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# Influence of fungal diversity and production of cellulolytic enzymes on decay of stored bagasse

by

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Submitted in fulfillment of the requirements for the degree of

**MAGISTER SCIENTIAE: MICROBIOLOGY**

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Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
University of Pretoria

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I, Nashveer Singh, declare that the thesis which I hereby submit for the degree M.Sc: Microbiology, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed in Pretoria on the 12th day of March 2008.

SIGNATURE: ..……..

In the attitude of silence, the soul finds the path in a clearer light, and what is elusive and deceptive, resolves itself into crystal clearness…..Our life is a long and arduous quest after truth.

Mahatma Gandhi

Dedicated to the loving memory of our little angel…….Orav.

# CONTENTS

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My greatest appreciation goes to the Divine Lord, without whom, none of this would be possible.

## PREFACE

Bagasse, a fibrous material derived from sugar cane, is an important alternative fibre for the pulp and paper industry (Atchinson, 1987; Venter, 1978; Salaber & Maza, 1971; Marshall 1938). The increasing demand for paper and the shortages in wood has set off research into various alternative non-wood sources of fibre (Venter, 1978), but bagasse is well established as a raw material in paper-making (Atchinson, 1988). Bagasse is suitable for use in the pulp and paper industry, because it is a waste material of an existing industry with transport and collection facilities already in place (Venter, 1978). However, sugar cane is a seasonal product and bagasse would need to be stored for extended periods of time. Having a residual sugar content of two to 3 %, and being a lignocellulosic residue, bagasse in storage is an ideal substrate for microbial colonisation (Ramaswamy *et al.*, 1989; Cusi, 1979). Severe losses of fibre are sustained due to decay, and large quantities become unsuitable for the pulping process (Atchison 1987; Rangamannar *et al.*, 1993). The role of bagasse in paper-making, and the storage and deterioration of bagasse, was reviewed in Chapter 1. The primary goal of the present study was to gain more comprehensive information on the environmental conditions, the microbial populations and their distribution patterns, and the holocellulose-degradation of stored bagasse. An understanding of the microbial succession and the environment could lead to better management of the bagasse pile. This could be achieved by putting into place procedural steps that would modify the immediate environment to minimize bagasse degradation by microbes. In doing so, the decay of fibre can be minimized and the current losses in bagasse yield can be alleviated.

Studies by Rangamannar *et al.* (1993) and Ramaswamy *et al.* (1989) concluded that fungi contributed largely to the losses in bagasse, therefore, the present study focused on the effects of fungi on bagasse. The environmental parameters inside and on the surface of a pile determine the presence and activity of fungi. Therefore, the temperature, pH and moisture content were measured throughout the study period. These data, presented in Chapter 2, have shown how the environment changes over time, and how the surface environment compares with the environment inside the bagasse pile. Since

different groups of microorganisms function optimally at specific temperatures, pH and aeration (Pelczar *et al.*, 1977), the profile of the environmental parameters in the bagasse pile were compared to prescribed conditions, in order to make recommendations for improved fibre preservation.

The environment on the studied bagasse pile was conducive to fungal growth, therefore, the fungi growing on the surface and the inside of the pile were isolated and enumerated, and the structural diversity evaluated. This approach was taken to gain deeper insight into the populations existing in and on the surface of the bagasse pile, and was discussed in Chapter 3. Microbial populations and their progression was compared to established diversity models to understand the status of bagasse decay (Magurran, 1988). Also, a- and ß-diversity calculations were done in order to determine where the most diverse and dominant microbial populations were present, and to compare the emerging populations at the surface and inside the pile over time.

The microbial numbers indirectly reflect potential decay of bagasse, while the influence of the environment on the pile is more indicative of microbial succession. The enzyme activities of a bagasse pile represent some of the biochemical processes that occur and, therefore, the main holocellulolytic enzymes, cellulases and xylanases, were evaluated in Chapter 4. The activities of the enzymes were thus applied as a more direct estimate of fibre damage in a bagasse pile.

The raw data collected in this present study have been presented as appendices in support of the summarised data in the individual chapters. A study of this nature has, to my knowledge, not been published, therefore, making the information presented here a significant contribution in understanding decay and preservation of bagasse for the pulp and paper industry.

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## SUMMARY

**Influence of fungal diversity and production of cellulolytic enzymes on decay of stored bagasse**

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**Key words: Bagasse, biodiversity, cellulase, decay, fungi, lignocellulose, microclimate, xylanase**

Bagasse is the fibrous derivative of sugar cane, that is grown on a commercial scale in many tropical and sub-tropical countries, where ideal climatic conditions are experienced. The seasonality of sugar cane presents storage problems for bagasse, since this lignocellulosic material is susceptible to degradation by a diverse range of microorganisms, mainly fungi. The decay that is brought about contributes largely to the losses of fibre in a bagasse pile. The surrounding microclimate, and conditions within the pile, needs to be carefully monitored in order to understand the factors that support the fungal populations and biochemical activity.

The microclimate at the surface and inside the bagasse pile at a paper mill in Stanger (South Africa) was carefully monitored over a one-year storage period.

Significant changes were noted in temperature, pH and moisture content, between the surface and the inside of the pile, as the pile aged. The data were compared to established parameters for bagasse preservation, and it was found that the temperature was lower than expected, thus promoting fungal growth. The pH was much higher (promoting bacteria and actinomycetes) and the moisture content was too low to produce anaerobic conditions. The environmental conditions in the bagasse pile at Stanger, therefore, promoted the proliferation of microbes, and consequently decay.

Fungi that were present in the pile, were enumerated in order to investigate the diversity and fungal succession. There was a wider variety of species and higher numbers of fungi at the surface than inside the bagasse pile and the Shannon and Berger-Parker diversity confirmed these observations. Sorensons measure also showed that the types of fungal communities at the surface and inside the pile only started becoming similar toward the latter part of storage. When compared to models for abundance of species, conditions on the surface of the pile allowed maximum niche occupation at the beginning of storage, followed by the establishment of a mature community. The inside of the pile displayed minimal niche pre-emption followed by a state where most fungal species shared the domain. This study indicated that, as the storage time increased, the microbial communities became better established.

Bagasse is rich in holocellulose, the basic raw material used for paper-making. Since there were many species of holocellulolytic fungi found growing on the surface and the inside of the bagasse pile, the activity of cellulases and xylanases were determined. These enzymes were found to be active at the surface and inside the pile. However, higher activities of both enzymes were noted inside the bagasse pile than on the surface. The higher levels of activity inside the pile, despite lower fungal numbers, suggested that fungal counts were not a clear indication of biomass or biochemical activity. It appeared that the environment on the inside of the bagasse pile promoted the establishment of specific fungal populations that bring about a high degree of degradation to fibre inside the bagasse pile.

# CHAPTER 1

# UTILISATION OF BAGASSE IN PAPERMAKING AND THE IMPACT OF MICROBIAL ACTIVITY ON FIBRE QUALITY: A REVIEW

Vascular bundles of bagasse colonized by *Lenzites betulina* (Provided by C. Dunn)

**ABSTRACT**

Bagasse is the lignocellulosic residue derived from sugar cane. Its scientific name is *Saccharum officinarum* and is grown commercially in most of the tropical and sub-tropical countries worldwide, including South-East Asia, India, Egypt and parts of Latin America. Bagasse has a fibrous structure after the sucrose has been extracted from the sugar cane, and is a very valuable raw material to the pulp and paper industry. Due to sugar cane being a seasonal product, bagasse has to be stored for long periods of time, and the open structure and presence of residual sugars make the bagasse fibre susceptible to fermentation and degradation by a diversity of microorganisms. The extent of microbial degradation that occurs depends on the storage conditions. By establishing conditions that support fibre preservation, the wet-bulk storage system is very efficient in creating anaerobic conditions that limit fungal growth, and wet conditions that favour organic-acid bacteria that limit cellulose degradation. Therefore, microbes can either be beneficial or detrimental to fibre quality. The immediate environment of a bagasse pile is also highly influential on fibre quality. There is thus a need to study the effect of the environment on microbial activity in bagasse. The present study could ultimately contribute to the understanding of the role microorganisms play in the quality of bagasse, and how stored bagasse can be managed to ensure good quality fibre.

## INTRODUCTION

Sugar-cane bagasse has been well established as a valuable fibrous raw material for pulp and paper-making (Atchinson, 1987; Venter, 1978; Salaber & Maza, 1971; Marshall 1938), while its advent dates back more than 150 years (Atchison, 1988). The value of using this fibrous material was gained due to the quest for alternative fibrous materials to satisfy the growing need for paper (Misra, 1971). An increasing interest in using non-wood fibres as alternative sources of fibre for the pulp and paper industry has been noted in various studies (Hatakka *et al*., 1995; Vares *et al.*, 1995; Giovannozzi-Sermani *et al.*, 1994). This interest is due to the supply of wood being unable to cope with rapidly increasing fibre demands. Alternative non-wood sources of fibre that have been studied in addition to bagasse (Atchison, 1987; Venter, 1978; Salaber and Maza, 1971; Marshall, 1938) are reed canary grass and tall fesque (Hatakka *et al.,* 1995), wheat straw (Barassa *et al.,* 1995; Croon, 1995; Granick, 1979; Misra, 1971; Rostanci & Yalinkilic, 1988), rice straw (Bolton, 1996), kenaf (Sabharwal *et al.*, 1994) and hemp (Bolton, 1996). Bolton (1996) concluded that a major advantage of using non-wood fibres, is its short rotation time compared to the long rotation of forest trees. Increasing amounts of pulp are being produced globally using non-wood fibres, with China producing almost half of the worlds non-wood pulp (Table 1).

**Table 1:** Worldwide distribution of non-wood pulp mills based on capacity (Croon, 1995).

| Country | Non-Wood Pulp Mills (%) |
|---|---|
| China | 48.0 |
| India | 13.5 |
| Latin America | 7.0 |
| Asia & Oceania | 5.7 |
| U.S.S.R | 4.9 |
| U.S.A | 2.8 |
| Africa | 2.6 |
| Eastern Europe | 2.1 |

The first truly successful utilisation of bagasse as a fibrous material on a commercial scale, was for the manufacture of insulation board in 1920 by the Cellotex corporation in Marrero, Louisiana (West, 1920). This endeavour was successful and later in 1939, the initial bagasse-based pulp and paper mill operations were carried out in Peru, Taiwan and the Philipines (Atchison, 1987). The use of bagasse in the manufacture of pulp and paper was regularly considered over the last 150 years whenever fibre shortages presented themselves and much research has been done, and still continues, in the hope of strengthening the role of bagasse in the pulp and paper industry (Atchison, 1988).

The ratio of the amount of cane produced to the amount of bagasse produced varies from region to region, but as a rough estimate on a world-wide scale, for every ton of sugar produced, 1.2 to 1.3 tons of moisture-free bagasse is obtained (Atchison, 1968). Bagasse pulps are used in practically all grades of paper, including bags, wrappings, printing, writing, tissue paper, towelling, corrugating medium, linerboard, bleached boards and coating base stock (Atchison, 1987).

