The process recipe carried out after soaking is given in appendix I.

2.8.1 EFFECT OF DIFFERENT ENZYME TREATMENT ON PROPERTIES OF LEATHER

2.8.1.1 DETERMINATION OF TOTAL GLYCOSAMINOGLYCANS

The method used for the determination of total glycosaminoglycans was modified from Alexander *et al.* (1986).

Reagents:

Dimethyl sulphoxide solution:

5.10 g/l magnesium chloride

2.05 g/l sodium acetate trihydrate

Made up in DMSO

Papain digest buffer:

5.694 g sodium dihydrogen orthophosphate

4.833 g disodium hydrogen orthophosphate

Made up to 500 ml and pH 6.4 then add;

8.75 g sodium chloride

0.395 g cysteine hydrochloride

1.85 g di-sodium EDTA

(In the case of limed hides, 37.25 g di-sodium EDTA was added)

GAG stain:

1.0 g Alcian Blue 55%

5.1 g magnesium chloride

1.025 g anhydrous sodium acetate

250 ml 96% ethanol

250 ml distilled water

GAG destain:

20.4 g magnesium chloride

4.1 g anhydrous sodium acetate

1 litre 96% ethanol

1 litre distilled water

Sample preparation:

The sample was cut into very small pieces. In the case of raw hide and soaked hides, the hair was sheared off. Then they were dried in an oven at 100 °C to constant weight. Approximately 1 g of the dry sample was weighed into small necked sevoril tubes and then 5 ml/g dry weight of papain digest buffer was added to each of the tubes followed by 5 µl/ml of papain enzyme. Samples were mixed well with a rotary mixer. Samples were left to digest in an incubator at 60-70 °C over night. After that, the samples were mixed on the rotary mixer again, a further 2.5 µl/ml of papain enzyme was added to each tube and they were left in the incubator until digestion was completed. Afterwards, 1 ml of diethyl ether was added to each tube with a pipette, solutions were mixed well on the rotary mixer and then centrifuged for 30 minutes at 2000 *g* at 5 °C. Then, the upper fat containing ether layer was pipetted off into a beaker in order to remove the natural fat from the samples. After that, the aqueous layer containing digested skin was pipetted off into a clean sample tube and left in the fridge for 12 hours to allow any remaining ether to evaporate off.

Procedure:

A standard containing 1 mg/ml dermatan sulphate was prepared (0.025 g dermatan sulphate in 25 ml) and then using a 1 µl syringe, 6 straight 1 cm lines of each sample and 6 lines of standard dermatan sulphate were put on the matt side of a sheet of cellulose acetate paper and they were left to dry. The remaining digest was kept in the fridge to use for hydroxyproline analysis. The staining solution was

filtered through 4 layers of No. 4 Whatman paper using suction filtration. Staining of the cellulose acetate paper was as follows:

1st stain: lie sheet face down on surface for 2 minutes, push under surface for 2 minutes.
2nd stain: push under surface for 11 minutes and agitate at regular intervals.

Destaining: lie face down on the surface for 2 minutes. Agitate in several 5 minutes changes of destain until a white background was obtained.

After these treatments, the sheets were hung to dry. Then, 3 lines of each of the samples and 3 lines of the dermatan sulphate standard were cut using a scalpel and they were dissolved in 1 ml of DMSO solution for 30 minutes at 30 °C. Also 4 background pieces were dissolved to act as a control. Optical density was measured at 678 nm using a spectrophotometer.

Calculation:
Total glycosaminoglycan content of hide was calculated by dividing the amount of GAG per ml of solution by the amount of collagen (see section 2.8.1.2) in per ml solution.

2.8.1.2 DETERMINATION OF HYDROXYPROLINE FROM PAPAIN DIGESTED HIDE SAMPLES

The standard BSLT method was used in this experiment.
Reagents:
0.1 g/l hydroxyproline
0.05 M copper sulphate

1.25 M NaOH

6% hydrogen peroxide

0.9 M sulphuric acid

5% *p*-dimethylaminobenzaldehyde (DMAB)

Procedure:

Four standard solutions, with concentrations from 5 mg/l to 20 mg/l, were prepared from 0.1 g/l hydroxyproline. From papain digested protein and from each standard solution, duplicate solutions were prepared in the following way:

One cm^3 of standard or protein solution with

2 cm^3 of 0.05 M copper sulphate

2 cm^3 of 1.25 M NaOH

2 cm^3 of 6% hydrogen peroxide

They were pipetted into 25 cm^3 volumetric flasks in the order given above. Blanks were prepared in the same way but using 2 cm^3 of distilled water. The flasks were shaken gently once a minute for 5 minutes, then placed in a water bath at 40 ^{o}C for 15 minutes to destroy excess hydrogen peroxide. After that, the flasks were cooled rapidly in water and 10 cm^3 of 0.9 M H_2SO_4 were added followed by 5 cm^3 of 5% DMAB solution. Then, samples were placed in a water bath at 70 ^{o}C for 30 minutes to develop the colour. Afterwards, the solutions were cooled and diluted to the mark using distilled water. Then, the absorbance of each solution was measured against the blank at 555 nm. A standard hydroxyproline graph was used to determine the concentration of hydroxyproline in the papain digested hide samples.

2.8.1.3 PHYSICAL TESTING

Before physical testing, leathers were conditioned at 20 °C and at 65 ± 2% relative humidity. Table 2.2 sets out the physical tests performed on leather samples.

Table 2.2: Physical tests were carried out on leathers

Physical Tests	**Method**	**Notes**
Colour Measurement	Minolta Colour Meter (CR-210b)	Grain surface
Measurement of Softness	ST-300 Softness Tester	Full thickness
Measurement of Tear Strength	IUP 7	Six samples parallel to backbone Six samples perpendicular to backbone
Measurement of Tensile Strength	IUP 6	Six samples parallel to backbone Six samples perpendicular to backbone
Measurement of Distension and Strength of Grain by the Ball Burst Test	IUP 9	Six samples parallel to backbone Six samples perpendicular to backbone

CHAPTER 3

RESULTS AND DISCUSSION

3.1 INTRODUCTION

The results presented in this chapter can be divided into five readily defined sections. These are:

Section 3.2 reports details of analysing dung materials and the lignocellulosic composition of dung is discussed. Then, the interaction between dung and hide is illustrated by microscopy.

Section 3.3 primarily deals with attempts to remove dung from hide by using a number of commercially available enzymes in order to establish the basis for successful removal. Then, a solubilisation study involving dung and enzymes is presented using the techniques of chemical oxygen demand and reducing sugar analysis. Also, the effects of enzyme treatment on individual components of dung are presented.

Section 3.4 reports the growth of the fungal species *Coriolus versicolor*, *Aspergillus niger* and *Trichoderma reesei* in different culture conditions for the production of cellulosic, xylosidic and lignocellulosic enzymes. Their activities are quantified and compared with commercial enzymes.

Section 3.5 covers the study of the mechanism of enzymatic dung removal using paper chromatography, gel permeation chromatography, solid state NMR and infrared spectroscopy.

Section 3.6 deals with pilot scale experiments in tannery drums for dung removal using fungal cultures and commercial enzymes. Rate of dung removal is described. Also, the effects of enzyme treatment on chemical and physical properties of leather are presented.

3.2 COMPOSITION OF PLANT MATERIALS

The composition of typical samples of dung from heavily clad hides was determined by conducting triplicate experiments. Table 3.1 shows three experimental results and average results. Whilst there will be variations in analysis, depending on diet, the results represented here are likely to be representative of the usual constitution. It is clear from the analysis that the majority of the material is made up of lignocellulosic compounds. The two major components are cellulose and hemicellulose forming together up to 58% by weight of the dung, with lignin the next major component at 21%. The remaining 20% by weight was composed of smaller amounts of ether and water soluble materials. In addition to that, 6% of protein was found in the dung samples.

It can be observed that the percentages of the different fractions do not add up to 100%. This is because chemical substances dealt with are not clearly defined (Jermyn, 1955). It may also be the case that lignin has interactions and chemical linkages with the hemicellulose fraction (Obst, 1982), so when the lignin fraction is isolated, it might contain some components from the hemicellulose. In the fractionation studies, first holocellulose was isolated, which contains hemicellulose and cellulose. According to Jermyn (1955) this method of delignification is the most successful. Cellulose and hemicellulose fractions were then isolated from the holocellulose component. However, even in the most successful scenario there is always some cross contamination between lignocellulosic components.

Figure 3.1 illustrates the possible relationship between lignocellulosic components. In this case, the percentage of cellulose component represented in Table 3.1 is called α-cellulose. It possibly contains some hemicellulose fraction. Similarly, hemicellulose and lignin fractions have some cross contamination between each other. In addition to that, some protein component of dung might be present in the ether and water soluble fractions of the samples, because of the easy solubility of some part of the protein.

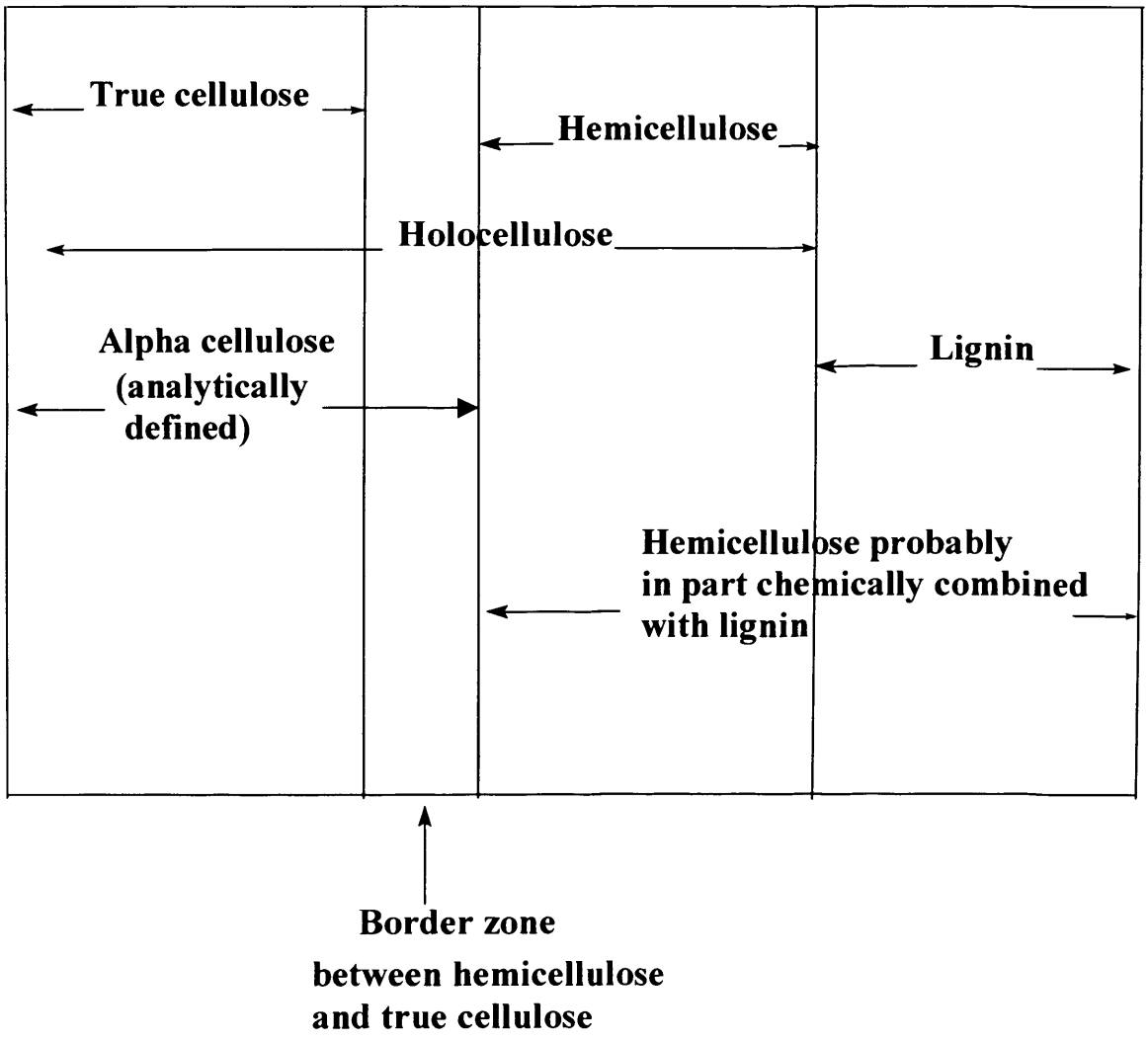
This experiment shows that up to 80% of dung composition is accounted by three major components, cellulose, hemicellulose and lignin, which reflect the lignocellulose composition of the animal feed. Traditional feeds such as grasses, contain mostly cellulose (46%) and hemicellulose (16%) in the plant cell walls, with a lesser amount of lignin (10%) (Jalc *et al.*, 1997). On digestion by ruminants, some of the cellulose and a small amount of hemicellulose are hydrolysed to glucose and other sugars by rumen microflora, but lignin remains undigested. As a result, a greater proportion of lignin relative to cellulose and hemicellulose remains in dung, compared with that in grasses. This would be accounted for by the unreactive nature of the lignin polymer.

From this study, it was concluded that in order to remove dung from hide, it is necessary to target those components which constitute the adhesive mechanism, cellulose and hemicellulose, using the enzymes cellulase and xylanase. In addition to that, the lignin component should be targeted by the polyphenol oxidases in order to enable access to cellulose and hemicellulose by their degrading enzymes.

Table 3.1: Composition of dung based on triplicate analysis of dung samples collected in November, 1996 (% based on dry weight basis).

Component	Sample I (% on dry weight)	Sample II (% on dry weight)	Sample III (% on dry weight)	Average	SD
Cellulose	30.4	30.2	29.4	**30.0**	0.5
Hemicellulose (contains mostly xylan)	27.0	28.1	27.7	**27.6**	0.6
Lignin	20.9	21.2	22.1	**21.4**	0.6
Protein	6.0	6.0	6.0	**6.0**	0.1
Ether soluble	9.8	10.1	10.1	**10.0**	0.1
Cold water soluble	5.9	5.6	5.9	**5.8**	0.2
Hot water soluble	4.0	4.1	4.2	**4.1**	0.1

Figure 3.1: Relationship between lignocellulosic materials (Brown, 1972).

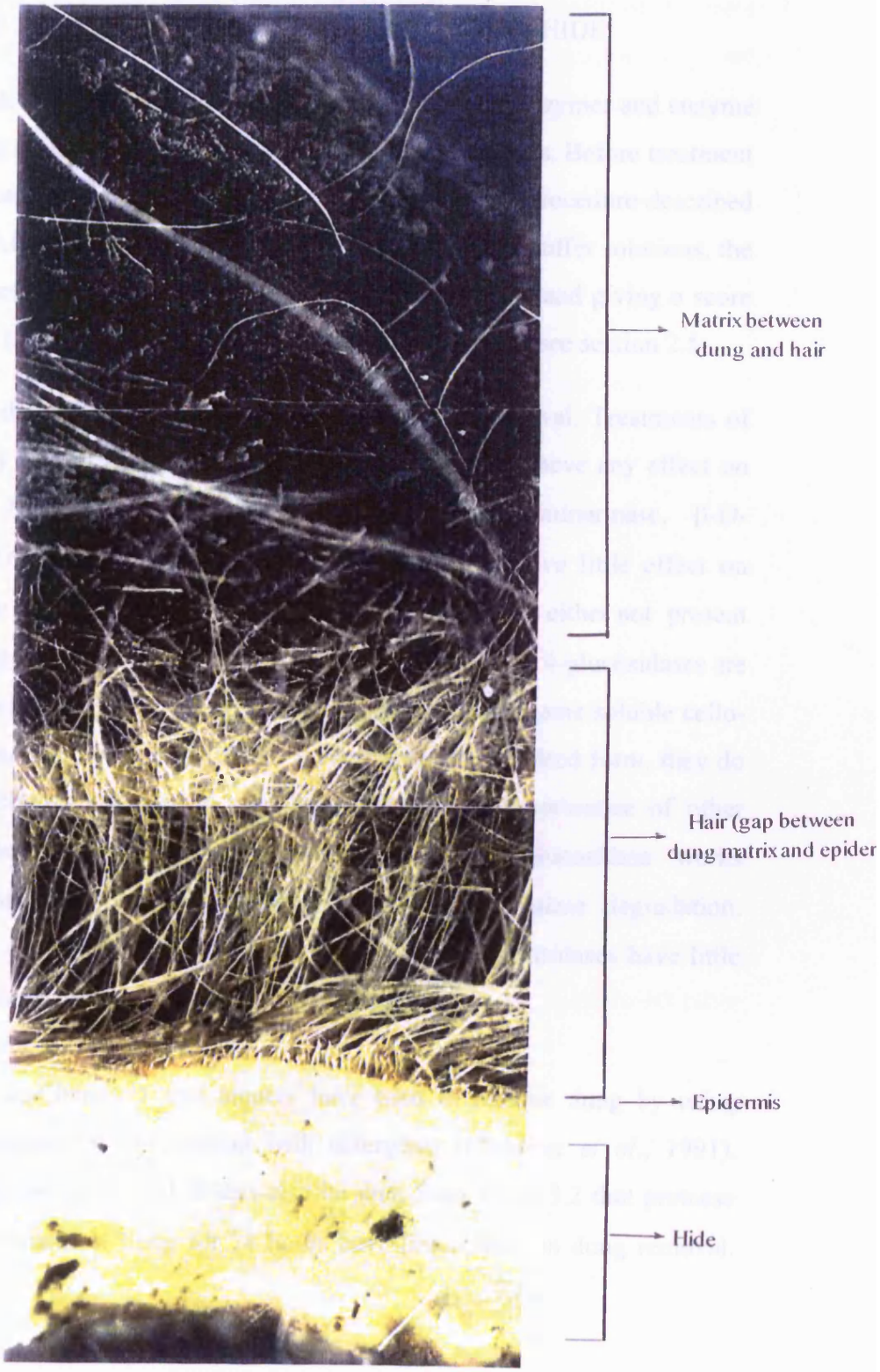


3.2.1 MICROSCOPIC EXAMINATION OF DUNG CLAD HIDE

It was useful to investigate the interaction between hide and dung. Microscopic examination of dung on hide revealed that adhesion was between the dung and the hair alone, see Figure 3.2; there was no sticking of the dung to the epidermis. This accounts for the difficulty in removing dry dung; the matrix of hair within the dung creates a strong composite material. Indeed, mixing wet cattle dung with hair is still used as a building material in the developing economies, in much the same way as the traditional filling of wattle and daub when dried.

Therefore the dung could be removed either by breaking the hairs or by degrading the cellulose-hemicellulose-lignin complex. Breaking the hair is feasible, but intact keratin is resistance to proteases, leaving only keratinase or conventional unhairing chemicals as degrading options. Keratinases are not favoured on grounds of the health risk to operatives, but unless unhairing is separated from the opening up part of the process, the dung will be solubilised into the liming liquor, inevitably contributing to the effluent loading.

Figure 3.2: The adhesion of dung to hair (magnification X10)



3.3 ASSAYING ENZYMATIC REMOVAL OF DUNG

3.3.1 ENZYME TREATMENT OF PIECES OF DUNG CLAD HIDE

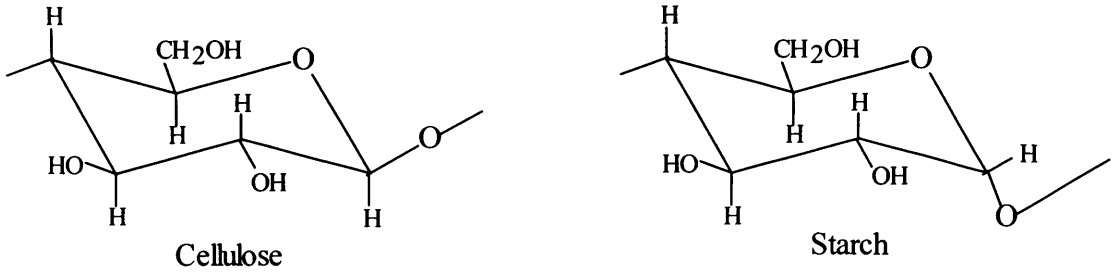
In this experiment, interest lay in finding out the most effective enzymes and enzyme mixtures for dung removal using commercial enzyme preparations. Before treatment of dung-clad hides, the enzymes were assayed according to the procedure described in section 2.4.8. After treatment of the samples with enzymes in buffer solutions, the samples were assessed by passing a spatula over the hair surface and giving a score on a scale of zero (no appreciable removal) to *** (easy removal) see section 2.5.

Table 3.2 shows the effects of different enzymes on dung removal. Treatments of samples with only buffer solution at pH 5.0 and pH 7.0 do not have any effect on dung removal. Different types of carbohydrases like laminarinase, β-D-galactosidase, β-D-1,4-mannosidase and β-D-1,4-glucosidase have little effect on dung removal, due to the fact that substrates for these enzymes are either not present in quantity in dung or are not present at all. It is known that β-D-1,4-glucosidases are members of the cellulase family. They hydrolyse cellobiose and some soluble cello-oligosaccharides to glucose (Wood, 1992). However, in their isolated form, they do not attack the cellulose polymer to any great extent. In the presence of other cellulosic enzymes (endo- and exo- cellulases), β-D-1,4-glucosidase works synergistically with other enzyme components to effect cellulase degradation. Therefore, in this experiment it is also proved that β-D-1,4-glucosidases have little effect on the cellulosic components of dung when used alone.

It was explained in Chapter I that tanners have tried to remove dung by using amylases and proteases in conjunction with detergents (Christner *et al.*, 1991). However, this was not successful. It can also be seen from Table 3.2 that protease and α-amylase treatment of dung for 24 hours have little effect on dung removal.

Starch, which is a substrate for α-amylases, possesses similar structural features to other carbohydrates like cellulose (see Figure 3.3). From the standpoint of chemical structure, starch differs from cellulose in two major ways: the glucose rings are linked together through carbons 1 and 4 by α rather than β linkages, and considerable chain branching occurs through carbon 6. However, like cellulose, complete hydrolysis yields D-glucose.

Figure 3.3: Structural representation of cellulose and starch



Composition analysis of dung showed that there was no starch fraction in the analysed plant materials. Therefore, treatment of dung with α-amylases, not surprisingly, produced little action in terms of dung removal. Moreover, it should be accepted that using α-amylases for cleaning hides in the soaking process will not affect the integrity of the dung, due to chemical differences between starch and other carbohydrates present in the dung.

In terms of protease treatment, it can be said that it leads to a small contribution to the dung removal, since analysed dung samples contain 6% of protein fraction.

Effective dung removal was obtained in 24 hours by using 5 U/ml of cellulase and xylanase separately. Attack on the lignin fraction by laccase leads to dung removal as well, but the effectiveness of the treatment was less than that of cellulase and xylanase treatment. It might be said from these finding that, if the major composition of dung is targeted by enzymes, to attack these major fractions, effective dung removal might be obtained easily within 24 hours. Moreover, it might also be said that adhesive properties of dung to the hide probably come from cellulose and xylan composition and their digestion products. Although the feed additives such as oligosaccharides, and rumen microflora producing a range of extracellular polysaccharides with adhesive properties, might contribute to the adhesion of the dung (Monson and Poul, 1995), treatment with a range of carbohydrases to attack these materials did not contribute any appreciable removal of dung. Therefore, it is unlikely that other polysaccharides, rather than cellulose and hemicellulose, are important in the mechanism of dung removal.

Before discussing effects of cellulase, xylanase and laccase treatments on dung removal, it should be pointed out that experimental conditions applied were chosen to present the most difficult conditions. Dry dungy hides were used along with very little mechanical action during the soaking process. It should be noted that in normal tannery practices, usually wet-salted hides with moisture contents about 45% are used along with very high mechanical actions.

The effect of cellulase and xylanase concentration on dung removal is illustrated in Table 3.3. With cellulase treatment, dung was removed within 24 hours when 2.5 units per ml enzyme was used. However, for complete removal of dung with xylanase within 24 hours, 5.0 units per ml enzyme concentration was required. Therefore, in terms of dung removal, cellulases are more effective at low concentration that xylanases.

Table 3.2 a: Effects of enzymes on dung removal from pieces of dung clad hides

Enzymes	Activity (units per ml)	pH	Removal score
No enzyme	0	5.0	0
Cellulase	5	5.0	***
Xylanase	5	5.0	***
Laminarinase	5	5.0	*
β-D-galactosidase	5	5.0	*
Laccase	0.2	5.0	**
No enzyme	0	7.0	0
Protease	5	7.0	*
α-amylase	5	7.0	*
β-D-1,4-mannosidase	5	7.0	*
β-D-1,4-glucosidase	5	7.0	*

*** Easy removal
** Removed with moderate difficulty
* Difficult and incomplete removal
0 No appreciate removal

Table 3.2 b: Definition of enzyme units

Enzymes	Definition
cellulase, xylanase, β-D- galactosidase, α-amylase, β-D-mannosidase	One unit of enzyme is defined as the amount of enzyme that liberates 1 μg of reducing sugar from substrate per minutes at 37 °C
β-D-glucosidase	One unit of enzyme is defined as the amount of enzyme which catalyses the release of 1 μmole of 4-nitrophenol per minutes at 40 °C.
Laccase	One unit of laccase causes a change in absorbance of 1.0 per minute at 25 °C
Protease	One unit of enzyme is defined as the amount of enzyme that releases 1 μmole of tyrosine per minute at 37 °C

When the rate of dung removal was investigated by using cellulase and xylanase, dung was removed within 18 hours with cellulases when either 2.5 or 5.0 units per ml were added to the hide pieces (see table 3.4). With xylanase treatment, dung was completely removed in 24 hours with 2.5 units per ml of xylanase and in 20 hours with 5.0 units per ml. Removal of dung with moderate difficulty was observed after 14 hours with these two enzymes. Increasing the enzyme concentration from 2.5 to 5.0 units per ml did not result in any shortening of reaction time for cellulase and only minimal reduction in reaction time for xylanase.

Using the laccase alone did not give a satisfactory result for dung removal. However, if cellulase, xylanase and laccase are added together to the dung clad hide, dung can be removed very effectively (see Table 3.5). Using 0.008 units per ml of laccase, 5.0 units per ml of cellulase and 5.0 units per ml of xylanase mixture removed the dung from hide within 6 hours. The dung was washed from the hide pieces without any additional mechanical action. By using three enzymes together, they act on substrates synergistically so their actions on substrates are more pronounced than when they are used separately. It is well known that lignification protects polysaccharides from hydrolytic enzymes and limits the digestibility of lignocellulosic materials by ruminant animals and by cellulases applied *in vitro* (Reid, 1995). There is also the case that, due to the hydrophobic and insoluble nature of lignin which is sequestered in a dense matrix of carbohydrates, this restricts the access of enzymes and contact with cellulose and hemicellulose polymers. However, when the lignin is modified by laccase, accessibility of other lignocellulosic enzymes increases and so the rate of carbohydrate digestion increases (Reid, 1995).

Table 3.3 The effect of concentration on dung removal

Enzyme Concentration (Units)	Cellulase	Xylanase
5.0	***	***
2.5	***	**
1.0	*	*
0.1	*	*
0	0	0

Table 3.4 Rate of dung removal from dung-clad hide pieces with cellulase and xylanase

Enzymes	**6 h**	**8 h**	**14 h**	**16 h**	**18 h**	**20 h**	**24 h**
Cellulase (5 U/ml)	*	*	**	**	***	***	***
Cellulase (2.5 U/ml)	*	*	**	**	***	***	***
Xylanase (5 U/ml)	0	*	**	**	**	***	***
Xylanase (2.5 U/ml)	0	*	*	**	**	**	***

Table 3.5 The influence of ligninase on dung removal

Enzyme(s) and Concentration	Score
0.016 units laccase	**
0.008 units laccase 5 units cellulase 5 units xylanase	****[a]
0.008 units laccase 2.5 units cellulase 2.5 units xylanase	***

[a] this score goes outside the scale defined, because the dung was removed without the need for any appreciable mechanical action.

3.3.2 EFFECT OF DIFFERENT ENZYME TREATMENT ON SOLUBILISATION RATE OF DUNG

In this section, the effects of enzyme treatment on solubilisation rate of dung are presented. As described in Chapter II, 0.25 –1.00 mm particle sized fibrous dry dung powder was used throughout the experiments. Total enzyme concentration used for each experiment was 2.0 units per ml.

The experiments were divided into three sections and the results have been evaluated in separate sections:

a- overall solubilisation rate of the substrate (chemical oxygen demand)

b- solubilisation of carbohydrates (reducing sugar)

c- effects of enzymes on the individual components of dung

3.3.2.1 CHEMICAL OXYGEN DEMAND

A suitable method to determine organic matter released into solution is the determination of chemical oxygen demand. After reacting the enzymes with the substrate, a number of organic materials are released into the solution. In our case, these organic materials may be listed in following way:

- Lignocellulosic components
- Protein
- Triglycerides
- Enzyme used in the experiments

Chemical oxygen demand is not specific in terms of the individual components of dung, however, whatever the solution contains can be measured by this method. Figure 3.4 shows the COD results of the control sample, treatment with buffer at pH 5.0, and individual enzymes, namely cellulase, xylanase and laccase. In this figure, "enzyme" stands for the COD of the enzyme mixture consisting of cellulase, xylanase, laccase and β-glucosidase mixture.

As can be seen from Figure 3.4, the enzymes used in this experiment do not give a big contribution to the COD of solution within 24 hours (average COD value was 4444 mg/l). The results are comparable with the results of the control sample. Therefore, the contribution of enzyme to the COD as an added material is so small that it may be ignored. The treatment of dung with 2 units per ml of cellulase showed an increase in the first eight hours, however, further treatment time did not show any

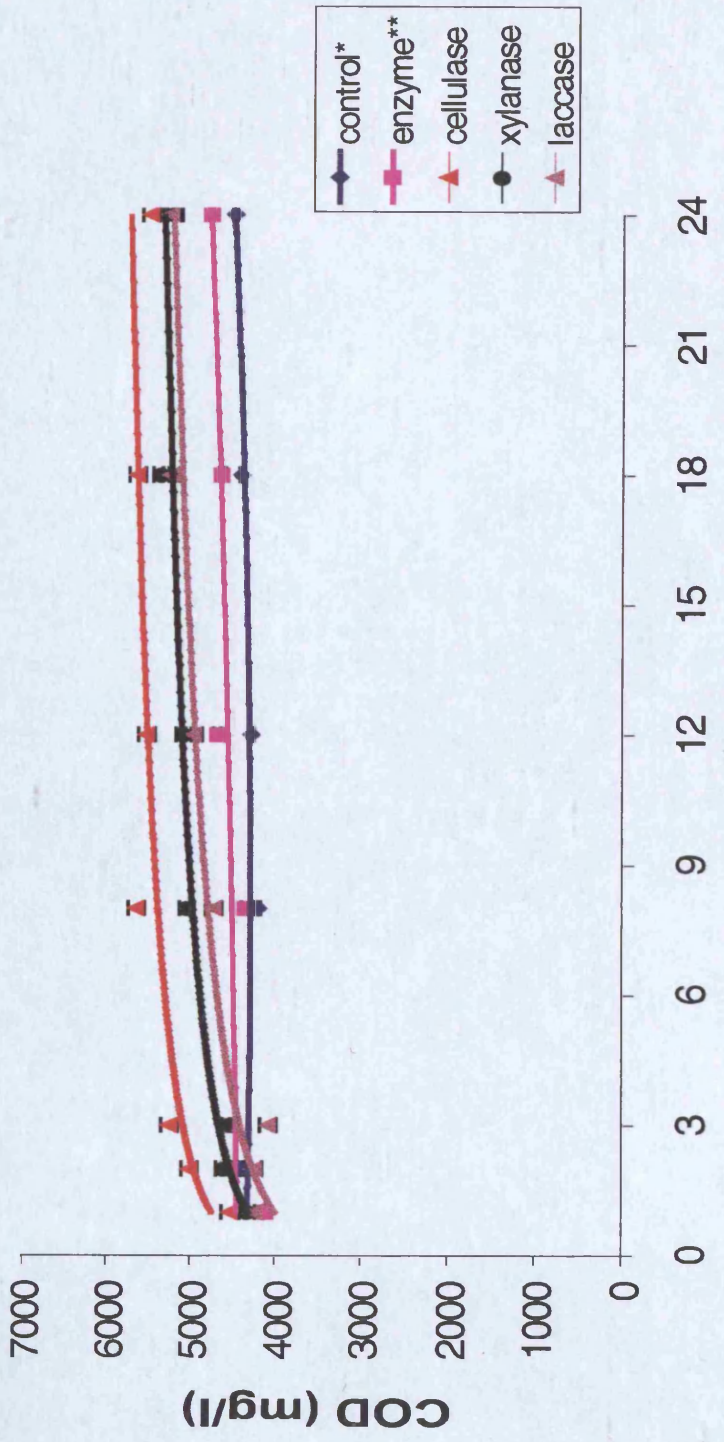
increase in the COD level. From the xylanase and laccase treatments, it can be said that overall contribution of these treatments to the COD is lower than the cellulase treatment. The xylanase treatment seems to be more effective than the laccase treatment. An increase in COD from the xylanase treatment continued over 18 hours and then the COD leveled off. A rise in COD from the laccase treatment continued to the end of the process time, but it was always lower than cellulase and xylanase treatment. These results may be explained in the following way. Viikari *et al.* (1994) investigated the enzymatic accessibility of xylan in lignocellulosic materials. They used *Trichoderma reesei* RUT C-30 culture filtrate for purification of endoxylanase, α-arabinosidase, β-xylosidase and xylan esterase. However, treatment of lignocellulosic material with these enzymes for 24 hours, even using high enzyme concentration, only 20% of fibre-bond xylan was hydrolysed. Whereas the isolated xylans of wood and kraft pulps were solubilised more extensively with a hydrolysis yield of 50- 65%. Limited accessibility of the enzyme in lignocellulosic materials may be due to the molecular organisation of xylan with other materials like cellulose and lignin (Viikari *et al.*, 1994), dependent on specific surface area (Stone *et al.*, 1969) and fibre porosity (Wong *et al.*, 1988). However, the effects of surface area and fibre porosity of the substrate may influence our experiment less, because a very fine particle size substrate was used and the plant material substrate had already been passed through a digestion process inside the animal's rumen, so the crystallinity of the substrate should be low. Therefore, it might be stated that treatment of substrate with xylanase leads to lower solubility than with cellulase, due to the fact that enzymatic accessibility of xylan is limited by other components present in the substrate.

In the case of laccase treatment, one cannot expect to have a substantial amount of degradation in a limited amount of time, in this case treatment time was 24 hours. Rosenberg (1979) studied lignin biodegradation with the thermotolerant fungi

Chrysosporium pruinosum and found that 50% of the lignin was degraded from dung after a treatment of 12 days. Due to the much higher quantity of cellulose in cattle dung and its easy accessibility by enzymes, treatment of dung with cellulosic enzyme produced the highest organic matter in the solution.

When the enzyme mixtures were used, the solubilisation rate of dung differed from the single enzyme additions (see Figure 3.5): the COD values of the solutions were higher in all cases. For two units per ml of cellulase and xylanase (50:50) the COD level was slightly higher than the single cellulase addition. Similarly, the same can be said for two units per ml of cellulase and β-glycosidase (80:20). For all cases, the solubilisation rate was much higher than xylanase addition alone. By using three or four enzyme components together, the solubilisation rate of dung was raised markedly especially by the addition of four enzymes together in the solution. From previous results of the use of cellulase, xylanase and laccase together for treatment of dung clad hide, dung was removed very easily within six hours under the experimental conditions. Similarly in this study, it was confirmed that using enzyme components together increases the solubilisation rate of dung and it is more pronounced when cellulase, xylanase, β-glycosidase and laccase are used together. The reaction of lignocellulosic material with laccase leads to some changes in the lignin-carbohydrate complexes of the substrate and further degradation of the substrate with cellulosic and xylosidic enzymes is enhanced. From these findings and previous findings, it can be said that the enzymes responsible for effective dung removal from hides should consist of at least three components, namely cellulase, xylanase and laccase.

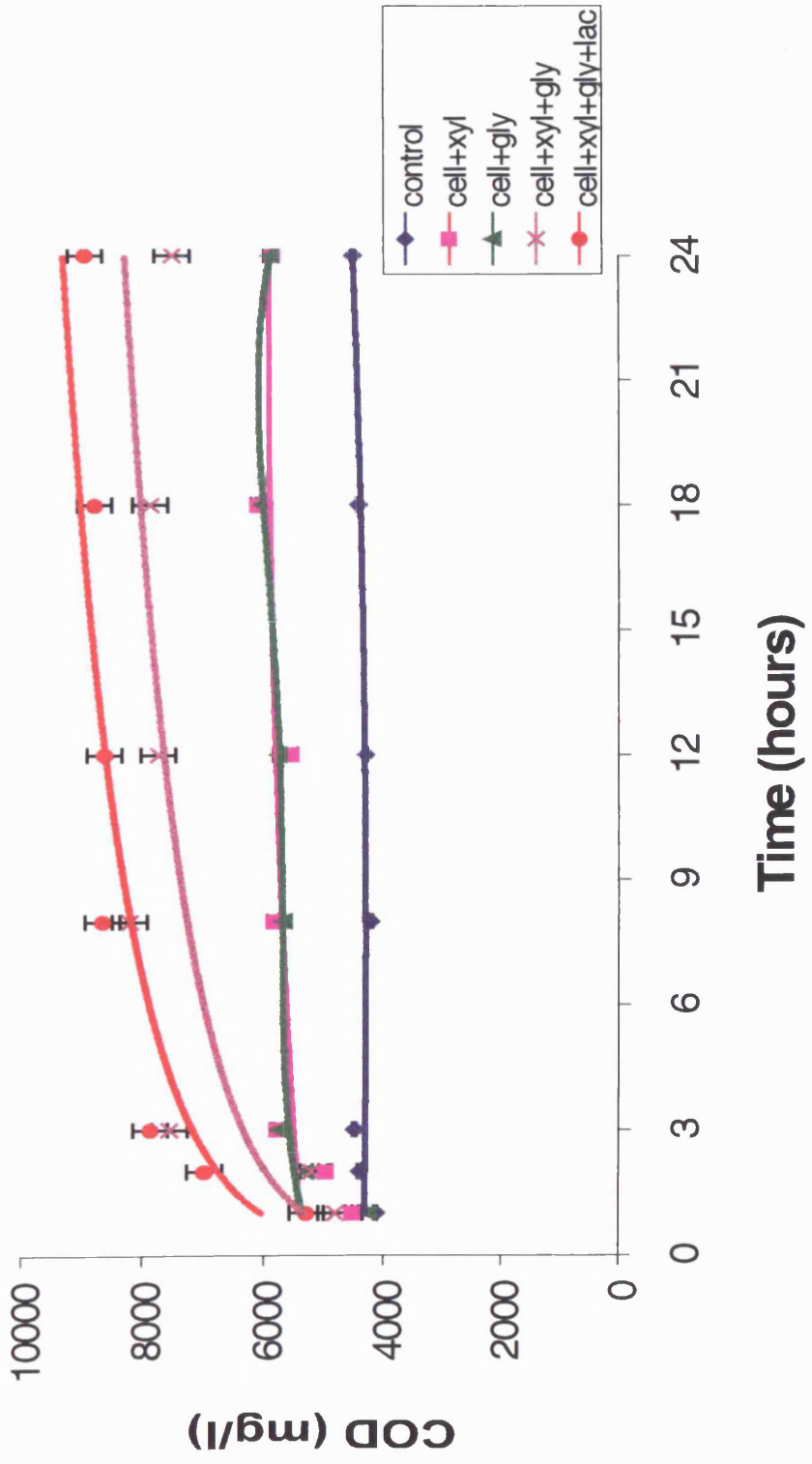
Figure 3.4: Chemical oxygen demand of dung samples treated with individual enzymes (error bars are based on standard error)



* control: mixture of dung and buffer

**enzyme: cellulase, xylanase and laccase mixture

Figure 3.5: Chemical oxygen demand of dung samples treated with mixture of enzymes (error bars are based on standard error)



3.3.2.2 REDUCING SUGAR IN DUNG SOLUTION

This experiment was more specific than COD determination of enzyme treated dung samples because sugars derived from the carbohydrate fractions of dung after enzyme treatment were determined. After treatment with enzymes, aliquots were taken from the solution to determine the sugar content. The experiments were conducted on the same aliquots used in COD determination.

Reducing sugar measurement is a common practice to evaluate the reaction of cellulosic and xylosidic enzymes with various lignocellulosic materials. For example, Puls *et al.* (1985) and Poutanen *et al.* (1986) investigated effects of a steaming pretreatment of birch wood on enzymatic degradation of this material. They used the reducing sugar measurement to evaluate the effectiveness of enzymatic hydrolysis of steam pretreated woods. Mandels *et al.* (1981) measured the saccharification efficiency of three cellulase-enhanced mutants of *Trichoderma reesei* on ball-milled pulp, avicel, ball-milled newspaper and absorbent cotton using the reducing sugar method.