From all of the non-wood fibres available to the pulp and paper industry in South Africa, bagasse seems the most favourable to use, since it is a by-product of an established industry. Systems exist, with transportation and collection facilities already in place and the technology for the manufacture of paper and chemical recovery systems are established. Further studies need to be carried out to optimise and maximise the potential of bagasse in the pulp and paper industry. Bagasse is increasingly being used as a raw material for pulp, paper and paperboard, and more than any other raw material since the 1950's (Atchison, 1988). In India, large and medium-scale mills are producing printing and writing paper from bagasse, with some mills even producing coated paper. Smaller-scale mills are being used to produce Kraft fluting medium for the packaging industry (Visvanathan *et al.,* 1998).

When considering bagasse and its applicability in paper making, the negative attributes need to be considered as well. Bagasse has to be stored for extended periods of time as a result of sugar cane being a seasonal product, and a large quantity

of bagasse is rendered unsuitable for pulping due to biodegradation (Rangamannar *et al.*, 1993). Contributing factors that make bagasse so vulnerable to microbial inhabitation, includes the presence of residual sugars, heterogeneity of the tissues, and various environmental parameters that are influential in a bagasse pile (Ramaswamy *et al*., 1989). The deleterious effects of microorganisms cause a significant loss of valuable raw materials where mainly bacteria, actinomycetes and fungi contribute to this loss (Ramaswamy *et al.*, 1989; Cusi, 1979). Many aerobic and anaerobic bacteria can degrade the polymeric structure of cellulose, hemicellulose and lignin (Rangamannar *et al.*, 1993). Environmental conditions also contribute significantly to bagasse storage problems. These conditions must be controlled in order to maintain unfavourable conditions for detrimental microorganisms whilst creating optimum conditions for those that are beneficial. Also, bagasse requires well controlled bleaching and refining conditions to avoid damage to the fibre (Misra, 1980). This comparative view of bagasse, coupled with the negative aspects of this fibrous material, provides greater insight into the potential of its use in the pulp and paper industry.

It is evident that much attention should be placed on perfecting the preservation of bagasse, which will improve its use to the pulp and paper industry. The aim of this chapter is to review the role of bagasse in the paper-making industry, and to focus on how microbes and the environment influence bagasse preservation.

## BAGASSE COMPOSITION

Bagasse is the fibrous residue that remains as a waste product from the sugar milling process, and is often used to fuel boilers at sugar mills (Visvanathan *et al*., 1998). However, the ability of bagasse to be used as an alternative fibre source in the pulping process was recognised more than a century ago (Atchison, 1988). Bagasse mainly has a 51 % moisture content, 47 % consists of fibrous solids, and 2 % is water soluble (Fernandez *et al*., 1989). These figures could vary considerably, depending on the variety of sugar cane, geographic source, maturity, harvesting and the milling process used (Granick, 1979).

Bagasse, which is a very fibrous solid, has three components (Misra, 1980), of which the first includes the epidermis, cortex and pericycle. The second component is the vascular fibre bundles and the third is the parenchyma or pith cells. The first component, which is thick-walled, strong, and absorbs cooking chemicals slowly, makes up the rind fibres and is very important in determining the pulp tearing strength. The rind fibres alone represent a superior raw material for the manufacture of pulp and paper (Atchison, 1987).

The vascular fibre bundles are divided into two different classes, viz, central and rind vascular bundles. The central fibre bundles are used to transport nutrients and the rind vascular bundles are thin walled and larger than the wood fibres (Misra, 1980). Figure 1 shows a single layer of epidermis cells followed by bundle sheaths embedded in layers of schlerenchymatic tissue. Phloem, protoxylem and metaxylem vessels are contained in the bundle sheaths. The smaller bundle sheaths are noted to be closer to the epidermis, whilst the larger ones are more centralised and embedded in the parenchymatic tissue or pith (Rodriguez-Vasquez *et al*., 1992). Pith cells are of no use to the pulping process and are removed as much as possible, as they can swell up to 30 times their own size and thus consume large quantities of pulping liquor (Atchison, 1988; Granick, 1979; Venter, 1978).

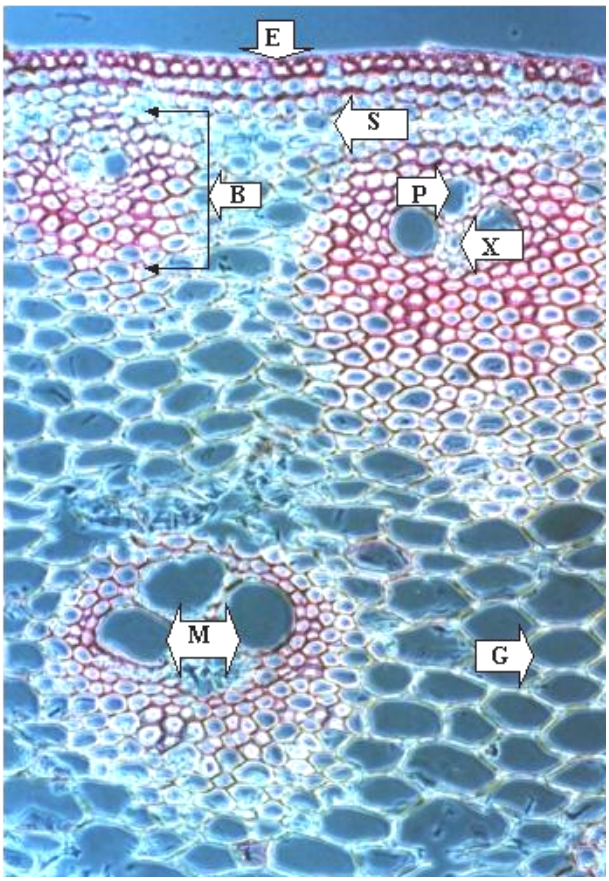


**Figure 1:** Light micrograph of a cross section through sugar cane, showing the epidermis layer (E), the schlerenchymatic tissue (S), the phloem (P), protoxylem (X) and metaxylem (M), and the parenchymatic tissue or pith cells (G). (Micrograph provided by C. Dunn).

The length to diameter (L/D) ratios of bagasse fibre are similar to that of hardwoods, but smaller when compared to softwoods and straw (Table 2). Softwoods have long fibres and provide stronger pulps for tough wrapping and packaging paper grades, whilst hardwood pulps are used for mass-produced printing and writing grades (Atchison, 1987; Misra, 1980; Granick, 1979). Bagasse fibres, thus having similar properties to hardwood pulps, are generally used for printing and writing grade paper (Visvanathan *et al.*, 1998; Atchison, 1987).

**Table 2:** Comparison of fibre dimensions between bagasse and other commonly used raw materials (Visvanathan *et al.*, 1998).

| Raw Materials | Length (mm) | Diameter ( m) | L/D |
|---|---|---|---|
| Depithed Bagasse | 1 | 20 | 50 |
| Bamboo | 2.3 | 18 | 127 |
| Softwood (Pine) | 2.7 to 4.6 | 32 to 43 | 84 to 106 |
| Hardwood (Eucalyptus) | 0.7 to 1.6 | 20 to 40 | 35 to 40 |
| Rice Straw | 1.5 | 8.5 | 176 |
| Wheat Straw | 1.5 | 15 | 100 |

**Biochemical composition**

Lignin, cellulose and hemicellulose are three natural polymers that occur together in lignocellulosic materials (Sarikaya & Ladisch, 1997). Plant cells are distinguished from animal cells by the presence of true cell walls, containing polysaccharides as the major structural material. Cellulose is the major structural unit of plant cell walls, and exists as long thread-like fibres, called microfibrils (Ghose, 1987). The cellulose microfibrils are embedded in a matrix formed through covalent interactions between lignin and hemicellulose (Cote, 1965) that will bring about the hydrolysis of cellulose to glucose units, if removed. Cellulose (Figure 2), is a linear polysaccharide consisting of glucose units with 1,4-ß-D-glycosidic linkages, and is usually found in the primary and secondary walls of plants, and also in the middle lamellae, where they act as a connection between cells (Sarikaya & Ladisch, 1997).

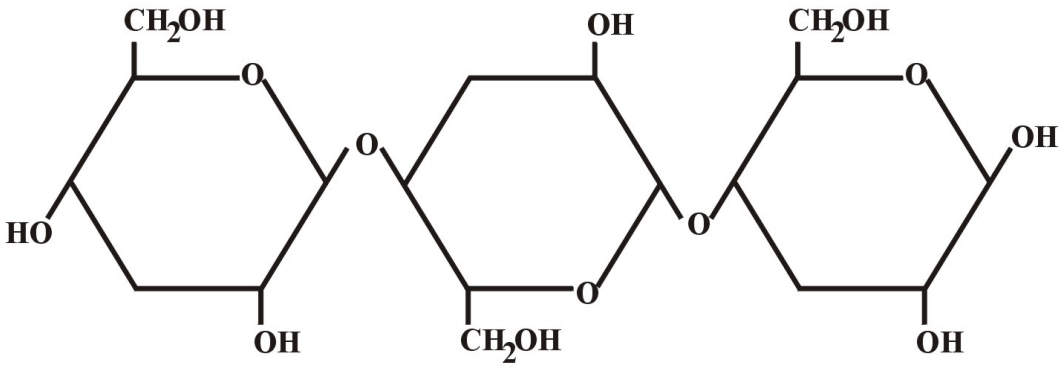


**Figure 2:** Chemical structure of cellulose.

Lignin arises from the polymerisation of trans-?-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Figure 3). Units of lignin are linked by non-hydrolysable carbon-carbon bonds, that are rarely formed, and ether bonds. Carbon-carbon bonds link aromatic nuclei whilst ether bonds link propyl side-chains to aromatic nuclei (Odier & Artaud, 1992).

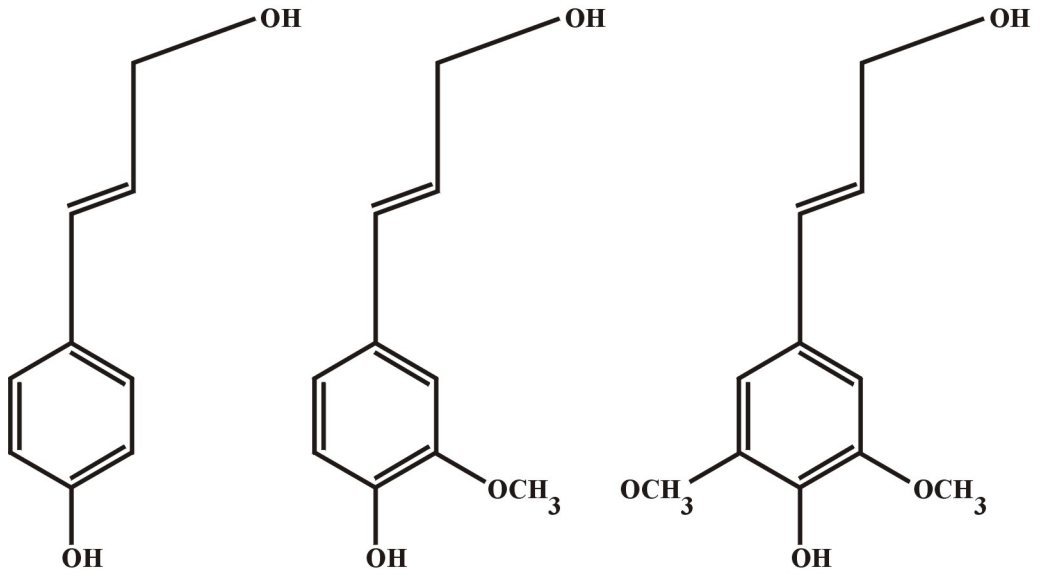


**Figure 3:** The chemical structures of the precursors of lignin: (1) trans-?-coumaryl alcohol (b) trans-coniferyl alcohol (c) trans-sinapyl alcohol (Sarikaya & Ladisch, 1997).

Hemicellulose, which has a more complex structure than cellulose, consists of xylans, mannans, galactans and glucans (Figure 4). This polymer would hence require more complex enzyme sytems to break down its structure. Hemicellulose does not only exist on its own, but is bound to lignin through covalent interactions (Beguin & Aubert, 1994).

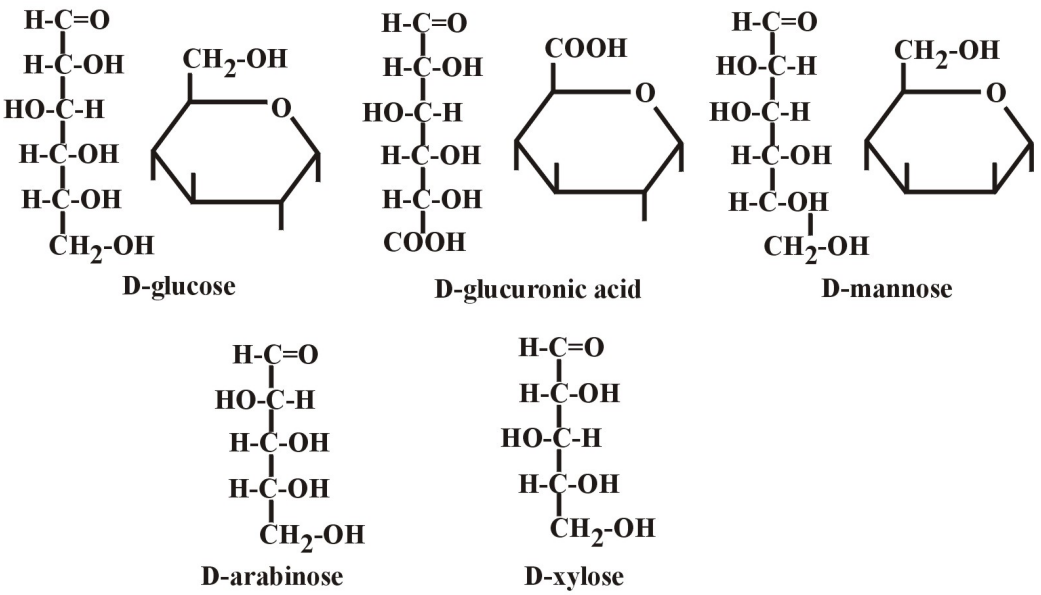


**Figure 4:** Chemical structure of the monomers making up hemicelluloses (Robards, 1970).

The process of lignocellulose degradation is complex and still not completely understood (Donaldson, 2001) because of the difficulty in obtaining pure forms of lignin, cellulose and hemicellulose, without breaking the covalent bonds between these polymers (Odier & Artaud, 1992). Cellulase enzymes, produced by microorganisms, have been widely studied because of their economic importance (McGinnis & Shafizadeh, 1980). When a fungal spore germinates on lignocellulosic substrates, enzymes are secreted by the fungal hyphae as they penetrate the cell wall. This results in the general dissolution of the primary cell wall (Kewalramani *et al.*, 1987). The cellulases and hemicellulases initially attack at the lumen wall, and gradually work outward as they erode each successive wall layer of the polysaccharides. In contrast to the internal fibre damage caused by fungi, bacterial attack seems to be restricted to the surface of the fibres (Kewalramani *et al.*, 1987; Reese, 1971).

**Other chemical properties**

The chemical composition of bagasse varies somewhat from country to country depending on the soil, the climate and the length of the growing season (Atchison, 1987; Misra, 1980; Granick, 1979). Various studies have been carried out to compare different sources of fibre (Table 3) such as bagasse, hardwood, softwood, wheat, straw and bamboo (Visvanathan *et al.*, 1998; Atchison, 1987; Misra, 1980).

**Table 3:** Comparison of some characteristics of bagasse with other commonly used raw materials (Visvanathan *et al.*, 1998).

| Property | Bagasse depithed | Bamboo | Softwood Pine | Hardwood Eucalyptus | Wheat Straw | Rice Straw |
|---|---|---|---|---|---|---|
| **Hot Water Solubility** | 5.9 | 11.1 | 2.0 | 5.24 | 10.5 | 15.5 |
| **1-% NaOH solubility** | 31.6 | 28.0 | 12.2 | 14.55 | 40.1 | 49.4 |
| **A/B Extraction**** | 1.7 | 8.0 | 2.0 | 2.6 | 5.3 | 7.4 |
| **Lignin** | 26.9 | 32.0 | 28.8 | 29.90 | 17 | 10.6 |
| **Holocellulose** | 66.1 | 56.6 | 69.0 | 69.4 | 70.7 | 70.2 |
| **Pentosans** | 28.9 | 20.2 | 8.6 | 19.3 | 25 | 24 |
| **Ash** | 2.6 | 3.4 | 0.2 | 0.08 | 7 | 14.8 |
| **Silica** | 2.0 | 3.0 | 0.13 | 0.05 | 5 | 11.5 |

* All Figures in %

**A/B extraction: Alkyl-Benzene extraction

Bagasse has a high 1-% NaOH solubility (Table 3) indicating that bagasse is susceptible to deterioration during storage (Misra, 1980). This can be minimised by ensuring that the bagasse is stored in the most efficient way. Bagasse has a lower value for Alkyl-Benzene extraction and this illustrates that the presence of wax and resins are much lower in bagasse (Atchison, 1987). The lignin content in bagasse is lower than that of bamboo and wood, therefore, the chemical requirement for its removal is less (Table 3). The structure of bagasse is much more open for the cooking chemicals to degrade lignin. The holocellulose content is comparable to that of wood and straw, thus showing that high-yield pulp can be obtained from depithed bagasse under optimal pulping conditions (Misra, 1980).

## BAGASSE DEPITHING

The importance of depithing bagasse, before utilising it for the manufacture of paper and pulp, has been recognised for more than 80 years, and a tremendous effort has been made to develop efficient depithing methods (Atchison, 1987; Atchison, 1963; Cusi, 1959; Knapp *et al.*, 1957; Keller, 1956). Pith cells are extremely thin-walled, and occur with many other short, thin-walled parenchymatic cells that partly originate from the vascular bundles. They decrease strength properties and increase chemical consumption in the cooking stage (Hahn, 1979). These thin-walled cells increase drainage resistance, increase sheet resistance and will not act as stress-distributing fibres in the fibre network (Giertz & Varma, 1979). Therefore, pith separation from the fibre is imperative.

Depithing can be carried out in a two stage process with preliminary screening to provide adequate pith separation. The location of the depithing stations in the two stage process is determined by the locations of both the pulp and the sugar mill (Misra, 1971). Whole bagasse contains between 48 and 52 % moisture when released from the sugar mill. It is then screened to remove free pith prior to the depithing operation. The primary depithing is called 'moist' depithing (Misra, 1971). In all cases, bagasse for pulp production should be moist depithed as soon as possible and stored before microbial deterioration occurs. The bagasse is preferably moist depithed at the sugar mill, before proceeding to the pulp mill (Hurter, 1991).

The second depithing stage is carried out to complete the pith separation at the pulp mill. Both dry depithing and wet depithing could be used, however, it has been shown that wet depithing is preferable, due to the final depithed bagasse having better pulping properties and a uniform moisture content. Wet depithing also prevents the problem of dust, that causes 'Bagassosis" amongst workers in proximity of the bagasse processing. It has been shown that wet depithing is more effective in removing pith embedded in the fibre bundles, and very efficient in removing water soluble constituents and residual sugars (Giertz &Varma, 1979).

**PULPING**

Many of the common wood pulping processes have been adopted for the pulping of bagasse (Hurter, 1991). These include the soda, sulphate, mono-sulphate or neutral sulphate semi-chemical (NSSC) processes, as well as the pomilio process to produce pulp suitable for writing and printing paper grades (Hurter, 1991; Atchison, 1971). Pulping, using the soda or sulphate process, largely depends on availability of the chemicals and their relative prices (Hurter, 1991). Sulphate or Kraft pulping is commonly used to obtain unbleached pulp due to the fact that the yield and strength properties are higher, and cooking time is shorter. However, it is disadvantageous, because it consumes large amounts of chemicals. To obtain fully bleached pulp, either the soda or sulphate process is used (Misra, 1971). Soda pulp requires less bleaching chemicals to attain high brightness (Hurter, 1991) and is, therefore, preferred. Mills around the world use different pulping procedures that suit them best. Examples of this include the use of mechanical pulping at Tamil Nadu in India (Rangan, 1996), sulphate pulping at the Ping-tung Pulp factory (Wang & Tao, 1978), and the caustic soda process in Argentina (Salaber & Maza, 1971).

The mono-sulphate process is equally suitable for pulping of bagasse, but is less preferred due to its lack of chemical recovery systems that present serious effluent disposal problems (Misra, 1980). Mills that are well equipped for effluent disposal can handle this process very easily. The mono-sulphate process provides a higher yield and requires less chemicals for bleaching. It also provides a five to 10 % higher brightness to pulp, than that obtained using the soda or sulphate process (Misra, 1971). This process requires less bleaching chemicals to attain the same brightness as that obtained using the soda or the sulphate methods (Atchison, 1971).

The Pomilio process had been practiced in many straw and bagasse pulp mills (Misra, 1971), however, its use became very uncommon due to the high chemical requirement and effluent disposal problem (Misra, 1980). In many cases, installation of an electrolytic plant is necessary to reduce the cost of caustic soda and chlorine.

Chemical pulping is the most common method of pulping used worldwide, with kraft and soda processes being used most often (Atchison, 1988). Mechanical

pulping incorporates a fractionating procedure, thereby releasing thermo-mechanical pulp (Fine fractions) that is cleaned and thickened, and chemi-mechanical pulp (Course fractions) that is mixed with liquor (NaOH and $Na_2SO_3$) and passed through steaming tubes. The chemi-mechanical and thermo-mechanical pulp fractions are mixed in a 1:1 ratio, bleached by using peroxide, and thus form the bleached mechanical bagasse pulp (Misra, 1980). Thermo-mechanical pulp has very low strength characteristics and, therefore, most bleached mechanical pulp is produced using chemi-thermo mechanical processes (Atchison, 1987). These processes have drastically improved strength properties (Visvanathan *et al.*, 1998) and is being used in various proportions for the production of standard newsprints and other grades of printing paper in the USA, Hawaii and India (Visvanathan *et al.*, 1998; Atchison, 1987; Hinrichs *et al.*, 1962; Wethern & Captein, 1961). Unbleached chemical bagasse pulp is prepared similarly to bleached bagasse pulp, which is used for high quality printing and paper grades. Bagasse is washed and blended with kraft cuttings for the production of low strength kraft paper, that is used as fluting medium in the packaging industry (Visvanathan *et al.*, 1998).