Figures 3.6 and 3.7 demonstrate the results of reducing sugar (glucose) analysis after exposing the dung sample to the enzymatic reaction. Figure 3.6 shows that apart from cellulase addition, none of the single enzyme additions demonstrated an increase in the reducing sugar content. The reason might again be the inaccessibility of xylan to xylanolytic enzymes. In the case of laccase, carbohydrate components of substrate are not reaction sites for lignin degrading enzymes so laccase treatment caused no substantial changes in the sugar content of the hydrolysing solution. However, it should be noted that the reaction of dung with cellulase produced a substantial amount of reducing sugar compared with other treatments. The reason for this was not only the abundance of cellulose and its easy accessibility by the enzyme but also the cellulose mixture used in this experiment contained components that

were endo and exo-glucosidase and β-glycosidase (Wood, 1992; Eveleigh, 1987). These components are the most suitable mixture for producing glucose from cellulose due to their synergistic action on cellulosic components.

Figure 3.7 demonstrates the effect of enzyme mixtures on dung in producing reducing sugar. It should be noted that treatment with enzyme mixtures produced larger amounts of sugar compared with control sample. However, in some cases after three hours, the amount of glucose in the solution reduced. If the first three hour period is considered, addition of cellulase and other enzyme mixtures produced higher amounts of sugar than the control sample without enzymes.

Table 3.6 shows the sugar produced by cellulase and enzyme mixtures within three hours. The reason why the cellulase addition produced more sugar than enzyme mixtures in the one hour period might be that the amount of cellulase in the mixtures was not enough to produce a higher glucose content in the given period. In the case of enzyme mixtures, after three hours, nearly the same amount of glucose was produced compared to cellulase application. However, the cellulase and xylanase enzyme mixture (50:50) did not work as well as the other conditions.

The cause of a reduction in sugar content observed in some cases after 3 hours might be explained as follows. Even though all the dung samples used in these experiments were air dried and before treatment were put in an oven at 65 ^{o}C until constant weight was obtained, there might be some bacterial action that could consume the glucose produced by enzyme reactions. Also it should be noted that dung is a substrate that contains a very large bacterial population, even treatments with heat may not have any effect on some bacteria and they would use the available glucose. Table 3.7 shows the number of microorganisms found in fresh dung.

Figure 3.6: Effect of single enzyme addition to dung on the amount of reducing sugar production (error bars are based on standard error)

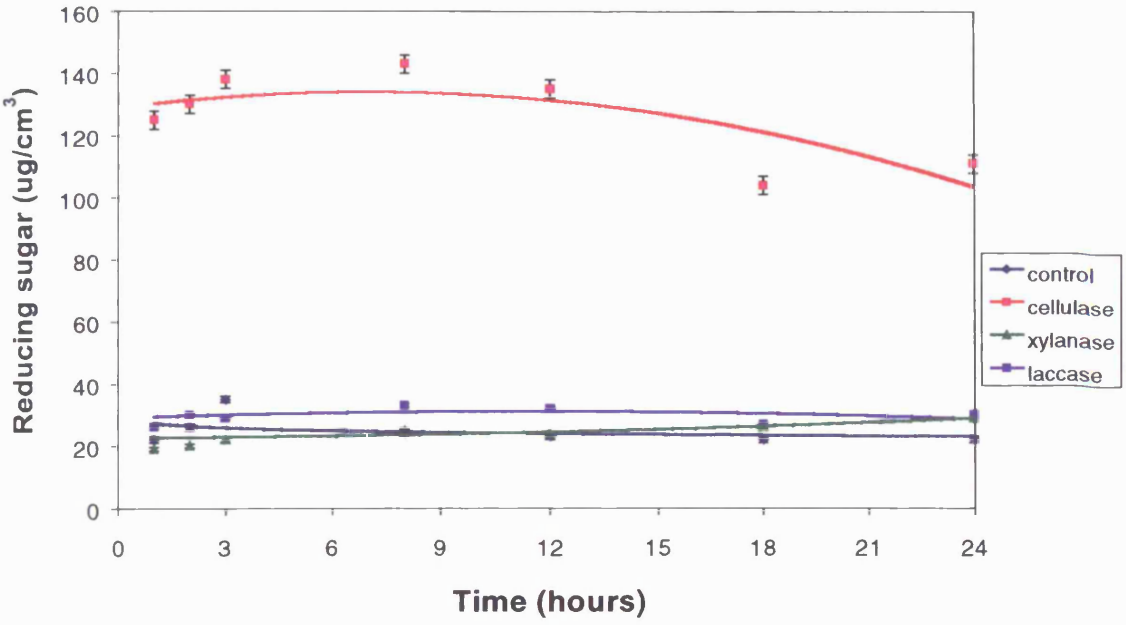


Figure 3.7: Effect of enzyme mixtures' addition to dung on reducing sugar production (error bars are based on standard error)

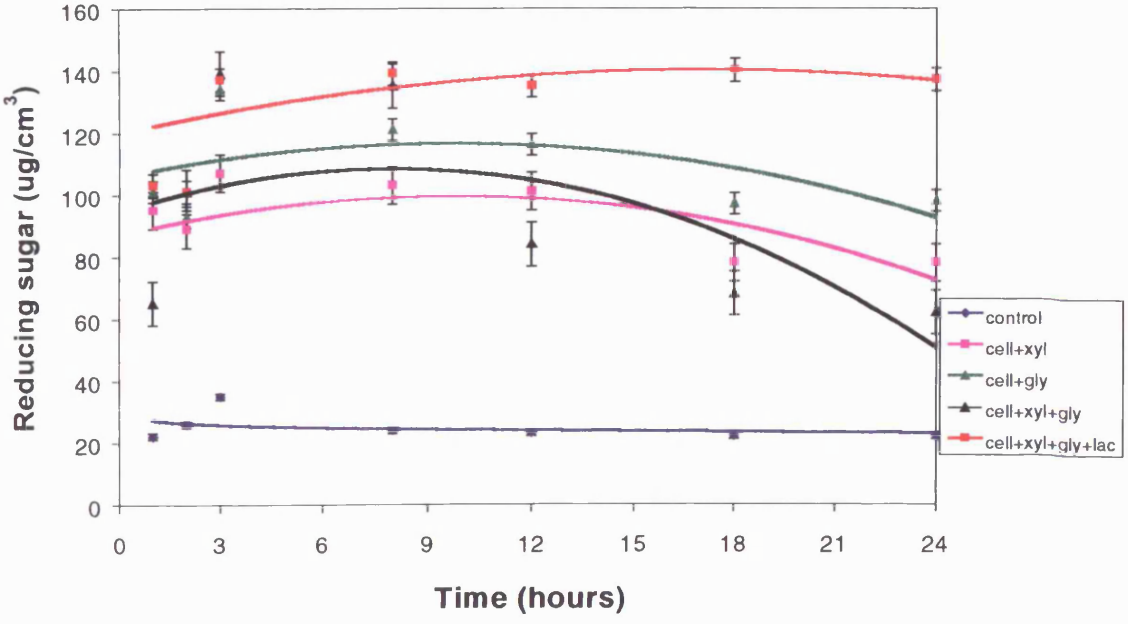


Table 3.6 Amounts of sugar production

Treatment	Reducing sugar in 1 hour ($\mu g/cm^3$)	Reducing sugar in 2 hours ($\mu g/cm^3$)	Reducing sugar in 3 hours ($\mu g/cm^3$)
Control	22	25	34
Cellulase	126	130	138
Cellulase+xylanase	91	90	107
Cellulase+ β-glycosidase	102	89	132
Cellulase+xylanase+ β-glycosidase	67	101	138
Cellulase+xylanase+ Laccase+β-glycosidase	101	101	134

Table 3.7 Microorganisms in fresh dung (modified from Brown, 1972)

Number of species of bacteria	9
Number of bacteria per gram	8.0×10^8
Number of cellulolytic bacteria per gram	5.6×10^4

3.3.2.3 EFFECT OF ENZYMES ON THE INDIVIDUAL COMPONENTS OF DUNG

In this section, the effect of enzymes on the individual components of dung is discussed. These components are cellulose, hemicellulose and lignin. After treatment of dung with enzymes, samples were dried and fractionation of individual components was conducted. In section 3.2, hemicellulose and cellulose fractions were determined by first isolating holocellulose and then holocellulose was further fractionated into cellulose and hemicellulose components. However, conducting the same type of experiment requires a large amount of sample. For this reason, enzyme treated samples were hydrolysed with acid and then they were subjected to sugar analysis as described in section 2.6.3. Cellulose and hemicellulose fractions were not separated from each other and they were assigned as the carbohydrate part of dung.

Table 3.8 demonstrates the remaining carbohydrate and lignin parts of dung after enzyme degradation. The untreated sample consisted of 59% carbohydrate and 22% lignin. The remaining part was protein, fat (both probably hydrolysed during acid treatment) and ash. Treatment of the control sample with water did not change the amount of lignocellulosic component present in the sample. It should be noted that small differences in lignin and carbohydrate content of samples should not be regarded as significant, because of the inhomogeneity of substance. Although the untreated sample and control sample did not show big differences in carbohydrate content, treatment with cellulase reduced the carbohydrate content of dung by about 7%. The lignin fraction remained unchanged with this treatment. About 3% of carbohydrate was removed by treatment with xylanase. The lignin content of dung was slightly reduced by laccase addition, however it is unclear whether the 2% reduction of carbohydrate content was due to either the action of laccase or inhomogeneity of the analysed sample. Treatment of samples with enzyme mixtures

led to about 7- 8% fall in the carbohydrate content. Their lignin contents showed no change, apart from the sample treated with four enzymes. Involvement of laccase in the enzyme mixture resulted in 2- 3% reduction in lignin content.

It may be concluded from this study that treatment with enzyme and enzyme mixtures reduce both carbohydrate and lignin contents of dung. These reductions are more pronounced when using cellulase, xylanase and β-glycosidase together and adding laccase into this mixture. Moreover, there are indications that components of dung are not preferentially solubilised, effectively any reaction results in dung degradation, hence, analysis of residual dung is largely unchanged.

Table 3.8 Effect of enzyme treatment on the carbohydrate and lignin content of dung

Enzyme Treatment of Dung	Carbohydrate Content (%)	SD	Lignin Content (%)	SD
Initial	59.2	3.4	22.0	0.4
Control	59.5	2.1	23.4	0.3
Cellulase	52.0	2.0	22.8	0.2
Xylanase	55.7	3.0	22.0	0.4
Laccase	57.0	2.8	19.2	0.1
Cellulase+xylanase	53.0	1.8	23.4	0.1
Cellulase+β-glycosidase	52.7	1.1	21.8	0.2
Cellulase+xylanase+β-glycosidase	51.2	2.0	22.3	0
Cellulase+xylanase+ β-glycosidase+laccase	51.6	2.1	20.1	0.1

3.4 EXTRACELLULAR ENZYMES PRODUCTION FROM FUNGAL ORGANISMS

Before presenting the results of experiments with enzymes produced from *Coriolus versicolor*, *Aspergillus niger* and *Trichoderma reesei*, it is worthwhile giving some brief background information on the production of cellulases, xylanases and laccases from fungal organisms.

3.4.1 CELLULASE PRODUCTION

Several authors have reviewed the numerous published papers dealing with cellulase production, for example Coughlan (1985), Mandels (1982), Persson *et al.* (1991) and Esterbauer *et al.* (1991).

For most fungal organisms, cellulose is the best substrate for enzyme production. However, it should be noted that some cellulosic substrates are readily degraded and they are consumed quickly, which leads to yields of only low levels of enzyme and some pure insoluble substrates such as cotton are not broken down readily enough to support adequate growth. The optimal balance between starvation and an excess of energy is essential for high cellulase production (Esterbauer *et al.*, 1991).

For all microorganisms examined to date, the synthesis of cellulases is induced by the presence of cellulose, lactose or sophorose and all are repressible by dextrose or other readily metabolised sugars in the growth medium (Coughlan, 1985 and Mandels, 1982). However, the natural inducer of cellulase is not known. The most widely accepted view of the induction process is that the organisms produce low constitutive levels of cellulase. This effects some breakdown of cellulose in the

medium and a soluble molecule then enters the cell and effects induction. Cellobiose is a likely candidate since it is a product of cellulase degradation and growth on cellobiose will lead to cellulase production (Mandels, 1982). However, it is effective only at high concentration. Lactose is also an inducer in some species, but again only at high concentration. Sophorose, 2-O-β-glucopyranosyl-D-glucose, is a powerful inducer at low concentration for endo- and exocellulase synthesis by *T. reesei*. Sophorose has two regulatory roles in *T. reesei*; it induces the synthesis of endo- and exocellulases and it also represses the production of β-glucosidase, since that would decrease the rate of sophorose synthesis and effectively enhance its ability to induce cellulase production (Coughlan, 1985).

Fungal cellulolytic enzyme production can be divided into three main groups, according to the carbon source used in the cultivation. These are soluble substrates, solid-soluble substrates and lignocellulosic substrates (Persson *et al.*, 1991). The most commonly used soluble carbon sources are lactose and glucose. A variety of solid purified cellulose sources can be used for cellulase production. By changing the carbon source from soluble to solid purified cellulose, the enzyme concentration increases dramatically. However, the use of purified cellulose substrates for enzyme production is very costly in large scale processes. When lignocellulose is the carbon source for cellulosic enzyme production, generally enzyme concentration levels are similar to those from purified substrates and they are very cost effective (Doppelbaur *et al.*, 1987 and Persson *et al.*, 1991).

3.4.2 XYLANASE PRODUCTION

Xylanases are generally produced along with cellulases during fungal growth on cellulose and xylan substrates (Wong and Saddler, 1992). However, it is commonly believed that synthesis of xylanases and cellulases is separately regulated. Like cellulases, the natural inducer of xylanases is not known and small soluble fragments formed from low constitutive levels of cellulase action penetrate the cell membrane. Following uptake by the organisms these compounds provoke the formation of larger quantities of the enzymes (Haltrich *et al.*, 1996). However, the most prominent inducer molecule is xylobiose that has been found to be an effective inducer in a range of microorganisms. Induction of xylanase synthesis can also be achieved by β-methyl- D- xylopyranoside.

Senior *et al.* (1989) proposed that the xylanase to cellulase ratio in fungal growth medium is directly proportional to the xylan to cellulose ratio in the growth substrate. Although it has been shown that for some organisms the synthesis of xylanase and cellulase is separately regulated, there are other organisms in which xylanase formation is clearly linked to that of cellulase. In *T. reesei*, xylan and xylobiose selectively induce the formation of xylanase, whereas sophorose induces production of both cellulase and xylanase (Hrmova *et al.*, 1986). In *Aspergillus terreus* selective induction of xylanase could be achieved by employing xylan, xylobiose or D-xylose as substrates, whereas in the presence of cellulose or cellobiose both cellulase and xylanase were formed (Hrmova *et al.*, 1989).

3.4.3 LACCASE PRODUCTION

Laccase is particularly abundant in wood destroying white-rot fungi that are able to degrade lignin, but absent in brown-rot fungi that are unable to decompose lignin (Kirk, 1987).

Lignin extracted from wood cannot serve as the substrate for growth of *Basidiomycetes* because the rate of lignin degradation is too low to provide energy for the culture. Therefore, lignin metabolisation by fungi occurs only in the presence of an alternative carbon and energy source (Elisashvili, 1993). Under natural conditions, white-rot fungi obtain energy and carbon through degradation of cellulose and hemicellulose by the enzymes of cellulase and hemicellulase complexes. This might be the reason why degradation of wood lignin by most fungi proceeds simultaneously with utilisation of polysaccharides.

It has been demonstrated that inoculation of *Coriolus unicolor* and *Coriolopsis villosus* with a carbon source in the form of citrus husks, produced laccase and ligninase. Increasing the concentration of citrus husks from 2 to 4-5% caused a 3.8-4.5 fold increase in laccase activity. The addition of readily utilized carbohydrates, glucose and cellobiose increased laccase production. Laccase from *C. versicolor* can be induced with 2,5-dimethylaniline (2,5-xylidine) as shown by Fähraeus and Reinhammer (1967).

3.4.4 RESULTS OF EXTRACELLULAR ENZYME PRODUCTION FROM FUNGAL ORGANISMS

Table 3.9 represents the various enzyme activities from fungal cultures and commercial enzyme preparations. For larger scale production of enzymes, ten or fifteen flasks, each containing 200 ml liquid medium, were incubated with microorganisms and at the end of the incubation time they were mixed and their activities were measured.

Laccase was produced from *C. versicolor* by the method of Fähraeus and Reinhammer (1967). *C. versicolor* was induced for laccase production by 2,5-dimethylaniline added on day six of growth. The culture medium contained glucose for the carbon source and L-asparagine and DL-phenylalanine for nitrogen sources. Under these conditions, *C. versicolor* showed 0.158 U/mg laccase activity along with 0.137 and 0.0174 U/mg of β-glucosidase and β-xylosidase activity respectively. When *C. versicolor* was growth on Abrams medium (Abrams, 1948), supplemented with 5% carboxymethylcellulose, a range of enzymes were produced. However, the activities of laccase and β-glucosidase were lower than when these enzymes produced from Fahraeus medium. The reason for low laccase production might be attributed to absence of induction process with 2,5-dimethylaniline. The presence of cellulolytic enzymes from *C. versicolor* was studied by Evans (1985b).

One of the cheapest way to produce extracellular enzymes by fungi is to use readily available byproducts such as lignocellulosic wastes. It was thought that cattle manure may be efficiently used as a carbon source for this reason. Furthermore, fungal culture grown on dung might produce enzymes that are more suitable for removing dung from hide. For this reason, Abrams medium used for extracellular enzyme production from *C. versicolor* was supplemented with 3% cattle dung as a carbon source. In the present study, higher enzyme activities were obtained with this

modification compared to the Abrams medium supplemented with 5% carboxymethylcellulose. Leontievsky and co-workers (1997a and 1997b) showed that growth of *Coriolus versicolor*, *Panus tigrinus*, *Phlebia radiata* and *Phlebia tremellosa* on wheat straw produced yellow-brown laccases in contrast to blue colour from submerged cultures and their laccase activities were higher than those produced in submerged cultures. Therefore, higher laccase activity obtained from *C. versicolor* grown in the presence of dung may be attributed to this phenomenon of white-rot fungus. The higher cellulase, xylanase and β-glucosidase activities from *C. versicolor* grown on the cattle dung may be due to the higher amount of carbon sources compared to Abrams medium.

Aspergillus niger and *Trichoderma reesei* were grown in Abrams medium (Abrams 1948) supplemented with 15 g/l carboxymethylcellulose and 5g/l xylan in order to produce both cellulosic and xylanolytic enzymes together. It is well known that *T. reesei* and *Aspergillus niger* are amongst the best producers of cellulosic and xylanolytic enzymes. *T. reesei* showed higher cellulase activity than *A. niger*. However, xylanase, β-glucosidase and β-xylosidase activities were higher in the *A. niger* culture. It may be seen that these two enzymes represent the same properties in terms of xylanolytic and cellulosic enzyme production. Therefore it was decided that for further studies with these fungal cultures, *T. reesei* should be omitted. For this reason, *Aspergillus niger* culture medium was used in the tannery experiment for treatment of dung clad hides.

Commercial enzyme preparations were also assayed in order to compare the activity results from fungal preparations and commercial enzymes. It should be noticed that commercial xylanase, cellulase and β-glucosidase showed the highest xylanase, cellulase and β-glucosidase activities respectively compared with fungal enzyme preparations. The higher enzymatic activities from such preparations may be

attributed to different growing media used in their preparation. However, due to the commercial nature of these enzymes, there was no information available regarding their growing conditions, nor what quantity of culture filtrate had been dried.

Commercial enzymes and fungal culture broths were assayed by using casein as substrate in order to determine whether they had any protease activity. None of the samples showed any activity against casein. This means that these enzymes can be safely used in the soaking process because they are unlikely to have any effect on skin proteins.

Table 3.9: Activities of enzymes

a. Activities of laboratory cultured enzymes

	Protein (mg/ml)	Cellulase (U/mg)	Xylanase (U/mg)	Glucosidase (U/mg)	Xylosidase (U/mg)	Laccase (U/mg)
C.versicolor (Fahraeus medium)	0.755	-	-	0.137	0.0174	0.158
C.versicolor (Abrams medium)	0.316	17.59*	10.63	0.487	-	0.006
C.versicolor (Abrams medium + dung)	1.950*	3.236	5.432	0.149	0.009	0.225
Aspergillus niger	0.680	10.82	30.5*	2.58*	6.26*	-
Trichoderma Reesei	1.023	11.36	10.26	0.193	0.998	-

*Represents the highest enzyme activity among the growth culture.

b. Activities of commercial enzymes

	Cellulase (U/mg)	Xylanase (U/mg)	Glucosidase (U/mg)	Xylosidase (U/mg)
Cellulase**	48.6	16.3	5.3	2.96
Xylanase**	12.9	33.45	3.65	8.2
Glucosidase**	6.12	9.8	17.7	4.53

**These enzymes were obtained from chemical companies (Biocatalyst and Sigma)

3.4.5 EFFECTS OF SODIUM CHLORIDE AND NONIONIC DETERGENT ON ACTIVITY OF ENZYMES

The majority of the raw hides and skins processed in the leather industry are in either the wet or dry salted condition. The preservative salt, in the form of sodium chloride, has to be removed in the soaking process in order to obtain proper rehydration. Nonionic surface active agents are commonly used in the soaking process to obtain better and quicker rehydration and to disperse natural fats inside the hides. Therefore, it was necessary to find out whether these agents and salt have any effects on the activities of enzymes used for dung removal. For this reason, commercial cellulase and xylanase enzymes were assayed in the presence of up to 3.0 M sodium chloride. The effect of nonionic detergent on enzyme activity was determined in the presence of 2% fatty alcohol ethoxylate.

Figure 3.8 shows the effect of sodium chloride concentrations on the activities of xylanase and cellulase. Cellulase and xylanase activities were increased by approximately 20% and 100% respectively by the presence of sodium chloride. This means that increasing the activity of the enzymes should result in less enzymes being required for the same action.

Figure 3.9 shows the effect of nonionic fatty alcohol ethoxylate on cellulase and xylanase activities. It was found that there were no significant changes in the enzyme activities in the presence of nonionic detergent.

Figure 3.8: The effect of sodium chloride concentrations on the activity of cellulase and xylanase

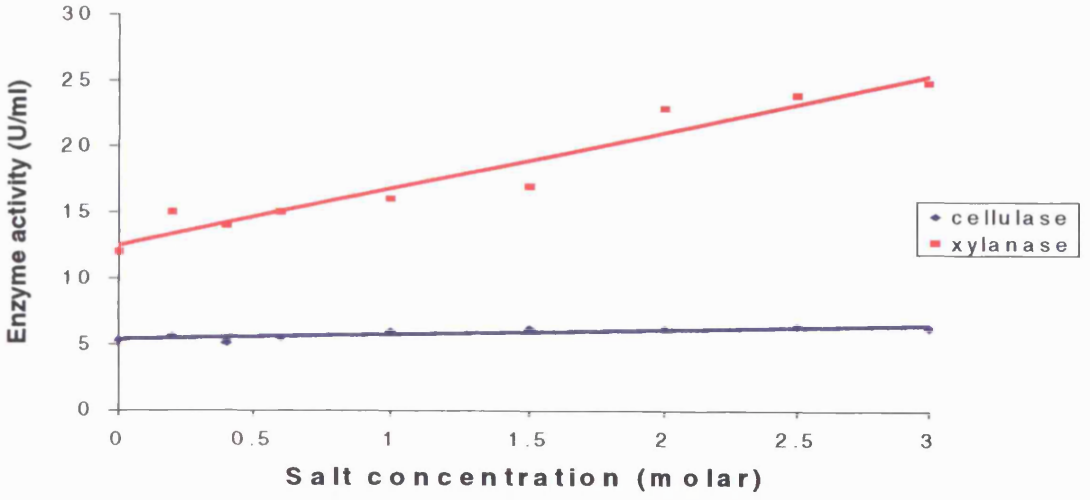
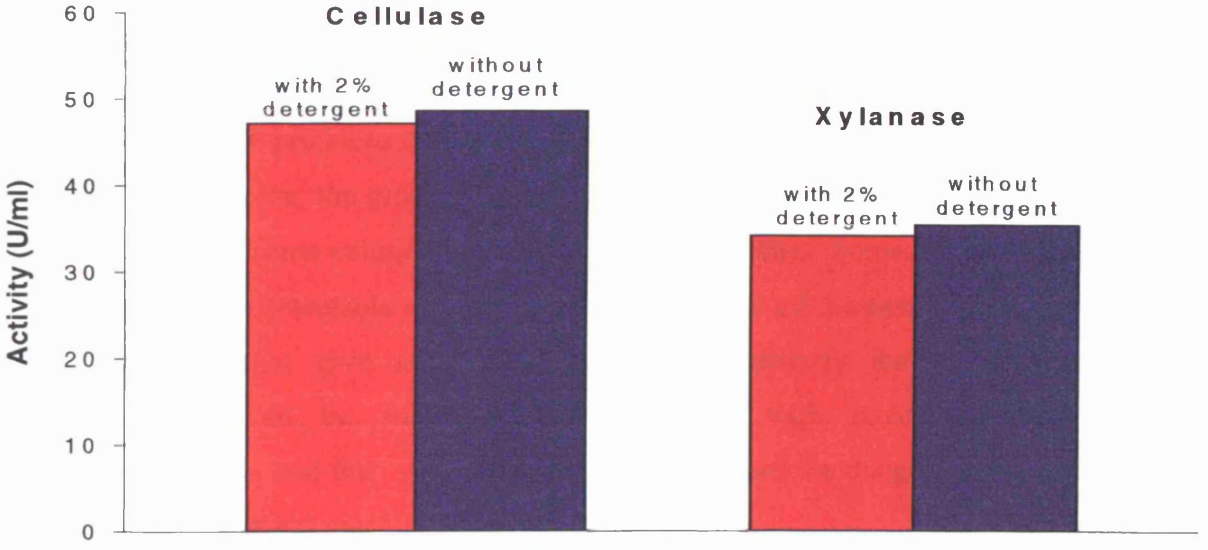


Figure 3.9: Effect of nonionic fatty alcohol ethoxylate on the activity of xylanase and cellulase



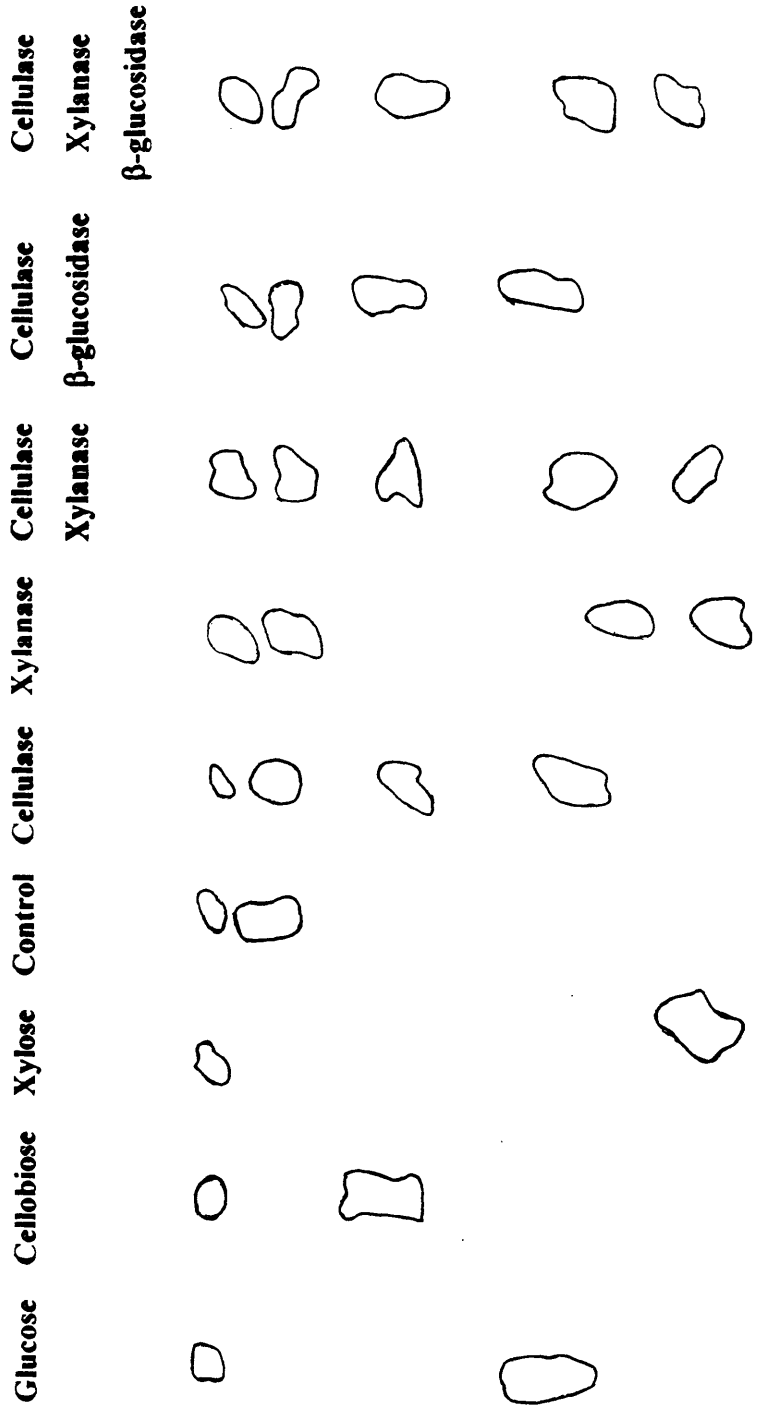
3.5 MECHANISMS OF ENZYMATIC DUNG REMOVAL

This part can be divided into three sections. In the first section, paper chromatography is used to study dung degradation. The second section covers the study of dung degradation using gel permeation chromatography. ^{13}C/MAS solid state NMR and IR spectroscopy techniques for the study of dung degradation are covered in the final section.

3.5.1 PAPER CHROMATOGRAPHY OF THE CARBOHYDRATES

The traditional way to analyse the enzymatic hydrolysis products of cellulose and xylan in by paper chromatography (Biely *et al.*, 1992). Also paper chromatography provides a reliable and inexpensive method for analysing the sugar from faecal extracts (Smith, 1969). Therefore, reaction solutions from enzyme degradation were analysed by descending chromatography, using butan-1-ol/pyridine/water (60/40/50) mixture. Moreover, solutions of glucose, cellobiose and xylose were analysed by paper chromatography in order to provide reference chromatograms. Figure 3.10 shows the paper chromatography results of different enzyme applications. It can be seen from the figure that the cellulase mainly produces cellobiose and glucose, xylanase treatment produces xylose and glucose: the glucose is detectable here because xylanolytic enzymes usually exhibit some cellulosic and β-glycosidic activities. Some big molecular components are detectable in paper chromatography in all the samples, but their Rf values cannot give accurate information to identify the components. However, it can be suggested that they are high molecular weight oligosaccharides and they must be easily removed from the dung because even the control sample shows the same type of component.

Figure 3.10: Paper chromatography of enzyme degraded dung products



3.5.2 GEL PERMEATION CHROMATOGRAPHY OF ENZYMATIC DUNG REMOVAL

Gel permeation chromatography (GPC) involves the size separation of molecules in solution. The separation of sample molecules is based on differences in their "effective size" in solution, effective size being closely related to molecular weight. Separation is accomplished in a column containing closely controlled pore sizes. Molecules with small effective sizes (low molecular weight) will penetrate more pores than molecules with larger molecular weight and therefore will take longer to emerge from the column. If the column packing material covers the right range of molecular sizes, the result will be a size separation with the largest molecules coming out first.

Based upon this background, it was thought that different enzyme treatments of dung samples would produce different molecular weight components in different amounts. Measuring the molecular weight distribution of the solution might give valuable information regarding the progress of enzyme treatment. For this reason, water extracts obtained after enzymatic treatments were analysed with GPC using polysaccharides as a reference. Figures 3.11 and 3.12 demonstrate the GPC results of water extracts of individual enzymes and enzyme mixture treatments respectively. Although the chromatograms may at first appear complex and difficult to deconvulate, some general considerations can substantially simplify the system. It should be noted that liquid extracts from dung show a great complexity and heterogeneity, hence this complexity is reflected in the GPC results. All of the chromatograms show high and low molecular components with different intensities. Moreover, they represent the similar patterns, except the chromatogram from laccase treatment. In this case, there is a distinct middle molecular weight product. In order to simplify the GPC results, the calculated relative areas under the chromatograms are given in Table 3.10, corrected for diluting. The intensity increase is higher with

the enzyme mixture treatment than it is for individual enzyme treatments, especially the enzyme mixture containing four different enzyme components. These findings are consistent with the results obtained by other analyses such as chemical oxygen demand and reducing sugar.

Figure 3.11: Gel Permeation Chromatography results of individual enzyme treatments

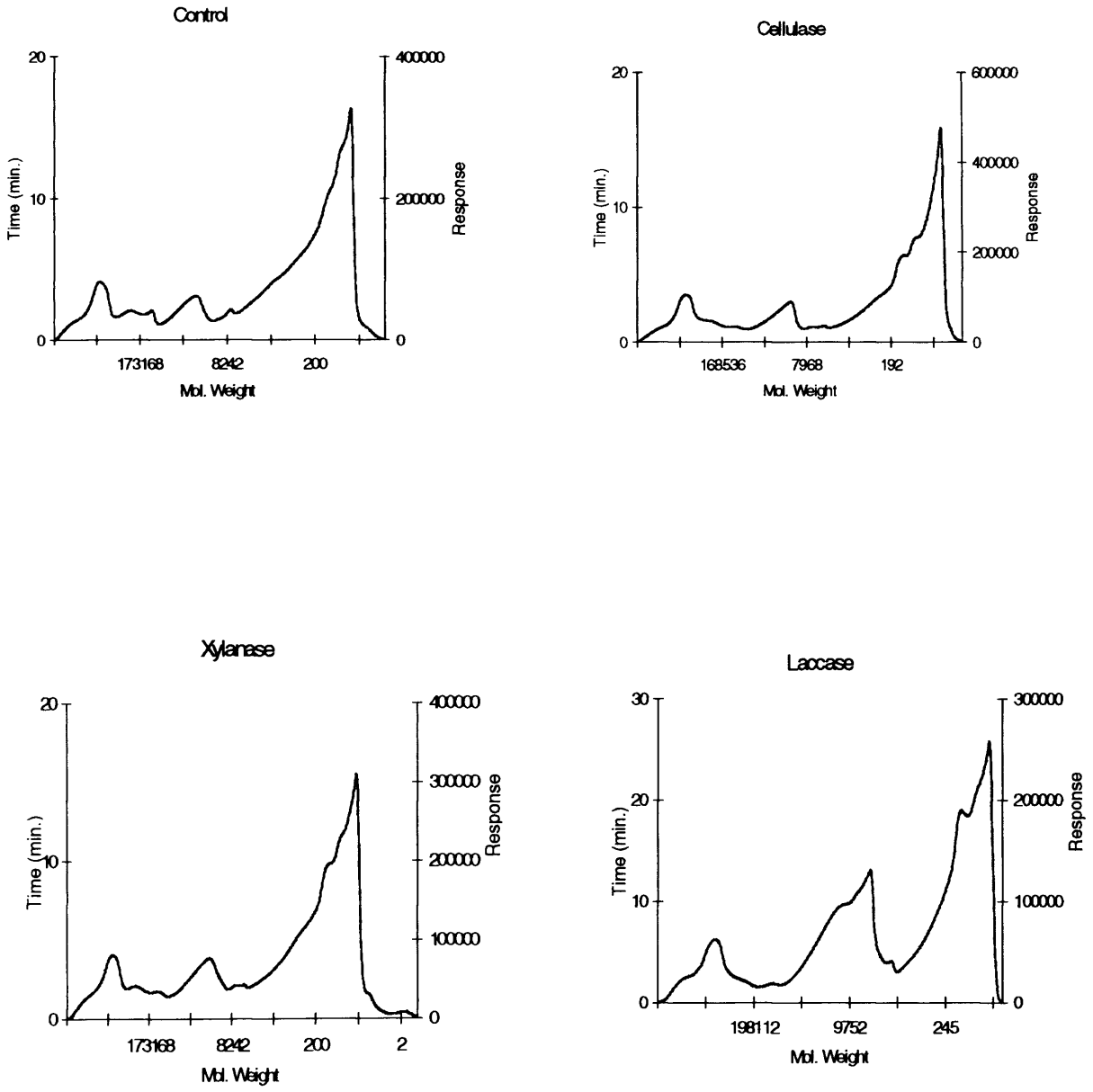


Figure 3.12: Gel Permeation Chromatography results of enzyme mixture treatments

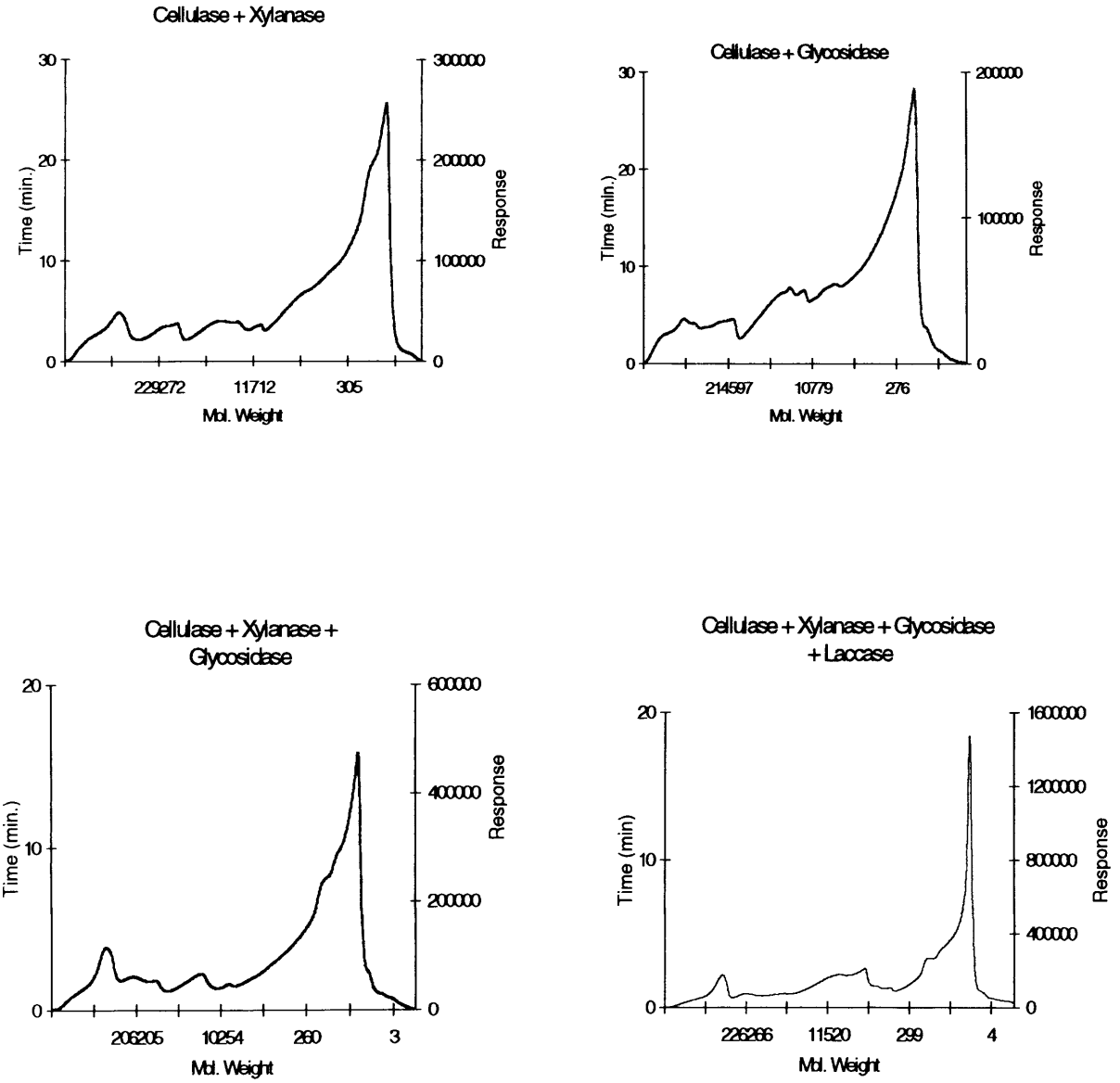


Table 3.10: Comparison of different enzyme treatment of their relative areas under the GPC chromatogram

Enzyme Treatment	**Relative Area under the GPC Chromatogram**
Control	1.00
Cellulase	1.20
Xylanase	1.06
Laccase	1.05
Cellulase+Xylanase	1.56
Cellulase+Glycosidase	1.29
Cellulase+Xylanase+Glycosidase	2.82
Cellulase+Xylanase+Glycosidase+Laccase	4.77

3.5.3 ^{13}C CP/MAS SOLID STATE NMR AND IR SPECTROSCOPY OF ENZYME TREATED DUNG SAMPLES

There are several literature references concerning the application of ^{13}C cross-polarisation/magic angle spinning (CP/MAS) NMR to investigate lignocellulosic materials. There is also a recent review of solid-state NMR studies of wood and other lignocellulosic materials (Gil and Neto, 1999).