**BAGASSE STORAGE**

The regular supply of bagasse to pulp and paper mills is a major problem due to the seasonal restriction of sugar cane in many parts of the world. During a season, the cane is grown until it reaches its optimum sugar content and is then cut and sent for crushing at the sugar mill. Once the harvesting season ends, the sugar mill is closed until the next season, and bagasse must be stored for use until the start of the next season (Granick, 1979). Large quantities of bagasse is stored to ensure an on-going supply to the paper mills during the non-cutting season. In South Africa, cutting usually takes place during eight months of the year (Venter, 1978), while in other countries it is restricted to three to six months, and must be stored for the rest of the year (Atchison, 1987). The bagasse can be stored in several ways, including dry baling and stacking, or wet bulk storage (Granick, 1979). The difficulties in storage result from biochemical deterioration, microbial degradation, and the danger of fire from either accidental fires or spontaneous combustion (Hurter, 1991; Atchison, 1971) .

**Bale storage**

The classical method of bale storage was developed after extensive research by the Cellotex corporation in the 1920's and 1930's (Atchison, 1987), resulting from a compromise between two extreme conditions that led to excellent preservation (Hurter, 1991). This method was patented by Lathrop & Munroe (1933).

Since microorganisms rely on water for many of their metabolic functions, research proved that the immediate drying of the bagasse, after leaving the sugar mill, minimised the degree of degradation (Atchison, 1987). This procedure was used when the bagasse was wet-depithed to remove the residual sugars present, and then dried. In cases where the bagasse was not depithed, significant fungal attack was noted on end products such as particle board (Atchison, 1987). However, in the manufacture of pulp, residual sugars are dissolved in the pulping process (Hurter, 1991, Misra, 1980). Large-scale drying of bagasse was considered to be too costly and a cheaper means of bagasse preservation was sought. Later, research investigated the wetting of the bagasse pile in order to fill up the air spaces in the pile (Atchison, 1987; Lathrop & Munroe, 1926). By displacing air with water, the oxygen supply in the pile was reduced, and fermentation was virtually eliminated (Atchison, 1987). Once again, it was realised that it was too costly to keep the bagasse pile wet throughout the storage period. The problem was then alleviated when an optimum bale storage method was developed. This method included an optimum sized bale stored at optimum conditions. The heat generated from the natural metabolism of microorganisms, as well as acid fumes, would be dissipated without damaging the fibre. The heat from this natural fermentation process can be used to effectively dry the bagasse (Atchison, 1987; Lathrop & Munroe, 1933).

Several other methods of storing bagasse in baled form have been described, involving small bales that can be managed by a single person, dry storage in 800 kg bales after moist and wet depithing, and moist storage in 600 kg bales with holes in the centre to allow heat dissipation (Hurter, 1991).

**Wet-bulk storage**

The first known attempts to store bagasse in a bulk form were made by Ritter in 1930 and is now being used in many new bagasse pulp and paper mills (Wang & Tao, 1978; Salaber & Maza, 1971, Moebius, 1966; Macdonald, 1963). The Ritter process for wet-bulk storage appears to have many advantages over any of the bale storage methods. In 1937, O. Kuhne investigated the method at "Preusissche Holzforschungsinstitut" near Berlin and his findings were published in 1937 and 1938. However, world war two prevented more research (Moebius, 1966).

The general principle of the Ritter process involves impregnation of the bagasse with a 'biological liquor', and flushing the bagasse suspension to a bulk-storage area through elevated channels (Atchison, 1987). This modified wet-bulk storage process is a very effective system in which a bacterial culture, mainly *Lactobaccilus* is added to a bagasse pile. Rapid fermentation is promoted, and this is coupled with a rapid decrease in sugars and water solubles. This method of storage is mainly based on the production of lactic acid that reduces the pH, and hence results in better preservation of the bagasse (Wang & Tao, 1978). By adding a bacterial culture, the lag phase of fermentation is increased to the extent where sugars are quickly converted into alcohol, carbon-dioxide and organic acids. This action prevents any other microorganisms, including thermophylic cellulolytic-microorganisms, from becoming dominant, and therefore, minimises the deterioration to bagasse fibre (Atchison, 1988). This process of storage to promote the growth of lactic acid bacteria, creates an anaerobic, thermophilic, low-pH environment within the micro-environment of a bagasse pile.

Salaber & Maza (1971) conducted a study whereby bagasse was treated by the Ritter process over a period of ten months, and it was found that maximum preservation was obtained when the moisture was maintained at approximately 80 %, the temperature within the pile was maintained at approximately 50°C, and the pH at 4. The stored bagasse also showed a 10 % decrease in pith content and a stable holocellulose content. Because of the superior results of wet- bulk storage systems of various types, including the Ritter biological treatment of the bagasse suspension, the

trend has been towards this type of storage. Practically all new bagasse mills globally, are now using some type of wet-bulk storage system (Hamilton & Leopold, 1987).

Molasses is sometimes added to promote the growth of lactobacilli when temperatures increase, favouring thermophilic cellulolytic microorganisms (Atchison,1988). This addition of molasses, reproduces sufficient biological liquor for the treatment of incoming bagasse, and refortification of the liquor used for flushing the bagasse to storage (Salaber and Maza, 1971). The storage area consists of a receiving slab of special construction (concrete coated with an epoxy resin or a special asphalt on compacted earth) that is traversed in one direction by a number of parallel channels with removable wooden or concrete covers (Atchison, 1971). These channels provide a drainage system for recovery of the flushing liquor when opened, and if closed, they provide a channel for reclaiming the bagasse (Moebius, 1966). This method of storage, or variations thereof, has been used successfully in South Africa, Argentina, Brazil, Mexico, Taiwan, Cuba, Iraq and Iran (Atchison, 1988).

The first mill to use the Ritter process was the Ngoye Paper Mill at Felixton in South Africa, and started storing bagasse in bulk form in 1956. Shortly afterwards in 1962, the Piet Retief paper mill in South Africa started storing bagasse in a similar manner (Moebius, 1966; Macdonald, 1963). Mills in Argentina and in Brazil, Ledesma mill and Refinadora Paulista mills respectively, followed shortly afterwards, adopting the same storage techniques (Venter, 1978; Salaber & Maza, 1971; Moebius, 1966).

The choice of the type of storage method employed depends on the number of sugar mills involved, transport distance, means of delivery and the length of the cane season. However, the wet-bulk storage system is preferred to the baling storage system. This is due to handling and storage costs being lower, especially if the bale system uses smaller bales. The threat of bagasossis is also minimal, and there is a reduced risk of fire (Wen-ching *et al.*, 1973; Moebius, 1966).

## BAGASSE DETERIORATION

Bagasse deteriorates at a rapid rate during ineffective storage (Cusi, 1979; Misra, 1971). This is brought about by the deleterious effect of microorganisms, which render considerable amounts of bagasse unsuitable for pulping (Rangamannar *et al.,* 1993; Ramaswamy *et al.,* 1989). Ramaswamy *et al.* (1989) concluded that bacteria, actinomycetes and fungi (Table 5) contribute largely to the deterioration of bagasse in storage. This is attributed to the high residual sugar content in bagasse, presence of water solubles making bagasse a fertile substratum, the openness of the pile, heterogeneity of the tissues and the vast exposed surface area of a bagasse pile (Ramaswamy *et al.*, 1989; Cusi, 1979).

Ramaswamy *et al.* (1989) also noted that fungi play a major role in bagasse deterioration, and over 40 fungal species were isolated in his study on bagasse. *Aspergillus niger, A. terreus, A. grisea, A. fumigatus,* as well as species of *Rhizopus, Mucor* and *Humicola* were commonly found throughout the trial period (Figure 5). The intensity of microbial degradation is also dependant on various environmental parameters such as temperature, pH and aerobicity, whilst the growth and metabolic activity of the prevailing microorganisms result in the biochemical degradation of bagasse that ultimately leads to a reduction in yield and physical strength properties (Ramaswamy *et al.*, 1989). Many aerobic and anaerobic bacteria also catabolise cellulose, hemicellulose and to a smaller degree, lignin (Rangamannar *et al.*, 1993), therefore, making bagasse very suitable for microbial colonisation and degradation (Schmidt & Walter, 1978).

**Table 5:** The relative abundance of microorganisms in stored bagasse (Ramaswamy *et al.*, 1989).

| Organisms | Relative Abundance |
|---|---|
| **Bacteria** | +++ |
| **Actinomycetes** | + |
| **Fungi** | |
| *Mucor* spp. | ++ |
| *Rhizopus* spp. | ++ |
| *Humicola* spp. | ++ |
| *Saccharomyces* spp. | +++ |
| *Penicillium* spp. | ++ |
| *Paecilomyces* spp. | ++ |
| *Aspergillus niger* | ++++ |
| *A. fumigatus* | ++++ |
| *A. grisea* | +++ |
| *A. terreus* | +++ |
| *A. sydowi* | +++ |
| *A. cervicus* | ++ |
| *Neurospora* spp. | ++ |
| *Drechslera* spp. | ++ |
| *Fusarium* spp. | ++ |
| *Trichoderma* spp. | +++ |
| *Vulvariella* spp. | ++ |

++++ Abundant +++ Frequent
++ Present + Infrequent

The main reason for microbial success lies in their physiological ability to proliferate in varying environments. Fungi are obligate aerobes that inhabit environments optimally at temperatures lower than 45°C, and can function in more acidic environments (Table 6). Yeasts, however, function only at temperatures between 18 and 22°C. Actinomycetes are facultative aerobes, and can function in a wide temperature range. They grow optimally in very alkaline environments. Since there are a vast number of known bacterial species, their range of growth varies across a wide pH and temperature range as shown in Table 6 (Lengeler *et al.*, 1999; Pelczar *et al.*, 1977).

**Table 6:** Optimal environmental parameters and the effects of fungi, bacteria and actinomycetous organisms on stored bagasse (Pelczar *et al*., 1977).

| Parameters | Fungi | Bacteria | Actinomycetes |
|---|---|---|---|
| Temperature Optima | < 45°C | Thermotolerant | Thermotolerant |
| pH Optima | < 6 | > 7 | >8 |
| Aerobic/Anaerobic | Obligate aerobes | Aerobic and Anaerobic | Facultative aerobes |
| Effects on bagasse | Most Damaging | Damaging | Damaging |

In Figure 5, at moderate temperatures (18°C to 22°C), yeasts dominate in bagasse piles that have a 2.5 to 3 % residual sugar content (Hurter, 1991; Rangamannar *et al.*,1993; Cusi, 1979). Sucrose is hydrolysed into glucose and fructose, that produces ethanol and carbon-dioxide under anaerobic conditions, and water and carbon-dioxide under aerobic conditions (Cusi, 1979). These two exothermic reactions generate approximately 1350 calories of heat per gram mole of sucrose (Rangamannar *et al.*, 1993) and thus bring about an increase in the temperature of the bagasse pile and the micro-environment within it.

The distinct temperature increase brings about a decrease in the activity of yeasts, whilst other mesophilic fungi and bacteria continue to catabolise the available sugar. Once the sugar content starts to decrease, cellulolytic microorganisms break down cellulose to soluble compounds that provide the nutrient requirement for the non-cellulolytic microorganisms (Rangamannar *et al.*, 1993). The simultaneous effects of such biological activity result in the decreased availability of sugars, significant increases in temperature, followed by increases in the microbial population within a bagasse pile (Cusi, 1979).