However, there have been few studies on solid state NMR of dung and rumen forages. Liang and co-workers (1996) reported a CP/MAS NMR study to

compare the structural differences between composted and stockpiled manure. Mcbride (1991) studied the influence of cellulose crystallinity on the extent of cellulose digestibility in the rumen of steers using this technique.

Most of the studies in CP/MAS NMR of lignocellulosics are concentrated on the chemical differences in plant material species and changes due to chemical treatments and biodegradation.

Himmelsbach *et al.* (1983) studied the ratio of lignin and carbohydrate between grass species from single spectra of the whole plant material. Newman (1994) used resolution enhanced NMR spectra obtained from seven different tree species to investigate crystallinity of the hardwood and softwood components.

Several authors have investigated the structural changes due to chemical treatments by the application of the CP/MAS NMR technique (Wallbacks *et al.*, 1991; Dunn *et al.*, 1991; Kimura *et al.*, 1992; Boonstra *et al.*, 1996 and Guadalix *et al.*, 1997).

Because the interest in the present study is the biodegradation of lignocellulosic material, it is useful to discuss some publications concerning the use of the NMR technique in lignocellulose biodegradation studies.

Ali *et al.* (1996) studied a series of laboratory-aged papers using solid-state NMR spectroscopy. They found that the aged papers represent different spectra pattern than the unaged sample. Gilardi *et al.* (1994 and 1995) studied the biodegradation of spruce (*Pinus sylvestris*) and beech wood samples (*Fagus sylvatica*) with both brown-rot fungi (*Coniophora puteana*) and white-rot fungi (*Coriolus versicolor*) using NMR spectroscopy. In their first paper, using wide line solid-state NMR, spin-lattice (T_1) and spin-spin (T_2) relaxation times measurements of wood samples subjected to fungal attack were reported. T_1

showed an increase up to 8.8 fold for white-rotted and 1.5 fold for brown-rotted samples. T_2 showed an increase of up to 2.1 fold for white-rotted and 1.7 fold for brown-rotted samples. In their second paper, they looked at the CP/MAS NMR spectra of the biodegraded samples and it was found that there were big differences between the NMR signals of the control sample and decayed samples. In particular, in the aliphatic and aromatic region of spectra corresponding to cellulose, hemicellulose and lignin, resonances are altered by biodegradation. Also it was shown from the NMR signals, that brown-rot fungi degrade polysaccharides, leaving a modified lignin network, whereas white fungi extensively degrade both polysaccharides and lignin. A similar study conducted by Martinez *et al.* (1991) showed that the natural decay of ulmo wood (*Eucryphia cordifolia*) by *Ganoderma australe* gave rise to decreased lignin ^{13}C NMR signals. Huang *et al.* (1998) monitored the biomacromolecular degradation of *Calluna vulgaris* through qualitative and quantitative changes in lignin, polysaccharide, aliphatic, cutin and protein fractions collected at three intervals (0.5, 7 and 23 years) investigated by solid-state NMR and pyrolysis-GC/MS. Akin and co-workers (1995) were interested in the effects of enriched CO_2 on the biodegradation of wheat with white-rot fungus. They used solid-state NMR to evaluate the changes due to CO_2 content. Their results were also supported by other analytical methods and electron microscopy. Hemmingston and Morgan (1990) investigated the CP/MAS ^{13}C NMR pattern of photodegraded lignocellulosic material. Kimura *et al.* (1992) studied irradiated unbleached and alkaline hydrogen peroxide bleached black spruce (*Picea mariana*) by NMR spectroscopy.

Several authors have also investigated lignocellulosic components by applying infrared (IR) spectroscopy. Sarkanen and Ludwig (1971) used synthetic and isolated lignin for the study of absorption band assignments. Gillardi *et al.* (1995) used FT/IR technique to study cell wall degradation by white-rot fungi.

They concluded that due to the considerable complexity of cell wall components, IR spectral studies could provide overall information on molecular changes during degradation.

It may be concluded from these studies that CP/MAS solid state NMR technique provides useful detail information on the biodegradation of lignocellulosic components by enzymes. However, IR spectra can provide overall information at the molecular level during the biodegradation of lignocellulosic components.

On the basis of the background, the application of ^{13}C CP/MAS NMR and IR spectroscopy were used in this research to illustrate the structural differences between enzyme treated dung samples and control samples. Moreover, the solution from enzyme treatments, which were freeze dried, were also subjected to solid-state NMR and IR experiments to understand the mechanism of dung degradation.

3.5.3.1 CP/MAS NMR SPECTRA OF INTACT DUNG

The carbon-13 CP/MAS NMR spectrum of untreated dung is given in Figure 3.13. Chemical shift assignments have been carried out according to Atalla *et al.*, 1980; Maciel *et al.*, 1981; Kolodziejski *et al.*, 1982. Data are reported in Table 3.11.

The peak at 66 ppm and the shoulder at 63 ppm is assigned to C-6 carbon of the cellulose and hemicellulose. The overlapping peaks at 70-80 ppm are due to C-2, C-3 and C-5 carbons of cellulose and the broad background is C-2, C-3 and C-5 carbons of hemicelluloses. The peak at 105 ppm is assigned to the C-1 atom of cellulose and the broad peak at 103 ppm, appearing on the side of the 105 ppm peak of cellulose, is the C-1 carbon of the hemicellulose. Between 100 and

140 ppm is assigned to C-2, C-5 and C-6 aromatic rings and side chain carbons of lignin. The C-1 carbon of aromatic rings in lignin is at 135 ppm. The peak at 148 ppm is assigned to C-4 carbon of the lignin aromatic ring containing free phenolic groups and at 154 ppm, C-3 and C-5 carbons of the lignin aromatic ring containing methoxy groups. Hemicellulose exhibits many spectral features similar to cellulose, but its signals tend to be broader, reflecting the heteropolymeric nature of these carbohydrates.

The spectral features obtained from untreated dung sample are consistent with previous literature data (Atalla *et al.*, 1980; Earl and VanderHart, 1980 and Gilardi *et al.*, 1995). When the spectral result is compared with cellulose, lignin and whole pine cell wall (Gilardi *et al.*, 1992) (see Figure 3.14), a close similarity can be observed.

Table 3.11: Assignments of ^{13}C chemical shifts of CP/MAS NMR spectra of lignocellulosic compounds (modified from Atalla *et al.*, 1980; Earl and VanderHart, 1980 and Gilardi *et al.*, 1995).

Chemical Shifts (ppm)	**Assignments**
21.5	Acetate groups in hemicelluloses
56	Aryl methoxyl carbons of lignin
63	C-6 carbon of amorphous cellulose or C-6 on the surface of fibers
66	C-6 carbon of crystalline cellulose
63-66	Background of C-6 carbon of hemicelluloses
70-80	Overlapped signals of C-2, C-3 and C-5 in cellulose; broad background of C-2, C-3 and C-5 carbons of hemicelluloses
84	C-4 carbon of amorphous cellulose
89	C-4 carbon of crystalline cellulose
84-98	Background of C-4 carbon of hemicellulose
103	Shoulder of C-1 carbon of hemicelluloses
105	C-1 carbon of cellulose
110-140	C-2, C-5 and C-6 carbon of aromatic rings and side-chains carbons of lignin
135	C-1 carbon of aromatic ring carbons of lignin
148	C-4 carbon of lignin aromatic ring containing free phenolic groups attached
154	C-5 carbon of lignin aromatic ring containing methoxy groups
150-160	Oxygen substituted aromatic ring carbons (C-3 and C-4)
174	Carbonyl groups of lignin and carboxyl groups of hemicellulose

Figure 3.13: CP/MAS carbon-13 NMR spectra of untreated dung

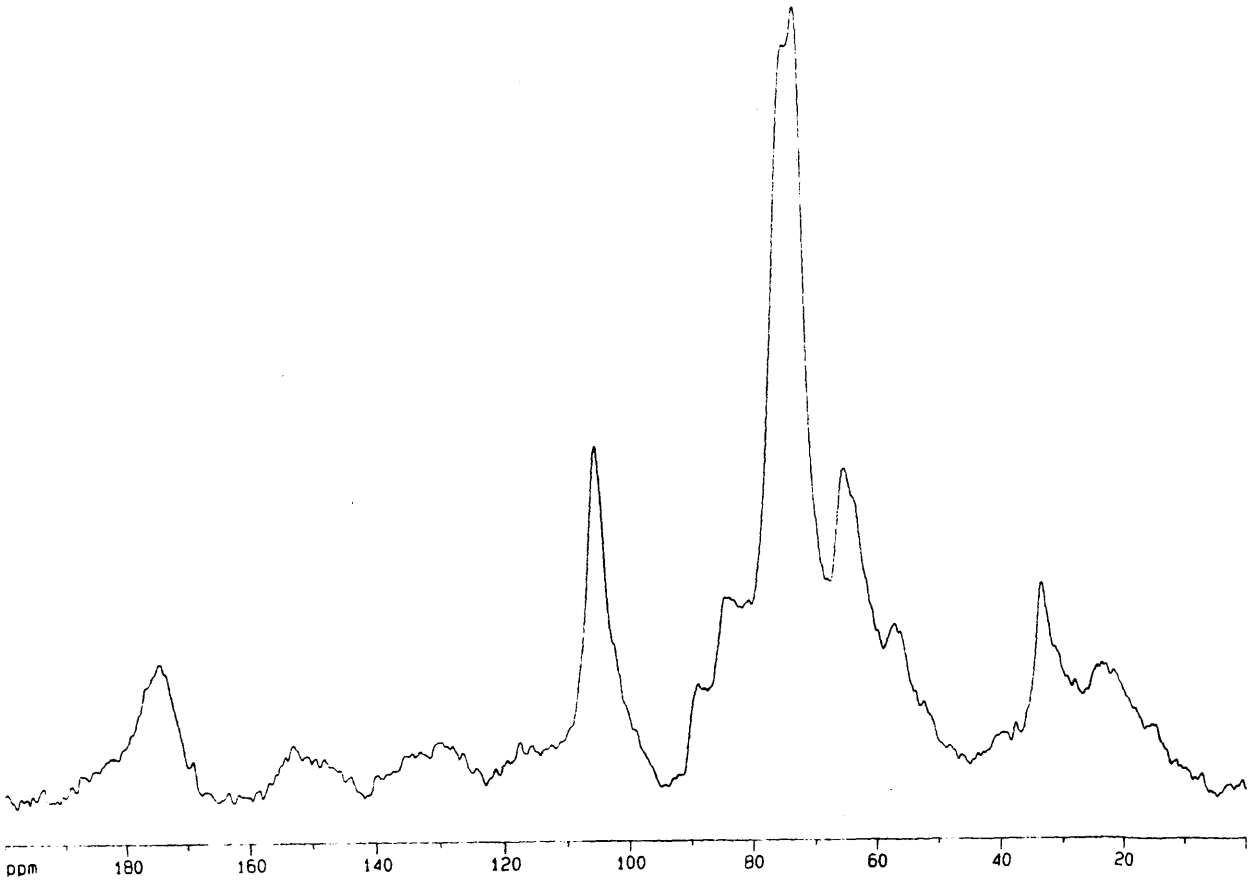
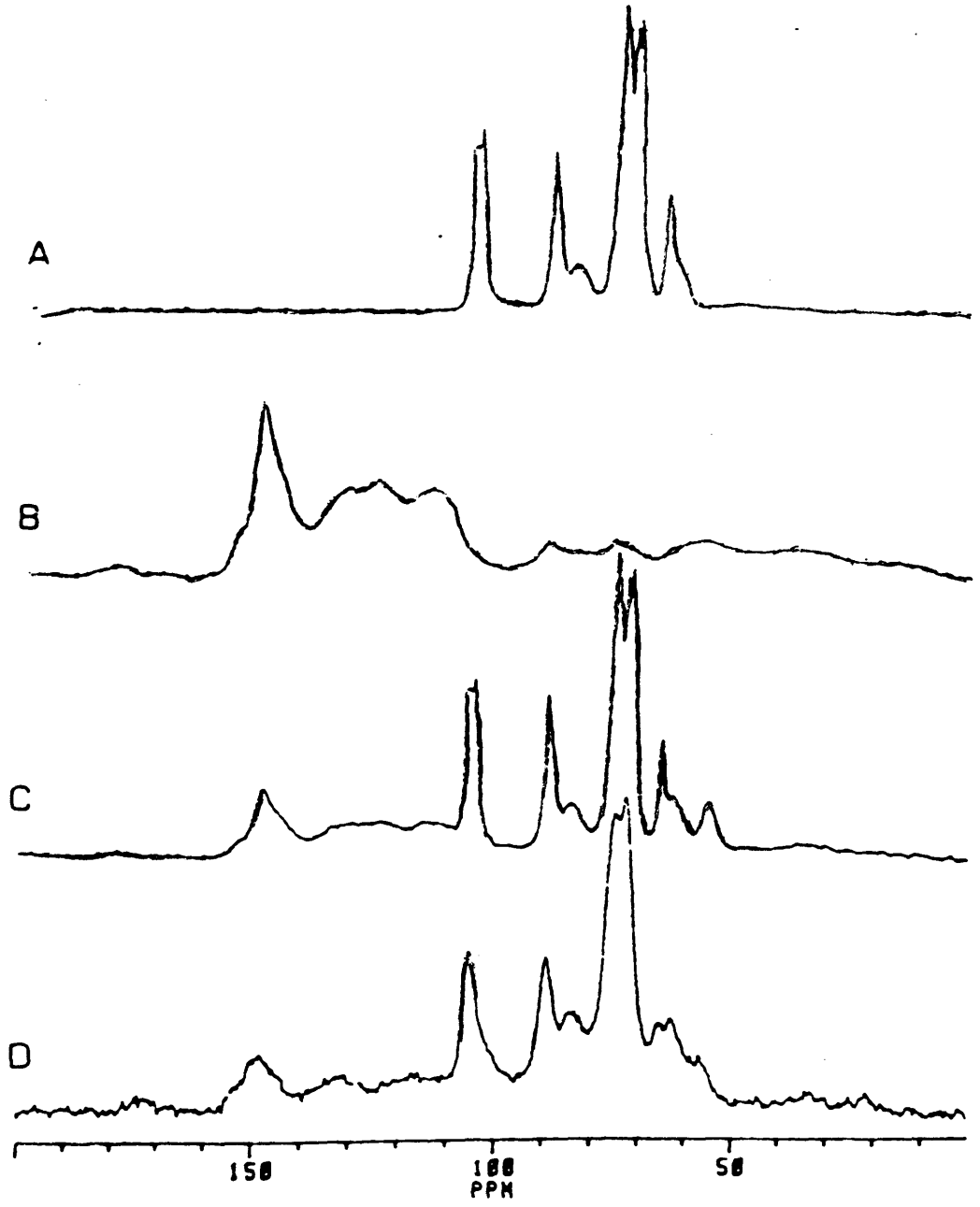


Figure 3.14: CP/MAS carbon-13 spectra of (a) cellulose, (b) spruce lignin, (c) addition of (a + b) and (d) whole cell walls from pine wood (Gilardi, 1992)



3.5.3.2 CP/MAS SPECTRA OF DEGRADED DUNG

Dung samples were studied by CP/MAS technique after treatment by different enzymes and enzyme mixtures for 24 hours. The control sample's spectrum is presented in Figure 3.15. The enzyme treated samples' spectra are presented in Figures 3.16, 3.17, 3.18 and 3.19.

As can be seen from these figures, there are no differences in peak intensities observed when control and enzyme treated samples are compared. However, it might be said that C-1 carbon of the hemicellulose at 103 ppm on the side of the 105 ppm peak of cellulose disappears compared with untreated and control samples. This may be due to some loss occurred in the hemicellulose part of the dung during the enzyme treatments.

The results from this experiment can be explained as follows: the cellulosic, xylanolytic and lignolytic enzyme treatments remove the dung from hides without changing the fundamental structure of the dung. The enzymes probably attack the structural parts in lignocellulose, which are most susceptible to solubilising with enzymes and the amorphous parts of the substrate. From other studies on the enzymatic degradation of lignocellulosic materials, this degradation period of up to 24 hours is short to observe significant changes at the molecular level: other studies have been conducted for periods of weeks.

Figure 3.15: CP/MAS carbon-13 NMR spectra of control sample

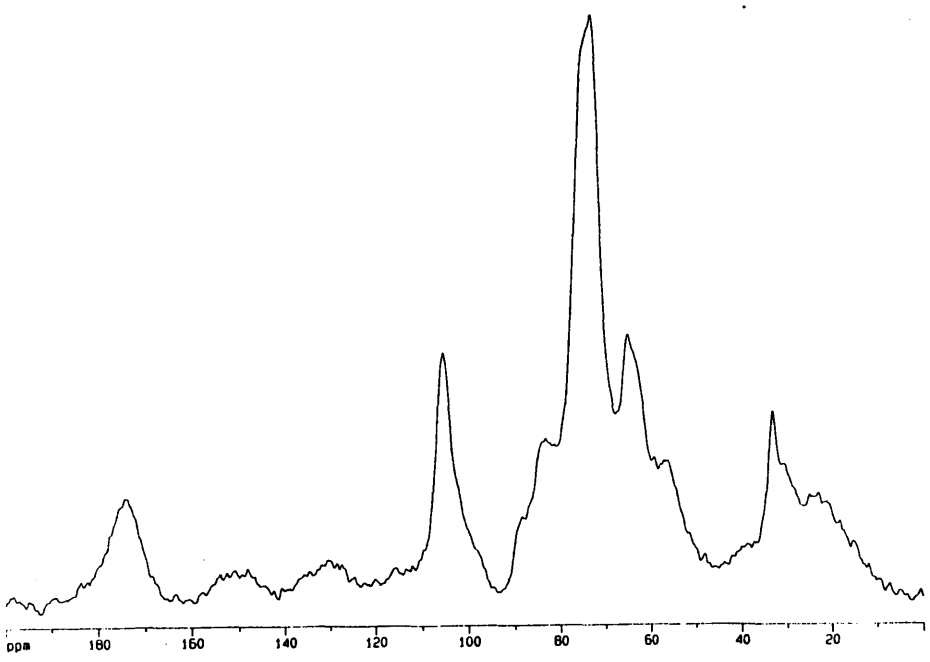


Figure 3.16: CP/MAS carbon-13 NMR spectra of cellulase treated dung

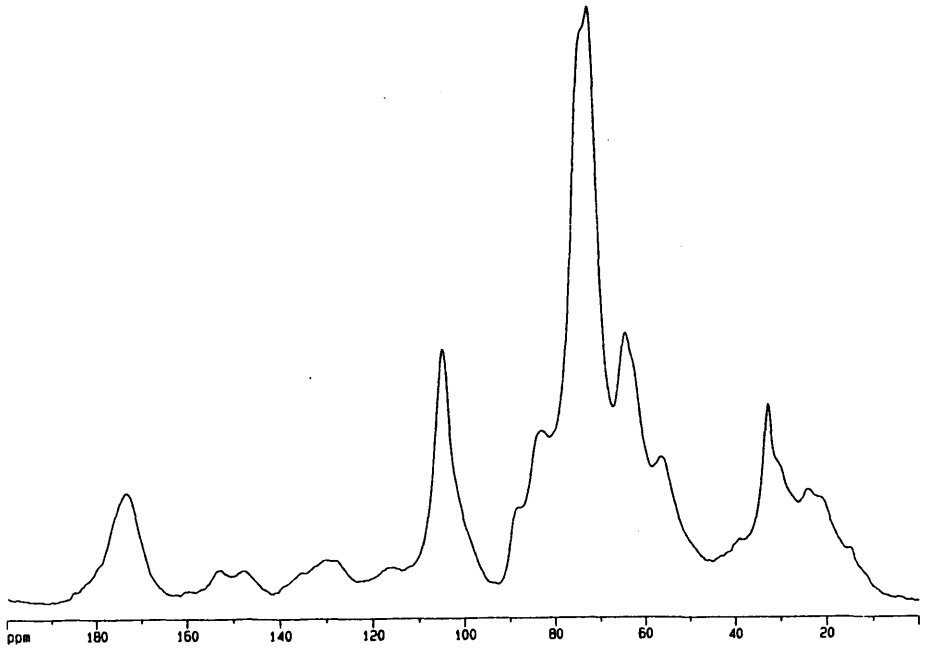


Figure 3.17: CP/MAS carbon-13 NMR spectra of xylanase treated dung

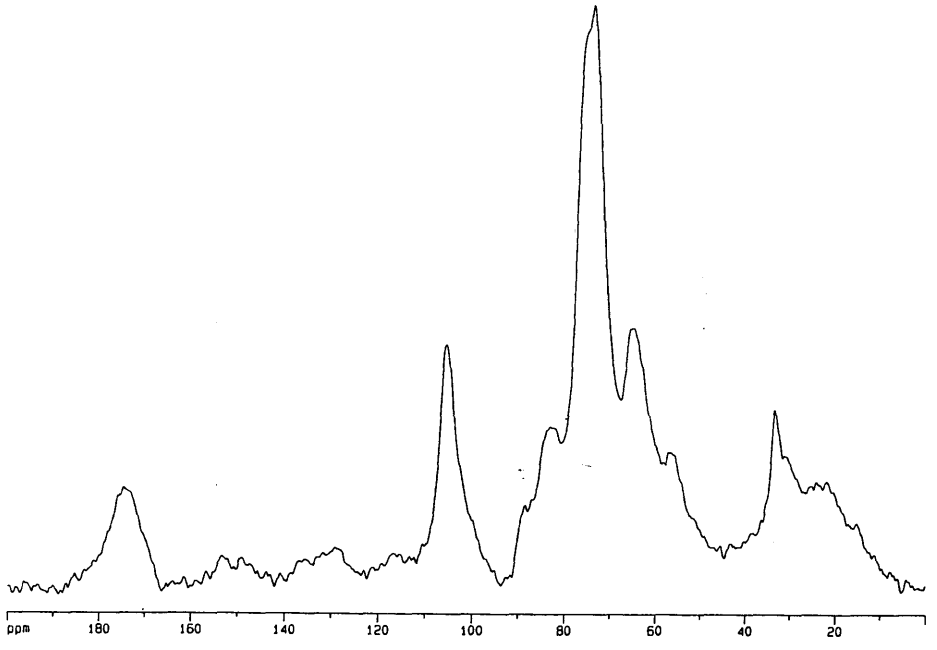


Figure 3.18: CP/MAS carbon-13 NMR spectra of laccase treated dung

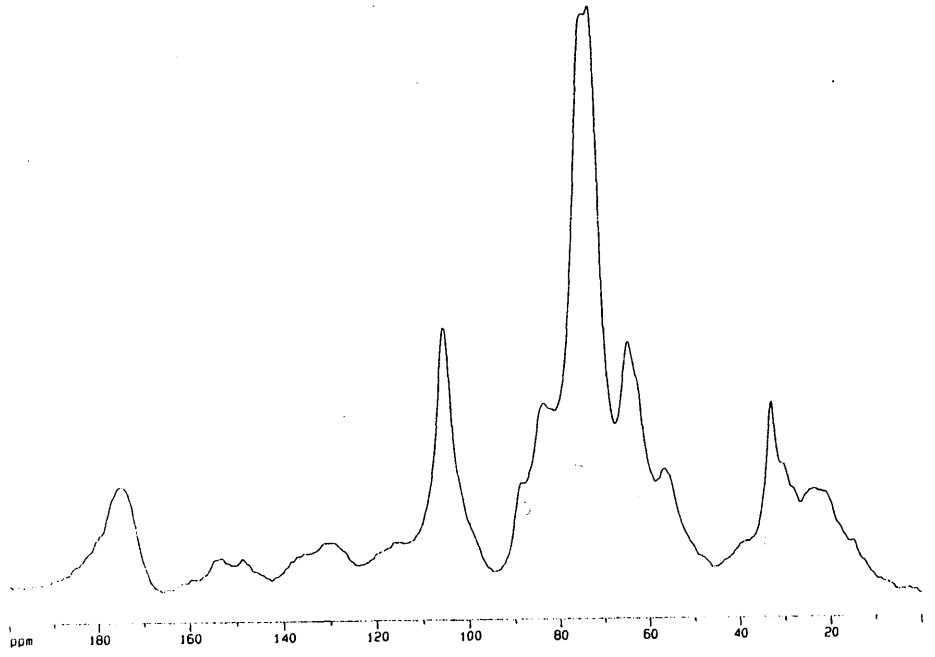
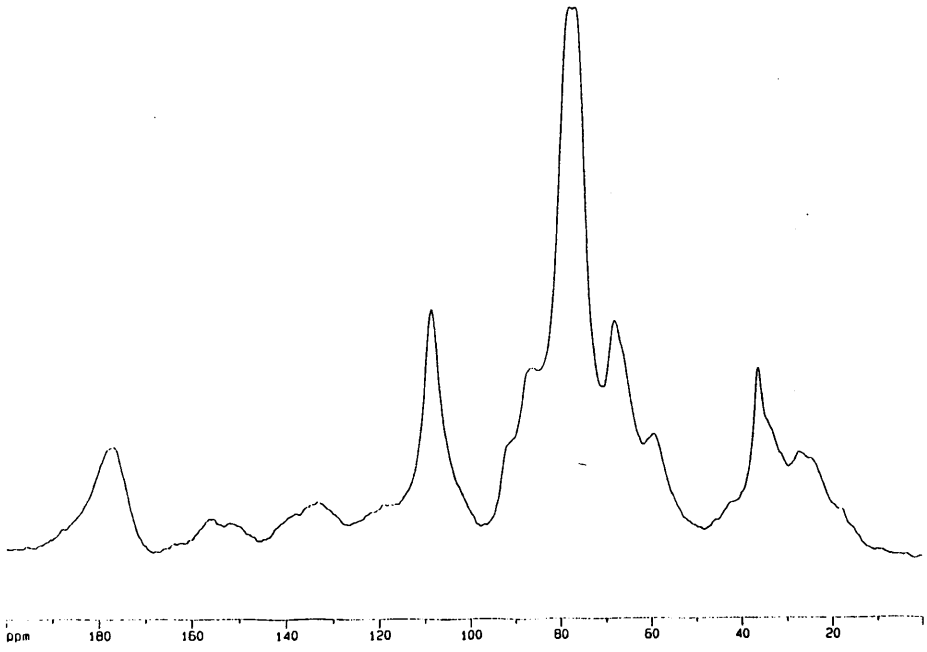


Figure 3.19: CP/MAS carbon-13 NMR spectra of cellulase+xylanase+laccase+β-glucosidase treated dung



3.5.3.3 CP/MAS SPECRA OF FREEZE-DRIED WATER EXTRACTS FROM DUNG

Table 3.12 shows the signal assignments which are derived from literature data (Liang *et al.*, 1996). The solid-state ^{13}C NMR of water extract from the control sample exhibits major signals at 26, 72 and 182 ppm (see Figure 3.20). The signal in the 16 to 40 ppm region is most likely due to CH_3 and CH_2 groups in normal, branched and cyclic alkanes or fatty acid material (Liang *et al.*, 1996). The signal at 72 ppm could be assigned to carbohydrates. The signal at 182 ppm is assigned to carboxylic acids.

Figures 3.21, 3.22 and 3.23 show the water extracts from cellulase, xylanase and laccase treated dung samples. The water extract derived from cellulase treatment exhibits signals at 26, 33, 56, 70, 82, 126, 175 and 182 ppm. As in the control sample, signals at 16 and 33 ppm are due to CH_3 and CH_2 groups of alkanes or fatty acids. The signal at 56 ppm might be C of proteins or C in OCH_3. Signals at 70 and 82 ppm can be assigned to be C from carbohydrates, mainly cellulose and hemicellulose. Signals at 126 ppm might be due to C derived from aromatic compounds like lignin. Finally, signals at 175 and 182 ppm can be assigned to be C of carboxylic acids. The spectrum from xylanase treatment shows the same features as cellulase treatment apart from showing the signal at 72 ppm in the carbohydrate region. In terms of the water extract from laccase treatment, signals at 21, 55, 69, 100, 128, 176 and 183 ppm can be observed. The signal at 21 ppm can be assigned to aliphatic C of alkanes and fatty acids. The signal at 55 ppm might be derived from protein C and C in OCH_3. In the carbohydrate region of the spectra, two peaks can be observed at 69 ppm and 100 ppm. The peak at 69 ppm does not appear in other spectra. This inconsistency might be explained as follows; treatment of dung with laccase enzyme leads to small destruction to the lignin component, because some lignin components are

released which can be seen from the signal at 128 ppm. Due to the interaction of lignin with cellulose and hemicellulose carbohydrates, this modification may lead to splitting the bonds between lignin and carbohydrate and this may produce the additional carbohydrate signal at 69 and 100 ppm of the spectrum. The same feature can be observed in the GPC experiments, that the laccase treatment produces a very distinctive response at the medium molecular weight region of the chromatogram (see Figure 3.11). It is thought that these may be the same component of carbohydrates.

From this study, it can be said that cellulase and xylanase treatment do attack mostly the carbohydrate components of the dung and due to the interaction with carbohydrate and lignin, some aromatic components can also be released. The laccase treatment made small changes in the lignin composition (see Table 3.8) and also some carbohydrate components were released by this treatment.

Table 3.12: Solid-state ^{13}C of CP/MAS NMR spectra characteristics of freeze-dried water extracts from dung (Liang *et al.*, 1996).

Chemical Shifts (ppm)	**Assignments**
0-40	Aliphatic C (alkanes + fatty acids)
41-60	Protein C, peptide C, amino acid C, C in OCH_3
61-105	Carbohydrate C
106-150	Aromatic C
151-170	Phenolic C
171-190	Carboxylic C
0-105	Aliphatic C
106-187	Aromatic C

Figure 3.20: CP/MAS carbon-13 NMR spectra of water extract of control dung sample

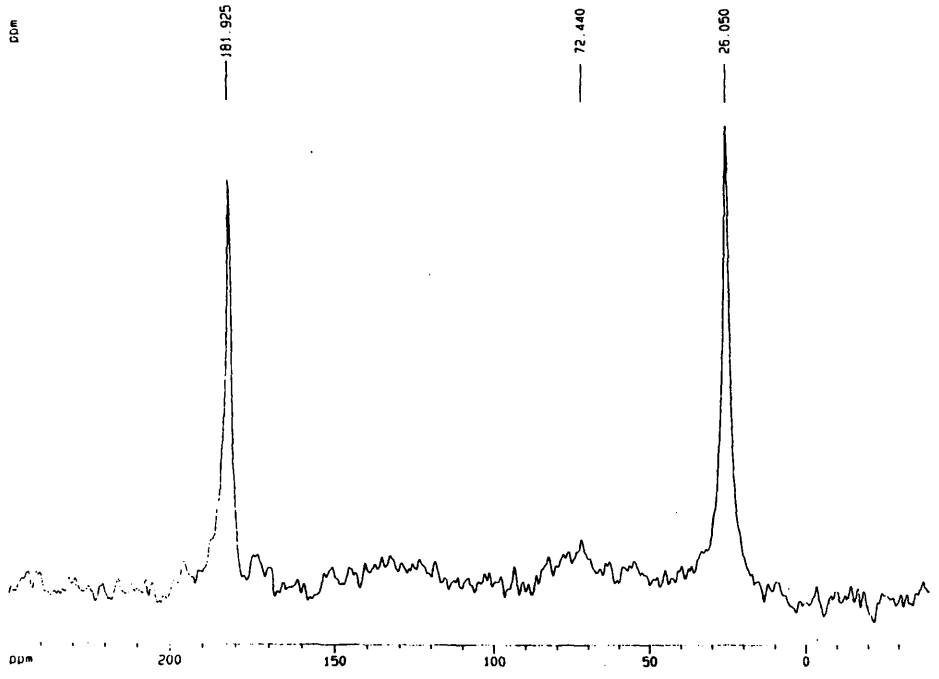


Figure 3.21: CP/MAS carbon-13 NMR spectra of water extract of cellulase treated dung

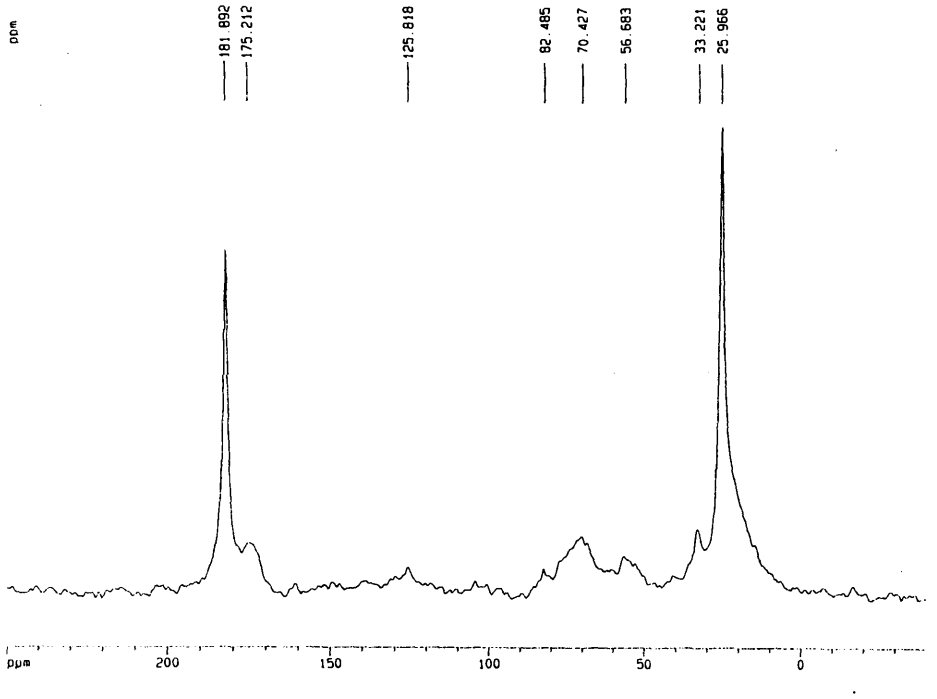


Figure 3.22: CP/MAS carbon-13 spectra of water extract of xylanase treated dung

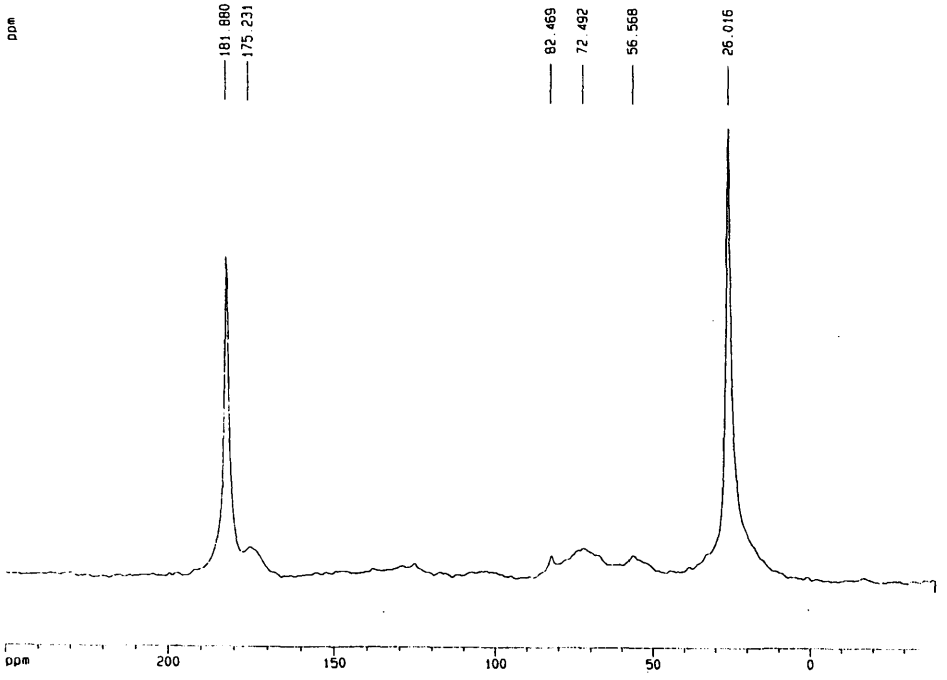
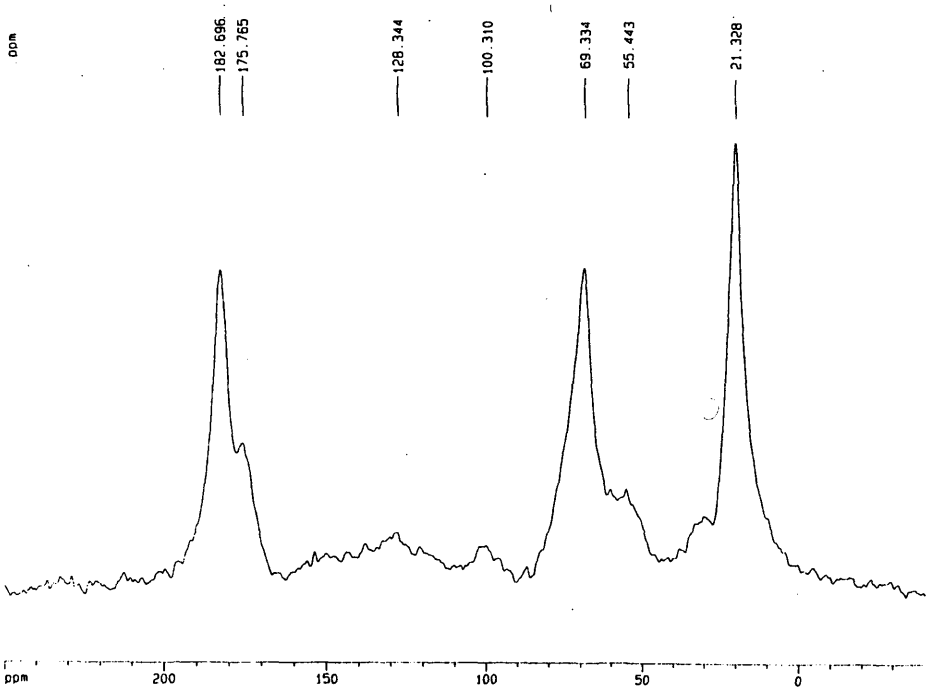


Figure 3.23: CP/MAS carbon-13 spectra of water extract of laccase treated dung



3.5.3.4 INFRARED (IR) SPECTROSCOPY STUDIES ON DUNG DEGRADATION

In this research infrared spectroscopy was applied to the enzyme degraded dung samples and freeze dried water extracts from enzyme degradation. Table 3.13 shows the signal assignments of IR spectra made by using literature data (Gilardi, 1992).

Figure 3.24 shows the IR spectra of the control sample. Figures 3.25, 3.26, 3.27 and 3.28 show the IR spectra of dung samples which received different enzymatic treatments. Due to the complex structure of the substrate, it is not surprising to observe that the spectra show a considerable overlap of absorption bands. In general, the fingerprint regions of the all spectra are very similar and there are no differences between control and enzyme treated samples. These observations are paralleled by CP/MAS NMR data, that also showed no differences between samples. The same conclusion can be drawn from this study that the enzyme responsible for dung degradation can attack the structural parts in the substrate that are most susceptible to solubilisation, without changing the fundamental structure of the dung.

Figure 3.29 shows the spectra of freeze dried water extract of the control sample. Figures 3.30, 3.31, 3.32 and 3.33 show the spectra of water extracts of various enzyme treated samples. Alcohol, carbonyl and carboxyl groups are formed during cellulose and hemicellulose biodegradation. Alcohol group formation can be observed in the spectra of cellulase, laccase and enzyme mixture treated samples' water extracts at 800- 1200 cm^{-1} region. Carbonyl and carboxyl group formations were not observed in the IR spectra due to very broad spectra pattern. Oxidation of the aromatic structure of lignin produces phenolic

OH, carbonyl and carboxyl C=O groups, aromatic C=C groups and alkene RCH=CHR structures. Aromatic group formation is observed in all spectra at 1500-1600 cm^{-1}. However, this is attributed to easy solubility of some aromatic structure in the substrate, because the same peak is observed in the control sample. The IR spectra study of water extracts, obtained after enzymatic treatment, does not give a clear picture about biodegradation pathway of dung due to the complexity of the solution. The CP/MAS NMR spectras of the same samples produced more informative results than the IR spectra.