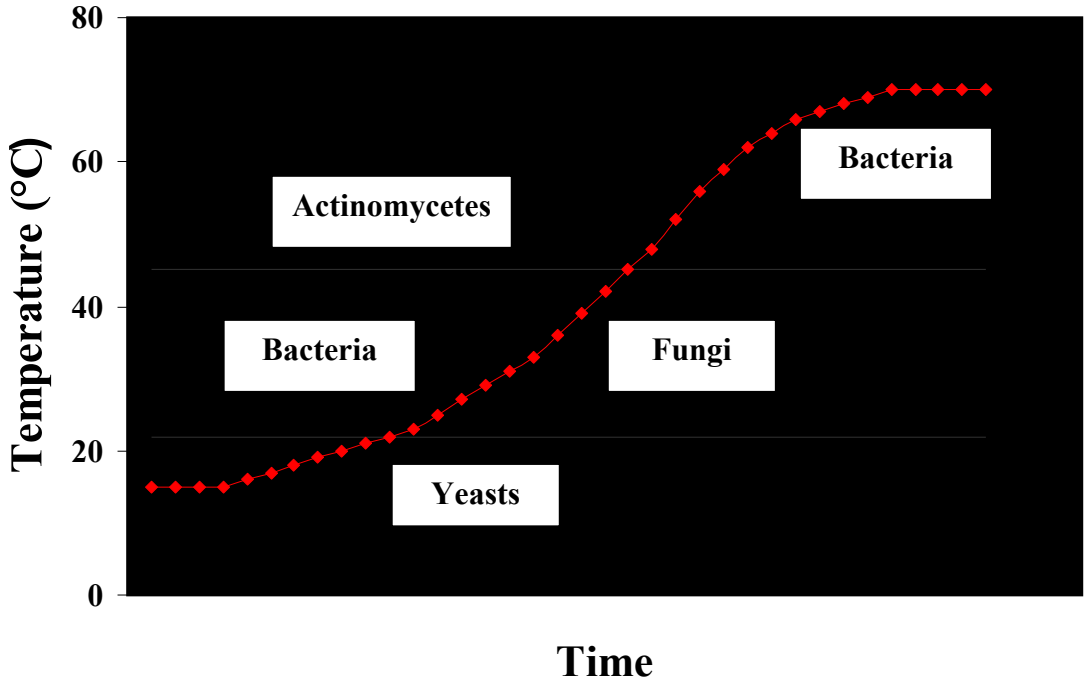


**Figure 5:** Changes in temperature in a bagasse pile over time and its influence on the succession of different microbial guilds. The horizontal lines differentiate the thermophiles, the thermotolerant microorganisms and the mesophiles (Pelczar *et al*., 1977).

Thermophylic microorganisms (mainly bacteria) are more adapted to higher temperatures, and start to proliferate as the temperature approaches 45°C, and organisms that dominated the pile previously, become inactive (Ramaswamy *et al.,* 1989). Cellulolytic action is intensified by this stage and the thermo-tolerant organisms (bacteria and actinomycetes) continue to develop until a maximum temperature is reached (65°C). This inactivates the different cellulases and thus brings about a halt in the nutritional supply. Usually this temperature is reached at the inner core of the pile, where heat loss is minimal (Rangamannar *et al.,* 1993). According to Ramaswamy *et al.* (1989), the pH at the top of a pile is 5.5 to 6.5 and the core pH is 3.5 to 4.0. At approximately 50°C (Rangamannar *et al.*, 1993), lactobacilli can produce lactic acid that can further reduce the pH and inactivate other microorganisms that bring about the degradation of bagasse. Provided that soluble nutrients are available, and that the temperature and other environmental factors are

favourable, the thermophiles that will initially predominate are the lactic acid-producing bacteria, and they will bring about a decrease in pH that will in turn inhibit cellulolytic microorganisms (Cusi 1979).

The discolouration of bagasse is strongly related to the biochemical reactions caused by microorganisms (Rangamannar *et al.,* 1993). The brightness of bagasse has been noted to vary from the surface of the pile to the inner regions of the pile. The top layers are the darkest with a brightness of between 15 to 18 % ISO, whilst the core displays a brightness of between 38 to 40 % ISO (Rangamannar *et al.,* 1993). This is due to the action of sunlight and heat at the surface, and the lack of compaction, which results in oxygen reacting with residual sugars to generate heat (Rangamannar *et al*., 1993).

**CONCLUSIONS**

Bagasse is a valuable source of fibre in the pulp and paper industry (Hatakka *et al*., 1995; Vares *et al.*, 1995; Giovannozzi *et al.*, 1994). Previous studies have shown that the physical, optical and surface strength properties of bagasse are adequately suited for the production of high quality pulp and paper (Visvanathan, 1998). Various storage techniques have been developed over the years (Granick, 1979; Cusi, 1979; Moebius, 1966; Mcdonald, 1963) and the Ritter process has proven to be most successful in the preservation of bagasse (Granick, 1979). However, due to bagasse having to be stored extensively, microbial degradation plays a significant role on the quality of bagasse used for paper-making. According to Ramaswamy *et al.* (1989), fungi in particular play a major role in the deterioration of bagasse, and this must be taken into consideration when developing suitable storage techniques.

Decay, due to microbial activity, is apparently reflected by changes in pH and temperature (Ramaswamy *et al.*, 1989). Proper control of pH, temperature and aeration of a bagasse pile, can play a significant role in minimising microbial decay of bagasse (Rangamannar *et al.,* 1993). The presence of holocellulolytic microorganisms is also a cause for concern, since the main component of bagasse

fibre is holocellulose (Duenas *et al*., 1995). According to Atchison (1987), losses of atleast 5 % of a bagasse pile, is caused by the activity of cellulolytic microorganisms. Studies have shown that bagasse as a substrate, supported high activities of cellulases (Ojumu *et al.*, 2003; Guitierrez-Correa & Tengerdy, 1997; Duenas *et al.*, 1995).

As a direct result of fibre decay due to long term-storage of bagasse, the pulp and paper characteristics are severely altered (Hurter, 1991; Ramaswamy *et al.*, 1989). Studies on microbial interference and decay of bagasse have never been carried out in South Africa in order for appropriate preservation techniques to be developed. A complete understanding of the environmental parameters of a bagasse pile relative to the South African climate, the succession of microbial populations and the effect of holocellulolytic microbes, will ensure that suitable recommendations be made and practiced, for the maximum preservation of bagasse.

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# CHAPTER 2

# RELATIONSHIP BETWEEN THE AGEING OF A BAGASSE PILE, AND THE ENVIRONMENTAL PARAMETERS IN THE PILE

**ABSTRACT**

The microclimate surrounding and within a bagasse pile is very influential in determining the degree of degradation to bagasse fibre. The most important environmental parameters concerned include, the temperature, pH and moisture content. An understanding of the changing environment in a bagasse pile would allow the manipulation of certain parameters to reduce microbial degradation. The aim of this study was to monitor the microclimate at the surface and inside a bagasse pile at the Sappi Stanger mill in Kwazulu Natal, South Africa. Significant changes of the different parameters between these two sampling points, as well as significant changes over time, were observed. It was concluded that the environment in the bagasse pile at the Stanger mill, promotes microbial attack of the fibre. The temperatures measured at the two sampling points were generally lower than the ideal for preservation, the pH was much higher, and the moisture levels were too low to produce anaerobic conditions. Various recommendations have been posed to maintain the various environmental parameters at levels for reduced microbial interference. It is recommended that temperatures should be carefully monitored and managed in order for lactobacilli to grow and in turn produce lactic acid that will maintain the pH at desired levels. By compacting the pile further, and by feeding the pile with leached water from overhead sprinkling systems, moisture levels can be maintained at the optimum for bagasse preservation. The pile should also be stacked higher in order to reduce the surface area to lower surface losses.

## INTRODUCTION

The degradative effect of microorganisms is a major factor contributing to fibre deterioration of bagasse in storage (Rangamannar *et al.*, 1993; Ramaswamy *et al.*, 1989; Cusi, 1979; Schmidt & Walter, 1978). Therefore, if proper storage techniques are not employed, unfavourable fermentation is inevitable, and sufficient bagasse is not efficiently utilised for the pulping processes. Underlying environmental parameters, contributing to the perpetuance of microbial populations, include the temperature on the surface and within the pile, pH and the degree of oxygenation at points within the pile (Ramaswamy *et al.*, 1989). Environmental factors indirectly bring about deterioration of bagasse fibre, by establishing conditions that are favourable for microbial proliferation.

Bacterial development is favoured by higher temperatures, higher moisture and neutral to alkaline pH. Actinomycetes will be favoured at high temperatures and high pH but lower moisture, whilst fungi will be favoured at lower temperatures, acidic pH and lower moisture (Pelczar *et al.*, 1977). Therefore, temperature, pH and moisture content are influential in predicting the levels of microbial activity in a bagasse storage pile. Rangamannar *et al.* (1993) identified two important temperature zones, between 18 and 43°C and between 45 and 70°C. The first zone allows for yeasts and fungi to react with residual sugars until approximately 22°C, where mesophilic microorganisms start to prosper until temperatures approach 45°C (Ramaswamy *et al.*, 1989). From 45°C and above, the thermophiles start becoming active and thermophilic microbial activity allows the temperature to soar to a maximum of approximately 70°C. At this point, the microbial activity starts decreasing and in turn causes the temperatures to decrease (Rangamannar *et al.*, 1993). At approximately 50°C, lactobacilli can proliferate and hence produce lactic-acid, that would in-turn reduce the pH and ultimately prevent further microbial decay. Therefore, the pH of the pile is very influential and needs to be maintained below pH 4 (Rangamannar *et al.*, 1993). The pH is thus dependant on temperature and if the temperature is maintained optimally, then the pH would also be maintained optimally, and microbial activity would be minimal.

The consistency of bagasse, bulk density and height of the bagasse pile determine the degree of aerobicity in the bagasse pile (Rangamannar *et al.*, 1993). Since oxygen plays an important role in many chemical reactions (Pelczar *et al.*, 1977), its presence in the pile needs to be minimised. Spontaneous combustion of bagasse is also a severe threat, and is caused by sufficient heat and a high presence of oxygen within the pile (Moebius, 1966). By compacting the pile, anaerobic conditions can be established. This would greatly inhibit the growth of fungi, since they are obligate aerobes, and reduce other chemical reactions requiring oxygen (Rangamannar *et al.*, 1993). It is, therefore, necessary to maintain anaerobic conditions within a bagasse pile.