Table 3.13: Assignments of IR bands of lignocellulosic materials (Gilardi, 1992)

Band Position cm^{-1}	**Functional group**
3500- 3200	O-H Alcohols
2960- 2850	O-H Methyl and methylene groups
1780- 1670	C=O Carbonyls
1670- 1650	C=C Alkenes
1600- 1500	C=C Aromatic
1265- 1235	O-H Phenolic
1200- 800	O-H Alcohols (primary and secondary) and aliphatic ethers
910	C=C Alkenes

Figure 3.24: IR spectra of control sample

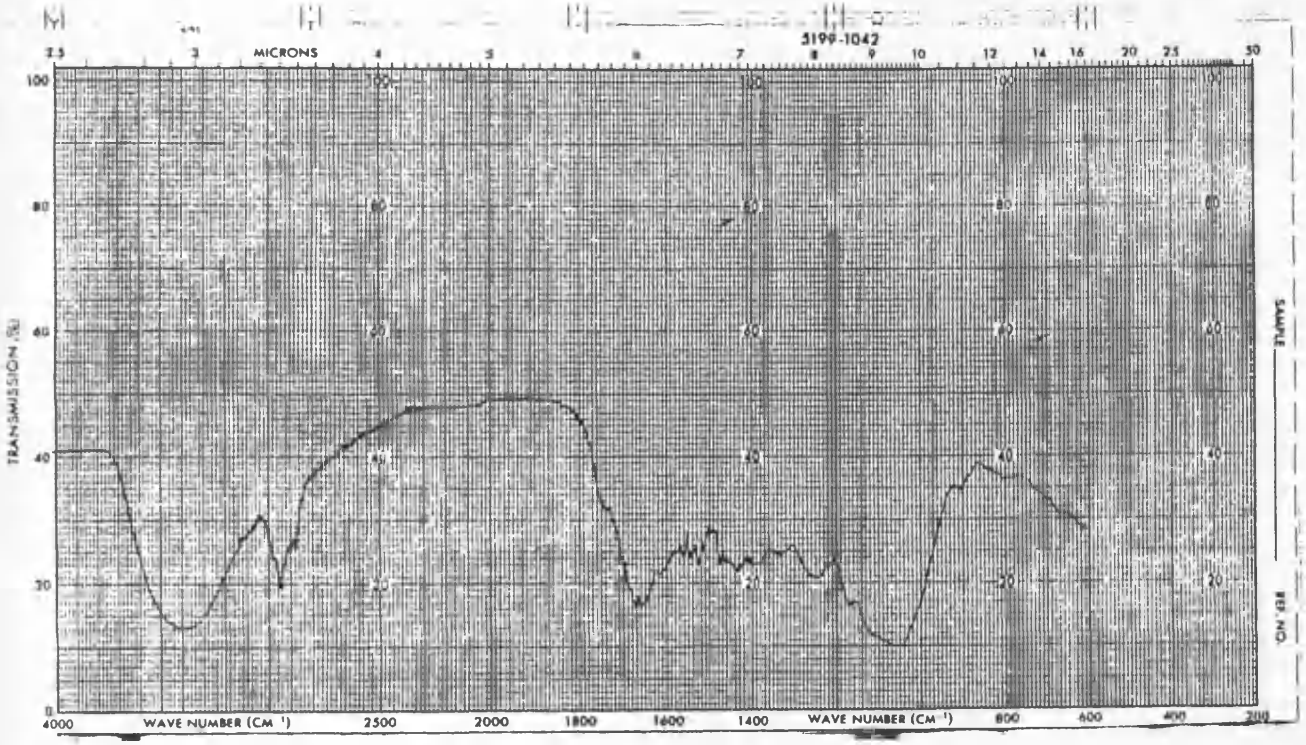


Figure 3.25: IR spectra of cellulase treated dung

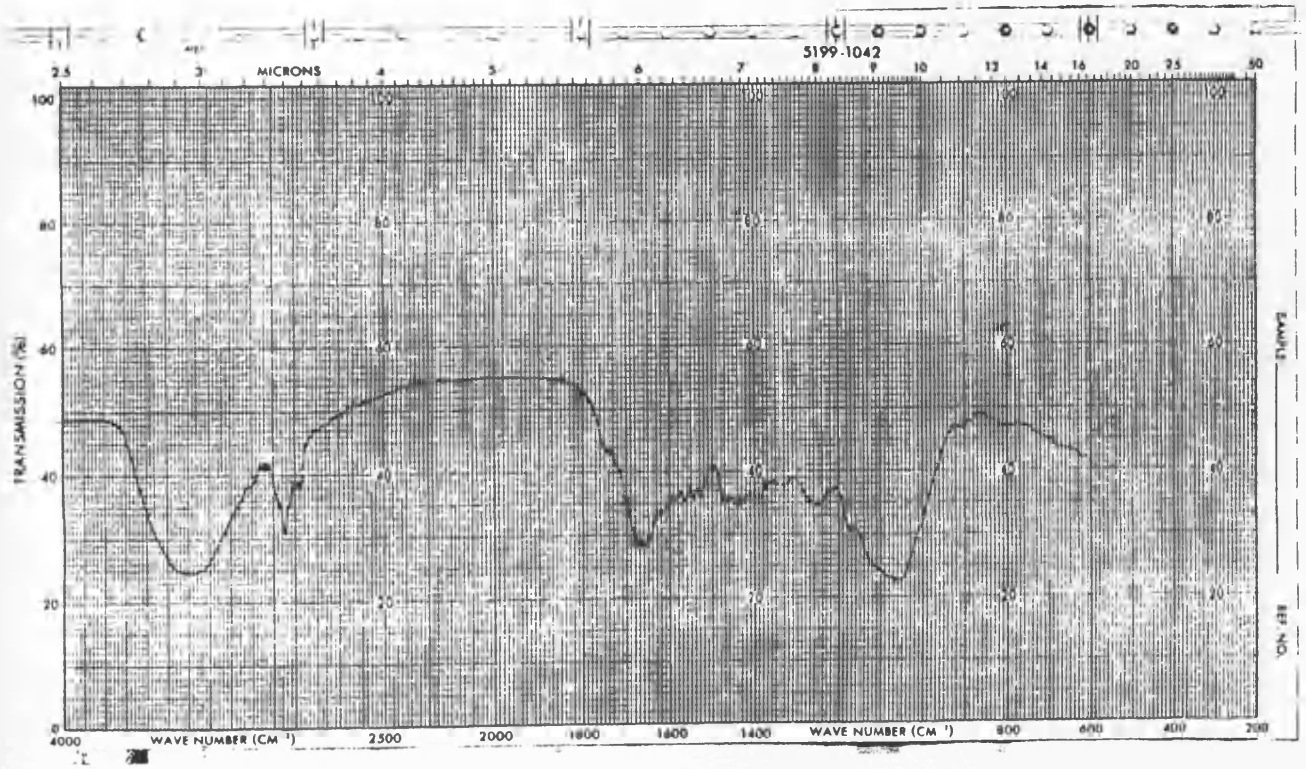


Figure 3.26: IR spectra of xylanase treated dung

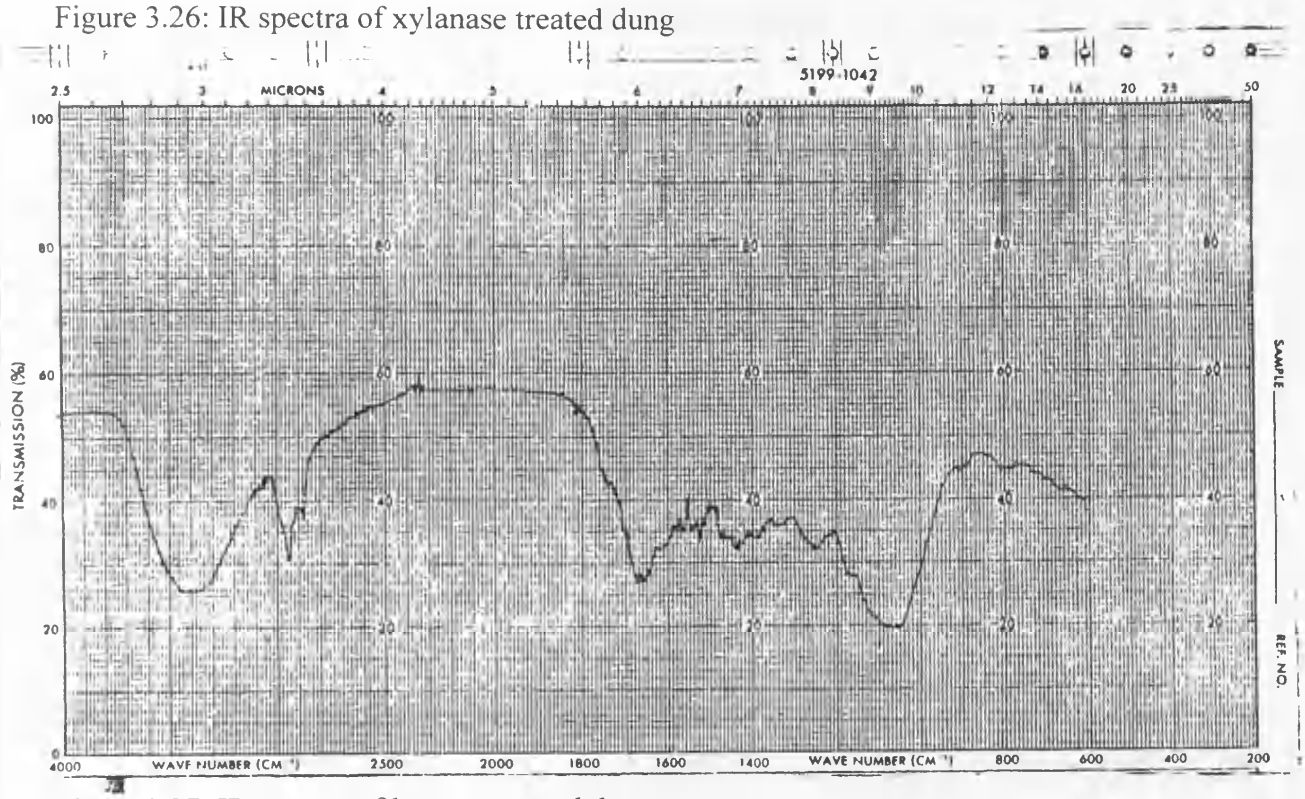
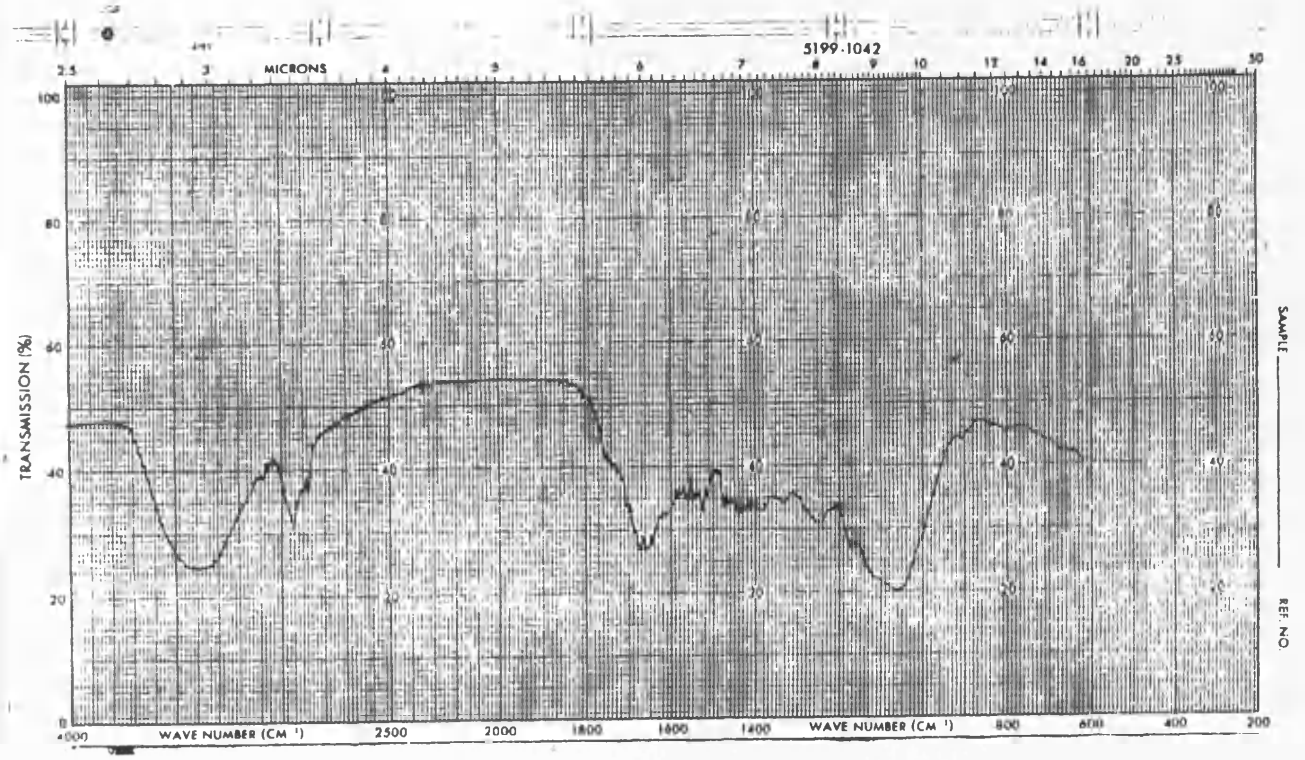


Figure 3.27: IR spectra of laccase treated dung



3.28: IR spectra of cellulase+xylanase+lacase+β-glucosidase treated dung

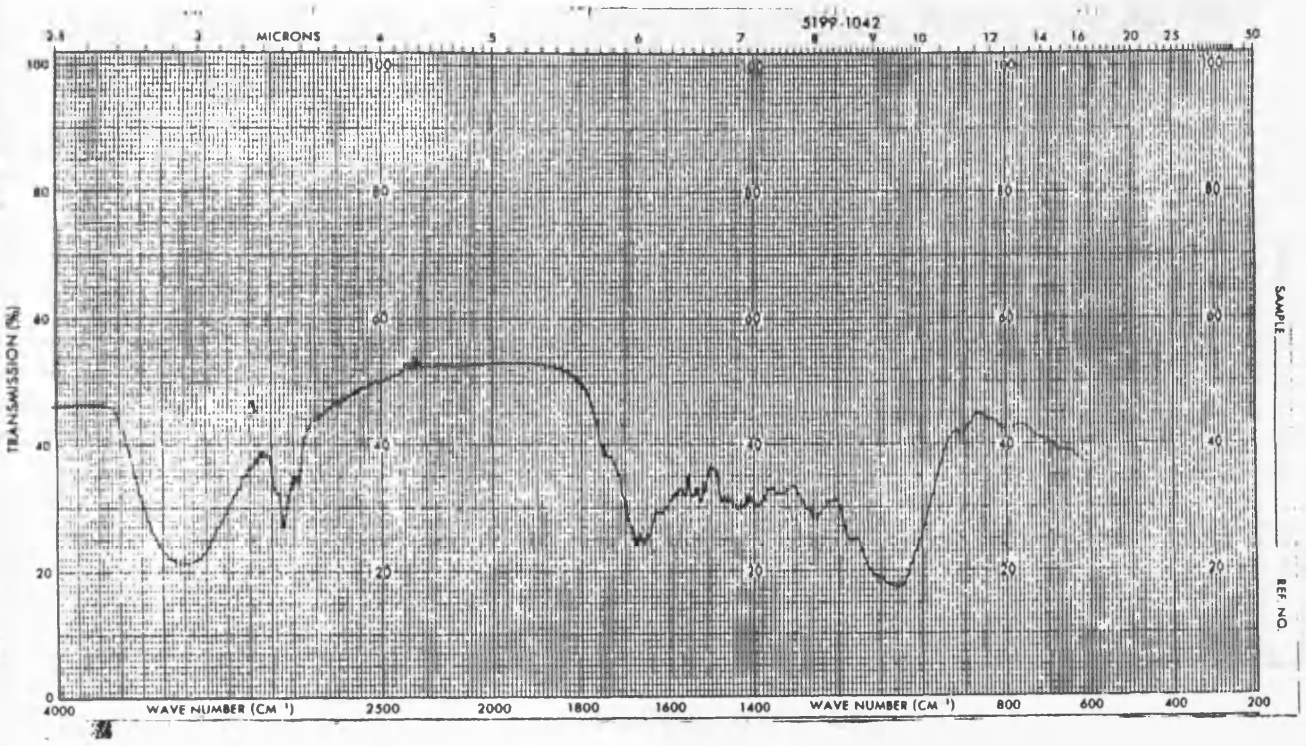


Figure 3.29: IR spectra of water extract of control dung sample

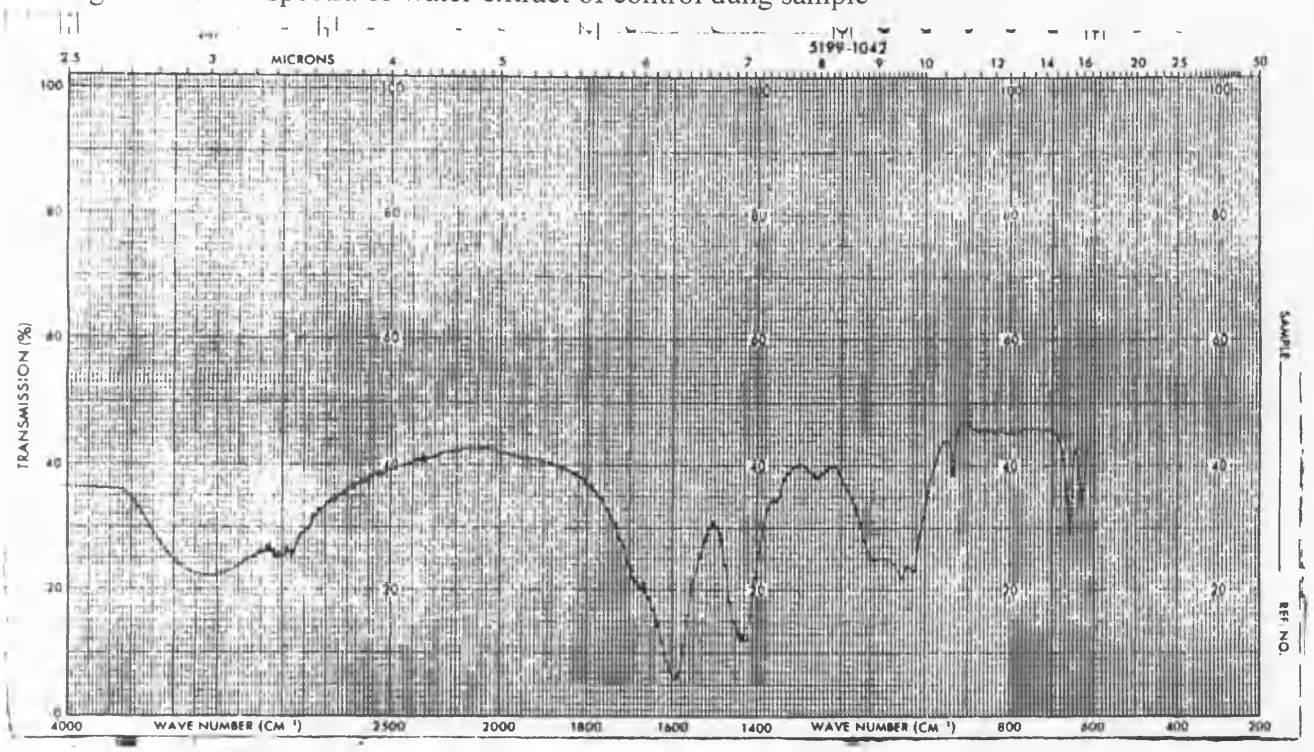


Figure 3.30: IR spectra of water extract of cellulase treated dung

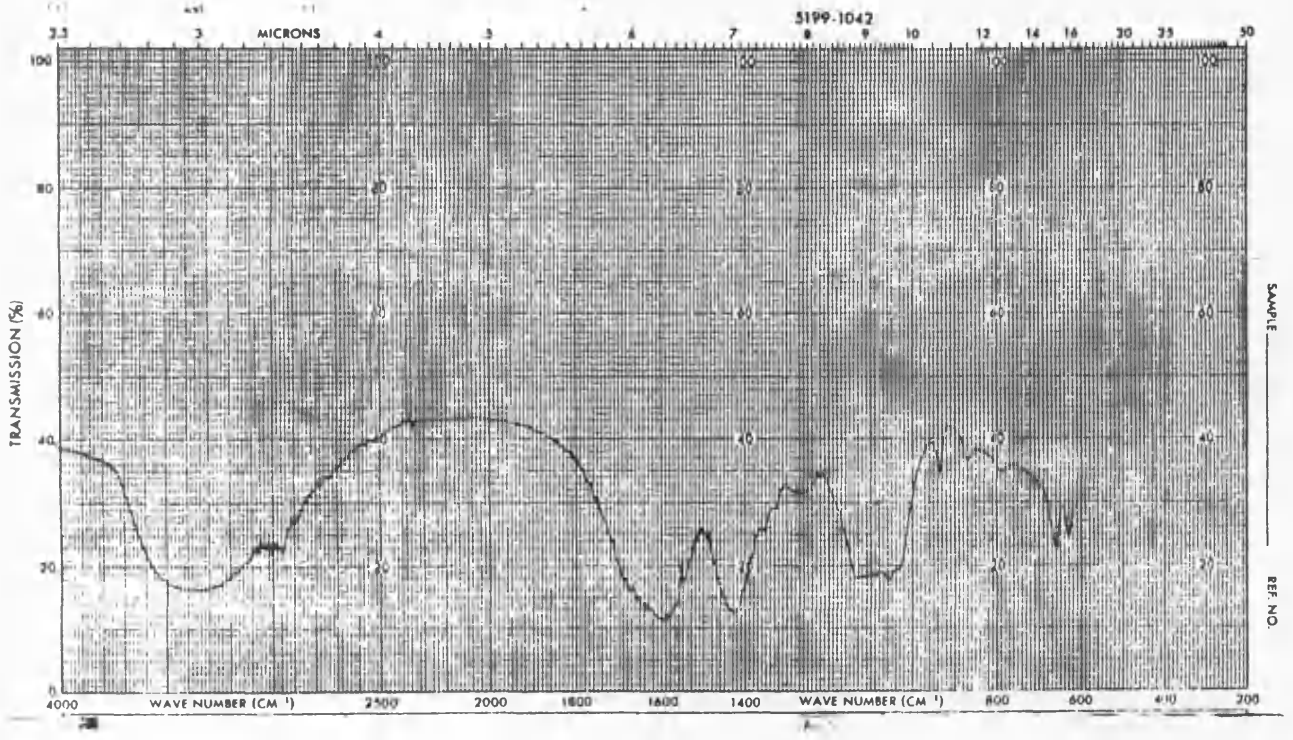


Figure 3.31: IR spectra of water extract of xylanase treated dung

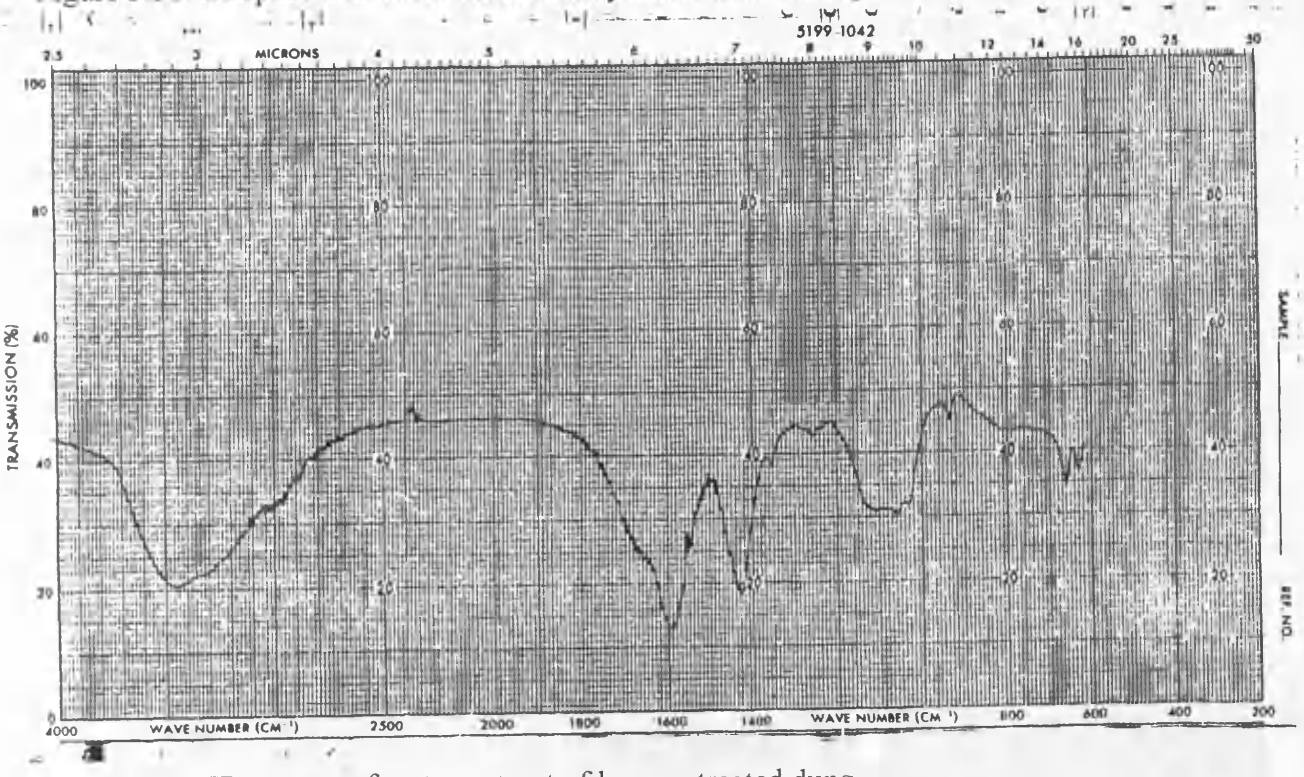


Figure 3.32: IR spectra of water extract of laccase treated dung

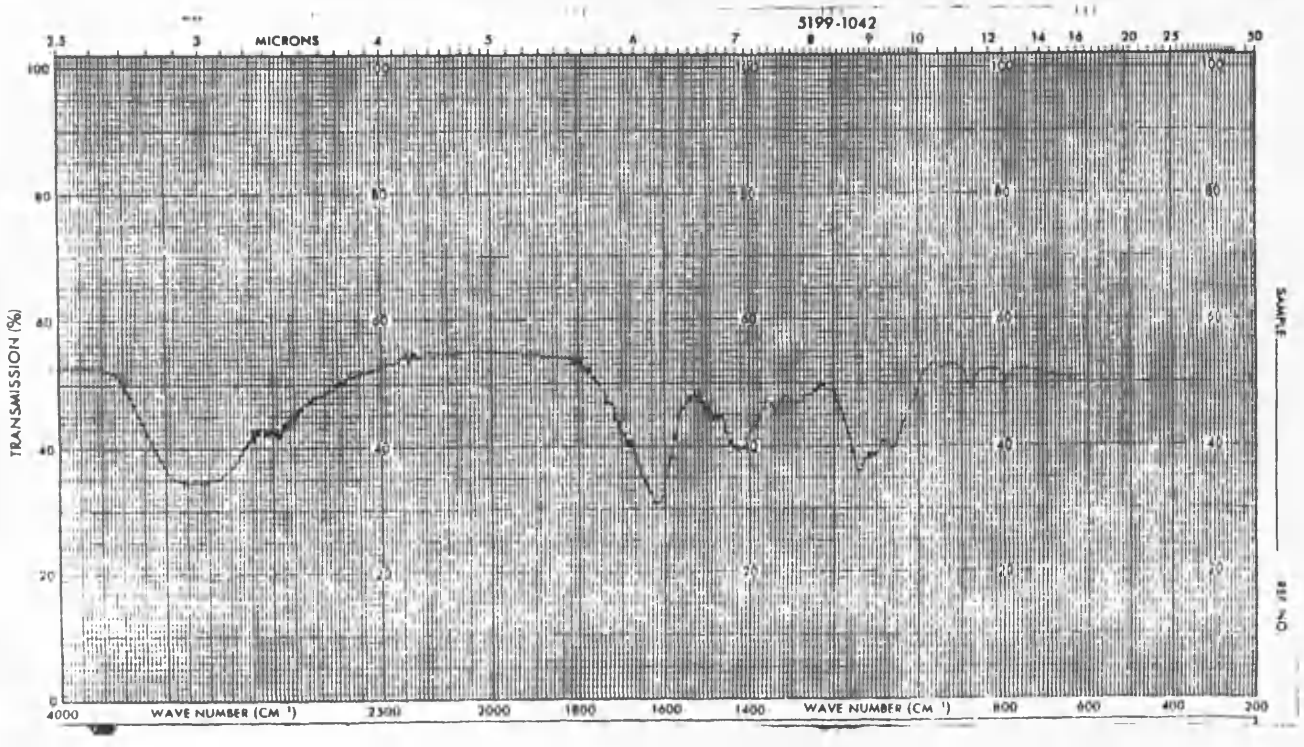
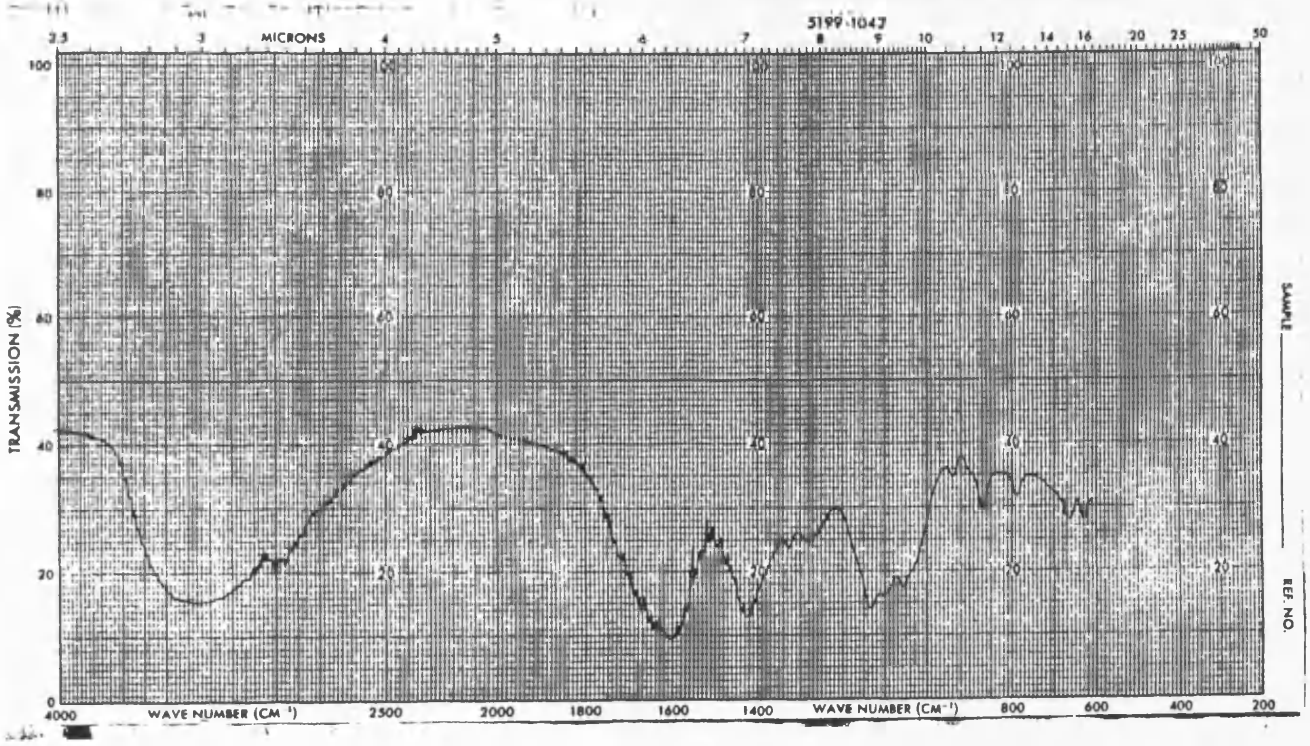


Figure 3.33: IR spectra of water extract of cellulase+xylanase+laccase+β-glucosidase treated dung



3.6 PILOT SCALE TRIAL OF DUNG REMOVAL

Dung clad hides were treated with enzymes in the soaking process in pilot scale tannery drums. Before processing each hide was sided into two halves. The left half was used as a control sample and right half was used for enzymatic soaking. Four hides were used for four different treatments in this trial. It should be noted that the hide samples used throughout in these experiments were heavily dung clad. In addition, attempts were made to remove the dung physically before processing, however, apart from removing dung from within hair matrix, most of the time it merely resulted in hair being separated from the hides. It should be noted that the hide samples used showed no hair slip, defined as removing hair easily from hide, due to putrefaction of the hide by bacterial action.

All four control samples were placed into the same drum for the soaking process. Only water and the nonionic detergent were used for the control soaking process, but, for enzymatic soaking processes, enzymes were included in these mixtures.

After the soaking process, all the sides were placed into the same drum and processed through to the crusting stage. Therefore, differences in the chemical and physical properties of the leathers can be attributed to the different soaking processes applied to the hides.

3.6.1 EFFECTS OF CELLULASE, XYLANASE AND LACCASE TREATMENT ON DUNG REMOVAL

The enzyme concentration used in this experiment was 2.5 U/ml, made up with cellulase, xylanase and laccase in the ratios of 50: 30: 20 respectively (concentration was based on separate enzyme activities). Enzymes used for this trial were obtained from Biocatalyst Ltd, UK, except laccase which was obtained by growing *C. versicolor* on Fähraeus medium. The effects of this treatment on dung removal are represented in Table 3.14.

Table 3.14: Effect of cellulase, xylanase and laccase enzyme mixture treatment on removal of dung from hide

Treatment		No appreciable removal	Difficult and incomplete removal	Removed with moderate difficulty	Easy removal	Removed without any appreciable action
Control	1 hours	*				
	3 hours	*				
	6 hours		*			
	18 hours			*		
Enzyme	1 hours					*
	3 hours					*
	6 hours					*
	18 hours					*

3.6.2 EFFECT OF *ASPERGILLUS NIGER* BROTH TREATMENT ON DUNG REMOVAL

Dung clad hide was treated with *Aspergillus niger* broth, which was grown on Abrams medium supplemented with 15 g/l carboxymethylcellulose and 5 g/l xylan. The enzyme concentration used was based on the cellulase activity in the culture fluid, which was 2.5 cellulase U/ml. The dung removal results obtained from this treatment are represented in Table 3.15. Dung was easily removed by treatment of fungal broth within one hour. However, in this case, physical application was necessary to remove the dung by using a spatula. After six hours treatment with fungal broth, no dung particles were observed on the hide, they had passed into the float.

Table 3.15: Effect of *Aspergillus niger* broth treatment on removal of dung from hide

Treatment		No appreciable removal	Difficult and incomplete removal	Removed with moderate difficulty	Easy removal	Removed without any appreciable action
Control	1 hours	*				
	3 hours	*				
	6 hours		*			
	18 hours			*		
Enzyme	1 hours				*	
	3 hours				*	
	6 hours					*
	18 hours					*

It can be seen that even after eighteen hours process with the control treatment effective dung removal was not obtained.

3.6.3 EFFECT OF *CORIOLUS VERSICOLOR* BROTH TREATMENT ON DUNG REMOVAL

Coriolus versicolor was grown on Abrams medium that was supplemented with 1% glucose and 5% carboxymethylcellulose. The enzyme concentration used was 2.5 cellulase U/ml. Treatment of dung clad hide with this culture broth also led to effective dung removal, see Table 3.16.

Table 3.16: Effects of *Coriolus versicolor* treatment on removal of dung from hide

Treatment		No appreciable removal	Difficult and incomplete removal	Removed with moderate difficulty	Easy removal	Removed without any appreciable action
Control	1 hours	*				
	3 hours	*				
	6 hours		*			
	18 hours		*			
Enzyme	1 hours		*			
	3 hours			*		
	6 hours			*		
	18 hours				*	

It can be seen from the table that in three and six hours treatment time, dung was removed with moderate difficulty. Effective dung removal could only be obtained after eighteen hours treatment time. The results of treatment by *Coriolus versicolor* may be explained as follows; the properties of cellulases and xylanases obtained from this culture broth may be different than those from

Aspergillus niger and commercial enzyme preparation in terms of their effects on dung removal. However, when the control treatment is compared with culture broth treatment, it is clear that enzyme components from this culture were effective on the dung components.

3.6.4 EFFECT OF *CORIOLUS VERSICOLOR* CULTURE BROTH SUPPLEMENTED WITH CATTLE MANURE ON DUNG REMOVAL

Coriolus versicolor, grown on Abrams medium supplemented with 3% dry cattle dung, was used for treatment of hide. The enzyme concentration was 2.5 cellulase U/ml. From section 3.4.4, using the cattle dung in culture growth medium resulted in increases of cellulase, xylanase and laccase activity of the culture broth, compared with the same organism grown on Abrams medium. Easy dung removal was obtained with this enzyme within six hours treatment, see Table 3.17. One hour of enzyme treatment produced difficult and incomplete dung removal, however, after three hours dung was removed with moderate difficulty. The dung removal obtained by this treatment was more effective than the previous treatment. This may be due to the fact that *C. versicolor* grown on dung produces enzymes that are more useful for dung removal. In other words, during the growth of the fungus, it must break down the components of dung in order to provide energy to survive, so this process may produce enzymes that are more effective in terms of dung removal than fungal culture grown on any other carbon sources.

Table 3.17: Effects of *Coriolus versicolor* broth supplemented with cattle manure on removal of dung from hide

Treatment		No appreciable removal	Difficult and incomplete removal	Removed with moderate difficulty	Easy removal	Removed without any appreciable action
Control	1 hours	*				
	3 hours	*				
	6 hours		*			
	18 hours			*		
Enzyme	1 hours		*			
	3 hours			*		
	6 hours				*	
	18 hours					*

3.6.5 EFFECT OF DIFFERENT ENZYME TREATMENTS ON PROPERTIES OF LEATHER

In this section properties of leather produced from different enzyme treatments were investigated, in terms of chemical and physical properties. The chemical properties were considered in terms of proteoglycans removal due to the enzyme action. The physical properties were considered in terms of the effects of enzyme treatment on strength, softness and colour properties of leathers. All the properties of enzyme treated samples were compared with the samples without any enzyme treatment.

3.6.5.1 EFFECT OF ENZYME TREATMENTS ON PROTEOGLYCAN REMOVAL

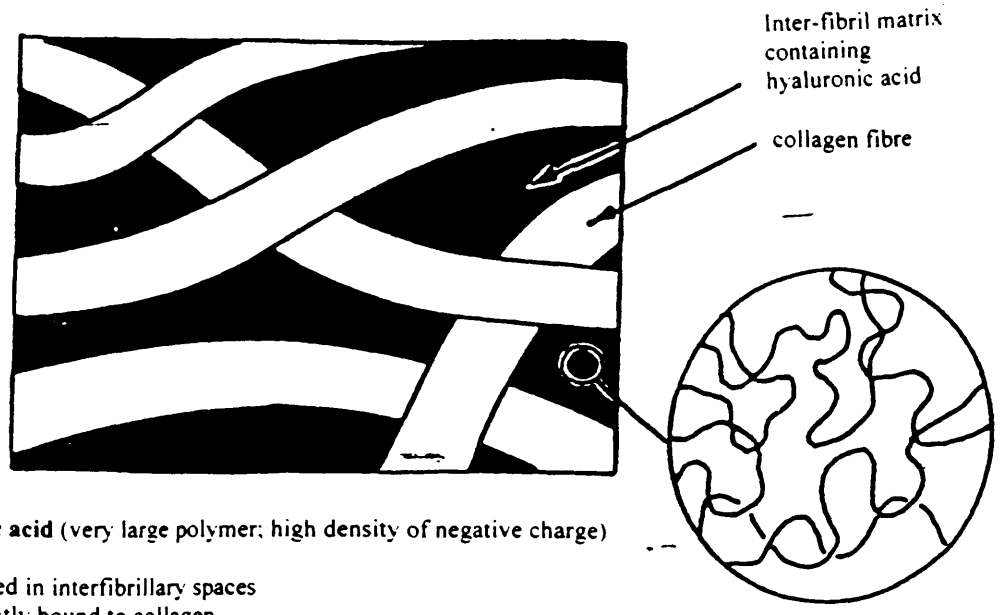
It is well known that the opening up of the fibre structure of leather is related to the removal of cementing substances and mucopolysaccharides from hide and skins during beamhouse operations. There are two principal cementing substances, hyaluronic acid and dermatan sulphate (Alexander *et al.*, 1986 and Alexander, 1988). Hyaluronic acid does not bind to the collagen and is largely removed in soaking in the presence of electrolyte and mechanical action. However, dermatan sulphate proteoglycan is chemically bonded to the collagen and it is extensively removed from hides under the alkali conditions of sulphide unhairing/liming (Alexander *et al.*, 1986) Hyaluronic acid and dermatan sulphate structures and properties are represented in Figures 3.34 and 3.35.

It was assumed that, due to glycosidic linkages between repeating polysaccharide units in proteoglycan, linkage between L-iduronic acid and N-acetyl-D-galactosamine-4-sulphate in dermatan sulphate and D-glucuronic acid and N-acetyl-D-glucuronic acid in hyaluronic acid, these molecules might be removed by the action of enzymes used in this project, due to action of these enzymes on the glycosidic linkages. Effective removal of hyaluronic acid and dermatan sulphate in beamhouse processes, in this case in soaking, represents a number of advantages to the tanner, such as reduction of process time and chemicals.

For this reason, samples were collected from hide before any enzyme treatment, after enzyme treatment and after the liming process. Their total glycosaminoglycan contents were determined by the method described in section 2.8.1.1. The results are presented in the Table 3.18.

The samples taken from hides in each processing step were from the butt region of the hides. In addition to that they were taken from the same area of the hides, in order to eliminate the topographical differences between the samples. Total GAG (glycosaminoglycan) of the raw hide from Table 3.18 represents the initial glycosaminoglycan content of each hide. As the hides were soaked, the hyaluronic acid polysaccharide was completely extracted due to the presence of electrolyte, in this case sodium chloride and strong mechanical action. According to Alexander e*t al.* (1986), what remains after effective soaking is dermatan sulphate proteoglycan. Moreover, under practical tannery conditions over 50% dermatan sulphate is removed during the sulphide unhairing/liming step. From the results in Table 3.18, the total glycosaminoglycan contents of control and enzyme treated samples exhibit consistency. In other words, there were no significant differences between samples, which means that the dermatan sulphate remains unchanged whether the enzymes are applied or not. Furthermore, the use of enzymes does not facilitate dermatan sulphate removal in liming. In the light of these results, it can be said that enzymes used in this project for effective dung removal do not affect the proteoglycan components of the hide.

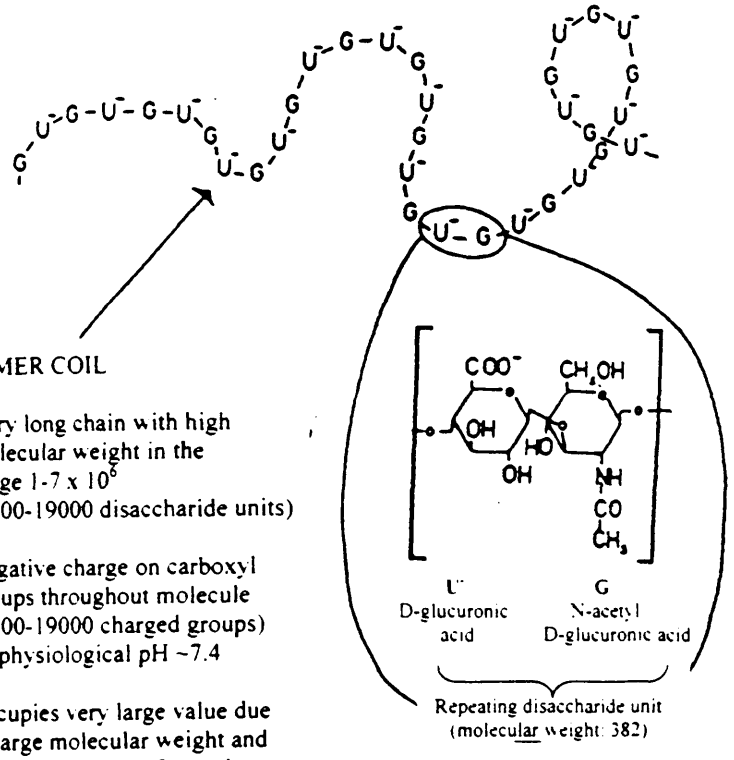
Figure 3.34: Structure of hyaluronic acid (Alexander *et al.*, 1986)



Hyaluronic acid (very large polymer; high density of negative charge)

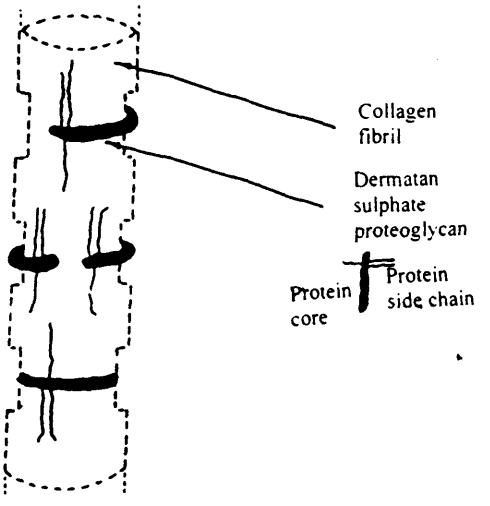
1. Localised in interfibrillary spaces
2. Not tightly bound to collagen
3. Occupies a very large volume
4. Polymers entangle to form viscous gel that immobilises water in skin
5. Inhibits diffusion of soluble molecules
6. Increase osmotic pressure, inflating the collagen matrix

Representation of entangled hyaluronic acid polymers forming an extensive network between fibres

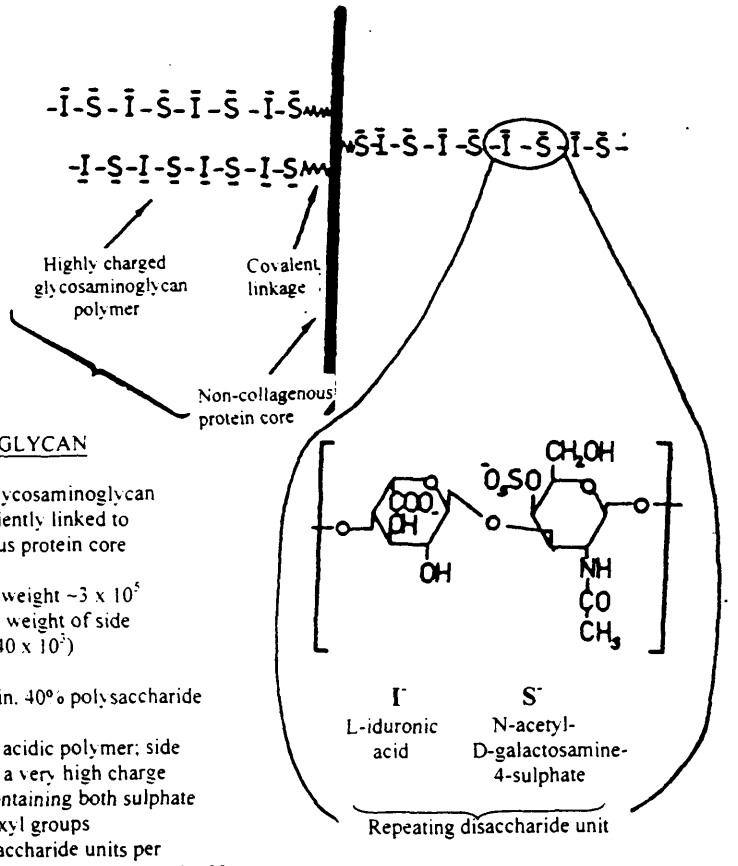


1. Very long chain with high molecular weight in the range $1\text{-}7 \times 10^6$ (2700-19000 disaccharide units)
2. Negative charge on carboxyl groups throughout molecule (2700-19000 charged groups) at physiological pH ~7.4
3. Occupies very large value due to large molecular weight and mutual repulsion of negative charges

Figure 3.35: Structure of dermatan sulphate (Alexander *et al.*, 1986)



Schematic representation to illustrate electrostatic bonding of dermatan sulphate proteoglycan with collagen fibril



PROTEOGLYCAN

Two to three glycosaminoglycan Polymers covalently linked to Non-collagenous protein core

1. Molecular weight ~3 x 10^5 (molecular weight of side chain: 15-40 x 10^3)
2. 60% protein, 40% polysaccharide
3. Extremely acidic polymer; side chain with a very high charge density containing both sulphate and carboxyl groups (35-90 disaccharide units per glycosaminoglycan polymer; 70-180 charged groups per glycosaminoglycan polymer) all fully ionised at physiological pH~7.4

I^- L-iduronic acid

S^- N-acetyl-D-galactosamine-4-sulphate

Table 3.18: Removal of total glycosaminoglycan from hides by the enzyme actions

Treatment	Process stage				
	Raw	Soaked		Limed	
	Total GAG (%on collagen weight)	Total GAG (% on collagen weight)	%GAG removed	Total GAG (% on collagen weight)	%GAG removed
Enzyme	0.63	0.42	33.3	0.24	61.9
Control	0.71	0.47	33.8	0.25	64.7
A. Niger	0.67	0.49	26.8	0.27	59.7
Control	0.74	0.52	29.7	0.28	62.2
C.versicolor	0.79	0.56	29.1	0.29	63.2
Control	0.71	0.51	28.1	0.24	66.1
C.versicolor (supplemented with dung)	0.79	0.57	27.8	0.30	62.0
Control	0.74	0.53	28.3	0.27	63.5

3.6.5.2 HISTOLOGICAL EXAMINATION OF HIDE SAMPLES

Scanning electron microscopy is a valuable technique that provides information for both process monitoring and fault diagnosis in leather technology (Garwood, 1995). Opening up and splitting of fibre structure of leather, due to beamhouse operations, can be monitored with this technique. It is well known that one of the aims of beamhouse process is to remove the proteoglycans from collagen, which leads to opening up of the fibre structure. It has been demonstrated that leather samples which received proper beamhouse operation exhibited a well split and opened up structure, in contrast, leather which received no liming operation exhibits unsplit or unopened up structure when leather samples were investigated with scanning electron microscopy (Covington and Alexander, 1993). Poor opening up can prevent the penetration of fatliquors into the hierarchy of the collagen structure, consequently promoting resticking when leather is dried. Moreover, it leads to poor softness properties in the final leather.

In section 3.6.5.1, it was demonstrated that enzymes used for dung removal from hide had no effect on the glycosaminoglycan components of hide. Therefore, it should be expected that opening up of fibre structure is not affected by such treatments. In order to confirm this result, scanning electron microscopy investigation was conducted. The crust leathers, which received different soaking treatments, were examined with SEM. The magnification used was x1300.

The control leather sample exhibited fibre bundle separation, shown in Figure 3.36. When the fibre bundles were focused using high magnification, it may be seen that fibril bundles were separated from each other. The control samples, which were soaked with water and nonionic detergents and received sulphide

unhairing/liming process, showed a well split and opening up structure. Figures 3.37, 3.38, 3.39, and 3.40 show enzyme treated leather samples. In each case, fibre bundle and fibril bundle splitting were observed and leathers showed good splitting and opening up as well. Therefore, it can be concluded from this study that enzymatic dung removal process does not make any substantial contribution to fibre splitting and opening up. The results observed in this experiment were probably due to the effects of normal beamhouse operations, not due to the enzymatic effects.

Figure 3.36: Scanning electron microscopy of control leather sample, demonstrating the degree of opening up of fibre structure. (magnification X1300)

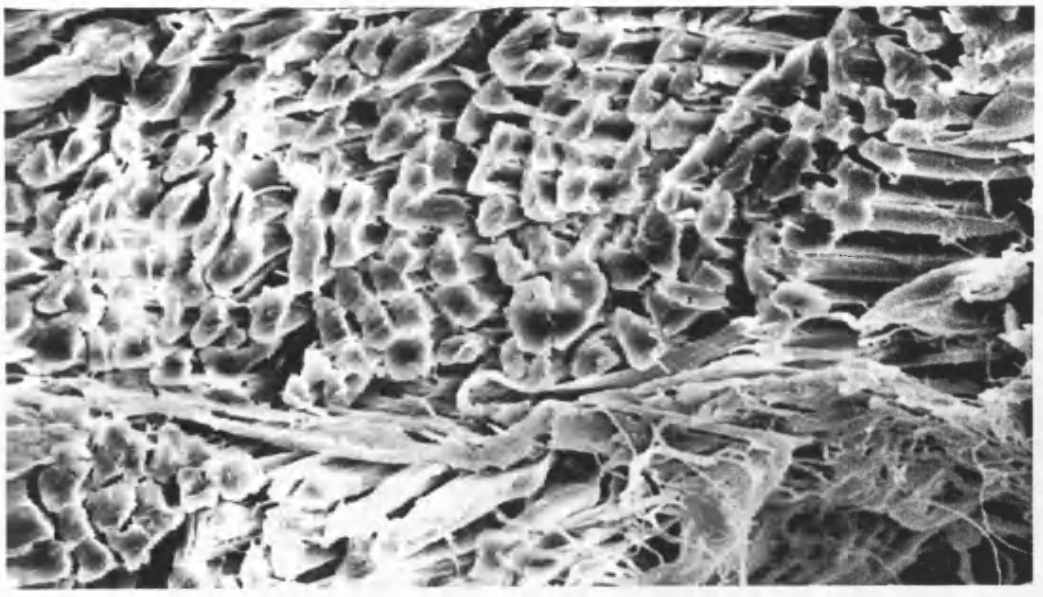


Figure 3.37: Scanning electron microscopy of enzyme mixture treated leather sample (cellulase+xylanase+laccase), demonstrating the degree of opening up of fibre structure. (magnification X1300)

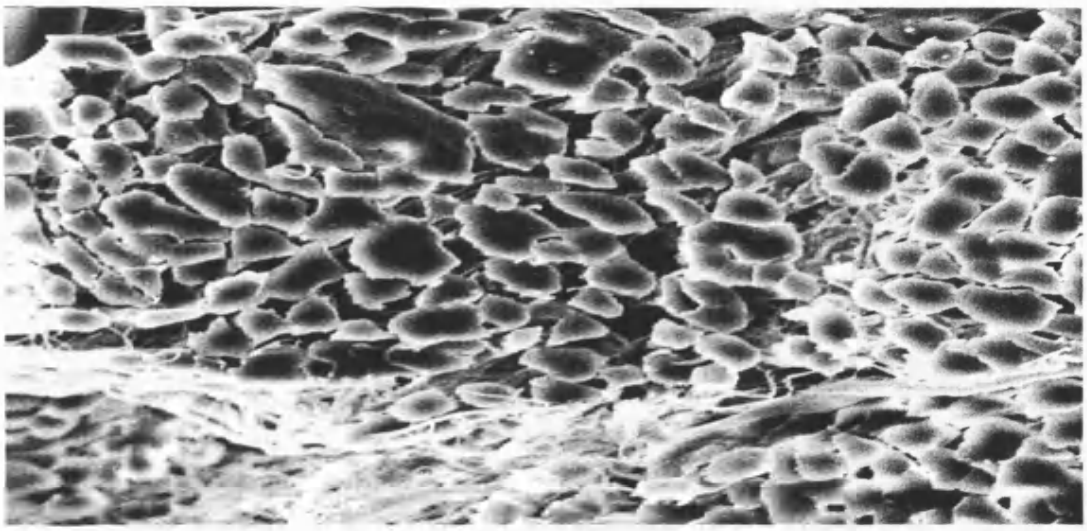


Figure 3.38: Scanning electron microscopy of leather treated with *Aspergillus niger* culture broth, demonstrating the degree of opening up of fibre structure. (magnification X1300)

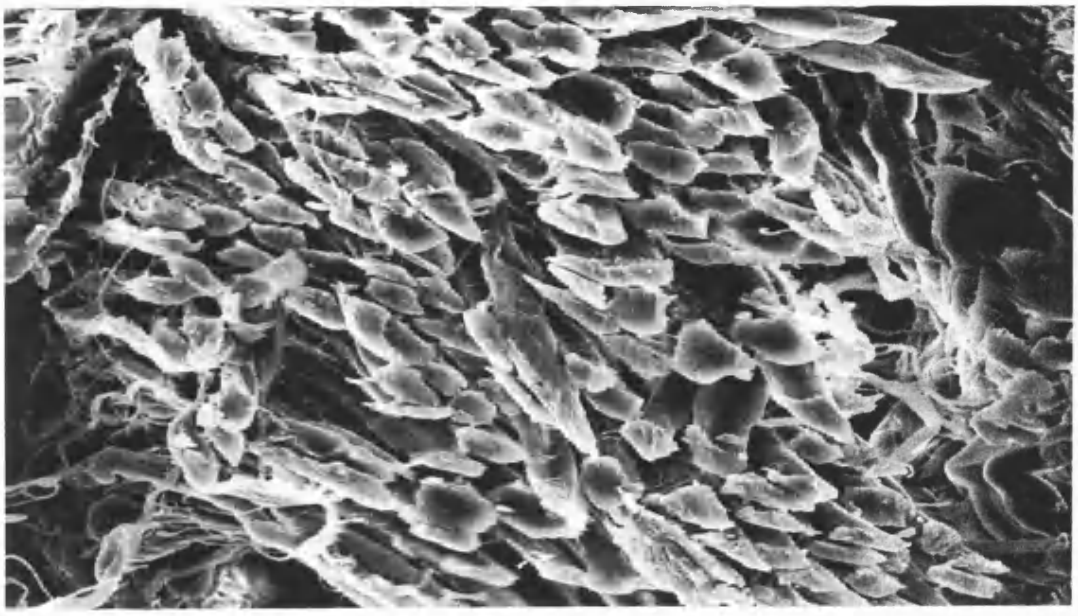


Figure 3.39: Scanning electron microscopy of leather treated with *Coriolus versicolor* cultural broth supplemented with dung, demonstrating the degree of opening up of fibre structure. (magnification X1300)

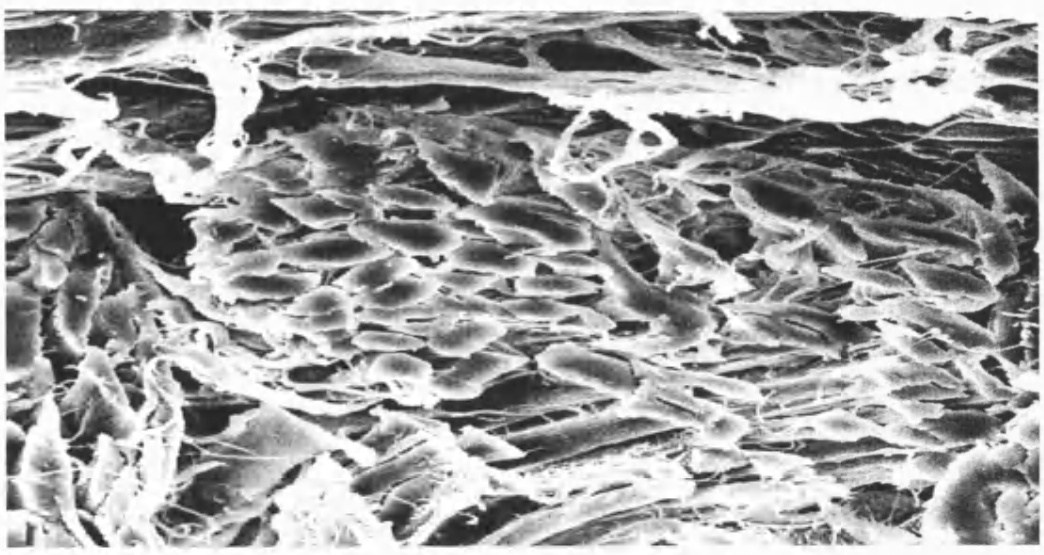
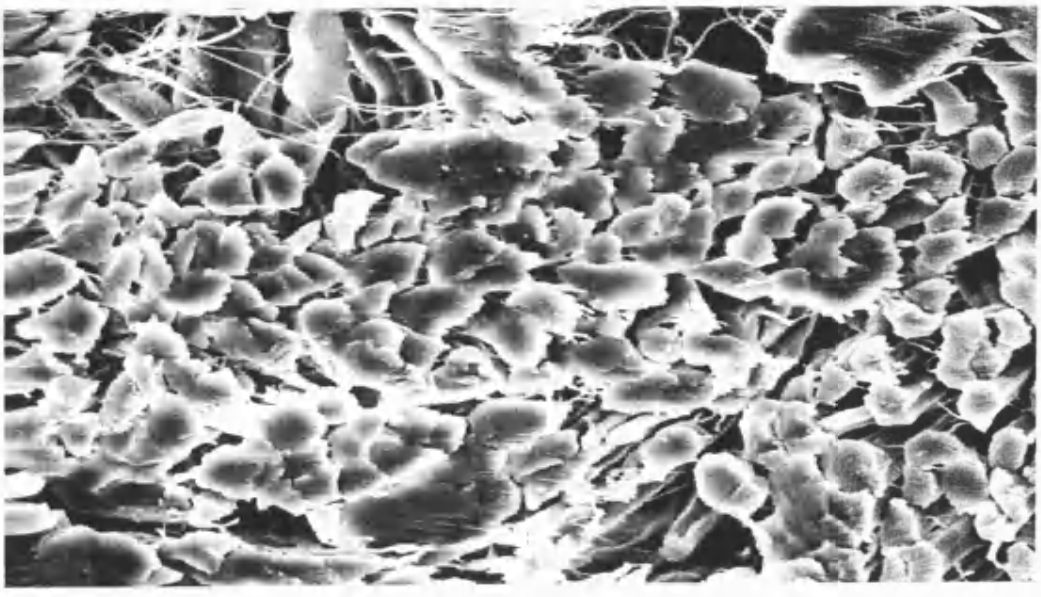


Figure 3.40: Scanning electron microscopy of leather treated with *Coriolus versicolor* cultural broth, demonstrating the degree of opening up of fibre structure. (magnification X1300)



3.6.5.3 EFFECTS OF ENZYME TREATMENTS ON PHYSICAL PROPERTIES OF LEATHER

This section can be divided into three sections as follows; effects on softness, strength and colour properties of leather. The softness test and colour differences tests were performed over the whole area of hides, using the BLC softness gauge and Minolta Colour Tester respectively. In the case of strength tests, samples tested were taken from official sampling position according to the method IUP 2. This position provides a more uniform specimen to minimise the differences in test results, when two different leathers are compared.

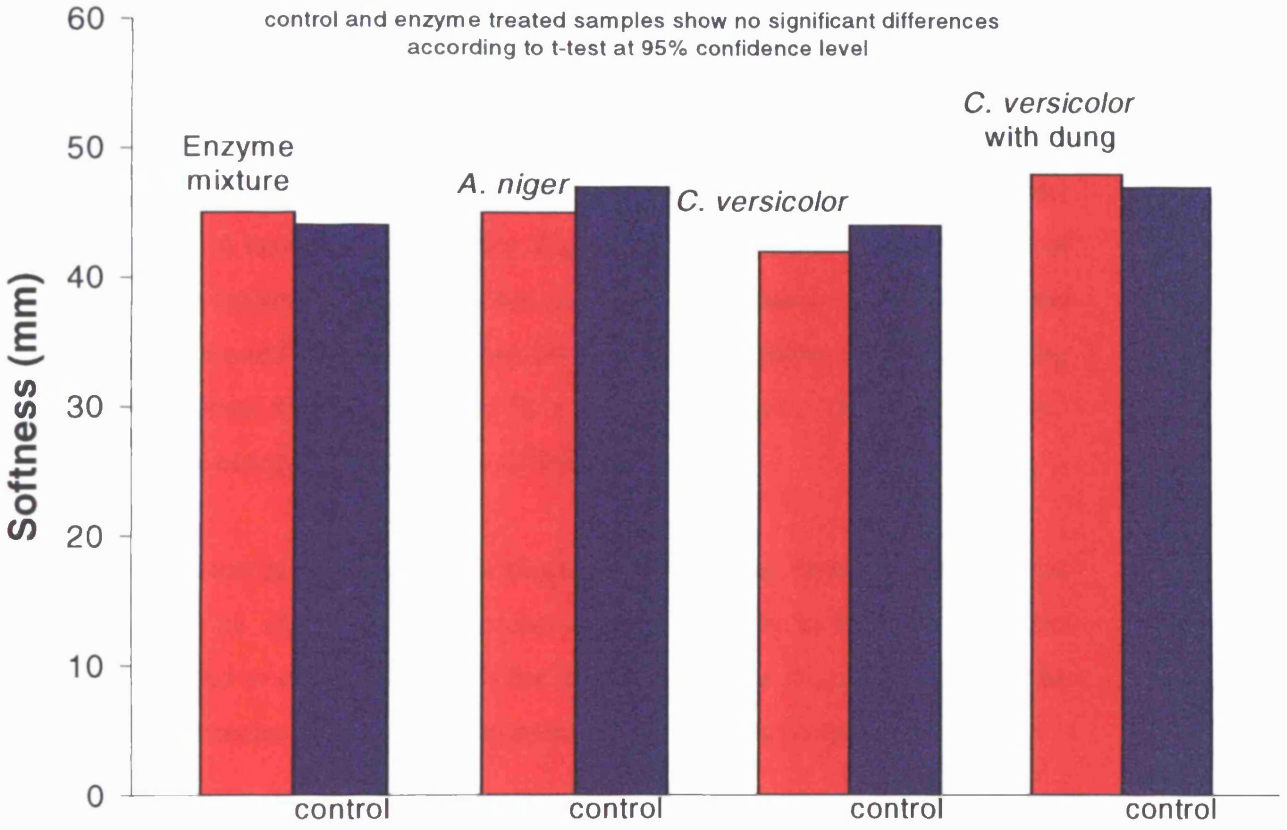
3.6.5.3.1 EFFECTS OF ENZYME TREATMENT ON SOFTNESS PROPERTIES OF LEATHER

A series of papers concerning softness properties of and production of soft leather was published by Covington and Alexander (1993), Alexander *et al.* (1993a and 1993b). They found that production of quality soft leather is only possible, if splitting occurs at the fibril bundle level of the hierarchy of the fibre structure and removing the glycosaminoglycans is done in beamhouse operation. In subsequent fatliquoring, oils can penetrate down the hierarchy of the structure and prevent the fibre structure resticking during the drying operation. Those authors emphasised the importance of softness development in the dry mechanical operations.

It has already been shown that enzymes responsible for dung removal do not affect fibre structure and components of hide. Therefore, it was not expected that there would be any differences in the softness properties of enzyme treated and

control samples. Figure 3.41 represents the softness properties of the leathers. There are no significant differences between control samples and enzyme treated samples.

Figure 3.41: Effect of enzyme treatments on softness properties of leather



Standard deviations of BLC softness test

Treatment	Standard deviation
Enzyme mixture	2.6
Control	3.1
Aspergillus niger	2.1
Control	3.9
C. versicolor	2.9
Control	3.2
C. versicolor supplemented with dung	2.9
Control	2.3

3.6.5.3.2 EFFECTS OF ENZYME TREATMENT ON STRENGTH PROPERTIES OF LEATHER

Tensile strength, percentage elongation, tear strength and lastometer properties of leathers were investigated under this heading. Tensile strength is one of the fundamental tests for leather performance and it is used commonly as a quality specification of leather especially if the application of leather involves direct pull, such as in lasting upper leather. Figure 3.42 shows the tensile strength of control and enzyme treated leather samples. Elongation at break is also important in a number of applications and variable elongation may affect lasting and the resultant top line position in a shoe (Landmann, 1999). Figure 3.43 represents the elongation properties of leathers.

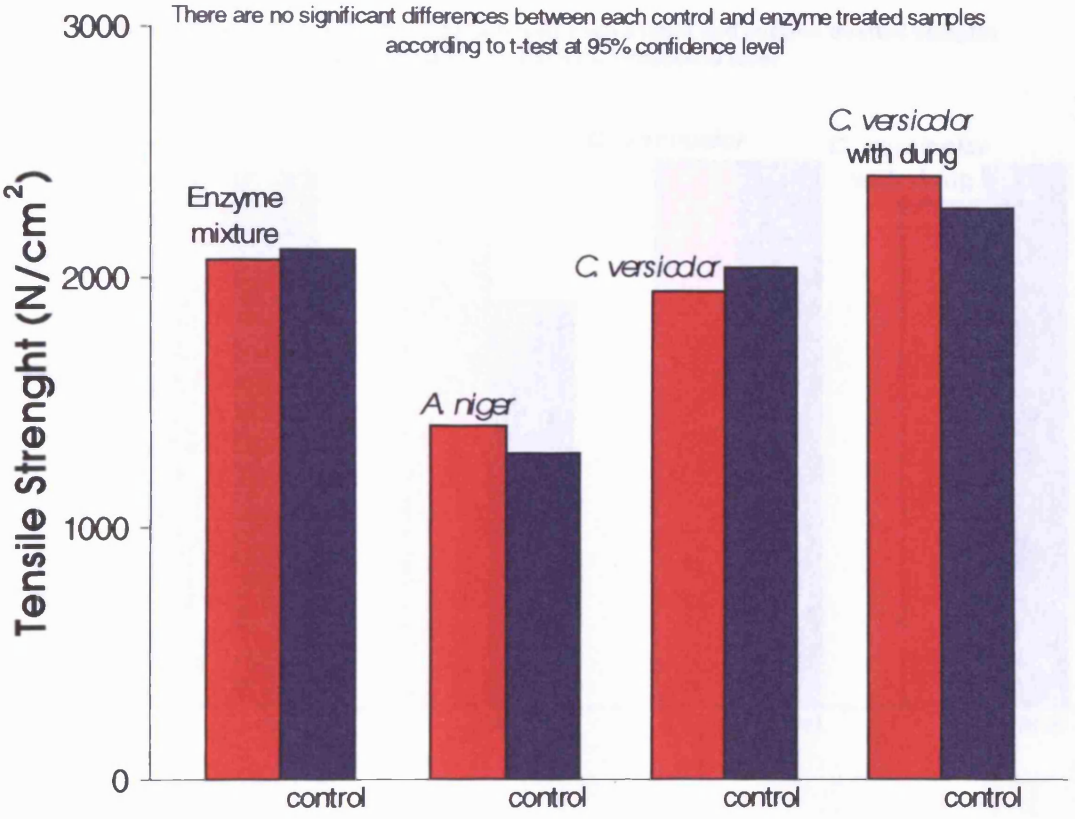
Tear strength test is one of the most frequently carried out physical tests because it simulates one of the most common forms of failure in service. It is also commonly used to compare leathers for the same end use. Figure 3.44 shows the tear strength results of control and enzyme treated leather samples.

The lastometer is a widely used test for footwear leathers. The principle is that a circular specimen of leather is clamped in an apparatus and a ball on a rod is pressed against the flesh side under increased pressure until a crack is seen on the grain surface. At this point, both the extension and the load are recorded giving the distension and strength at grain crack (Landmann, 1999). The guideline performance requirements are generally quoted as 7.0 mm distension at grain crack and a load of 20 kg. Figures 3.45 and 3.46 represents the lastometer test results of leather samples.

It can be seen that there are no significant differences in strength results between control and enzyme treated samples. There are slight differences in some results, however, that should be attributed to the heterogeneous character of leather. Therefore, it can be said from this series of experiments that enzyme treatments for dung removal do not affect the strength properties of leathers produced in these trials.

The colour differences between enzyme treated and control samples were measured by using the Minolta colour measurement machine. The results are presented in Figure 3.47. It was found that colour properties of leathers are not affected by enzyme treatments used for dung removal.

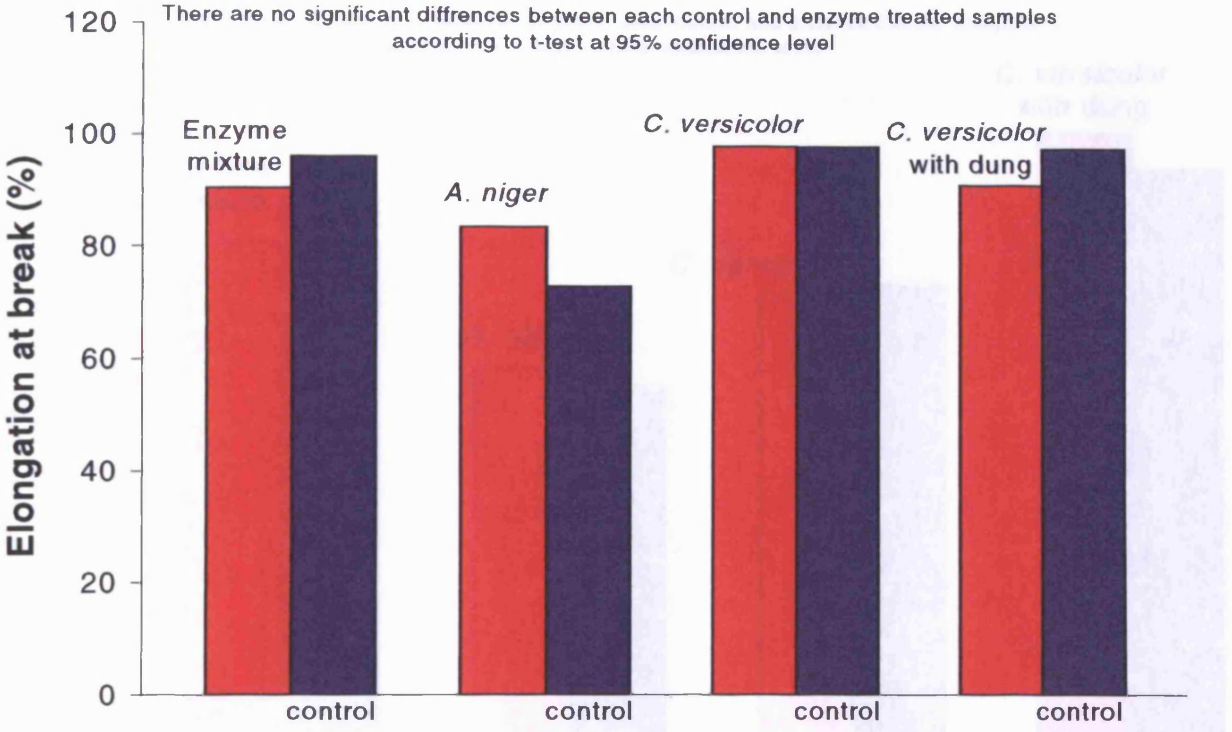
Figure 3.42: Effects of enzyme treatment on tensile strength of leathers



Standard deviations for tensile strength test

Treatment	Standard deviation
Enzyme mixture	660
Control	592
A. niger	348
Control	384
C. versicolor	377
Control	528
C. versicolor supplemented with dung	466
Control	335

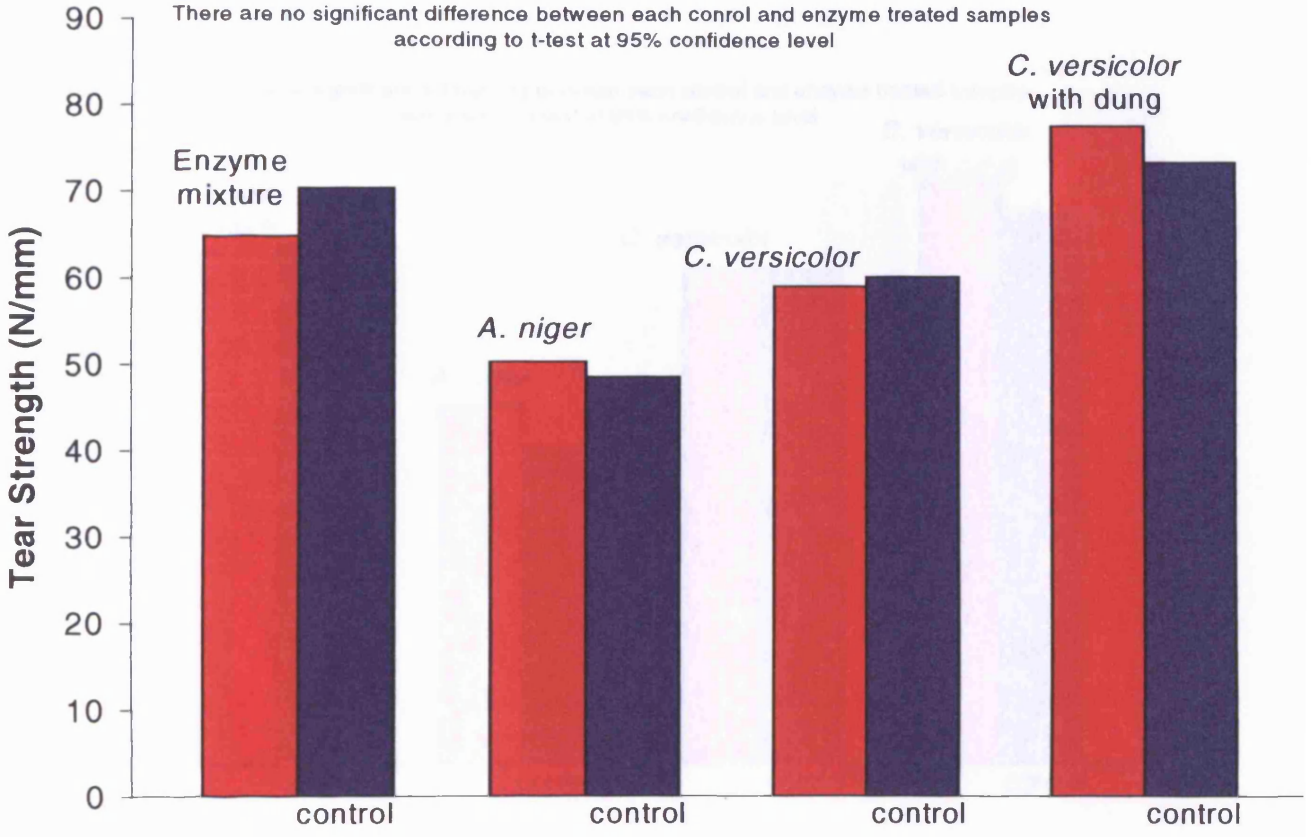
Figure 3.43: Effects of enzyme treatment on elongation at break



Standard deviations for elongation test

Treatment	Standard deviations
Enzyme mixture	7.9
Control	25.9
A. niger	15.8
Control	9.9
C.versicolor	23.0
Control	23.0
C. versicolor supplemented with dung	14.9
Control	6.3

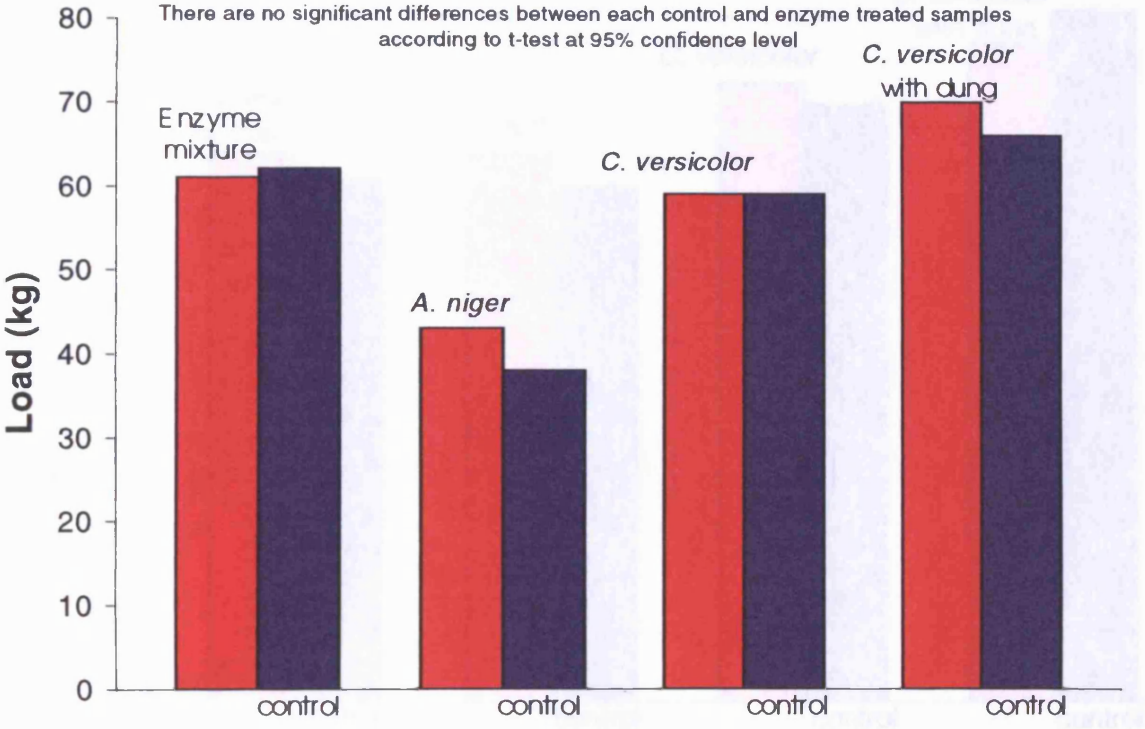
Figure 3.44: Effect of enzyme treatment on tear strength of leathers



Standard deviations for tear strength test

Treatment	Standard deviation
Enzyme mixture	9.2
Control	13.4
A. niger	10.3
Control	7.9
C. versicolor	8.6
Control	6.2
C. versicolor supplemented with dung	5.9
Control	7.6

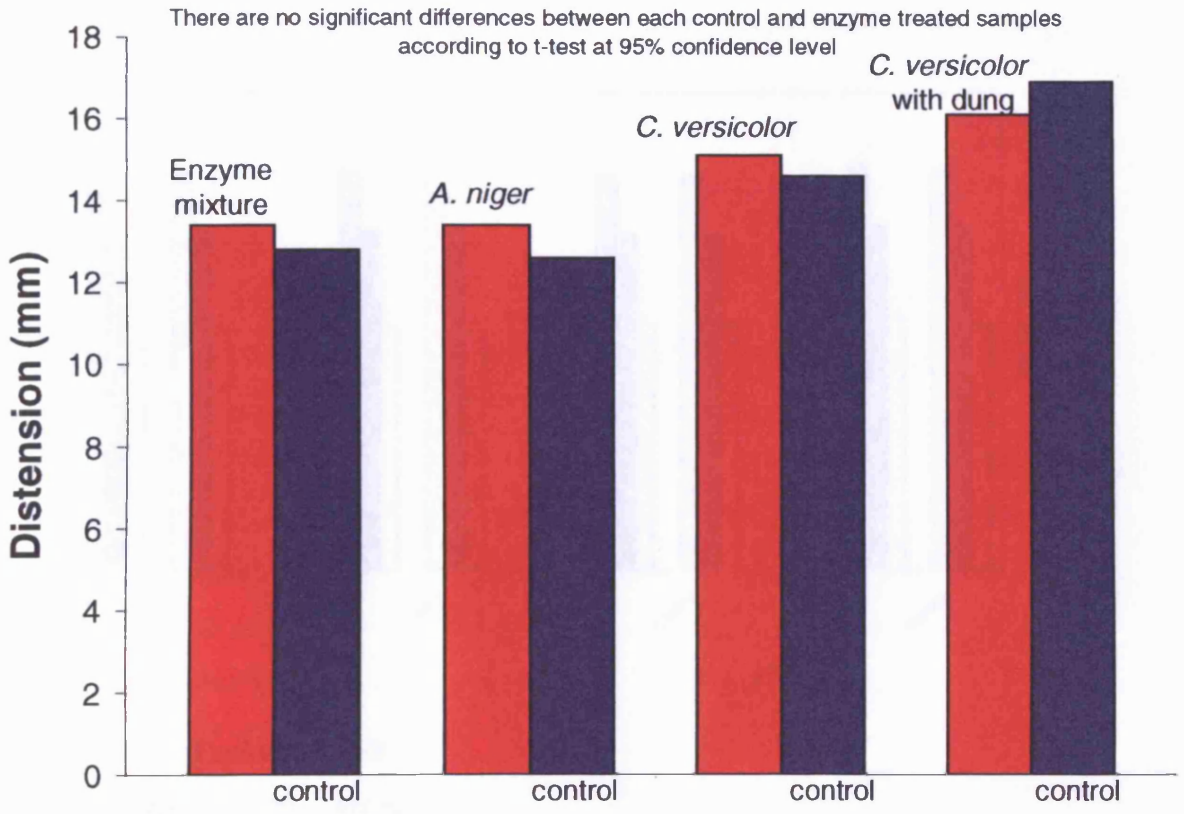
Figure 3.45: Effects of enzyme treatment on grain strength properties with lastometer



Standard deviations of lastometer test

Treatment	Standard deviation
Enzyme mixture	8.3
Control	7.6
A. niger	4.5
Control	6.1
C. versicolor	6.6
Control	4.0
C. versicolor supplemented with dung	4.5
Control	9.4

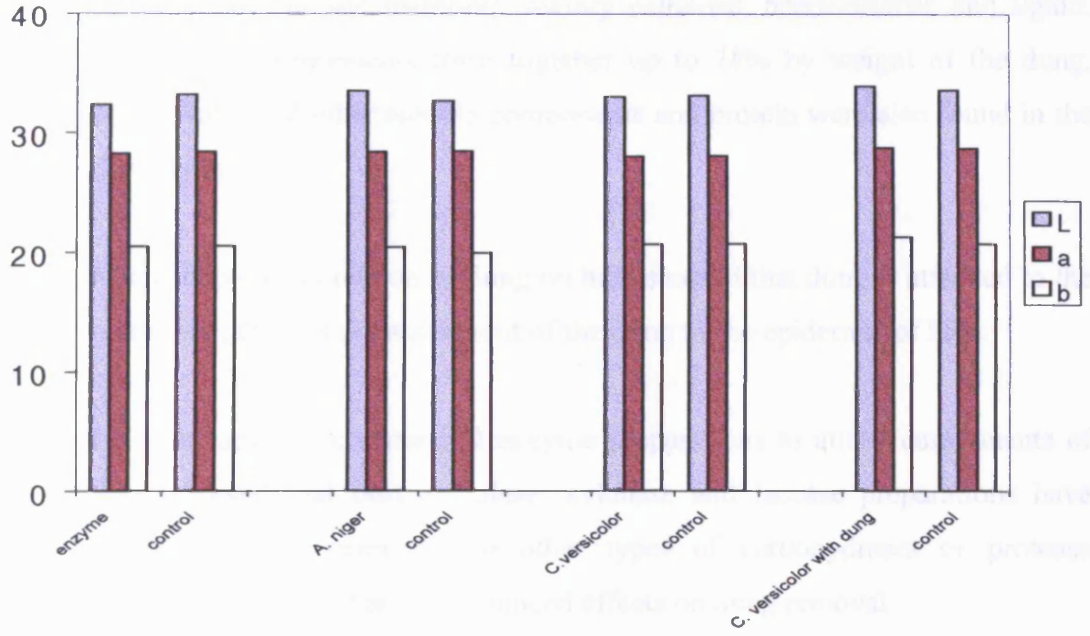
Figure 3.46: Effects of enzyme treatment on grain distension



Standard deviation of grain distension measurement with lastometer

Treatment	Standard deviation
Enzyme mixture	1.62
Control	1.09
A. niger	1.00
Control	1.77
C. versicolor	1.38
Control	1.37
C. versicolor supplemented with dung	0.83
Control	1.64

Figure 3.47: Effects of enzyme treatment on colour of leather (L, a, b values in the CIELAB colour space)



L: lightness of colour (0- 100)

a: Red to green (+ red, - green)

b: yellow to blue (+ yellow, - blue)

Standard deviations from colour measurement test

Treatment	L	a	b
Enzyme	0.29	0.26	0.27
Control	0.28	0.26	0.24
A.niger	0.21	0.20	0.21
Control	0.24	0.24	0.23
C.versicolor	0.15	0.14	0.18
Control	0.09	0.12	0.09
C. versicolor with dung	0.12	0.15	0.13
Control	0.30	0.19	0.26

CHAPTER 4

CONCLUSIONS

1. The composition of typical samples of dung from heavily clad hide is made up of lignocellulosic materials, mainly cellulose, hemicellulose and lignin. These three components form together up to 78% by weight of the dung. Some water and ether soluble components and protein were also found in the dung samples.