The trends in the development of bagasse storage methods has been directed toward control of the fermentation of the residual sugars present in bagasse, so as to preserve fibre quality to the greatest possible extent. It has been shown that bagasse in storage for six months and longer between cutting seasons, could lead to minimum losses of 10 % in fibre yield (Atchison, 1987). More than 75 % of this loss is due to the fermentation of sugars and fibre deterioration. Such losses can reach between 20 and 30 % if proper storage practices are not implemented (Atchison, 1987). The Ritter process is based on a storage method in which an acid medium and anaerobic conditions are combined to provide an environment that prevents the development of undesirable microorganisms (Wang & Tao, 1978; Salaber & Maza, 1971, Moebius, 1966; Macdonald, 1963). This process facilitates the storage of bagasse over long periods of time with minimum fibre deterioration and minimum overall losses. The sugars present in bagasse are metabolised by the lactic-acid producing microorganisms, which maintains the pH between 3.8 and 4.2, the temperature at approximately 50°C, and the moisture between 75 and 80 %. These conditions thus appear to be ideal for the preservation of cellulose in bagasse (Bruijn *et al.*, 1974).

Ramaswamy *et al.* (1989) found gross changes in the temperature and pH of the bagasse pile studied. These changes were attributed to the developing microbial flora in the bagasse pile, and to fluctuating enzymic activities against the main bagasse

components. The environment, therefore, influences microbial activity in the bagasse pile, and plays a major role in fibre decay. The aim of this study was to measure the temperature, pH and moisture content in a bagasse pile in order to study the relationships between the various environmental parameters and the storage time of bagasse, and to evaluate the storage practices for the preservation of bagasse.

## MATERIALS AND METHODS

### Site selection and sampling

The bagasse pile at the Sappi Stanger mill in Kwazulu Natal, South Africa, was surveyed to characterise the microclimate at two positions over the one-year storage period. Six sampling points were established, three at the surface of the pile, and three at 50 cm below the surface. Surface samples were obtained by scraping away superficial bagasse at the three sites and removing the test sample into brown paper bags. The inner pile samples were dug up by hand and also placed in brown paper bags. Both the inner and surface samples were brought back to the laboratory for processing. Sampling points did not include the highest point of the pile or the core of the pile, due to the lack of specialised equipment to reach these areas. During the May to December 2004 cutting season, samples were collected when the pile was 73, 117, 141, 169, 212, 268, 308 and 360 days old, and during the May to December 2005 cutting season, a sample was collected when the pile was 21 days old. This was done due to sampling not being possible until 73 days during the May to December 2004 cutting season.

**Temperature and pH**

The temperature of each of the six samples was determined at their location of sample isolation in the Stanger mill. This was done using a hand-held probe (Hl98128). A small sample of bagasse (1 g), from each of the six sampling points, was shaken vigorously in 100 ml distilled water. The pH was then measured with a pH meter (Consort C533).

**Moisture Content**

Fresh bagasse was collected at three surface sites, and at the three sites 50cm below the surface of the bagasse pile. Samples were collected in tightly sealable plastic bags to prevent evaporation of water. Samples were weighed and then dried at 100$^{o}$C for 24 hours. The moisture content was determined gravimetrically by weighing the samples again after the drying process.

**Experimental design and statistical analysis**

A randomized factorial experiment was conducted, with the postion in the pile and storage time as factors. The data was analysed using one-way analysis of variance (ANOVA), and the means of the different factors were compared using Tukey's test (Winer, 1971) at a 95% confidence level.

**RESULTS AND DISCUSSION**

**Temperature**

Significant differences in temperature between the surface and inside the bagasse pile were observed throughout the one year storage period (Figure 1). The inside of the pile had higher temperature readings (average 10.4°C higher) than the surface. The data representing the pile age at 21 days may not be representative since it was collected from bagasse of a different cutting season. As storage time increased until 73 days, there had been a significant increase in temperature on the surface and within the pile. However, the inner regions of the pile show a higher rate of increase than the peripheral regions.

This could be reflected by a higher rate of microbial activity in the inner regions of the pile or the escape of heat to the surrounding environment from the surface.

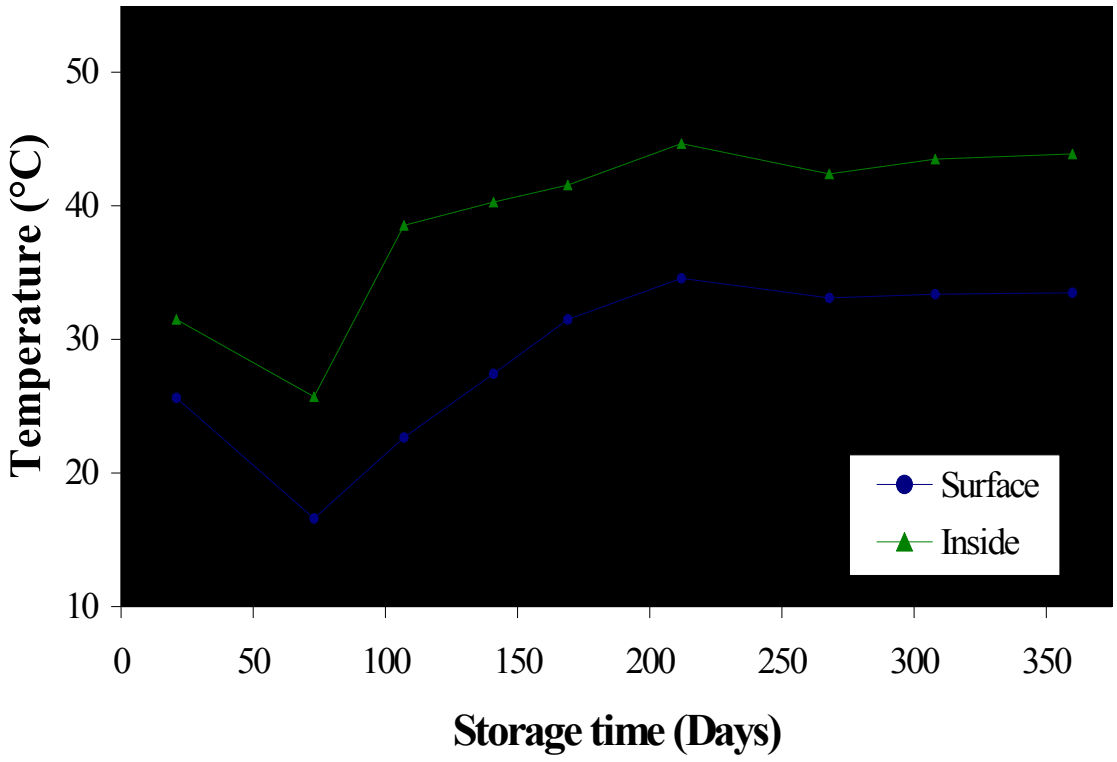


**Figure 1:** The mean temperatures of bagasse at the surface and inside the bagasse pile at different storage times over one year (Appendix A 1).

Error bars are set at Q = 2.464644 for the surface and Q = 3.247817 for inside (p=0.05; Tukey's test).

According to Rangamannar *et al.* (1993), the mesophilic fungi and yeasts start their attack on the residual sugars between 18 and 22°C, and this would account for the increase in temperature at the surface and inside the pile. Microorganism activity within the pile thus generates the heat that increases the temperature of the bagasse pile. The pile continued to show significant increases in temperature up to a maximum of 35°C and 45°C after 212 days, at the surface and inside the pile, respectively.

After 212 days, the pile showed non-significant decreases (decreases of 2°C) in temperature at the surface and within the bagasse pile. This decrease in temperature could be due to the residual sugars and other metabolites being metabolised. As a result, there is a decrease in cellular activity and the microbial numbers should start decreasing. When the microbial numbers decrease, microbial activity decreases and heat generation is slowed down. The bagasse pile is, therefore, unable to reach the temperatures required for the growth of lactobacilli (50°C). By promoting conditions for the growth of *lactobacillus,* the lag phase of fermentation is increased to the extent where sugars are quickly converted into alcohol, carbon-dioxide and organic acids. This prevents any other microorganisms, including thermophylic cellulolytic microorganisms, from becoming dominant, and thus bringing about too much deterioration of the bagasse (Atchison, 1988). If the growth of lactobacilli is hindered, preservation of bagasse is impeded.

**pH**

There were significant differences in pH between the surface of the bagasse pile, and inside the pile (Figure 2). These differences were noted throughout the study except for the last sampling where the pH at the surface of the pile, and the pH inside the pile were very similar (pH 5.5). The pH at the surface layers of the pile was generally higher (Average 1.0 unit) than the pH on the inside of the pile. The more acidic environment inside the pile could possibly be attributed to a higher microbial activity within the pile. The higher microbial activity can be supported by the higher temperatures recorded within the pile. Significant reduction in pH at the surface of the pile was only noted after 360 days. These results indicate that there was a relatively neutral pH (Average of 6.8) up until 308 days of storage. A neutral pH is ideal for microbial progression and the temperature profiles of the pile (Figure 1) are in accordance with an increasing rate of microbial activity. However, the sharp decrease in pH after 360 days (pH 5.5) was likely attributed to the production of organic acids by microorganisms other than Lactic-acid bacteria, since the recorded temperature of the pile was still too low for the development of lactic-acid bacteria.

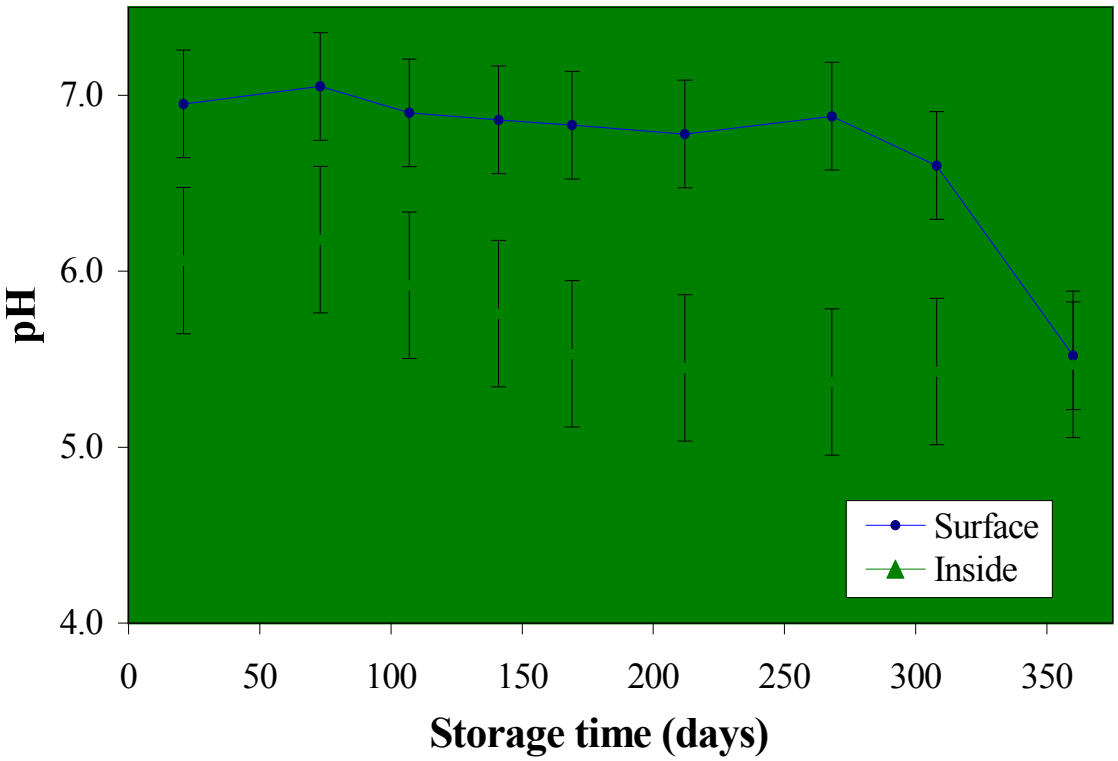


**Figure 2:** The mean pH profile of bagasse showing the pH at the surface of the bagasse pile, and the pH at 50cm into the bagasse pile at varying time intervals over one year (Appendix A 2)

Error bars are set at Q=0.305904 for the surface and Q=0.416262 inside the bagasse pile (p=0.05; Tukey's test).

Significant decreases in pH were noted after storage at 169 days (0.46 units) inside the pile. Although the temperature of the pile was increasing at this time, the desired temperatures for the growth of lactobacilli (50°C) was not reached, and it could be assumed that the decrease in pH was reflective of other acid-producing microorganisms. Further analysis showed that the pH inside the bagasse pile did not change significantly and remained at approximately pH 5.5 during the rest of the storage period. If the growth of lactobacilli was promoted by the conditions existing in the bagasse pile, the lactic acid that would be produced could result in a more acidic pH (Wang & Tao, 1978). Most microorganisms would not be able to survive under such acidic conditions that lead to better preservation of bagasse (Rangamannar *et al.*, 1993; Ramaswamy *et al.*, 1989; Atchison, 1987).

**Moisture Content**

The moisture content in the bagasse pile is inversely proportional to the amount of oxygen present, in and around, the bagasse pile. Since oxygen plays an important role in many biochemical reactions (Pelczar *et al.*, 1977), its presence should be kept to a bare minimum in order to facilitate the preservation of bagasse. Significant differences in moisture content were noted both at the surface of the pile, and inside the bagasse pile. The moisture content of the bagasse inside the pile was always on average 19.3 % higher than the surface of the pile (Figure 3). This was due to the superficial layers being influenced by outside heat and wind. The moisture content at the surface of the pile remained more or less constant over the first 212 days of storage, with fluctuations up to a maximum of 6 %.

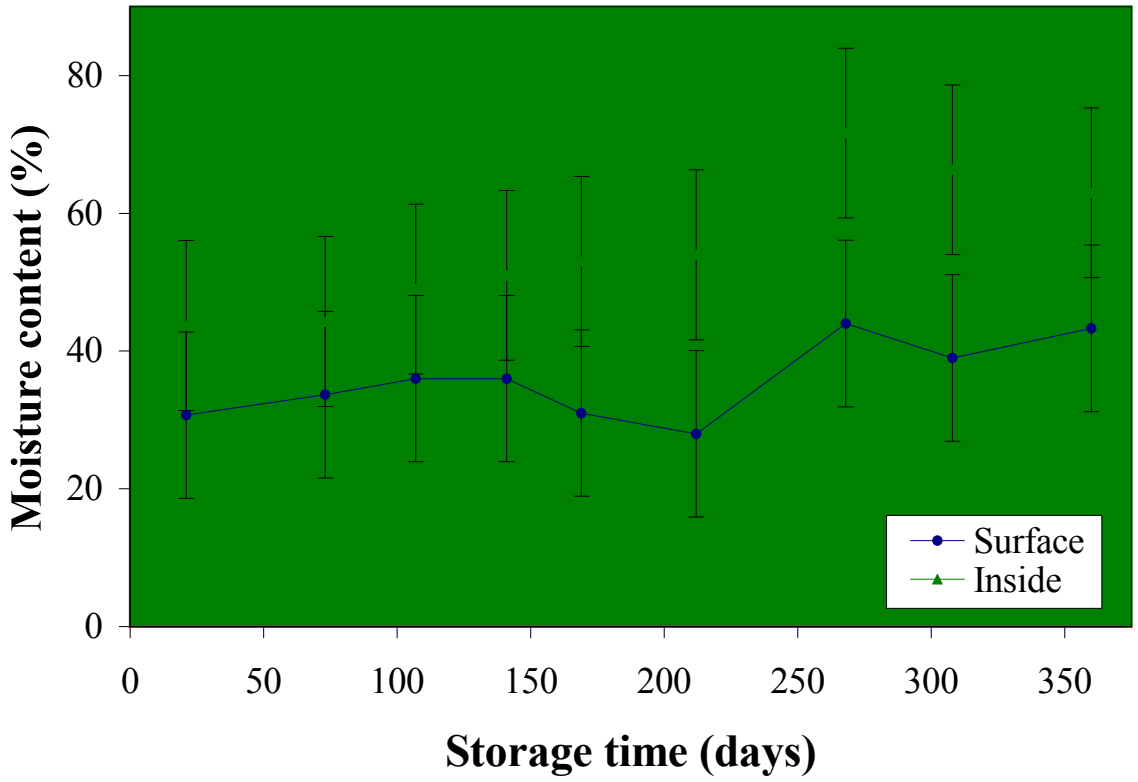


**Figure 3:** The mean moisture content at different positions in the bagasse pile, over the one year storage period (Appendix A 3).

Error bars are set at Q=12.08681 for the surface and Q=12.33554 inside the pile (p=0.05; Tukey's test).

The same trend was noted inside the pile where the moisture content showed no significant changes and fluctuated up to a maximum of 10 %. This lack of change in moisture could be attributed to rainfall replenishing the moisture, followed by heat dissipation from the inner pile. The heat from the inner regions of the pile could be dissipated to the outer regions of the pile, hence, the outer regions of the pile would maintain a relatively constant moisture. This theory is supported by the increasing temperatures experienced at the surface and inside the pile (Figure 1) that had no apparent effect on the moisture content.

Significant differences from the initial moisture were noted 268 days after the commencement of the study, both at the surface and inside the pile. This could possibly be attributed to rainfall. Moisture levels reached a maximum of 44 % and 72 % at the surface and inside the pile after 268 days of storage, respectively. According to Rangamannar *et al.* (1993), compacted, anaerobic storage has proven to produce high quality bagasse, due to the inhibition of chemical reactions associated with oxygen. In order to maintain these conditions, the moisture content needs to be maintained above 80 %. The bagasse pile in Stanger clearly does not maintain the desired moisture levels and, therefore, does not promote preservation of bagasse. There were no significant changes in moisture after 268 days, and the moisture was maintained at 42 % and 67 % at the surface and inside the bagasse pile, respectively.

## CONCLUSIONS

The environment surrounding and within a pile of bagasse is critically important to the end quality of bagasse (Rangamannar *et al.*, 1993, Ramaswamy *et al.*, 1989). In this study, the three most influential environmental parameters were measured in the same areas of the bagasse pile, at intervals, over the one year storage period. This study has shown that changes occur in a bagasse pile immediately after stacking. There were significant changes in temperature, continually throughout the storage period, whilst the changes in pH and moisture content were smaller. Unfortunately these changes, even

though sometimes minimal, do have an effect on microbial succession in a bagasse pile (Wang & Tao, 1978; Pelczar *et al.*, 1977; Moebius, 1966). Temperatures are 5°C lower within the pile, and 15°C lower on the surface of the pile (Figure 1), than the ideal, whilst the pH measured on the surface and inside the pile (Figure 2) was too high (up to 2 pH units). Moisture levels on the surface were lower than the ideal by 30 % (Figure 3). The temperature, pH and moisture, within and surrounding the pile, were in the range suitable for attack by most groups of microorganisms (Pelczar *et al.*, 1977) resulting in a greater degree of damage to bagasse fibres.

In order to achieve lower losses of bagasse when in storage, the temperature in a bagasse pile should be carefully monitored and maintained at 50°C and above, in order for lactobacilli to proliferate (Rangamannar *et al.*, 1993). Since lactobacilli produce lactic acid, the desired acidic pH levels should be reached (Ramaswamy *et al.*, 1989, Salaber & Maza, 1971). Lactobacilli can also be added at strategic points on the bagasse pile, and allowed to profilerate. The excess leached water should be drained and returned to the pile in order to maintain lower pH levels and minimise aerobicity . The bagasse pile should be wet regularly by existing overhead sprinkling systems, in order to keep the pile as moist as possible and reduce aerobicity. Bagasse should be further compacted in order to reduce oxygen from penetrating the core layers. Studies on cereal straw (Misra, 1983) and reeds (Wierdermann, 1983) have indicated that low-moisture, high-compaction bale-storage has provided the best form of protection from degradation (Misra, 1983; Wierdermann, 1983). Problems posed by the environment to reeds is very similar to the problems associated with wood chips (Wolfaardt & Rabie, 2003), which are also stored in piles. The rise of the inside temperature due to slow oxidation and high moisture, creates the ideal conditions for rapid degradation by fungi (Wolfaardt & Rabie, 2003; Wierdermann, 1983). It can, therefore, be concluded that these environmental parameters, if not controlled well, could contribute significantly to the degradation of all types of fibrous material.

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# CHAPTER 3

# THE INFLUENCE OF AGEING OF STORED BAGASSE ON THE DIVERSITY OF FUNGAL POPULATIONS

**ABSTRACT**

An understanding of the microbial populations present in a bagasse pile is influential in determining the degree of losses of bagasse when in storage, as well as in putting forward strategies to minimize the activities of these microbes. There are both detrimental and beneficial microbes present in stored bagasse, and in order to gain more insight into the developing communities in the ageing pile, periodic isolations can be carried out from the pile. The aim of the present study was to enumerate the fungal species proliferating on the surface and inside the bagasse pile, during a storage period of one year. The data were then subjected to various biodiversity calculations in order to model the abundance of species, and to use the α-diversity and ß-diversity indices to study fungal succession on the surface and inside the bagasse pile. It was discovered that more species of fungi were present on the surface of the pile, than inside the bagasse pile, and that the fungal diversity was higher at the surface. Due to the inner pile having a more specialized environment, dominance of the most abundant species was higher. When the different communities were compared using Sorenson's measure, it was found that the fungal communities occurring at the surface and inside the pile were completely different in the early stages of storage, but became more similar toward the end of storage. The efficiency of preservation of bagasse was thus compromised, because the microbial communities established themselves better as the bagasse aged.

## INTRODUCTION

Bagasse has established itself as a major alternative non-wood fibre for the manufacture of pulp and paper, but its storage has created an undeniable problem. Wet-bulk storage techniques have been employed, and have proven very successful, however, the core of the bagasse pile preserves itself very well while the peripheral zones are susceptible to biodegradation (Rangamannar *et al.*, 1993; Ramaswamy *et al.*, 1989; Atchison 1987; Cusi, 1979; Moebius, 1966). Ramaswamy *et al.* (1989) concluded in a study on a bagasse pile at the Tamil Nadu Pulp Mill in India, that bagasse in the peripheral regions, is constantly associated with a diverse range of fungi. Bacteria and acinomycetes are more prevalent and influential in the core regions of the pile (Rangamannar *et al.*, 1993). Artificial inoculation with the deleterious organisms (fungi) resulted in a 8% reduction in yield (Ramaswamy *et al.*, 1989). This reduction in yield illustrates the need to control the proliferation of these fungi, if bagasse is to be preserved for extended periods.