2. Microscopic examination of dung on hide showed that dung is attached to the hair alone, there is no attachment of the dung to the epidermis of hide.

3. Using a range of commercial enzyme preparations to attack components of dung showed that only cellulase, xylanase and laccase preparations have effect on dung removal. No other types of carbohydrases or protease preparations showed any pronounced effects on dung removal.

4. Treatment of dung clad hide pieces with cellulase at 2.5 units per ml enzyme produced effective dung removal within 24 hours. However, xylanase treatment required 5.0 units per ml enzyme in order to have effective dung removal within 24 hours.

5. When laccase was used alone in order to attack lignin component of dung, it was not effective in terms of dung removal. However, when 0.008 units per ml of laccase, 5.0 units per ml of cellulase and 5.0 units per ml of xylanase mixture were used together, enzymes showed synergistic effects on the substrate and effective dung removal was obtained within 6 hours.

6. Synergistic effects of enzymes can also be observed in the solubilisation rate of dung. Enzyme mixtures containing cellulase, xylanase, laccase and β-glucosidase gave higher chemical oxygen demand and reducing sugar values than individual enzyme.

7. Treatment of dung with individual enzymes and enzyme mixtures reduced both carbohydrate and lignin contents of dung. These reductions were more pronounced when using cellulase, xylanase and β-glucosidase together.

8. Fungal organisms produced a range of lignocellulosic enzymes under the experimental conditions used in this project. *Coriolus versicolor* produced laccase, β-glucosidase and β-xylosidase when it is grown in Fähraeus medium. This organism also produced cellulase and xylanase along with laccase, β-glucosidase and β-xylosidase when Abrams medium was used. When the Abrams medium was modified with 3% cattle dung as a carbon source, higher enzyme activities were obtained compared with unmodified Abrams medium. *Aspergillus niger* and *Trichoderma reesei* also produced a range of lignocellulosic enzymes except laccase. When they were grown in Abrams medium, *T. reesei* showed higher cellulase activity than *A. niger*. However, *A. niger* produced higher xylanase, β-glucosidase and β-xylosidase activities than *T. reesei*.

9. Presence of sodium chloride up to 3 M concentration increased xylanase and cellulase activities by approximately 20% and 100% respectively. However, the presence of nonionic fatty alcohol ethoxylate did not change the enzyme activities.

10. Paper chromatography showed that treatment of dung with cellulase produced cellobiose and glucose, xylanase treatment produced xylose and glucose. In addition, all the enzyme treatments produced unidentified big molecular components.

11. Gel Permeation Chromatography showed that more degradation is produced by the enzyme mixture treatment than it is by individual enzyme treatments.

12. ^{13}C CP/MAS solid state NMR spectra of dung contain similar features to spectra obtained from cellulose and lignin materials and other lignocellulosic materials.

13. ^{13}C CP/MAS solid state NMR spectra of control and enzyme treated samples showed no differences in peak intensities. The same features were also observed from IR spectra of the control and enzyme treated samples. Therefore, it can be said that the lignocellulosic enzyme treatments remove the dung from hides, without changing the fundamental structure of dung, by attacking structural parts in dung which are most susceptible to solubilising with enzymes.

14. ^{13}C CP/MAS solid state NMR spectra of freeze-dried water extracts from dung showed that the carbohydrate parts of dung are mostly attacked by cellulosic and xylanosic enzyme treatments. In addition, some aromatic components from dung were released by these treatments. The spectra from laccase treated sample showed both aromatic signals and additional carbohydrate signals.

15. IR spectroscopy of freeze-dried extracts from dung did not provide a clear picture about the mechanism of dung removal.

16. When dung clad hide is treated with cellulase, xylanase and laccase mixture in the tannery drum, very effective dung removal is obtained within 1 hour with the help of nonionic detergent. When dung clad hide is treated with *Aspergillus niger* broth, most of the dung can be removed from hide within 1 hour. However, *Coriolus versicolor* broth treatment requires 18 hours for effective dung removal. When dung clad hide is treated with dung supplemented *C. versicolor* broth, dung can be removed from hide within 6 hours.

17. Enzyme treatment for dung removal does not affect components of hide such as hyaluronic acid and dermatan sulphate. Moreover, enzyme treatments do not have any effect on physical properties of leather produced by these treatments. Therefore, using these enzymes to affect dung removal is a very safe process as far as leather quality is concerned.

4.1 FUTURE WORK

1. This new enzyme technology should find an application for treating dung clad live animals in farms or in abattoirs. New studies should concentrate on application techniques of lignocellulosic enzymes to cattle and sheep, particularly employing immobilisation techniques of enzymes on live animals.

2. New fungal organisms and growing conditions can be used to produce cost effective lignocellulosic enzymes for dung removal. Along with this line, one of this project supervisors, Professor Christine Evans, has been already involved to enhance production yields of enzymes for dung removal purpose and it has been found that production yield of *Coriolus versicolor* can be increased several times if the correct conditions are applied.

CHAPTER 5

OVERVIEW

Contamination of hide with dung is a common problem in the meat and leather industries in many parts of the world. The dung clad hides cause many problems in the leather processing steps. In this project, the dung problem in the leather industry has been addressed and the solution to this problem has been presented.

It has been shown that using the right enzymes for targeting components of dung under the right conditions, dung can be removed within 1 hour during soaking process without affecting any properties of the hide. These findings are very important from tanners' point of view, because they can conduct the dirt and main soaking processes without putting their hides at risk of bacterial damage due to the prolonged process time. In addition, the risk of damaging hides during the mechanical fleshing operation can be also eliminated if dung is removed effectively in the previous operation. Effective dung removal in the early stage of leather processing can lead to another innovative technology in leather industry, green splitting i.e. splitting before chemical processing is initiated. Green splitting will produce clean and unmodified collagen splits, that can be effectively utilised by food and animal feeding industries.

This research has developed a solution to deal with the dung problem for tanners. What remains is the application of this technology or similar technologies to meat industry in order to reduce the risk of contamination of meat with faceal microorganisms. This technology can be adapted to remove the dung from live animal before slaughtering process, to eliminate one of the more dangerous problems associated with meat.

BIBLIOGRAPHY

Abrams, E. (1948). Microbiological deterioration of organic materials; its prevention and methods of test. National Bureau of Standards Misc. Publ. No: 188 U.S. Department of Commerce, Washington D.C.

Adams, G.A. (1965). Lignin determination. In: *Methods in Carbohydrate Chemistry.* Vol. V. (Ed: Whistler, R.L) Academic Press, New York, 185-187.

Adler, E. (1977). Lignin chemistry- past, present and future. *Wood Science and Technology* **11**, 169-218.

Akin, D.E.; Rigsby, L.L.; Gamble, G.R.; Morrison, W.H.; Kimball, B.A.; Pinter, P.J.; Wall, G.W.; Garcia, R.L.; Lamorte, R.L. (1995). Biodegradation of plant-cell walls, wall carbohydrates and wall aromatics in wheat grown in ambient or enriched CO_2 concentrations. *Journal of the Science of Food and Agriculture* **67**, 399-406.

Alexander, K.T.W.; Haines, B.M.; Walker, M.P. (1986). Influence of proteoglycan removal on opening-up in the beamhouse. *Journal of the American Leather Chemists Association* **81**, 85-100.

Alexander, K.T.W. (1988). Enzymes in the tannery- catalysts for progress? *Journal of the American Leather Chemists Association* **83**, 289-315.

Alexander, K.T.W.; Walker, M.P.; Webster, R.M.; Stanley, A.M.; Bugby, A.; Smith, Y. (1991). Selective use of enzyme during leather processing. *Proceedings of XXI. IULTCS Congress*, Barcelona.

Alexander, K.T.W.; Corning, D.R.; Covington, A.D. (1993a). The production of soft leather. Part II: Drying and stress softening. *Journal of the American Leather Chemists Association* **88**, 254-269.

Alexander, K.T.W.; Covington, A.D.; Stosic, R.G. (1993b). The production of soft leather. Part III: Measuring softness. *Journal of the American Leather Chemists Association* **88**, 270-277.

Ali, M.; Apperley, D.C.; Eley, C.D.; Emsley, A.M.; Harris, R.K. (1996). A solid-state NMR study of cellulose degradation. *Cellulose* **3**, 77-90.

Ander, P. and Eriksson, K.E. (1976). The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum. Archives of Microbiology* **109**, 1-8.

Aspinall, G. (1970). *Polysaccharides*. Pergaman Press, Oxford.

Atalla, R.H.; Gast, J.C.; Sindorf, D.W.; Bartuska, V.J.; Maciel, G.E. (1980). ^{13}C NMR spectra of cellulose polymorphs. *Journal of the American Chemical Society* **102**, 3249-3251

Bailey, D.G. (1994). Recovering hide value. *Leather*, April, 82.

Bajpai, P (1997). Microbial xylanolytic enzyme system: properties and applications. *Advances in Applied Microbiology* **43**, 141-194.

Bayerlein, F.; Habereder, P.; Franche, G. (1973). Soaking mixtures for hides. *German Patent* Ger. Offen. 2,161,824.

Beldman, G.; Leeuwen, M.F.S.V.; Rombouts, F.M.; Voragen, F.G.J. (1985). The cellulase of *Trichoderma viride* purification, characterisation and comparison of all detectable endoglucanases, exoglucanases and beta-glucosidases. *European Journal of Biochemistry* **146**, 301-308.

Beldman, G.; Voragen, F.G.J.; Rombouts, F.M.; Pilnik, W. (1988). Synergism in cellulose hydrolysis by endo-glucanases and exo-glucanases purified from *Trichoderma reesei*. *Biotechnology and Bioengineering* **31**, 173-178.

Bell, R.G. (1997). Distribution and sources of microbial contamination on beef carcasses. *Journal of Applied Microbiology* **82**, 292-300.

Betts, W.B.; Dart, R.K.; Ball, A.S.; Pedlar, S.L. (1991). Biosynthesis and structure of lignocellulose. In: *Biodegradation: Natural and Synthetic Materials*. (Ed: Betts, W.B.) Springer-Verlag, London, 137-155.

Biely, P. (1985). Microbial xylanolytic systems. *Trends in Biotechnology* **3**, 286-290.

Biely, P.; Vrsanska, M.; Kucar, S. (1992). Identification and mode of action of endo-1,4-β-xylanase. In: *Xylans and Xylanases*. (Eds: Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J.) Elsevier Science Publisher, Amsterdam, 81-95.

Blaich, R. and Esser, K. (1975). Function of enzyme in wood destroying fungi. II. Multiple forms of laccase in white-rot fungi. *Archives of Microbiology* **103**, 271-277.

BLC- Dung Survey (1998). BLC Leather Technology Centre, June. Restricted circulation.

Boonstra, M.G.; Pizzi, A.; Tekely, P.; Pendlebury, J. (1996). Chemical modification of Norway spruce and Scots pine- A ^{13}C NMR CP/MAS study of the reactivity and reactions of polymeric wood components with acetic anhydride. *Holzforschung* **50**, 215-220.

Bose, S.M.; Krishna, W.M.; Das, B.M. (1955). Mechanism of unhairing skins and hides by means of certain proteolytic or amylolytic enzymes. *Journal of the American Leather Chemists Association* **50**, 192-199.

Bourbonnais, R. and Paice, M.G. (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Letters* **267**, 99-102.

Brady, D.; Duncan, J.R.; Russell, A.E. (1990). A model for proteolytic depilation of skins. *Journal of the American Leather Chemists Association* **85**, 334-343.

Brown, S.E. (1972). A study of the digestive processes in dung beetles and some related aspects of the biology of two species of *Scorabaeidae* (*Typhaeus typhoeus* and *Oryctes boas*). Ph.D. Thesis, University of Leicester.

Burton, D.; Reed, R.; Flint, F.O. (1953). The unhairing of hides and skins without lime and sulphide. The use of mucolytic enzymes. *Journal of the Society of Leather Trades' Chemists* **37**, 82-87.

Buswell, J.A. and Odier, E. (1987). Lignin biodegradation. *CRC Critical Reviews in Biotechnology* **6**, 1-60.

Camaioni, D.M. and Franz, J.A. (1984). Carbon-hydrogen vs carbon-carbon bond cleavage of 1,2-diarylethane radical cations in acetonitrile-water. *Journal of Organic Chemistry* **49**, 1607-1613.

Chanda, S.K.; Hirst, E.L.; Jones, J.K.N.; Percival, E.G.V. (1950). The constitution of xylan from esparto grass (*Stipa tenacissima*, L.). *Journal of Chemical Society* **1950**, 1289-1297.

Chen, C.L. (1988). Characterisation of lignin by oxidative-degradation. Use of gas chromatography-mass spectrometry technique. *Methods in Enzymology* **161**, 110-136.

Christner, J. (1995). Modern enzyme applications in the beamhouse. *World Leather*, October, 60.

Christner, J.; Pfeiderer, E.; Taegar, T. (1991). Enzymatically aided soaking process for skins and hides. *UK Patent Application* GB 2,233,665.

Corbett, W.M. (1963). Determination of the alpha-cellulose content of cotton and wood cellulose. In: *Methods in Carbohydrate Chemistry*. Vol. III. (Ed: Whistler, R.L.) Academic Press, New York, 27-28.

Cordon, T.C. (1955). Unhairing of hides and skins by amylase preparations. *Journal of the American Leather Chemists Association* **50**, 270-274.

Cordon, T.C.; Everett, A.L.; Jones, H.; Windus, W. (1961). Elastase activity of some enzymes as related to their depilatory action. *Journal of the American Leather Chemists Association* **56**, 68-77.

Corning, D.R. (1982). Seasonal differences in leather. Part II: Pollution from dung. *BLMRA Journal*, December, 319-321.

Coughlan, M.P. (1985). The properties of fungal and bacterial cellulases with comment on their production and application. *Biotechnology and Genetic Engineering Reviews* **3**, 39-109.

Covington, A.D. and Alexander, K.T.W. (1993). The production of soft leather. Part I: The mechanism of chemical softening. *Journal of the American Leather Chemists Association* **88**, 241-253.

Covington, A.D. (1997). Modern tanning chemistry. *Chemical Society Reviews* **26**, 111-126.

Crawford, R.L. (1981). *Lignin Biodegradation and Transformation.* Wiley-Interscience, New York.

Cristescu, D. and Bratulescu, V. (1979). Enzyme preparation. *Romanian Patent* Rom. 66,056.

Daniels, R.P. (1995) COD: Cost of dung. *World Leather*, November, 16.

Dekker, R.F.H. (1980). The hemicellulase group of enzymes. In: *Polysaccharides in food.* (Eds: Blanshard, J.M.V. and Mitchell ,J.R.) Butterworths, London, 93-108.

Dekker, R.F.H. and Richards, G.N. (1976). Hemicellulases: their occurrence, purification, properties and mode of action. *Advances in Carbohydrate Chemistry and Biochemistry* **32**, 277-352.

Dhar, S.C. (1974). Production and applications of enzymes in the pretanning processes of leather manufacture. *Leather Science* **21**, 39-47.

Dianova, O.P.; Mortakov, A.A.; Shiskhova, A.N.; Vdovina, L.D. (1977). Treatment of fur hides. *Russian Patent* U.S.S.R 549,474.

Dodson, P.J.; Evans, C.S.; Harvey, P.J.; Palmer, J.M. (1987). Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses C_{α}-C_{β} cleavage in a lignin model compound. *FEMS Microbiology Letters* **42**, 17-22.

Donkersgoed, J.V.; Jericho, K.W.F.; Grogan, H.; Thorlakson, B.(1997). Preslaughter hide status of cattle and the microbiology of carcasses. *Journal of Food Protection* **60**, 1502-1508.

Doppelbauer, R.; Esterbauer, H.; Steiner, W.; Lafferty, R.M.; Steinmuller, H. (1987). The use of lignocellulosic wastes for production of cellulase by *Trichoderma reesei*. *Applied Microbiology and Biotechnology* **26**, 485-494.

Dunn, L.A.; Newman, R.H.; Hemmingson, J.A. (1991). Bleaching of eucalypt pulps studies by C-13 NMR spectroscopy. *Journal of Wood Chemistry and Technology* **11**, 93-103.

Earl, W.L. and VanderHart, D.L. (1980). High resolution magic angle sample spinning ^{13}C NMR of solid cellulose I. *Journal of the American Chemical Society* **102**, 3251-3252

Eggert, C.; Temp, U.; Eriksson, K.E.L. (1996). Laccase- producing white-rot fungus lacking lignin peroxidase and manganase peroxidase. Role of laccase in lignin biodegradation. *ACS Symposium Series* **655**, 130-150.

Eggert, C.; Temp, U.; Eriksson, K.E.L. (1997). Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. *FEBS Letters* **407**, 89-92.

Elisashvili, V.I. (1993). Physiological regulation of ligninolytic activity in higher basidium fungi. *Microbiology* **62**, 480-487.

Eley, A. (1997). *Escherichia coli* 0157: an increasingly significant food poisoning pathogen. *Nutrition and Food Science* **97**, 96-100.

Erikson, M. and Miksche, G.E. (1974). Characterization of gymnosperm lignins by oxidative degradation. *Holzforschung* **28**, 135-138.

Esterbauer, H.; Steiner, W.; Labudova, I.; Hermann, A.; Hayn, M.(1991). Production of *Trichoderma cellulase* in laboratory and pilot scale. *Bioresource Technology* **36**, 51-65.

Evans, C.S. (1985a). Laccase activity in lignin degradation by *Coriolus versicolor*: in vivo and in vitro studies. *FEMS Microbiology Letters* **27**, 339-343.

Evans, C.S. (1985b). Properties of the β-D-glucosidase (cellobiase) from the wood-rotting fungus, *Coriolus versicolor*. *Applied Microbiology and Biotechnology* **22**, 128-131.

Evans, C.S. (1991). Enzymes of lignin degradation. In: *Biodegradation: Natural and Synthetic Materials*. (Ed: Betts, W.B.) Springer-Verlag, London, 175-184.

Evans, C.S. (1996). Personal communication.

Eveleigh, D.E. (1987). Cellulase: a perspective. *Philosophical Transactions of the Royal Society of London Series A: Mathematical and Physical Sciences* **321**, 435-447.

Fahraeus, G. and Reinhammar, B. (1967). Large scale production and purification of laccase from the fungus *Polyporus versicolor* and some properties of laccase. *Acta Chemica Scandinavica* **21**, 2367-2378.

Freudenberg, K. (1968). The constitution and biosynthesis of lignin. In: *The constitution and biosynthesis of lignin.* (Eds: Neish, A.C.; Freudenberg, K.) Springer-Verlag, New York, 47-122.

Gallagher (1989). Immunocytochemical localization of lignocellulosic enzymes in Basidiomycetes. Ph.D. Thesis. Thames Polytechnic, London.

Garwood, R. (1995). Uses and applications of microscopy in the leather industry- A practical approach to process control and leather manufacture. *Journal of the American Leather Chemists Association* **90**, 337-346.

Gascoigne, J.A. and Gascoigne, M.M. (1960). *Biological Degradation of Cellulose.* Butterworths, London.

Gil, A.M. and Neto, C.P. (1999). Solid-state NMR studies of wood and other lignocellulosic materials. *Annual Reports on NMR Spectroscopy* **37**, 75-117.

Gilardi, G. (1992). Spectroscopic studies of lignin biodegradation. Ph.D. Thesis, Imperial College.

Gilardi, G.; Abis, L.; Cass, A.E.G. (1994). Wide-line solid-state NMR of wood-proton relaxation time measurements on cell walls biodegraded by white-rot and brown-rot fungi. *Enzyme and Microbial Technology* **16**, 676-682.

Gilardi, G.; Abis, L.; Cass, A.E.G. (1995). ^{13}C CP/MAS solid-state NMR and FT/IR spectroscopy of wood cell-wall biodegradation. *Enzyme and Microbial Technology* **17**, 268-275.

Gillespie, J.M. (1953). The depilation of sheepskins with enzymes. *Journal of the Society of Leather Trades' Chemists* **37**, 344-353.

Glenn, J.K.; Morgan, M.A.; Mayfield, M.B.; Kuwahara, M.; Gold, M.H. (1983). An extracellular H_2O_2- requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium. Biochemical and Biophysical Research Communications* **114**, 1077-1083.

Goetz, A.W. *(1956).* Washing and soaking. In: *The Chemistry of Leather Technology.* Vol: I. (Eds: O'Flaherty, F.; Roddy, W.T.; Lollar, R.M.) Krieger Publishing Company, Florida, 219-228.

Gold, M.H. and Alic, M. (1993). Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Microbiological Reviews* **57**, 605-622.

Green, G.H. (1956). Investigations on bating. Part I: A historical and critical review of the literature. *Journal of the Society of Leather Trades' Chemists* **40**, 195-204.

Grimm, O. (1966). Soaking method of hides and skins. *German Patent* Ger. Offen. 1,230,169.

Guadalix, M.E.; Almendros, G.; Martinez, A.T.; Gonzalezvila, F.J.; Lankes, U. (1997). A C-13 CP/MAS NMR evaluation of the structural changes in wheat straw subjected to diffrent chemical and biological pulping conditions. *Bioresource Technology* **60**, 245-249.

Hadley, P.J.; Holder, J.S.; Hinton, M.H. (1997). Effects of fleece soiling and skinning method on the microbiology of sheep carcases. *Veterinary Record* **140**, 570-574.

Haider, K. and Trojanouski, J. (1975). Decomposition of specifically ^{13}C- labelled phenols and dehydropolymers of coniferyl alcohol as models for lignin degradation by soft and white-rot fungi. *Archives of Microbiology* **105**, 33-41.

Haltrich, D.; Nidetzky, B.; Kulbe, K.D.; Steiner, W.; Zupancic, S. (1996). Production of fungal xylanases. *Bioresource Technology* **58**, 137-161.

Hameed, A.; Natt, M.A.; Evans, C.S. (1996a). Comparative studies of a new microbial bate and the commercial bate "Oropon" in leather treatment. *Journal of Industrial Microbiology* **17**, 77-79.

Hameed, A.; Natt, M.A.; Evans, C.S. (1996b). Production of alkaline protease by a new *Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. *World Journal of Microbiology and Biotechnology* **12**, 289-291.

Heidemann, E. (1993). *Fundamentals of Leather Manufacture*. Eduard Roether KG, Darmstadt.

Hemmingson, J.A. and Morgan, K.R. (1990). A CP/MAS ^{13}C NMR study of photodegraded newsprint. *Holzforschung* **44**, 127-131.

Higuchi, T. (1993). Biodegradation mechanism of lignin by white-rot basidiomycetes. *Journal of Biotechnology* **30**, 1-8.

Higuchi, T.; Shimada, M.; Nakatsubo, F.; Tanahashi, M. (1977). Differences in biosynthesis of guaiacyl and syringyl lignin in woods. *Wood Science and Technology* **11**, 153-167.

Himmelsbach, D.S.; Barton, F.E.; Windham, W.R. (1983). Comparison of carbohydrate, lignin and protein ratios between grass species by cross polarization magic angle spinning C-13 nuclear magnetic resonance. *Journal of Agricultural and Food Chemistry* **31**, 401-404.

Hrmova, M.; Biely, P.; Vrsanska, M. (1986). Specificity of cellulase and beta-xylanase induction in *Trichoderma reesei QM 9414*. *Archives of Microbiology* **144**, 307-311.

Hrmova, M.; Biely, P.; Vrsanska, M. (1989). Cellulose and xylan degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme and Microbial Technology* **11**, 610-616.

Huang, Y.; Stankiewicz, B.A.; Eglinton, G.; Snape, C.E.; Evans, B.; Latter, P.M.; Ineson, P. (1998). Monitoring biomacromolecular degradation of *Calluna vulgaris* in a 23 year field experiment using solid-state ^{13}C NMR and pyrolysis GC/MS. *Soil Biology and Biochemistry* **30**, 1517-1528.

IHATIS Congress- Amsterdam, April 1975.

Ishihara, T. (1980). The role of laccase in lignin biodegradation. In: *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*. Vol. II. (Eds: Kirk, T.K.; Higuchi, T.; Chang, H.) CRC Press, Florida, 17-31.

Jalc, D.; Siroka, P.; Ceresnakova, Z. (1997). Effect of six species of white-rot basidiomycetes on the chemical composition and rumen degrability of wheat straw. *Journal of General and Applied Microbiology* **43**, 133-137.

Jermyn, M.A. (1955). Celluloses and hemicelluloses. *Modern Methods in Plant Analysis* **2**, 197-225.

Jones, H.W.; Cordon, T.C.; Naghski, J.; Windus, W. (1961). Gelatine degradation by microbial and other enzymes as related to their unhairing activity. *Journal of the American Leather Chemists Association* **56**, 123-131.

Joseleau, J.P.; Comtat, J.; Ruel, K. (1992). Chemical structure of xylans and their interaction in the plant cell walls. In: *Xylans and Xylanases*. (Eds: Visser, J.;

Beldman, G.; Kusters-van Someran, M.A.; Voragen, A.G.J.) Elsevier, Amsterdam, 1-15.

Kantelinen, A.; Hatakka, A.; Viikari, L. (1989). Production of lignin peroxidase and laccase by *Phebia radiata*. *Applied Microbiology and Biotechnology* **31**, 234-239.

Kawai, S.; Umezawa, T.; Shimada, M.; Higuchi, T. (1988). Aromatic ring cleavage of 4,6-di (*tert*-butyl) guaiacol, a phenolic lignin model compound, by laccase of *Coriolus versicolor*. *FEBS Letters* **236**, 309-311.

Kersten, P.J.; Kalyanaraman, B.; Hammel, K.E.; Reinhammer, B.; Kirk, T.K. (1990). Comparison of lignin peroxidase, horseadish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochemical Journal* **268**, 475-480.

Kersten, P.J.; Tien, M.; Kalyanaraman, B.; Kirk, T.K. (1985). The ligninase of *Phanerochaete chrysosporium* generates action radicals from methoxybenzenes. *Journal of Biological Chemistry* **260**, 2609-2612.

Kimura, T.; Kimura, F.; Argyropoulos, D.S.; Gray, D.G. (1992). C-13 CP/MAS NMR study of photodegraded stoneground wood pulp. *Holzforschung* **46**, 331-336.

Kirk, T.K. (1983). Degradation and conversion of lignocellulose. In: *The Filamentous Fungi*. Vol: 4 Fungal Technology. (Eds: Smith, J.E.; Berry, D.R.; Kristiansen, B.) Edward Arnold, London, 266-295.

Kirk, T.K. (1987) Lignin- degrading enzymes. *Philosophical Transactions of the Royal Society of London Series A – Mathematical and Physical Sciences* **321**, 461-474.

Klyosov, A.A. (1990). Trends in biochemistry and enzymology of cellulose degradation. *Biochemistry* **29**, 10577-10585.

Koeppen, R. (1991). Quantification of hide defects today's leather. *Journal of the American Leather Chemists Association* **86**, 8-13.

Kolodziejski, W.; Frye, J.S.; Maciel, G.E. (1982). ^{13}C Nuclear magnetic resonance spectrometry with cross polarization and magic-angle spinning for analysis of lodgepole pine wood. *Analytical Chemistry* **54**, 1419-1424.

Kudva, I.T.; Blanch, K.; Hovde, C.J. (1998). Analysis of *Escherichia coli* 0157:H7 survival in ovine or bovine manure and manure slurry. *Applied and Environmental Microbiology* **64**: 3166-3174.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. *Journal of General Physiology* **30**, 291-310.

Kyriacou, A.; Neufeld, R.J.; MacKenzie, C.R. (1989). Reversibility and competition in the absorption of *Trichoderma reesei* cellulase components. *Biotechnology and Bioengineering* **33**, 631-637.

Landmann, A. (1999): Physical testing. In: *Leather Technologists Pocket Book.* (Ed: Leafe, M.K.) The Society of Leather Technologists and Chemists, Withernsea, 229-240.

Leontievsky, A.A.; Vares, T.; Lankinon, P.; Shergill, J.K.; Pozdnyakova, N.N.; Myasoedova, N.M.; Kalkkinen, N.; Golovleva, L.A.; Cammack, R.; Thurston, C.F.;

Hatakka, A. (1997a). Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters* **156**, 9-14.

Leontievsky, A.; Myasoedova, N.; Pozdnyakova, N.; Golovleva (1997b): "Yellow" laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS Letters* **413**, 446-448.

Liang, B.C.; Gregorich, E.G.; Schnitzer, M.; Schulten, H.R. (1996): Characterization of water extracts of two manures and their adsorption on soils. *Soil Science Society of America Journal* **60**, 1758-1763.

Lindroth, E.G. (1961). A study of a pancreatic enzyme unhairing system. *Journal of the American Leather Chemists Association* **56**, 31-41.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. (1951). Protein measurement with the Folin- phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.

Maciel, G.E.; Odonnell, D.J.; Ackerman, J.J.H.; Hawkins, B.H.; Bartuska, V.J. (1981). A C-13 NMR study of 4 lignins in the solid and solution states. *Macromolecular Chemistry and Physics* **182**, 2297-2304.

Malathi, S.; Chakraborty, T. (1991). Production of alkaline protease by a new *Aspergillus flavus* isolated under solid substrate fermentation conditions for use as a depilation agent. *Applied and Environmental Microbiology* **57**, 712-716.

Mandels, M. (1982). Cellulases. *Annual Reports on Fermentation Processes* **5**, 35-77.

Mandels, M.; Medeiros, J.E.; Andreotti, R.E.; Bissett, F. (1981). Enzymatic hydrolysis of cellulose: Evaluation of cellulase culture filtrates under use conditions. *Biotechnology and Bioengineering* **23**, 2009-2026.

Martinez, A.T.; Gonzalez, A.E.; Valmaseda, M.; Dale, B.E.; Lambregts, M.J.; Haw, J.F. (1991). Solid-state NMR studies of lignin and plant polysaccharide degradation by fungi. *Holzforschung* **45**, 49-54.

Mcbride, B.W. (1991). ^{13}C CP/MAS NMR spectroscopy of cellulose and forages. *New Zealand Journal of Agricultural Research* **34**, 257-262.

McHale, A. and Coughlan, M.P. (1981). The cellulolytic system of *Talaromyces emersonii*. Purification and characterization of the extracellular and intracellular β-glucosidase. *Biochimica et Biophysica Acta* **662**, 152-159.

Meat Hygiene Service- Clean livestock Policy, August, 1997.

Monson, P.F. and Poul, F. (1995). Oligosaccharide feed additives. In: *Biotechnology in Animal Feeds and Animal Feeding*. (Eds: Wallace, R.J.; Chesson, A.) VCH Verlagsgellscaft, Weinheim, 233-246.

Morohoshi, N. (1991). Laccase of the ligninolytic fungus *Coriolus versicolor*. *ACS Symposium Series* **460**, 207-224.

Newman, R.H. (1994). Crystalline forms of cellulose in softwoods and hardwoods. *Journal of Wood Chemistry and Technology* **14**, 451-466.

Newton, K.G.; Harrison, J.C.L.; Wauthers, A.M. (1978). Sources of psychrotrophic bacteria on meat at the abattoir. *Journal of Applied Bacteriology* **45**, 75-82.

Obst, J.R. (1982). Frequency and alkali resistance of lignin-carbohydrate bonds in wood. *Tappi Journal* **65**, 109-112.

Oertel, H.; Feigel, T.; Reich, G.; Wettengel, J.; Koehler, W.; Ozegowski, J.H.; Mueller, P.J.; Hess, W.; Linde, H. (1989). Enzymatic hide softening and swelling process using *Streptomyces rimosus* fermentation broths. *East German Patent* Ger.(East) DD264,710.

O'Flaherty, F.; Roddy, W.T.; Lollar, R.M. (1956). *The Chemistry and Technology of Leather*. Vol. I. Krieger, Florida.

Padmavathi, N.K.; Chandrababu, S.; Chitra, S.; Chandrakasan, G. (1995). Application of the extracellular proteases from the culture filtrate of *Streptomyces ssp.* G_{157} in bating. *Journal of the Society of Leather Technologists and Chemists* **79**, 88-89.

Palmer, J.M.; Harvey, P.J.; Schoemaker, H.E. (1987). The role of peroxidases, radical cations and oxygen in the degradation of lignin. *Philosophical Transactions of the Royal Society of London Series A- Mathematical and Physical Sciences* **321**, 495-505.

Pearson, R.P. (1994) Dung: how it damages. *Leather*, May, 14.

Pearson, R.P. (1995). The fifth quarter- the challenge and opportunity for improving hides and skins. *World Leather*, May, 26.

Persson, I.; Tjerneld, F.; Hahn-Hagerdal, B. (1991). Fungal cellulolytic enzyme production: A review. *Process Biochemistry* **26**, 65-74.

Pfannmuller, J. (1956). Bating. In: *The Chemistry and Technology of Leather*. Vol. I. (Eds: O'Flaherty, F.; Roddy, W.T.; Lollar, R.M.) Krieger, Florida, 347-386.

Pfleiderer, E. and Reiner, R. (1988). Microorganisms in processing of leather. In: *Biotechnology*. Vol. 6b Special Microbial Processes. (Eds: Rehm, H.J.; Reed, G.) VCH Verlagsgesellschaft, Weinheim, 729-743.

Phillips, C.A. and Roscoe, N. (1996). Survival of *Escherichia coli* 0157:H7 in ground beef during normal cooking procedures. *Nutrition and Food Science* **96**, 23-26.

Poutanen, K.; Puls, J.; Linko, M. (1986). The hydrolysis of steamed birchwood hemicellulose by enzymes produced by *Trichoderma reesei* and *Aspergillus awamori*. *Applied Microbiology and Biotechnology* **23**, 487-490.

Procter, H.R. (1903). *The Principles of Leather Manufacture*. E and F.N. Spon Limited, London.

Puls, J.; Poutanen, K.; Korner, H.U.; Viikari, L. (1985). Biotechnical utilization of wood carbohydrates after steaming pretreatment. *Applied Microbiology and Biotechnology* **22**, 416-423.

Reid, I.D. (1995). Biodegradation of lignin. *Canadian Journal of Botany* **73**, S1011-S1018.

Reilly, P.J. (1981). Xylanases: structure and function. In: *Trends in the Biology of Fermentations for Fuels and Chemicals*. (Ed: Hollaender, A.E.) Plenum Press, New York, 111-129.

Ridell, J. and Korkeala, H. (1993). Special treatment during slaughtering in Finland of cattle carrying an excessive load of dung; meat hygienic aspects. *Meat Science* **35**, 223-228.

Rockhvarger, O.D. and Zubin, A.M. (1971). Intensification of the degree of soaking of furred hides by using enzymes. *Kozharska Obuvn. Prom-st.* **13**(6), 30-32.

Rosenberg, S.L. (1979). Physiological studies of lignocellulose degradation by the thermotolerant mold *Chrysosporium pruinosum*. *Developments in Industrial Microbiology* **20**, 133-142.

Rosenberg, S.L. and Wilke, C.R. (1980). Lignin biodegradation and production of ethyl alcohol from cellulose. In: *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*. Vol. II. (Eds: Kirk, T.K.; Higuchi, T.; Chang, H.) CRC Press, Florida, 199-212.

Ryu, D.D.; Kim, C.; Mandels, M. (1984). Competitive adsorption of cellulase components and its significance in a synergistic mechanism. *Biotechnology and Bioengineering* **26**, 488-496.

Sarkanen, K.V. and Ludwig, C.H. (1971). *Lignins: Occurrence, Formation, Structure and Reactions*. Wiley-Interscience, New York.

Schoemaker, H.E. and Leisola, M.S.A. (1990). Degradation of lignin *by Phanerochaete chrysosporium. Journal of Biotechnology* **13**, 101-109.

Schroer, T. (1995): Legacy of the intensive cattle environment. *World Leather*, November, 13.

Senior, D.J.; Mayers, P.R.; Saddler, J.N. (1989). Production and purification of xylanases. *ACS Symposium Series* **399**, 641-654.

Sharphouse, J.H. (1971). *Leather Technician's Handbook.* Leather Producers' Association, Northampton.

Smith, I. (1969). *Chromatographic and Electrophoretic Techniques. Vol: I Chromatography.* William Heinemann, Bath.

Stone, J.E.; Scallan, A.M.; Donefer, E. (1969). Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. *Advances Chemistry Series* **95**, 219-241.

Stosic, P.J. (1996). Dung contamination of cattle hides. Hide Improvement Project, Project Report I. The Leather Technology Centre.

Stosic, S. (1998). Dung contamination of cattle hides- 1997 Survey. *BLC Journal*, February, 419-420.

Sunna, A. and Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. *Critical Reviews in Biotechnology* **17**, 39-67.

Taeger, T. (1985). Enzymes in the tannery. *The Leather Manufacturer*, December, 21.

Taylor, M.M.; Bailey, D.G.; Feairheller, S.H. (1987). A review of the uses of enzymes in the tannery. *Journal of the American Leather Chemists Association* **82**, 153-165.

Thorstensen, T.C. (1993). *Practical Leather Technology*. Krieger, Florida.

Thurston, C.F. (1994). The structure and function of fungal laccases. *Microbiology* **140**, 19-26.

Tien, M. (1987). Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. *CRC Critical Reviews in Microbiology* **15**, 141-168.

Tien, M. and Kirk, T.K. (1983). Lignin- degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* (Washington D.C.) **221**, 661-663.

Timell, T.E. (1964). Wood hemicelluloses Part: I. *Advances in Carbohydrate Chemistry and Biochemistry* **19**, 247-302.

Timell, T.E. (1965). Wood hemicelluloses Part: II. *Advances in Carbohydrate Chemistry and Biochemistry* **20**, 409-483.

Toshev, T. and Esaulenko, L. (1972). Accelerated refreshing of dry, nonsalted, preserved sheepskins with the enzymes of *Aspergillus oryzae. Kozharska Obuvn. Prom-st.* **13**(4), 12-14.

Trabitzsch, H. (1966). Concerning the possibilities of employing enzymes in the beamhouse- A review. *Journal of the Society of Leather Trades' Chemists* **50**, 382-389.

Tsao, G.T. and Chiang, L. (1983). Cellulose and hemicellulose technology. In: *The filamentous fungi*. Vol. 4. (Eds: Smith, J.E.; Berry, R.D.; Kristionsen, B.) Edward Arnold, London, 296-326.

Tuck, D.H. (1981). *The Manufacture of Upper Leathers*. Tropical Products Institute, London.

Umezawa, T. and Higuchi, T. (1991). Chemistry of lignin degradation by lignin peroxidases. *ACS Symposium Series* **460**, 236-246.

Viikari, L.; Kantelinen, A.; Buchert, J.; Puls, J. (1994). Enzymatic accessibility of xylans in lignocellulosic materials. *Applied Microbiology and Biotechnology* **41**, 124-129.

Walker, L.P. and Wilson, D.B. (1991). Enzymatic hydrolysis of cellulose: an overview. *Bioresource Technology* **36**, 3-14.

Wallbacks, L.; Edlund, U.; Norden, B. (1991). Multivariate characterisation of pulp using solid state ^{13}C NMR, FT/IR and NIR. *Tappi Journal* **74**, 201-206.

Whistler, R.L. and Faether, M.S. (1965). Hemicellulose. Extraction from annual plants with alkaline solutions. In: *Methods in Carbohydrate Chemistry*. Vol. V. (Ed: Whistler, R.L.) Academic Press, New York, 144-145.

Whistler, R.L. and Richards, E.L. (1970). Hemicelluloses. In: *The Carbohydrates.* Vol. 2a. (Eds: Pigman, W.; Horton, D.) Academic Press, New York, 447-469.

Wilkie, K.C.B. (1979). The hemicelluloses of grasses and cereals. *Advances in Carbohydrate Chemistry and Biochemistry* **36**, 215-264.

Wilkie, K.C.B. (1983). Hemicellulose. *Chemtech*, May, 306-319.

Wilson, H.R. (1956). The practice of bating. In: *The Chemistry and Technology of Leather.* Vol. I. (Eds: O'Flaherty, F.; Roddy, W.T.; Lollar, R.M.) Krieger, Florida, 323-346.

Wong, K.K.Y.; Tan, L.U.L; Saddler, J.N. (1988). Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. *Microbiological Reviews* **52**, 305-317.

Wong, K.K.Y. and Saddler, J.N. (1992). Trichoderma xylanases, their properties and application. In: *Xylans and Xylanases.* (Eds: Visser, J.; Beldman,G; Kusters-von Someren, M.A.; Voragen, A.G.J.) Elsevier Science Publisher, Amsterdam, 171-186.

Wood, J.T. (1912). *Puering, Bating and Drenching of skins.* E and F.N. Spon Limited, London.

Wood, T.M. and McCrae, S.I. (1972). The purification and properties of the C1 component of *Trichoderma koningii* cellulase. *Biochemical Journal* **128**, 1183-1192.

Wood, T.M. and McCrae, S.I. (1978). The cellulase of *Trichoderma koningii. Biochemical Journal* **171**, 61-72.

Wood, T.M. and McCrae, S.I. (1979). Synergism between enzymes involved in the solubilisation of native cellulose. *ACS Symposium Series* **181**, 181-209.

Wood, T.M.; McCrae, S.I.; Macfarlane, C.C. (1980). The isolation, purification and properties of the cellobiohydrolase component of *Penicillium funicalosum* cellulase. *Biochemical Journal* **189**, 51-65.

Wood, T.M. (1985). Properties of cellulosic enzyme systems. *Biochemical Society Transactions* **13**, 407-410.

Wood, T.M. (1992). Fungal cellulases. *Biochemical Society Transactions* **20**, 46-53.

Woodward, J. (1984). Xylanases: functions, properties and applications. *Topics in Enzyme and Fermentation Biotechnology* **8**, 9-30.

Woodward, J. (1991). Synergism in cellulase systems. *Bioresource Technology* **36**, 67-75.

Yates, J.R. (1968). Studies in depilation V. Investigation of the relative depilatory efficiency of a number of commercially available enzyme systems. *Journal of the American Leather Chemists Association* **63**, 464-473.

Yates, J.R. (1972). Studies in depilation. Part X: The mechanism of the enzyme depilation process. *Journal of the Society of Leather Trades' Chemists* **56**, 158-177.

Zugno, L.A. (1992). The effect of trypsin on soaking of salt cured hides. *Journal of the American Leather Chemists Association* **87**, 207-220.

APPENDIX I: Process recipe for leather production

After treatment with enzymes in the soaking operations, enzyme treated and control hides were fleshed and weighed. All hides were put into the same tannery drum.

Operation	Chemicals	%	T °C	Run Time	Comments
Unhairing/Liming					
	water	80	25		
	sodium sulphide	0.5		60 min.	
	sodium sulphide	1		60 min.	
	sodium sulphide	1			
	lime	2			
Drain					
	water	140	25	over night	pH: 12.5
Wash	Water	100	25	5 min.	
Drain					
Wash	water	300	25	5 min.	
Drain					
% based on limed hides					
Deliming					
	water	250	37		
	ammonium sulphate	3			
	sodium metabisulphite	0.5		1.5 h	Clear with phenolphthalein
	bate powder	1		1 h	pH: 8-8.5
Drain					
Wash	water	150	25	10 min.	
Drain					
Pickle					
	water	80	25		
	Sodium chloride	10		10 min.	6 Boume
	Sulphuric acid	1.2			
	Formic acid	0.5		2 hours	pH: 2.8-3.2
Tanning					
	33% basic chrome powder	3.5		30 min.	
	33% basic chrome powder	3.5		5 hours	

Operation	Chemicals	%	T °C	Run Time	Comments
Basification					
	sodium bicarbonate	1.5		1	pH: 4.0
Horse up				1 day	
Sammying					
Setting out					
Splitting					
Shaving					1.2-1.4 mm
All % based on shaved weight					
Wash	water	150	30		
	formic acid	0.25		15 min	
Drain					
Neutralisation					
	water	150	40		
	sodium bicarbonate	1.5			
	sodium formate	0.5		1 hour	pH: 4.8-5.0
Drain					
Wash	water	200	40	10 min.	
Drain					
Dyeing/fatliquoring					
	water	150	55		
	ammonia	0.5		10 min.	
	Lowapel br R (dyestuff)	2		45 min.	
	Remsynol ESA (fatliquor)	4			
	Remsynol ESI (fatliquor)	4		1 hour	
	formic acid	1		15 min.	
Drain					
Wash	water	200	25	10 min.	
Drain					
Horse up				1 day	
Sammying					
Setting out					
Vacuum drying					
Hang drying					
Conditioning					
Staking					
Toggle drying					

APPENDIX II: Chemical Oxygen Demand of Samples

1. Treatment with buffer solution

Titration of blank sample: 28.1 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.8	4120
	17.2	4360
	17.8	4120
2	17.1	4400
	17	4440
	17.2	4360
3	16.9	4480
	17.2	4360
	17.2	4360
8	17.6	4200
	17.5	4260
	17.6	4200
12	17.4	4280
	17.8	4120
	17.6	4200
18	17.1	4400
	17.0	4400
	17.1	4400
24	16.9	4480
	17.0	4400
	16.9	4480

2. Treatment with cellulase

Titration of blank sample: 28.3 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.0	4520
	17.4	4360
	17.5	4320
2	16.9	5000
	15.8	4560
	16.2	4840
3	15.2	5240
	15.3	5200
	15.3	5200
8	14.2	5640
	14.1	5680
	14.2	5640
12	14.5	5520
	14.4	5560
	14.0	5720
18	14.3	5640
	14.2	5200
	14.3	5200
24	14.6	5480
	14.3	5200
	14.6	5480

3. Treatment with xylanase

Titration of blank sample: 28.8 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	18.0	4320
	18.1	4280
	18.1	4280
2	17.3	4600
	17.9	4360
	17.9	4360
3	17.5	4520
	17.7	4440
	17.7	4440
8	16.2	5040
	16.5	4920
	16.2	5040
12	16.1	5080
	15.9	5160
	16.0	5120
18	15.4	5360
	15.5	5320
	15.5	5320
24	15.9	5200
	16.0	5120
	15.9	5120

4. Treatment with cellulase + xylanase

Titration of blank sample: 28.5 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.2	4520
	17.3	4480
	17.3	4480
2	16.1	4960
	15.7	5120
	15.9	5040
3	14.1	5760
	14.2	5720
	14.1	5760
8	14.0	5800
	14.1	5760
	14.1	5760
12	14.7	5520
	14.5	5600
	14.4	5640
18	13.4	6040
	13.7	5920
	13.4	6040
24	14.0	5800
	14.4	5640
	13.9	5840

5. Treatment with cellulase + β-glucosidase
Titration of blank sample: 28.5 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.9	4240
	17.4	4440
	17.2	4520
2	15.3	5280
	15.4	5240
	15.4	5240
3	14.2	5720
	14.4	5640
	14.8	5480
8	14.4	5640
	14.5	5600
	14.5	5600
12	14.2	5720
	14.7	5520
	14.6	5560
18	13.6	5960
	13.7	5920
	13.7	5920
24	13.9	5840
	14.1	5760
	14.0	5800

6. Treatment with cellulase + xylanase + β-glucosidase
Titration of blank sample: 28.4 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	16.4	4800
	16.6	4720
	16.8	4640
2	15.4	5200
	15.5	5160
	15.5	5160
3	9.6	7520
	9.2	7680
	9.7	7480
8	8.0	8160
	8.1	8120
	8.1	8120
12	9.2	7680
	9.0	7760
	8.8	7840
18	8.9	7800
	9.0	7760
	9.0	7760
24	9.8	7440
	9.3	7640
	9.1	7720

7. Treatment with laccase

Titration of blank sample: 28 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.4	4240
	17.6	4160
	17.5	4200
2	17.4	4240
	17.8	4080
	17.9	4040
3	17.8	4080
	17.7	4100
	17.7	4100
8	16.2	4720
	16.9	4440
	16.7	4520
12	15.6	4960
	15.7	4900
	15.8	4880
18	14.9	5240
	15.5	5000
	15.5	5000
24	14.9	5240
	15.0	5000
	15.1	5160

8. Treatment with cellulase + xylanase + β-glucosidase + laccase

Titration of blank sample: 28.4 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	15.2	5280
	15.8	5040
	15.8	5040
2	11.0	6960
	11.6	6720
	11.9	6600
3	8.8	7840
	8.9	7800
	8.9	7800
8	6.9	8600
	7.4	8400
	7.0	8560
12	7.0	8560
	7.1	8500
	7.1	8500
18	6.6	8720
	6.8	8640
	5.8	9040
24	6.2	8880
	6.5	8760
	6.5	8760

9. Enzyme mixture (not contain any dung)
Titration of blank sample: 28.4 cm^3

Time (hours)	Titration Result (cm^3)	COD (mg/l)
1	17.9 18.0 17.9	4100 4160 4100
2	17.0 16.7 17.0	4560 4680 4560
3	17.2 17.0 17.7	4480 4560 4280
8	17.3 17.2 17.2	4350 4480 4480
12	16.7 17.1 16.9	4680 4520 4500
18	16.8 16.9 16.9	4640 4500 4500
24	16.5 16.4 -	4760 4800 -

APPENDIX III: Reducing sugar measurement

1. Control sample

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.062	22
2	0.071	25
3	0.098	35
8	0.075	27
12	0.063	22
18	0.063	22
24	0.061	22

2. Cellulase treatment

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.353	126
2	0.364	130
3	0.388	138
8	0.403	143
12	0.387	138
18	0.311	111
24	0.310	110

3. Xylanase treatment

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.050	18
2	0.058	21
3	0.063	23
8	0.070	25
12	0.067	24
18	0.070	25
24	0.078	28

4. Laccase treatment

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.074	26
2	0.080	28
3	0.082	29
8	0.089	32
12	0.092	33
18	0.082	29
24	0.084	30

5. Treatment with cellulase + xylanase

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.255	91
2	0.253	90
3	0.301	107
8	0.320	114
12	0.281	100
18	0.215	77
24	0.217	77

6. Treatment with cellulase + β-glucosidase

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.287	102
2	0.250	89
3	0.372	132
8	0.341	121
12	0.320	114
18	0.275	98
24	0.271	96

7. Treatment with cellulase + xylanase + β-glucosidase

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.188	67
2	0.284	101
3	0.387	138
8	0.379	135
12	0.242	86
18	0.196	70
24	0.174	62

8. Treatment with cellulase + xylanase + laccase + β-glucosidase

Time (hours)	Average Absorbance	Reducing sugar (μg/ml)
1	0.283	101
2	0.284	101
3	0.378	135
8	0.382	136
12	0.379	135
18	0.386	137
24	0.385	137

APPENDIX IV: Effect of enzyme treatment on the carbohydrate and lignin content of dung

1. Carbohydrate content

Treatment	Absorbance at 620 nm	Average Absorbance	Amount (corrected for dilution) ($\mu g/cm^3$)	Carbohydrate (%)
Initial	0.658 0.676 0.661	0.665	237	59.25
Control	0.674 0.668 0.662	0.668	238	59.5
Cellulase	0.583 0.590 0.579	0.584	208	52
Xylanase	0.620 0.632 -	0.626	223	55.75
Laccase	0.631 0.643 0.646	0.640	228	57
Cellulase+xylanase	0.592 0.592 0.601	0.595	212	53
Cellulase+ glycosidase	0.593 0.588 0.594	0.592	211	52.75
Cellulase+xylanase +glycosidase	0.572 0.580 -	0.576	205	51.25
Cell+xyl+gly+lacc	0.572 0.584 0.578	0.578	206	51.6

2. Lignin content

Treatment	Lignin measurement (%)	Average Lignin Measurement (%)
Initial	21.9 22.7 22.4	22.0
Control	23.2 23.8 23.1	23.4
Cellulase	22.6 22.8 23.0	22.8
Xylanase	21.9 22.5 21.6	22.0
Laccase	19.2 19.3 19.1	19.2
Cellulase+xylanase	23.3 23.5 23.4	23.4
Cellulase+β-glycosidase	21.9 22.0 21.6	21.8
Cellulase+xylanase+β-glycosidase	22.3 22.3 22.3	22.3
Cellulase+xylanase+laccase +β-glycosidase	20.0 20.2 20.2	20.2

APPENDIX V: BLC softness measurements

Enzyme (mm)	Control (mm)	*A. niger* (mm)	Control (mm)	*C.versi.* (mm)	Control (mm)	*C.versi.* With dung (mm)	Control (mm)
45	45	48	45	44	44	45	45
45	45	51	45	40	46	46	44
46	46	41	46	44	40	46	47
51	48	44	47	41	41	52	46
51	39	45	51	40	42	54	53
44	41	46	43	41	39	48	45
46	45	46	48	42	51	49	46
45	42	45	51	39	46	52	48
42	45	43	56	46	45	41	49
41	41	48	52	40	47	51	49
48	45	42	48	44	47	50	44
45	43	42	41	41	43	45	46
45	39	45	40	40	46	46	48
43	51	47	43	46	41	48	48
45	51	43	45	43	51	48	50
47	43	42	44	40	50	50	44
46	44	45	47	40	44	51	49
46	45	45	48	46	41	52	46
46	51	48	48	41	43	47	46
48	40	46	47	51	43	50	48
46	42	46	44	50	42	45	44
47	43	45	50	42	47	44	44
45	44	44	47	42	45	49	46
45	43	45	48	41	43	46	48
43	45	44	51	44	46	48	48
45	44	44	52	40	44	47	51
43	43	47	42	40	40	47	51
45	41	45	40	44	40	53	49
38	44	44	41	40	41	47	45
42	44	44	46	41	43	46	45
TOTAL							
1354	1322	1350	1396	1273	1321	1443	1412
MEAN							
45	44	45	46	42	44	48	47
STANDARD DEVIATION							
3	3	2	4	3	3	3	2
t-test							
t_{stat}	1.4370	t_{stat}	-1.8820	t_{stat}	-1.9936	t_{stat}	1.4826
t_{critc}	2.0032	t_{critc}	2.0141	t_{critc}	2.0017	t_{critc}	2.0040

APPENDIX VI: Tensile strength and elongation at break

1. Enzyme mixture treatment

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.44	325	2257	44.2	88
2	1.46	340	2329	45.7	91
3	1.60	465	2906	48.7	97
4	1.56	415	2660	45.6	91
5	1.59	460	2893	48.3	96
6	1.55	455	2935	46.0	92
7	1.55	245	1581	44.2	88
8	1.52	265	1743	52.4	104
9	1.59	220	1384	42.2	84
10	1.46	195	1336	39.0	78
11	1.49	200	1342	38.7	77
12	1.60	240	1500	47.5	95
MEAN					
			2072		90
STANDARD DEVIATION					
			660		8

Control sample

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.69	500	2959		
2	1.86	580	3118	43.8	87
3	1.81	430	2376	39.7	79
4	1.70	400	2353	37.3	74
5	1.65	360	2182	37.3	74
6	1.59	415	2610	39.6	79
7	1.68	225	1339	66.6	133
8	1.66	330	1988	65.2	130
9	1.56	290	1859	61.3	122
10	1.81	255	1409	68.8	137
11	1.53	230	1503	52.8	105
12	1.48	245	1655	63.0	126
MEAN					
			2113		105
STANDARD DEVIATION					
			592		26

t-test for tensile strength: t_{stat} –0.15782 $t_{critical}$ 2.073875

t-test for elongation at break: t_{stat} –1.74363 $t_{critical}$ 2.178813

2. *Aspergillus niger* treatment

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.57	255	1624	36.5	73
2	1.57	285	1815	40.1	80
3	1.51	260	1722	38.3	76
4	1.56	300	1923	40.4	81
5	1.52	235	1546	26.8	54
6	1.50	255	1700	33.5	67
7	1.79	215	1201	53.6	107
8	1.54	145	942	44.1	88
9	1.57	165	1051	43.9	88
10	1.65	180	1091	41.6	83
11	1.78	185	1039	54.3	109
12	1.52	190	1250	48.4	97
MEAN					
			1409		84
STANDARD DEVIATION					
			348		16

Control sample

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.36	165	1213	26.6	53
2	1.41	240	1702	35.1	70
3	1.33	185	1391	30.1	60
4	1.47	300	2041	38.9	78
5	1.41	240	1702	36.2	72
6	1.41	230	1631	39.5	79
7	1.45	120	828	34.2	68
8	1.43	140	979	38.7	77
9	1.50	155	1033	46.3	93
10	1.57	160	1019	37.5	75
11	1.31	130	992	39.5	79
12	1.46	155	1062	35.4	71
MEAN					
			1299		73
STANDARD DEVIATION					
			384		10

t-test for tensile strength: t_{stat} 0.730012 $t_{critical}$ 2.07875
t-test for elongation at break: t_{stat} 1.961152 $t_{critical}$ 2.093025

3. *Coriolus versicolor* treatment

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.52	310	2039	48.4	97
2	1.61	310	1925	41.7	83
3	1.54	220	1428	43.9	88
4	1.66	310	1867	39.3	79
5	1.54	275	1785	33.4	67
6	1.68	350	2083	30.9	62
7	1.67	200	1198	59.0	118
8	1.72	225	1308	68.7	137
9	1.69	225	1331	60.3	121
10	1.68	215	1280	55.4	111
11	1.73	205	1185	51.1	102
12	1.59	160	1006	56.2	112
MEAN					
			1536		98
STANDARD DEVIATION					
			377		23

Control sample

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.74	435	2500	48.4	97
2	1.72	385	2238	41.7	83
3	1.88	475	2526	43.9	88
4	1.84	500	2717	39.3	79
5	1.92	500	2604	33.4	67
6	1.77	435	2458	30.9	62
7	1.89	375	1984	59	118
8	1.78	320	1798	68.7	137
9	1.93	300	1554	60.3	121
10	1.79	265	1480	55.4	111
11	1.62	195	1204	51.1	102
12	1.59	235	1478	56.2	112
MEAN					
			2045		98
STANDARD DEVIATION					
			527		23

t-test for tensile strength: t_{stat} –2.71583 $t_{critical}$ 2.085962

t-test for elongation at break: t_{stat} 0 $t_{critical}$ 2.073875

4. Treatment with *Coriolus versicolor* supplemented with dung

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.46	410	2808	43.3	87
2	1.48	445	3007	43.1	86
3	1.49	400	2685	39.5	79
4	1.6	500	3125	41.9	84
5	1.6	455	2844	40.9	828
6	1.49	375	2517	37.4	75
7	1.54	305	1981	51.2	102
8	1.72	340	1977	55.4	111
9	1.79	355	1983	62.9	126
10	1.55	315	2032	42.2	84
11	1.49	275	1846	47.8	96
12	1.57	335	2134	41.3	83
MEAN					
			2411		91
STANDARD DEVIATION					
			466		15

Control sample

Sample	Thickness (mm)	Reading (N)	Tensile (N/mm^2)	Elongation	%Elongation
1	1.48	410	2770	52.5	105
2	1.54	370	2402	44.5	89
3	1.58	390	2468	46.1	92
4	1.52	335	2204	42.6	85
5	1.52	420	2763	47.5	95
6	1.56	425	2724	50.1	100
7	1.76	355	2017	49.8	100
8	1.66	340	2048	47.4	95
9	1.66	350	2108	52.4	105
10	1.72	320	1860	51.2	102
11	1.68	355	2113	49.7	99
12	1.72	325	1890	51.7	103
MEAN					
			2281		98
STANDARD DEVIATION					
			335		6

t-test for tensile strength: t_{stat} 0.787965 $t_{critical}$ 2.085962
t-test for elongation at break: t_{stat}-1.37203 $t_{critical}$2.131451

APPENDIX VII: Tear strength test

1. Treatment with enzyme mixture

Enzyme mixture				Control		
Sample	Mean Thickness (mm)	Load (N)	Tear Strength (N/mm)	Mean Thickness (mm)	Load (N)	Tear Strength (N/mm)
1	1.78	100	56.1	1.62	82	50.6
2	1.54	98	63.6	1.67	116	69.4
3	1.51	98	64.9	1.57	92	58.5
4	1.54	82	53.0	1.63	74	45.3
5	1.75	90	51.3	1.48	70	47.0
6	1.62	106	65.1	1.60	94	58.7
7	1.84	112	60.7	1.54	98	63.6
8	1.66	116	69.8	1.61	98	60.8
9	1.54	124	80.5	1.51	82	54.0
10	1.72	106	61.5	1.51	108	71.5
11	1.56	116	74.0	1.52	92	60.2
12	1.87	144	76.7	1.63	112	68.5
MEAN						
			64.8			70.3
STANDARD DEVIATION						
			9.1			13.3
t-test						
t_{stat}: -1.18004 $t_{critical}$: 2.093025						

2. Treatment with *A. niger*

A. niger treatment				Control		
Sample	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**
1	1.82	90	49.3	1.39	52	37.2
2	1.78	84	47.1	1.46	70	47.8
3	1.80	74	41.0	1.47	78	53.0
4	1.75	86	49.1	1.54	76	49.3
5	1.77	94	53.0	1.44	54	37.3
6	1.69	88	51.9	1.34	54	40.0
7	1.53	114	74.3	1.40	72	51.33
8	1.84	110	59.7	1.61	-	-
9	1.53	98	63.9	1.59	102	64.0
10	1.53	92	59.8	1.44	72	50.0
11	1.77	94	52.9	1.50	78	51.8
12	1.60	118	73.4	1.46	76	52.0
MEAN						
		50.2				48.5
STANDARD DEVIATION						
		10.2				7.9
t- test						
t_{stat}: 2.046905 t_{crit}: 2.085962						

3. Treatment with *C. versicolor*

C. versicolor treatment				Control		
Sample	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**
1	1.62	82	50.6	1.79	108	60.1
2	1.67	116	69.4	1.85	96	51.7
3	1.57	92	58.5	1.80	106	58.6
4	1.63	74	45.3	1.83	122	66.4
5	1.48	70	47.0	1.80	92	51.0
6	1.60	94	58.7	1.79	112	62.5
7	1.54	98	63.6	1.72	116	67.3
8	1.61	98	60.8	1.94	122	62.7
9	1.51	82	54.0	2.05	102	49.5
10	1.51	108	71.5	1.91	126	65.9
11	1.52	92	60.2	1.94	122	62.8
12	1.63	112	68.5	1.69	106	62.4
MEAN						
		59				60.1
STANDARD DEVIATION						
		8.6				6.2
t- test						
t_{stat}: -0.34679 t_{crit}: 2.085962						

4. Treatment *with C. versicolor* supplemented with dung

Treatment with C. versicolor supplemented with dung				Control		
Sample	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**	**Mean Thickness (mm)**	**Load (N)**	**Tear Strength (N/mm)**
1	1.61	128	79.3	1.57	130	82.8
2	1.63	122	74.8	1.64	128	77.7
3	1.59	122	76.4	1.62	100	61.6
4	1.67	142	85.0	1.62	111	68.2
5	1.60	142	88.3	1.67	114	67.9
6	1.59	104	65.4	1.60	-	-
7	1.62	118	72.6	1.58	132	83.1
8	1.66	128	77.1	1.73	128	73.9
9	1.61	120	74.2	1.58	110	69.3
10	1.59	130	81.4	1.49	106	70.8
11	1.62	126	77.7	1.43	121	84.4
12	1.62	128	78.8	1.54	104	67.5
MEAN						
		77.6				73.4
STANDARD DEVIATION						
		5.9				7.6
t- test						
t_{stat}: 1.470704 t_{crit}: 2.093025						

Enzyme mixture			Enzyme mixture control			*Aspergillus niger*			*Aspergillus niger* control		
L	a	b	L	a	b	L	a	b	L	a	b
32.19	28.17	20.13	33.18	28.89	20.17	33.09	28.54	20.13	32.78	28.49	20.13
32.16	29.47	20.69	33.27	28.85	20.56	33.12	28.63	20.16	32.76	28.56	20.28
32.13	28.16	20.58	32.19	28.85	20.88	33.56	28.62	20.19	32.76	28.23	19.56
29.89	28.19	20.46	32.89	28.12	21.16	33.75	28.56	20.59	32.56	28.86	20.12
32.21	28.17	21.07	32.96	28.19	20.65	33.69	28.55	20.69	32.63	28.69	20.16
32.13	26.23	20.45	33.09	28.52	20.68	33.56	28.36	20.95	32.52	28.64	19.63
34.01	27.89	20.45	33.26	28.65	20.78	33.51	28.33	20.66	32.43	28.16	19.66
32.16	27.49	20.56	33.28	28.16	20.77	33.76	28.47	20.35	32.46	28.56	19.96
32.46	28.17	20.17	33.27	27.89	20.46	33.78	27.89	20.33	32.28	28.55	19.95
33.04	28.58	20.47	33.27	27.89	20.77	33.53	27.90	20.35	32.77	28.56	20.32
32.16	28.19	20.15	33.56	28.45	20.45	33.48	28.45	20.06	32.98	28.69	20.33
30.18	28.64	21.48	32.78	28.40	20.57	33.56	28.56	20.45	32.95	28.76	20.45
32.15	28.12	20.45	32.19	28.44	20.23	33.26	28.66	20.56	32.45	28.36	19.56
32.56	28.12	20.16	33.17	28.63	20.22	33.66	28.52	20.66	32.56	28.31	19.55
31.56	28.09	20.89	33.65	28.66	20.89	33.49	28.63	20.36	32.81	28.17	19.46
32.17	28.45	20.85	33.65	28.03	20.90	33.49	28.77	20.46	32.29	28.38	19.89
32.65	28.17	20.77	33.18	28.06	20.88	33.89	28.75	20.46	32.45	28.61	19.96
33.12	28.45	20.46	33.17	28.56	20.58	33.66	28.32	20.38	31.86	28.53	19.99
32.14	28.56	20.17	33.33	28.59	20.15	33.48	28.30	20.45	32.29	28.12	20.11
33.41	29.01	20.32	33.41	28.45	20.15	33.53	28.26	20.36	32.65	28.16	20.10
32.07	28.19	20.34	33.09	29.01	20.18	33.61	28.25	20.38	32.79	28.36	20.46
32.87	28.02	20.66	33.10	28.98	20.23	33.45	28.24	20.42	32.98	28.28	20.51
32.56	28.25	20.59	33.11	28.54	20.57	33.44	28.56	20.42	32.65	28.45	20.10
32.56	28.27	20.45	33.16	28.54	20.09	33.31	28.58	20.63	32.45	28.59	20.09
32.18	26.96	20.19	33.26	28.63	20.18	33.29	28.65	19.68	32.21	28.75	19.88

Enzyme mixture			Enzyme mixture control			*Aspergillus niger*			*Aspergillus niger* control		
L	**a**	**b**	**L**	**a**	**b**	**L**	**a**	**b**	**L**	**a**	**b**
32.63	28.78	20.22	33.45	28.18	20.36	33.56	28.66	20.36	32.79	28.69	19.86
32.27	28.17	20.34	33.29	28.55	20.59	33.56	28.66	20.67	32.96	28.38	19.98
32.26	28.45	20.36	32.96	28.36	20.55	33.89	28.45	20.85	32.85	28.88	19.91
30.13	28.45	20.19	33.21	28.48	20.47	32.96	28.63	20.46	32.88	28.86	19.76
32.45	28.19	19.99	33.21	28.51	20.38	32.99	28.51	20.33	32.88	28.63	19.77
32.56	28.04	20.18	33.18	28.51	20.45	33.59	28.51	20.21	32.59	28.65	19.84
31.17	28.17	20.48	33.15	28.63	20.66	33.69	28.55	20.20	32.76	28.26	19.88
32.16	28.94	20.19	33.23	28.65	20.77	33.66	28.48	20.56	32.77	28.49	20.01
32.17	28.56	20.34	33.26	28.17	20.54	33.54	27.99	20.54	32.49	27.89	20.00
32.55	28.17	20.35	33.45	28.2	20.52	33.50	28.41	20.43	32.79	27.88	19.95
32.18	28.16	20.69	33.23	28.63	20.57	33.36	28.16	20.42	32.77	28.73	19.93
32.17	27.56	20.89	33.14	28.56	20.56	33.33	28.15	20.65	32.89	28.39	19.91
32.45	28.95	20.88	33.10	28.56	20.56	33.26	28.32	20.64	32.86	28.71	19.91
32.24	28.56	20.89	33.09	28.49	20.62	33.28	28.38	20.65	32.88	28.77	19.89
32.28	28.45	20.48	33.09	28.22	20.60	33.56	28.41	20.86	32.64	28.65	20.12
32.16	29.26	20.96	33.65	28.13	20.72	33.56	28.40	20.47	32.56	28.32	20.15
33.16	28.27	20.55	33.60	28.56	20.25	33.28	28.62	20.44	32.55	28.38	19.89
32.56	28.12	20.89	33.56	28.26	20.21	33.63	28.46	20.36	32.49	28.45	19.88
32.12	28.47	20.19	33.25	28.54	20.28	33.26	28.21	20.38	32.59	28.98	19.95
32.52	28.16	20.19	33.28	28.55	20.28	33.45	28.59	20.45	32.26	28.76	19.96
32.06	28.17	20.47	33.28	28.62	20.58	33.56	28.56	20.44	32.88	28.69	19.97
32.17	28.19	20.12	33.16	28.66	20.54	33.52	28.63	20.40	32.76	28.47	19.99
32.14	28.46	20.12	33.45	28.51	20.59	33.52	28.42	20.46	32.32	28.46	20.12
32.14	28.19	20.17	33.12	28.17	20.59	33.29	28.31	20.38	32.98	28.56	20.09
32.56	28.31	20.89	33.18	28.19	20.55	33.31	28.36	20.45	32.72	28.32	20.01
TOTAL											
1611.98	1413.21	1023.98	1660.04	1422.87	1025.94	1674.06	1422.18	1022.19	1632.23	1425.07	998.94
ARITHMETIC MEAN											
32.23	28.26	20.47	33.20	28.45	20.51	33.48	28.44	20.44	32.64	28.50	19.97
STANDARD DEVIATION											
0.706	0.502	0.312	0.280	0.261	0.238	0.205	0.20	0.211	0.241	0.242	0.226

Coriolus versicolor			*Coriolus versicolor* control			*Coriolus versicolor* with dung			*Coriolus versicolor* with dung control		
L	a	b	L	a	b	L	a	b	L	a	b
32.98	28.05	20.58	33.06	27.98	20.68	33.89	28.81	21.16	33.16	28.89	20.56
32.69	28.05	20.66	33.09	27.86	20.66	33.90	28.83	21.16	33.05	29.06	20.98
32.99	27.98	20.67	32.98	28.19	20.81	34.01	28.79	21.22	33.48	28.74	20.94
33.1	27.67	20.78	32.97	28.06	20.55	34.01	28.64	21.36	33.16	28.66	21.06
33.06	27.99	20.77	33.00	28.27	20.89	33.96	28.46	21.48	33.27	29.12	20.48
33.09	28.12	20.19	33.19	28.28	20.96	33.78	28.13	21.03	33.56	29.14	20.66
32.96	28.16	20.16	33.19	28.09	20.74	33.79	28.94	21.36	33.45	29.00	20.59
33.14	28.31	20.35	33.21	28.13	20.74	33.97	28.92	21.48	33.48	28.85	20.78
33.48	28.30	20.38	33.14	28.06	20.77	34.16	28.99	21.44	33.06	28.67	20.89
33.45	27.95	20.56	33.19	28.16	20.58	34.15	29.06	21.27	33.09	28.77	20.99
32.56	27.91	20.77	33.27	27.99	20.56	34.00	29.01	21.20	33.44	28.77	21.12
32.96	27.86	20.89	33.20	27.81	20.64	33.85	28.78	21.23	33.29	28.56	21.16
32.98	28.12	20.80	33.18	28.12	20.67	33.87	28.89	21.36	33.16	28.59	21.18
32.78	28.16	20.81	33.19	28.11	20.72	33.89	28.89	21.59	33.15	28.59	20.89
32.96	28.11	20.86	33.09	28.32	20.78	33.81	28.71	21.55	33.88	28.96	20.97
33.12	28.19	20.65	33.16	28.41	20.77	33.83	28.69	21.36	33.87	28.99	20.67
33.06	28.20	20.54	33.16	28.36	20.56	33.95	28.69	21.25	33.85	28.97	20.36
33.06	28.21	20.87	33.15	28.09	20.66	33.95	28.56	21.24	33.69	28.65	20.39
33	28.32	20.91	32.96	28.07	20.82	33.94	28.54	21.26	33.68	28.52	20.87
33.17	28.33	20.90	32.94	28.27	20.88	33.98	28.81	21.49	33.57	28.54	20.77
32.89	27.99	20.86	33.06	28.12	20.83	34.01	28.88	21.40	33.46	28.74	20.68
32.9	28.00	20.87	33.03	28.16	20.73	34.06	28.86	21.31	33.67	28.36	20.97
32.97	28.12	20.76	33.21	28.16	20.76	33.87	28.77	21.39	33.96	29.05	20.91
32.98	28.06	20.78	33.26	28.18	20.71	33.76	28.97	21.34	33.98	28.97	20.90
32.98	28.15	20.76	33.31	28.14	20.72	33.74	28.63	21.34	34.06	28.68	21.12
33.13	28.17	20.70	33.16	28.01	20.79	33.79	28.97	21.20	34.06	28.64	21.01
33.14	28.06	20.69	33.14	28.01	20.73	33.94	28.81	21.60	34.00	28.73	20.98
33.19	28.08	20.76	33.16	28.25	20.76	33.96	28.86	21.38	33.49	28.76	20.78
33.05	27.95	20.82	33.06	28.14	20.72	33.96	28.85	21.35	32.96	28.67	20.66
33.01	27.99	20.84	33.15	28.11	20.79	33.76	28.81	21.44	33.79	28.78	20.62
32.89	27.66	20.49	33.16	28.00	20.85	33.54	28.81	21.45	33.76	28.89	20.37

Coriolus versicolor			*Coriolus versicolor* control			*Coriolus versicolor* with dung			*Coriolus versicolor* with dung control		
L	**a**	**b**	**L**	**a**	**b**	**L**	**a**	**b**	**L**	**a**	**b**
32.89	28.15	20.51	32.99	28.35	20.76	33.52	29.00	21.44	33.52	28.87	20.89
32.96	28.06	20.59	33.00	28.01	20.81	34.01	28.69	21.56	33.59	28.85	20.89
32.77	28.34	20.94	33.16	27.99	20.81	34.06	28.71	21.31	33.87	28.64	21.00
33.00	28.31	20.88	33.32	27.97	20.76	33.96	28.87	21.30	33.69	28.48	20.64
33.03	28.21	20.76	33.30	28.06	20.74	33.99	28.84	21.12	33.87	28.46	20.49
33.10	28.06	20.71	33.08	28.12	20.76	33.92	28.86	21.16	33.48	28.62	20.49
33.17	28.07	20.68	33.14	28.21	20.76	33.95	28.84	21.16	33.89	28.60	21.19
32.99	28.01	20.66	33.17	28.16	20.62	33.92	28.80	21.26	33.68	28.48	21.17
32.95	28.00	20.48	33.17	28.14	20.66	33.69	28.80	21.34	33.66	28.89	20.56
32.95	28.12	20.52	33.16	28.31	20.86	33.67	28.99	21.36	33.67	28.89	20.57
32.84	28.15	20.52	33.22	28.28	20.84	33.95	28.95	21.31	33.48	28.93	20.36
33.10	28.16	20.52	33.28	28.06	20.87	33.92	28.94	21.39	33.77	28.95	20.38
33.12	28.06	20.76	33.07	28.11	20.74	33.90	28.86	21.41	33.98	28.95	20.52
33.01	28.15	20.70	33.06	28..10	20.77	34.00	28.79	21.17	34.03	29.02	20.56
33.06	28.14	20.72	33.06	28.04	20.76	33.97	28.82	21.39	34.00	28.54	20.82
33.11	28.06	20.74	33.16	28.07	20.71	33.90	28.87	21.56	33.45	28.59	20.88
33.13	28.08	20.83	33.18	28.09	20.76	33.92	28.83	21.55	33.48	28.71	20.54
33.01	28.17	20.82	33.14	28.14	20.74	33.89	28.83	21.54	33.63	28.70	20.56
33.00	28.05	20.31	33.17	28.16	20.73	33.96	28.80	21.41	33.77	28.76	20.23
TOTAL											
1650.91	1404.57	1034.08	1656.85	1406.28	1037.29	1695.18	1440.45	1067.43	1680.04	1438.24	1038.05
ARITHMETIC MEAN											
33.01	28.09	20.68	33.13	28.12	20.74	33.90	28.80	21.34	33.60	28.76	20.76
STANDARD DERIVATION											
0.153	0.141	0.184	0.092	0.121	0.086	0.127	0.157	0.134	0.299	0.189	0.256

APPENDIX IX: Published papers

1. Auer, N.; Covington, A.D.; Evans, C.S.; Natt, M.; Tozan, M. (1999). Enzymatic removal of dung from hides. *Journal of the Society of Leather Technologists and Chemists*, **83**, 215-219.

2. Covington, A.D.; Tozan, M., Evans, C.S. (1999). Enzymatic removal of dung from animal hides and skins. Proceedings of the XXV International Union of Leather Technologists and Chemists Congress, Chennai, India, 355-361. ISBN: 0-07-463562-X

Journal of the Society of Leather Technologists and Chemists Vol. 83 p. 215

ENZYMATIC REMOVAL OF DUNG FROM HIDES

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Summary

Dung cladding presents problems in the processing of hides into leather. Together, cellulose, hemicellulose and lignin comprise 80% of the composition of bovine dung, which reflects the lignocellulose content of vegetable fodder. Dung is attached to the hides by physical entrapment in the hairs forming a 3-dimensional matrix with the hair. Removal of dung from hide pieces was effected by the action of cellulase, xylanase and laccase separately over 20-24 hours. Mixtures of these enzymes removed dung in 6-8 hours demonstrating that synergistic activity is more effective than separate enzyme treatments. The preferred enzyme mixture for optimal removal of dung within 8 hours at 25-35 C, at an economic cost, is 5.0 units per ml of cellulase with 1.0 unit per ml of xylanase, under the laboratory experimental conditions. Sodium chloride, up to 3 molar concentration, enhanced enzyme activity by 20% for cellulase and 100% for xylanase.

Introduction

Dung clad hides pose problems for tanners in the production of high quality leathers. Dirty hides can carry an average of 3.7 kg of dung per hide[1], but it has been reported that the dirtiest hides can have up to 20 kg of dung on them.[2] Attempts have been made to clean dung from cattle before they enter the abattoir: these have ranged from warm showers to scrubbing with stiff brushes, the former was ineffective and the latter was both ineffective and scratched the skin, devaluing the hide.[3]

Tanners attempt to remove the dung in the earliest step of wet-processing, when the salted hides are washed in water to remove the preserving salt and as much of the extraneous dirt and debris as possible. However, much hardened dung can still remain on the hides after this process. Enzymes have been used to assist in soaking, including proteases, amylases and lipases in conjunction with detergents.[4] Typically with limited success in removing dung. For successful treatment, the choice of enzymes should depend on their appropriate specificity. Proteases are specific to the degradation of proteins, amylases are specific to the degradation of starch and lipases are specific to the degradation of triglyceride grease: none of these substrates are present in any great quantity in dung. Enzymes appropriate for the removal of dung should be capable of degrading the major components of the dung and breaking the attachment of the dung to the hides.

Enzymes have been traditionally used in the beamhouse for a long time, to effect removal of unwanted proteins, fats and cellular debris in bating, while new enzymes are being introduced to assist in unhairing reactions.[5] The role of biotechnology in the tanning industry was reviewed some years ago[6], when it was shown that biological agents can replace some of the chemicals used for leather production, leading to a reduced chemical loading in the effluent, shortening of process times and increased opening up of the fibre structure. Further use of enzymes should lead to cleaner hides with less wastage for the industry, while minimising problems of effluent clean up.[7,8] Complete removal of dung in soaking will reduce damage to the hides and permit hides to be available for green splitting[9] and allow the hair to be machine shaved.[10]

In this paper we describe the application of enzymes, new to the leather industry, which are successful in removing dung from heavily clad hides and demonstrate the potential for implementing this biological treatment into the earliest steps of leather processing.[11]

Presented by Prof. C. S. Evans at the SLTC Annual Conference at Windermere, September 1998.

Materials and methods

Analysis of dung

Proximate analysis was carried out because the complexity of the composition of plant materials makes complete analysis extremely difficult. When proximate analysis is used the percentages of the different fractions may not add up to 100. Before analysis, dung samples were broken up and dried to equilibrium in air.

Ether Soluble Fraction: 5 g of air dried dung was extracted with ether for 15 hours in a Soxhlet apparatus. The solvent was evaporated and the residue dried for 12 hours at 100 C and then weighed.

Cold water extract: The material left after ether extraction was transferred to a beaker and mixed with 150 ml distilled water. After 24 hours the aqueous extract was filtered. The plant residue was washed 2 or 3 times with distilled water. The filtrate and washings were evaporated on a water bath. The residue was dried at 100 C to constant weight.

Hot water extract: The same plant material was treated with 150 ml distilled water on a boiling water bath for 3 hours. The material was filtered and the extract treated in the same way as the cold water extract.

Holocellulose isolation from dung: Holocellulose was isolated from the dung using the method described by Jermyn.[12] The dung was first treated by boiling it for 30 min. in 85% ethanol. Then 5 g of sample was treated several times with glacial acetic acid and sodium chlorite. Isolated holocellulose was used for the determination of the cellulose and hemicellulose content of the dung.

Cellulose fraction: The method used to isolate cellulose from the holocellulose was based on the method

described by Corbett.[13] A weighed sample of about 3 g of air dried holocellulose was treated with 35 ml of 17.5% aqueous sodium hydroxide for 5 minutes and then macerated with a glass rod for 10 minutes. During the maceration 40 ml of alkali were added in 10 ml portions. Then the sample was allowed to stand for 30 minutes. 75 ml of distilled water were added and the residue was immediately filtered off and washed with distilled water. It was then soaked for 5 minutes in 40 ml of 10% acetic acid. Finally, the residue was washed with ethanol and ether and dried to constant weight at 100 C.

Hemicellulose fraction: The method used to isolate hemicelluloses was based on that described by Whistler and Feather.[14] Deoxygenated sodium hydroxide solution was prepared by passing nitrogen through Fieser's solution (20 g of potassium hydroxide and 2 g of anthraquinone-2-sulphonic acid in 100 ml of water) before bubbling through warm sodium hydroxide solution for at least two hours. About 2 g of holocellulose were first treated with 10% deoxygenated sodium hydroxide solution. Then the sample was shaken gently for 20 hours at 20°C, filtered and washed with alkali solution, followed by distilled water. The filtrate and washings were cooled in an ice bath. The solution was acidified to pH 5.0 with 50% acetic acid. This causes part of the hemicellulose (hemicellulose A) to precipitate. The precipitate was removed and dehydrated by washing with 95% ethanol, then acetone and then ether. The remainder of the hemicellulose (hemicellulose B) was precipitated out of the solution by adding acetone. The precipitate was centrifuged off and dehydrated by washing with ether. The two types of hemicellulose were combined and dried for 24 hours at 40°C.

Lignin fraction: The method described by Adams was used to isolate lignin from dung.[15] The dung sample was extracted in a Soxhlet extractor for 4 hours with 95% ethanol. The extraction process was continued for a further 4 hours using 1:2 v v ethanol and toluene. The cellulose was removed from the sample by treating it with 15 ml of 72% sulphuric acid. The sample was placed in a water bath at 20°C for 2 hours. After that, 560 ml of distilled water was added and the sample was boiled for 4 hours. The lignin was allowed to settle and then it was filtered off. The lignin was washed with hot water to remove acid and then it was dried at 100°C to constant weight.

Protein: The protein fraction of the dung was determined by the standard Kjeldahl method, applying the factor of 6.25 to convert the nitrogen value.[16]

Microscopic examination of dung-clad hide

The physical interaction between dung and hide was examined using conventional light microscopy.

Materials

All of the enzymes used were obtained from Sigma Chemical Co., Poole, UK with the exception of laccase, which was purified from liquid cultures of the white-rot fungus *Coriolus versicolor*.[17,18] Enzymes were prepared in buffer solution at pH 5.0 (50 mM acetic acid–sodium acetate) or pH 7.0 (50 mM potassium dihydrogen orthophosphate–dipotassium hydrogen orthophosphate), depending on the optimum pH of the enzyme activity, applying the assay methods as described by Sigma.

The enzymes were assayed by measuring the product of their reactions, reducing sugars, using DNS reagent (0.25 g 3,5-dinitrosalicylic acid, 75 g sodium potassium tartrate, dissolved in 50 ml 2 M NaOH, diluted to 250 ml with water). 1 ml DNS reagent was heated with 1 ml of test solution at 100°C for 10 min. After cooling, the absorbance at 570 nm was recorded and the amount of reducing sugar produced determined by reference to a calibration graph using glucose, xylose, mannose or galactose as appropriate. One unit of enzyme is defined as the amount of enzyme that will liberate 1 mole of reducing sugar, measured as glucose equivalents, from substrate per minute at 30 C.

For a protease, one unit of activity is defined by hydrolysing casein to produce colour equivalent to 1 μmole (181 μg) of tyrosine per minute at pH 7 at 37 C.[16]

For a laccase, 1 unit of activity produces a rate of change in absorbance at 530 nm of 0.001 per minute at pH 6.5 at 30°C in a 3 ml reaction volume, using syringaldazine as substrate.

Enzyme treatment of pieces of dung clad hide

Hides were purchased from Midland Meat Packers Ltd., Crick, UK. Pieces of dung clad hide, each 5 cm^2, were treated with different enzymes at pH 5.0 or 7.0, depending on their pH optima. 10 ml volumes of each of the enzymes, cellulase, xylanase, laminarinase, β-D-1,4-galactosidase, β-D-1,4-mannosidase, β-D-1,4-glucosidase and α-amylase were used, containing approximately equal activities, based on the production of reducing sugars, (50–100 units).

Samples of hide pieces and enzymes were agitated gently on a rotary shaker at ambient temperature for up to 24 hours. Dung removal was assessed by passing a spatula over the hair surface and giving a score on a scale of zero (no appreciable removal) to *** (easy removal).

Effect of sodium chloride on cellulase and xylanase activities

For cellulase, the release of glucose from carboxymethylcellulose, was measured with and without NaCl: the initial activity was 5.3 units of pH 5.0 in the absence of NaCl. For xylanase, the release of xylose from birch xylan, was measured with and without NaCl: the initial activity was 12 units at pH 5.0 in the absence of NaCl. Up to 3.0 M NaCl was added to the enzyme assays and activity was measured.

Results

Table I shows the composition of typical samples of dung from heavily clad hides: the two major components are cellulose and hemicellulose, together forming up to 58% by weight of the dung, with lignin the next major component at 21%. The remaining 20% by weight is composed of smaller amounts of ether and water soluble materials.

Microscopic observation of the attachment of dung to the surface of hides showed that the dung is entrapped by the hairs and does not normally penetrate to the surface of the epidermis (Fig. 1). It therefore seemed appropriate to attempt to remove the dung from hides with enzymes that digest the dung components, rather than those that might degrade the

TABLE I
Composition of dung

Component	Content (% on dry weight)
Cellulose	30
Hemicellulose	28
Lignin	21
Protein	6
Ether soluble	10
Cold water soluble	6
Hot water soluble	4

components of the animal's epidermis or the keratin of the hair. The physical attachment of dung to the hairs would be disrupted if the matrix of the dung was digested.

A comparison of enzyme activities showed that soaking with protease had little effect on removing the dung from pieces of hide over 24 hours under laboratory conditions of mild agitation (Table II). In contrast, the action of cellulase and xylanase (a hemicellulase that degrades xylans) produced easy dung removal. Laccase, a lignin degrading polyphenol oxidase, was the next most effective enzyme treatment. Other carbohydrases which attack starch (α-amylase), mannose or galactose containing hemicelluloses (β-D-mannosidase, β-D-galactosidase) or glucans (laminarinase, β-1,3-glucosidase or β-D-1,4-glucosidase) were no more effective than protease. Cellulase and xylanase treatments left the hairs intact and apparently clean and silky.

The reaction time for complete dung removal by cellulase was 18 hours, when either 2.5 or 5.0 units per ml were added to the hide pieces. With xylanase treatment, dung was completely removed in 24 hours with 2.5 units per ml of xylanase and in 20 hours with 5.0 units per ml. Removal of dung with moderate difficulty was observed from 14 hours with these two enzymes. Increasing the enzyme concentration from 2.5 to 5.0 units per ml did not result in any shortening of reaction time for cellulase and only minimal reduction in reaction time for xylanase. Combining the two enzymes reduced the time for removal of the dung to 8 hours at ambient temperature (Table III). The minimum enzyme concentrations to remove the dung in 8 hours with a combined enzyme treatment were 5.0 units per ml of cellulase and 1.0 unit per ml of xylanase.

The most rapid time achieved for dung removal was 6 hours, using a mixture of cellulase (5.0 units per ml), xylanase (5.0 units per ml) and laccase (0.008 units per ml), when the dung was washed from the hide pieces without any additional mechanical action. Increasing the temperature of the reaction to 35 C did not reduce the time for dung removal further.

Because the commonest rawstock is wet-salted hide, the effect of salt on the activities of the enzymes is relevant to the proposed process. Different concentrations of sodium chloride were added into the assay solutions of cellulase and xylanase, to observe the effect on their activities. Fig. 2 shows that increasing the salt concentration up to 3.0 molar produced a doubling of the measured xylanase activity (initially 1.2 units per ml) in 50 mM acetate buffer at pH 5.0. Cellulase activity (initially 5.3 units per ml) was enhanced by about 20% in the presence of 1.5–3.0 M NaCl.

Figure 1. Light micrograph of dung on hide (mag. × 10).

Discussion

Analysis of dung showed that up to 80% of its composition is accounted for by three major components, cellulose, hemicellulose and lignin, which reflect the lignocellulose composition of the animal feed. Traditional feeds such as grasses contain mostly cellulose (46%) and hemicellulose (16%) in the plant cell walls

TABLE II
Effects of enzymes on dung removal from pieces of dung clad hides

Enzyme	Activity (units per ml)	pH	Removal score
No enzyme	0	5	0
Cellulase	5	5	***
Xylanase	5	5	***
Laminarinase	5	5	*
β-D-galactosidase	5	5	*
Laccase	0.2	5	**
No enzyme	0	-	0
Protease	5	-	*
α-amylase	5	-	*
β-D-1,4-mannosidase	5	-	*
β-D-1,4-glucosidase	5	-	*

*** easy removal; ** removed with moderate difficulty; * difficult and incomplete removal; 0 no appreciable removal.

TABLE III
Rate of dung removal from dung-clad hide pieces with cellulase and xylanase

Enzymes	6 h	12 h	14 h	16 h	18 h	20 h	24 h
Cellulase (5 U ml)	*	*	**	**	***	***	***
Cellulase (2.5 U ml)	*	*	**	**	***	***	***
Xylanase (5 U ml)	0	*	**	**	**	***	***
Xylanase (2.5 U ml)	0	*	*	**	**	**	***

*** easy removal; ** removed with moderate difficulty; * difficult and incomplete removal; 0 no appreciable removal.

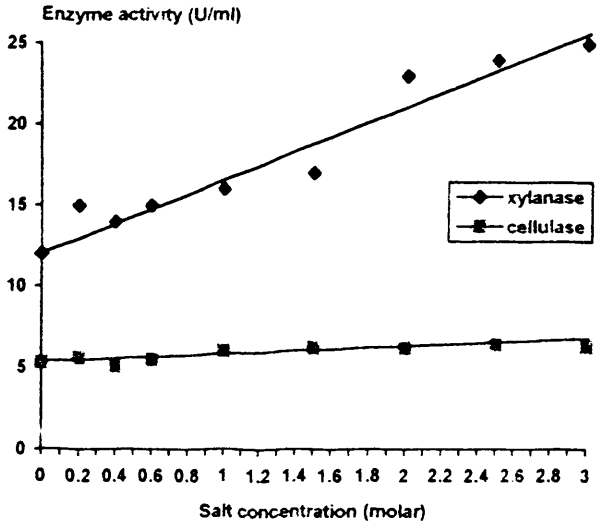

Figure 2. The effect of sodium chloride concentrations on the activity of xylanase and cellulase.

with lesser amounts of lignin (10%).[19] On digestion by ruminants, some of the cellulose and a small amount of hemicellulose are hydrolysed to glucose and other sugars by rumen microflora, but the lignin remains undigested. As a result, a greater proportion of lignin relative to cellulose and hemicellulose remains in the dung, compared with that in grasses. Lignin is a three-dimensional polymer, composed of phenylpropanoid units, which forms a protective matrix around the cellulose fibres and is covalently bound to hemicellulose. This "lignin-barrier" restricts access to cellulose and hemicellulose by microbial enzymes, so reducing their efficiency in abstracting glucose from the carbohydrate polymers.[20]

The attachment of dung to the hides is a physical process with the hairs forming a matrix around which the dung is embedded, producing a material similar to that used traditionally in wattle and daub when dried. Therefore the dung could be removed either by breaking the hairs or by degrading the cellulose-hemicellulose-lignin complex. Breaking the hairs is feasible, but intact keratin is resistant to proteases, leaving only keratinase or conventional unhairing chemicals as degrading options. Keratinases are not favoured on grounds of the health risk to operatives; conventional hair burning accomplishes the task, but unless unhairing is separated from the opening up part of the process, the dung will be solubilised into the liming liquor, inevitably contributing to the effluent loading.

Feed additives such as oligosaccharides, and rumen microflora producing a range of extracellular polysaccharides with adhesive properties, might contribute to the adhesion of the dung.[21] However, carbohydrases, other than cellulase and xylanase, were only marginally effective in removing the dung. So, it is unlikely that other polysaccharides are important in the mechanism of dung removal.

Although cellulase, xylanase and laccase separately removed the dung from the hide pieces, the time of reaction was between 18 and 24 hours. Cellulase hydrolyses the cellulose fibres, xylanase hydrolyses the xylan containing hemicelluloses, while breaking the covalent bonds linking lignin and hemicellulose, and laccase attacks the lignin component. The effect of combined enzyme treatments was to reduce the time to 6 to 8 hours, demonstrating that dung removal is greatly facilitated by the synergistic activities of the enzymes. Inclusion of laccase with cellulase and hemicellulase effected removal of dung most easily, demonstrating that degradation of the three major components of dung had occurred. Six to 8 hours is sufficient time for efficient reaction to be accommodated in the soaking steps in the beamhouse, although dung removal is likely to be accelerated by the greatly increased level of mechanical action experienced by the hides, even under the typical conditions of gentle agitation.

Cellulases are available as inexpensive commercial enzymes, for example used in detergents[22] and xylanases, although more expensive, are being used commercially in preparing animal feeds[23] and in some pulp and paper mills.[24] Laccase is the most expensive of the three enzymes with small, though increasing commercial use. It will be important to supply an enzyme treatment at minimal cost to the leather industry for economic viability, so the most effective treatment at a minimum price should be defined. To this end, the preferred enzyme mixture for optimal removal of dung within 8 hours at 25-35°C is 50 units of cellulase with 10 units of xylanase under laboratory experimental conditions.

As hides are salted for preservation, the first dirt soak is needed to remove salt. The effect of sodium chloride on cellulase and xylanase is to enhance the activity of the enzymes, by approximately 20% and 100% respectively. Increasing the activity of the enzymes should result in less enzymes being required for the same action. In our experiments the pieces of hides were gently washed with the enzyme solutions, whereas in the tanneries the de-salting wash occurs in a variety of vessels, all of which generate a higher level of agitation

and vigorous washing action. With increased agitation of the dung clad hides, the efficacy of dung removal should be increased, and either the time for removal will be reduced or less enzyme be required over the same time. In addition, the common use of nonionic detergents in industrial processes is likely to assist the process. These options need to be tested under industrial soaking conditions in the tanneries on whole hides to establish a commercially viable process. These trials are in progress and will be reported separately.

(Received January 1999)

References

1 Raw Material Quality - Dung Survey - 1996. *BLC Journal*. December 1996. Restricted circulation.
2 Dung Survey - 1998. BLC Leather Technology Centre, June 1998. Restricted circulation.
3 IHATIS Congress. Amsterdam. April 1975.
4 Christner, J., Pfleiderer, F. and Taeger, T. UK Patent Application GB 2,233,665, 1991.
5 Taylor, M. M., Bailey, D. G. and Feairheller, S. H., *J. Amer. Leather Chem. Ass.*, 1987, **82**, 153.
6 Alexander, K. T. W., *J. Amer Leather Chem Ass.*, 1988, **83**, 287.
7 Dhar, S. C., *Leather Science*, 1974, **21**, 39.
8 Simoncini, A., *J. Amer Leather Chem. Ass.*, 1987, **82**, 225.
9 Simone, G. D., *IULTCS Congress, Philadelphia, USA*, October 1989.
10 Covington, A. D., *IULTCS Congress, Chennai India*, January 1999.
11 Covington, A. D and Evans, C. S., UK Patent Application A2325241, November 1998.
12 Jermyn, M. A., *Modern Methods in Plant Analysis*, 1955, **2**, 197.
13 Corbett, W. M., Determination of the alpha-cellulose content of cotton and wood cellulose. In: Whistler, R. L. (ed.) *Methods in Carbohydrate Chemistry*, 3: 27-28 (1963).
14 Whistler, R. L. and Feather, M. S., Hemicellulose Extraction from annual plants with alkaline solutions. In: Whistler, R. L. (ed.) *Methods in Carbohydrate Chemistry*, 5: 144-145 (1965).
15 Adams, G. A., Lignin determination. In: Whistler, R. L. (ed.) *Methods in Carbohydrate Chemistry*, 5: 185-187 (1965).
16 Rosenberg, S. L., *Developments in Industrial Microbiology*, 1979, **20**, 133.
17 Fahraeus, G. and Reinhammar, B., *Acta Chem. Scand.*, 1967, **21**, 2367.
18 Evans, C. S., FEMS *Microbiol Letts*, 1985, **27**, 339.
19 Jalc, D., Siroka, P. and Ceresnakova, Z., *J Gen. Applied Microbiol.* 1997, **43**, 133.
20 Reid, I. D., *Canadian Journal of Botany*, 1995, **73**, S1011.
21 Monson, P. F. and Paul, F., Oligosaccharide feed additives In *Biotechnology in Animal Feed and Animal Feedstuffs*, eds. Wallace, R. J. and Chesson, A. Publ. VCH, 233-246 (1995).
22 Eriksen, N. In: "*Industrial Enzymology*" eds. Godfrey, T. and West, S. 2nd edition. London, Macmillan Press, 189-200 (1996).
23 Cowan, W. D. In "*Industrial Enzymology*" eds Godfrey, T. and West, S. 2nd edition. London, Macmillan Press, 71-86 (1996).
24 Viikari, L., Kantelinen, A., Poutanen, K. *et al.* In "*Biotechnology in pulp and paper manufacture*" eds. Kirk, T. and Chang, H-M. Butterworth-Heinemann, Boston, USA, 145-151 (1990).

Proceedings of the XXV IULTCS Congress, 1999
Cleaner Production: Experience Sharing **CPO-8**

ENZYMATIC REMOVAL OF DUNG FROM ANIMAL HIDES AND SKINS

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Summary

One of the major problems facing the meat and leather industries is dung cladding on hides. Dung dried onto hides is difficult to remove during the early stages of processing, resulting in the necessity for prolonging the soaking step, with the consequence of risking bacterial damage to the hides.

Microscopic examination of dung on hide reveals that adhesion is between the dung and the hair alone, there is no sticking of the dung to the epidermis. This accounts for the difficulty in removing dry dung; the matrix of hair within the dung creates a strong composite material.

Analysis shows that major components of the composition of dung are lignocellulosic materials (lignin, hemicellulose and cellulose). This project concerns solubilising the dung faster, to reduce soaking time by targeting these components with enzyme technology, using hemicellulases, cellulases, polyphenol oxidases and ligninases from commercial products and a range of fungal organisms (*C.versicolor*, *T. reesei* and *A. niger*).

The new technology may have application for dung removal from live animals prior to slaughter for hygiene purposes.

Introduction

Contamination of hide with dung is common problem in many parts of the world. It occurs mainly with beef cattle; milk cattle are kept cleaner for prevention of mastitis which can affect milk production[1]. The dung problem happens mainly in the winter period of the year when cattle are housed[2]. There is usually only limited space in the housing and so after the animal has defecated it cannot move away and at some stage will either sit or lie in the faeces which stick to the hide and are carried around by animal[1]. Dirty hides can carry an average of 3.7 kg of dung per hide[3], but it has been reported that the dirtiest hides can carry up to 20 kg of dung on them[4].

Contamination of animals with dung and dirt can cause a number of problems at the farm, the abattoir and the tannery. Excessive amounts of dung on animals affect the quality of milk, meat and the subsequent leather[5].

Tanners attempt to remove the dung in the earliest step of wet processing, when the salted hides are washed in water to remove the preserving salt and as much of the extraneous dirt

and debris as possible. However, much hardened dung can still remain on the hides after this process. Enzymes have been used to assist in dung removal, including proteases, amylases and lipases in conjunction with detergents, but with limited success. For successful treatment, the choice of enzymes should depend on their appropriate specificity. Proteases are specific to the degradation of proteins, amylases are specific to the degradation of starch and lipases are specific to the degradation of triglyceride grease; none of these substrates are present in any great quantity in dung, because the majority of the material is made up of lignocellulosic compounds[6-8] (see also Table I). Enzymes appropriate for removal of dung should be capable of degrading the major components of the dung and breaking the attachment of the dung to the hides.

It is well known that proteases have been used in the bating operation for several years to effect the removal of nonstructural proteins and residual epidermis, while new enzymes are being introduced for soaking, dehairing and degreasing[9]. Biological treatments can replace some of the chemicals used for leather production and lead to a reduced chemical load for effluent clean-up, shortening of processes time, increased opening up of the fibre structure. Further use of enzymes should lead to cleaner hides with less wastage for the industry, while minimising problems of effluent clean-up[10,11]. The role of biotechnology in the tanning industry was examined some years ago[12].

Complete removal of dung in soaking will reduce damage to the hides and permit more hides to be available for green-splitting[13]. In this paper we describe enzymes, novel to the tanneries, which are successful in removing dung from heavily-clad hides and demonstrate the potential for implementing this biological treatment into the earliest steps of leather processing.

Experimental

Analysis of dung

Before analysis, dung samples were dried to equilibrium in air and residual hair was removed. Proximate analysis was carried out because the complexity of the composition of plant materials makes complete analysis extremely difficult. When proximate analysis is used the percentage of the different fractions cannot be expected to add up 100%. Holocellulose was isolated from the dung using the method given by Jermyn[14] The isolated holocellulose was used for determination of cellulose and hemicellulose fractions of dung by using the methods given by Corbett[15] and Whistler et al[16]. The lignin fraction of dung was determined by using the method of Adams[17]. The protein fraction of dung was determined using the Kjeldahl method (Kjeldahl nitrogen value was 6.25)[18]. Details of the techniques and analytical methods are described in a forthcoming publication[19].

Enzymes

All enzymes were obtained from Sigma Chemical Co., Poole, UK with the exception of laccase, which was purified from liquid cultures of the white-rot fungus *Coriolus versicolor*[20,21].

Culture media for extracellular enzyme production

Coriolus versicolor, *Trichoderma reesei* and *Aspergillus niger* were maintained on a solid medium consisting of 2% (w/v) nutrient agar, supplemented with 3% (w/v) malt extract on

plates. Three different culture media were used for *Coriolus versicolor* to produce extracellular enzymes. The first medium was designed to produce laccase[21]. The second medium was designed to produce different lignocellulosic enzymes[22]. For this reason, 5% carboxymethylcellulose was added into the medium for modification. In the third medium, modified second medium was supplemented with 5% dry cattle dung. *Trichoderma reesei* and *Aspergillus niger* were incubated by using the medium of Abrams[23]. The medium was modified by addition of 15 g/l carboxymethylcellulose and 3 g/l xylan as a carbon source. All media were incubated for 7 days at 25°C and enzyme activities were assayed after this period.

Enzyme treatment of pieces of dung-clad hide and dung powder
Pieces of dung clad hide, each 5 cm^2, were treated with different enzymes, at pH 5.0 or 7.0 depending on their pH optima. Samples of hide pieces and enzymes were agitated gently on a rotary shaker at ambient temperature for up to 24 hours. Dung removal was assessed by passing a spatula over the hair surface and giving a score using the following scale:

***	easy removal;
**	removed with moderate difficulty;
*	difficult and incomplete removal;
0	no appreciable removal.

Experiments were also conducted to understand the detailed mechanism of dung degradation by using cellulase, xylanase and β-glucosidase. 5 g of moisture free dung samples were treated with different commercial enzymes and enzyme mixtures in rotary shaker at ambient temperature for up to 24 hours. Sample solution was taken from experimental bottles to analyse chemical oxygen demand.

Effect of sodium chloride on cellulase and xylanase activities:
For cellulase, the release of glucose from carboxymethylcellulose, was measured with and without NaCl. The initial activity was 5.3 units at pH 5.0 in the absence of NaCl. For xylanase, the release of xylose from birch xylan, was measured with and without NaCl. The initial activity was 12 units at pH 5.0 in the absence of NaCl. Up to 3 .0 M NaCl was added to the enzyme assays and activity measured.

Results and Discussion

The composition of typical samples of dung from heavily-clad hides was determined. Whilst typically there will be variations in analysis, depending on diet, the results represented in Table I are likely to be representative of the usual constitution. The two major components were cellulose and hemicellulose forming together up to 58% by weight of the dung, with lignin the next major component at 21%. The remaining 20% by weight was composed of smaller amounts of ether and water soluble materials. Hence, it is improbable that attacking the small protein content with proteases or applying amylase to attack starch that is not present in any substantial concentration would have a significant effect on solubilising or at least degrading the integrity of the dung.

Table 1: Composition of Dung (% based on dry weight basis)

COMPONENT	CONTENT
Cellulose	30%
Hemicellulose	28%
Lignin	21%
Protein	6%
Ether soluble	10%
Cold water soluble	6%
Hot water soluble	4%

Microscopical investigation of dung on hide revealed that adhesion is between the dung and hair alone; there is no sticking of the dung to the epidermis This accounts for the difficulty in removing dry dung; the matrix of hair within the dung creates a strong composite material. Indeed, mixing wet cattle dung with hair is still used as a building material in the developing economies, in much same way as the traditional filling of wattle and daub. This observation makes it possible to consider a treatment for removing dung from live animals. If the dung was stuck to the hide itself, any attempt to remove it would involve an invasive procedure, since the epidermis would be breached, and this would be inevitably cause distress to the animal. However, because the target is the interaction between the dung and the hair, enzyme treatments are non-invasive and the enzymes under investigation cannot target skin surface components, because they are specific to lignocellulosic components.

A comparison of enzyme activities showed that treatment with cellulase and xylanase produced easy dung removal (Table 2). Laccase, a lignin-degrading polyphenol oxidase, showed the next effective enzyme treatment in terms of dung removal. However, treatment with protease and α-amylase had little effect on removing the dung from hide over 24 hours. Other carbohydrases, like laminarinase, β-D-galactosidase, β-D-1,4-glucosidase and β-D-mannosidase, were no more effective than protease. Cellulase and xylanase treatment left the hairs intact and apparently clean and silky. During the digestion, rumen microflora produce a range of polysaccharides with adhesive properties, which might contribute to the adhesion of the dung. However, carbohydrates, other than cellulase and xylanase, were only marginally effective in removing the dung. Therefore, it is unlikely that other polysaccharides are important in the mechanism of dung removal[19].

Table 2: Effects of enzymes on dung removal from pieces of dung-clad hides

Enzyme	Units of activity ($U\ ml^{-1}$)	pH	Removal score
No enzyme	0	5	0
Cellulase	5	5	***
Xylanase	5	5	***
Laminarinase	5	5	*
β-D-galactosidase	5	5	*
Laccase	0.2	5	**
No enzyme	0	7	0
Protease	5	7	*
α- amylase	5	7	*
β-D-1,4-mannosidase	5	7	*
β-D-1,4 -glucosidase	5	7	*

The reaction time for complete dung removal by cellulase was 18 hours when either 2.5 or 5.0 units ml^{-1} were added to the hide pieces (Table 3). With xylanase treatment, dung was completely removed in 24 hours with 2.5 units ml^{-1} xylanase and in 20 hours with 5.0 units ml^{-1}. Removal of dung with moderate difficulty was observed from 14 hours with these two enzymes. Increasing enzyme concentration from 2.5 to 5.0 units ml^{-1} did not result in any shortening of reaction time for cellulase, and only minimal reduction in time for xylanase. Combining the two enzymes reduced the time for removal of the dung to 8 hours at ambient temperature. The minimum enzyme concentrations to remove the dung in 8 hours with a combined enzyme treatment were 5.0 units ml^{-1} of cellulase and 1.0 unit ml^{-1} of xylanase.

Table 3: Rate of dung removal from dung-clad hide pieces with cellulase and xylanase

Enzymes	6 h	12 h	14 h	16 h	18 h	20 h	24 h
Cellulase (5 U/ml)	*	*	**	**	***	***	***
Cellulase (2.5 U/ml)	*	*	**	**	***	***	***
Xylanase (5 U/ml)	0	*	**	**	**	***	***
Xylanase (2.5 U/ml)	0	*	*	**	**	**	***

The most rapid time achieved for dung removal was 6 hours using a mixture of cellulase (5.0 units ml^{-1}), xylanase (5.0 units ml^{-1}) and laccase (0.008 units ml^{-1}) when the dung was washed from the hide pieces without any additional mechanical action (Table 4).

Table 4: The influence of ligninase on dung removal

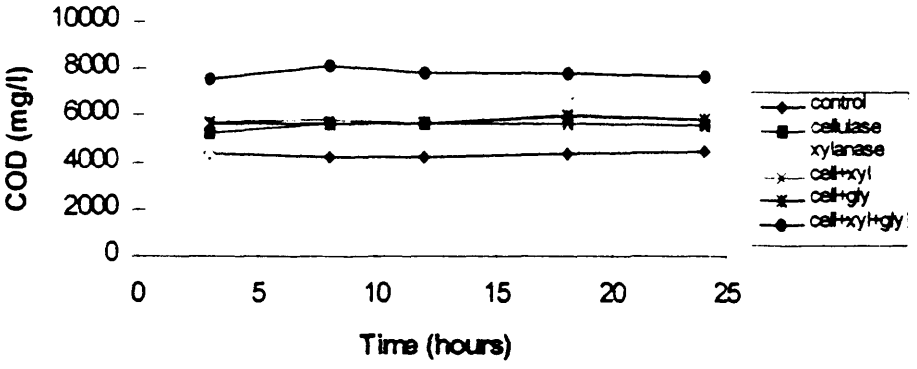
Enzyme(s) and Concentration	Score
0.016 units laccase	**
0.008 units laccase 5 units cellulase 5 units xylanase	****[a]
0.008 units laccase 2.5 units cellulase 2.5 units xylanase	***

[a] this score goes outside the scale defined, because the dung was removed the need for any appreciable mechanical action.

Although cellulase, xylanase and laccase separately removed the dung from the hide pieces, the time of reaction was between 18 and 24 hours. Cellulase hydrolyses the cellulose fibres, xylanase hydrolyses the xylan containing hemicelluloses, while breaking the covalent bonds

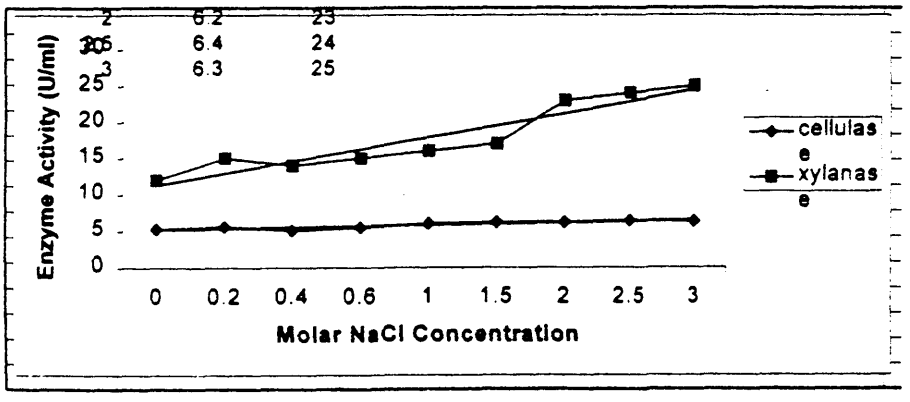
linking lignin and hemicellulose, and laccase attacks the lignin component. The effect of combined enzyme treatments reduced the time to 6 hours to 8 hours, showing that dung removal is greatly improved by the synergistic activities of the enzyme. Fig. 1 shows that there is synergism between cellulase, xylanase and β-glucosidase. When they are used together, they produced the highest COD results in solution.

Fig. 1: Effect of different enzymes and enzyme mixtures on solubilisation of dung



As hides are usually salted for preservation before delivery to the tanneries, the effect of salt on the activities of the enzymes is relevant to the proposed process. Varying concentrations of NaCl were added to assay mixtures of cellulase and xylanase respectively to observe the effect on their enzyme activities. Fig. 2 shows that increasing the NaCl concentration up to 3.0 M produced a doubling of the measured xylanase activity in the assay (initially 12 units $ml^{-1)}$ in 50 mM acetate buffer at pH 5.0. Cellulase activity (initially 5.3 units ml^{-1}) was enhanced by about 20% in the presence of 1.5 - 3.0 M NaCl. Increasing the activity of the enzymes should result in less enzymes being required for the same action. In the experiment the hides were tumbled with enzyme solution. However, in the tannery conditions, a variety of processing vessels are used in soaking process to generate a higher level of agitation. It should be expected that with increased agitation of the dung clad hides, the efficiency of dung removal would be increased; either time for removal will be reduced or less enzyme will be required over the same time. Moreover, using nonionic detergents is common practice in soaking process. Trials are in progress to determine the effect of nonionic detergents on dung removal and will be reported separately.

Fig. 2: Effect of sodium chloride concentration on the activity of xylanase and cellulase



One of the aims in this project is not only to use commercial lignocellulosic enzymes, but also produce enzymes from fungal cultures. We can modify fungal growth conditions in order to achieve the most appropriate enzyme mixture for dung removal. Table 5 shows that *Aspergillus niger* produces highest xylanase, β-glucosidase and β-xylosidase activity, however, *Trichoderma reesei* can produce the highest cellulase activity. The highest laccase activity was obtained when *Coriolus versicolor* was grown in Fahraeus medium. *C.versicolor*, which was grown in carboxymethycellulose medium supplemented with cattle dung, can produce the enzymes which are the most useful for dung removal from hide, with appropriate activity.

Table 5: Enzyme activities of different cultural filtrates

	Protein (mg/ml)	Cellulase (U/ml)	Xylanase (U/ml)	β-glucosidase (U/ml)	β-xylosidase (U/ml)	Laccase (U/ml)
C. versicolor (Fahraeus medium)	0.755	-	-	0.104	0.0132	0.12*
C. versicolor (CMC medium)	0.316	5.56	3.66	0.0154	-	0.002
C.versicolor (CMC medium + dung)	1.95	6.3	10.6	0.291	0.0178	0.044
Aspergillus niger (Abrams medium)	0.680	7.36	20.8*	1.755*	4.26*	-
Trichoderma reesei (Abrams medium)	1.023	10.6*	10.5	0.198	1.021	-

Experiments are still in progress to investigate the detailed chemistry and kinetics of dung removal using commercial enzymes and fungal cultures, and to investigate the effects of these enzymes on hide components and the properties of the leather produced.

Acknowledgement

The authors wish to thank Fungal Biotechnology Group in University of Westminster for their cooperation in this project and Turkish Ministry of National Education, General Directorate for Higher Education for their financial support.

References

1. *Leather*, May 1994, p.14.
2. Pearson, R.P. *World Leather*, May 1995, p.26.
3. Raw Material Quality- Dung Survey- 1996, BLC Journal, December 1996. Restricted circulation.
4. Dung Survey- 1998, BLC Leather Technology Centre, June 1998. Restricted circulation.
5. Stosic, P.J. Hide Improvement Project Report I: Dung Contamination of Cattle hides, BLC Leather Technology Centre, August 1996.
6. Bellamy,. W.D. *Chem. Eng. News*, 1972, **50**, 14.
7. Bellamy, W.D. Proc. Amer. Inst. Chem. Eng., Meeting, August 1972.
8. Rosenberg, S.L. and Wilke, C.R. Lignin Biodegradation and the Production of Ethyl Alcohol from Cellulose. In: Kirk, T.K. et al (eds.) *Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications.*, CRC Press, Florida, 1980, **2**, 199.
9. Taylor, M.M., Bailey, D.G. and Feairheller, S.H. *J. Amer. Leather Chem. Assoc.*, 1987, **82**, 153.

10. Alexander, K.T.W. *J. Amer. Leather Chem. Assoc.*, 1988, **83**, 287.
11. Dhar, S.C. *Leather Science*, 1974, **21**, 39.
12. Simoncini, A.J. *J. Amer. Leather Chem. Assoc.*, 1987, **82**, 225.
13. Simone, G.D. *Proc. IULTCS Congress, Philadelphia*, October 1989.
14. Jermyn, M.A. *Modern Methods in Plant Analysis*, 1955, **2**, 197.
15. Corbett, W.M. Determination of the alpha-cellulose content of cotton and wood cellulose. In: Whistler, R.L. (ed.) *Methods in Carbohydrate Chemistry*, 1967, **3**: 27.
16. Whistler, R.L. and Featler, M.S. *Methods in Carbohydrate Chemistry*, 1965, **5**, 144.
17. Adams, G.A. Lignin determination. In: Whistler, R.L. (ed.) *Methods in Carbohydrate Chemistry*, 5: 185, 1965.
18. Rosenberg, S.L. *Developments in Industrial Microbiology*, 1979, **20**, 133.
19. Auer, et al., *J. Soc. Leather Technol. Chem.*, in press.
20. Evans, C.S. *FEMS Microbial Letters*, 1985, **27**, 339.
21. Fahraeus, G. and Reinhammar, B. *Acta Chem. Scand.*, 1967, **21**, 2367.
22. Dodson et al., *FEMS Microbiology Letters*, 1987, **42**, 17.
23. Abrams, E. National Bureau of Standards Misc. Publ. No 188 US Dept. of Commerce, Washington, 1948.