Fungi function in many ways in different ecosystems, including mycorrhizal symbionts, endophytism, pathogenesis and most importantly as agents of degradation. These microorganisms are traditionally known to degrade carbon and nitrogen-rich substrates, however, their ability to participate in most of the biochemical pathways that characterize nutrient cycles is well understood (Wainwright, 1992). Fungi as decomposers are especially important in ecosystems where a large volume of the biomass is comprised of lignocellulosic material. The largest component of bagasse fibre consists of cellulose that is highly degradable by many microorganisms, especially fungi. The second most abundant component is lignin, a recalcitrant polyphenolic molecule that can only be effectively degraded by white-rot fungi (Trappe and Luoma, 1992). The succession of fungi, especially on woody materials, is well known (Frankland, 1992), however, the effects of fungi on non-wood fibres such as bagasse, is not well understood (Ramaswamy *et al.*, 1989). Some fungi are pioneer colonizers, and are later replaced by other fungal species. Frankland (1992) concluded that the diversity within the fungal kingdoms usually results in the presence of fungi through all successional stages despite

changes in the immediate environment. It now becomes more apparent, that in addition to characterizing the fungi that are present on bagasse, the biodiversity and population characteristics should also be measured.

Biological diversity is characterized essentially by two very important indicators, species richness and species evenness. These indicators encompass the two components of biodiversity, namely; variety and relative abundance (Magurran, 1988). It is very rarely found that all species are equally abundant because some species are better competitors and more prolific than others (Danoff-Burg, 2003). The Shannon index, and the Berger-Parker and Simpsons indices, were termed 'heterogeneity indices' because they encompass both species richness and evenness (Peet, 1974). The Shannon index seeks to characterize the diversity of a community by a single number, and is based on the proportional abundance of species (Magurran, 1988). The second group of heterogeneity indices (Berger-Parker index and Simpsons index) is referred to as dominance measures because they are weighted more toward the abundances of the most common species. As more data sets containing information on species richness and species evenness were generated, patterns of species abundance became apparent (Magurran, 1988). These patterns led to the development of species abundance models that were strongly advocated as providing the only acceptable criteria for the evaluation of species diversity (Magurran, 1988).

Magurran (1988) stated that a species abundance distribution utilizes all the information gathered in a community and is the most complete mathematical description of data. Diversity is thus examined in relation to four main models (Figure 1), a geometric series, log-normal distribution, log-series distribution and according to the MacArthur's broken-stick model (Magurran, 1988). A geometric series represents an environment where fewer species are abundant, whereas a log series distribution and a log normal distribution represent an environment where the intermediate species are more common. The broken stick model represents an environment where species abundance is

evenly distributed, therefore, species evenness is highest when the broken-stick model is fitted (Southwood, 1978, Magurran, 1988).

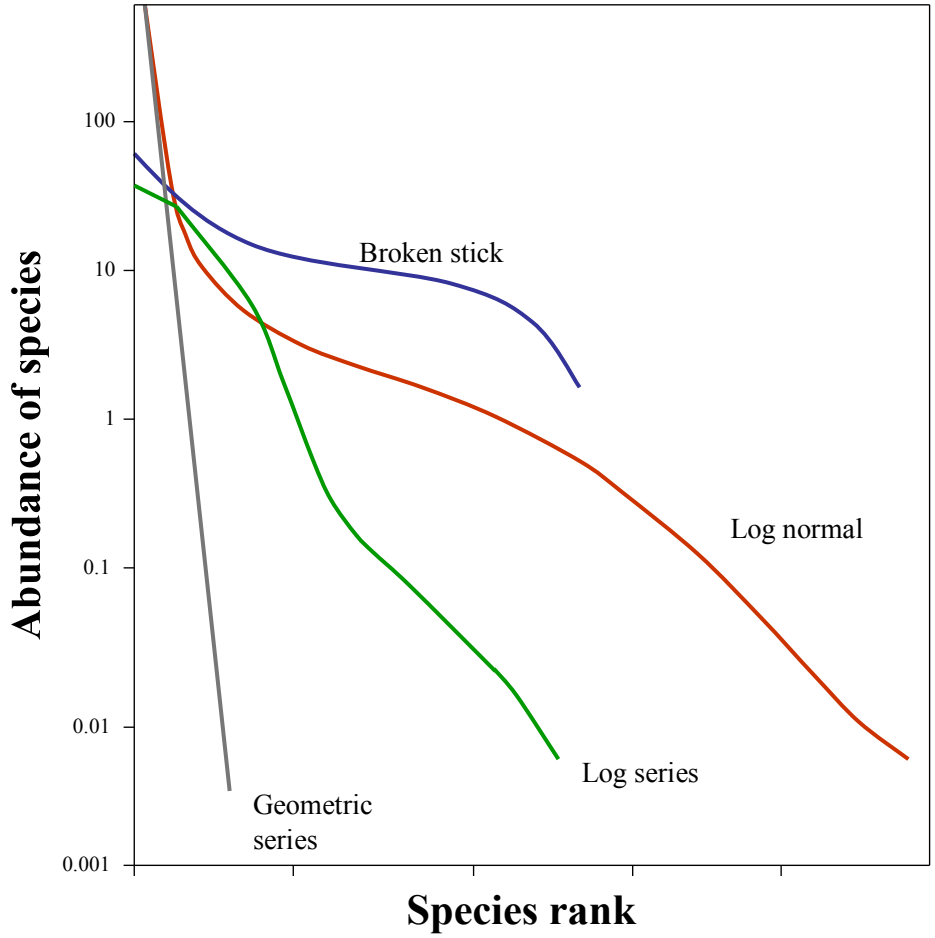


**Figure 1**: Rank abundance plots illustrating the typical shape of four species abundance models. (Adopted from Magurran, 1988).

Since large quantities of bagasse need to be stored for extended periods before pulping, microbial degradation is imminent. In order to make recommendations to improve the stacking practices for minimal biodegradation of bagasse, the communities of fungi that exist in a bagasse pile need to be characterised. The objective of this study was to enumerate the fungi on bagasse, in order to assess the structural diversity of communities at the surface and inside the bagasse pile, at the Sappi Stanger mill.

## MATERIALS AND METHODS

### Site selection

This study was conducted on an isolated part of the bagasse pile at the Sappi Stanger mill in Kwazulu Natal. There was no human or mechanical interference at this part of the pile for the duration of the project. Six areas were selected for sampling; three at the surface and three for sampling 50 cm into the pile. Bagasse from these areas was collected at intervals during the course of this study. During the May to December 2004 cutting season, samples were collected when the pile was 73, 117, 141, 168, 212, 268, 308 and 360 days old, and during the May to December 2005 cutting season, a sample was collected when the pile was 21 days old. This was done due to sampling not being possible until 73 days during the May to December 2004 cutting season.

### Sampling and statistical analysis

Surface samples were obtained by scraping away superficial bagasse at the three sites, and then removing the test sample. The samples were placed in brown paper bags, and stored for later use. The inner pile samples were dug up by hand, placed in brown paper bags, and also stored for later use. The samples were brought back to the laboratory for processing. A factorial experiment was conducted, with the position in the pile and storage time as factors. According to Magurran (1988), statistical analysis that is based on replicated samples (in this case three replicates) is superior to the *t*-test. The data was analysed using one-way analysis of variance (ANOVA), and the means of the different factors were compared using Tukey's test (Winer, 1971) at a 95% confidence level.

### Fungal enumeration

Potato Dextrose Agar (Biolab) containing chloramphenicol (250 mg/L), and Malt Salt Agar (Biolab) prepared with malt extract (20g /L) and salt (75 g/L) were prepared as described by Rabie *et al*. (1997) and poured into 90-mm petri dishes and left to solidify overnight. Five pieces of bagasse, approximately 5 to 10 mm in length, were placed in

each petri dish. In total, 20 petri dishes were used to represent 100 pieces of each replicate collected at the surface and inside the bagasse pile. Two sets of samples were prepared and placed at 25 and 45°C respectively, for up to two weeks. After incubation, all fungi present were identified based on morphological characteristics by microscopic examination. In some cases, certain fungi that could not be identified were transferred to fresh medium for further incubation. All specimens were identified to species level, except for nine *Penicillium* spp., two *Mucor* spp., two *Humicola* spp., and one species each of *Trichoderma, Alternaria, Acremonium*, *Monilia*, *Nigrospora*, *Paecilomyces*, *Rhizopus, Staphylotrichum*, *Sporotrichum, Eurotium* and *Fusarium,* as well as one coelomycetous fungus. All the fungi that were present on the bagasse pieces, including an unknown species, a white mycelial fungus and a yellow fungus, was counted and used in the calculation of diversity indices.

**Determination of structural diversity**

In order to assess the utilization of resources and the environmental impact on the microbial communities, the abundance of species was tested for a goodness of fit to the different distributions by using a $?^2$ test (Magurran, 1988). Species were placed in abundance classes according to the number of individuals, where the upper limit of each class was determined by :

$$U = 2^c + 0.5 \qquad (1)$$

Where $U$ = the upper limit of a class, and $c$ = the class number.

The different components of diversity, including species richness, evenness and dominance were calculated as described by Magurran (1988). The Shannon diversity index was calculated for each replication at the surface and inside the bagasse pile, in order to estimate the diversity of fungi in a bagasse pile as follows:

$$H' = ?\,p_i \ln p_i \qquad (2)$$

Where $H'$ = the Shannon index of species richness, and $p_i$ = the proportional abundance of the $i$th species and:

$$p_i = (n_i/N) \qquad (2.1)$$

Where $n_i$ = the number of individuals of the $i$th species, and $N$ = the total number of individuals.

The Berger-Parker index was used as a measure of dominance by the most abundant fungal species and was calculated using:

$$d = N_{max}/N \quad (3)$$

Where $d$ = the Berger-Parker index of dominance, $N_{max}$ = the number of individuals in the most abundant species and $N$ = the total number of individuals.

The similarity between the samples within, and between the surface and the inner pile, was calculated using Sorensons measure for quantitative data as follows (Magurran, 1988):

$$C_N = 2_{jN} / (aN + bN) \quad (4)$$

Where $aN$ = the total number of individuals at a site, $bN$ = the total number of individuals in a second site, and, $jN$ = the sum of the lower of the two abundances recorded for species found in both sites. The matrix of similarities thus obtained, was used to construct a dendogram with the "Unweighted Pair-Group Method Using Arithmetic Averages" (UPGMA).

## RESULTS AND DISCUSSION

### Fungi

A large number of fungi with the ability to reduce lignocellulosic polymers were found in bagasse. A total of 51 species were found at the surface of the bagasse pile, and 31 species were found inside the pile. These fungi included large numbers of *Aspergillus fumigatus* and *A. pennicillioides* inside the pile (Table 1), and *Cladosporium cladosporioides, Phoma sorghina* and *Penicillium* spp. at the surface of the pile (Table 2). *Penicillium* spp., *Aspergillus* spp. and *Trichoderma* spp. are well known opportunistic storage fungi (Domsch *et al*., 1980), therefore, accounting for their high prevalence on bagasse.

**Table 1:** Fungal numbers inside the bagasse pile at Stanger (Appendix B).

| Species | Sampling time (Days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **21** | **73** | **117** | **141** | **169** | **212** | **268** | **308** | **360** |
| *Acremonium* sp. 1 | | | 7 | | | 3 | | | |
| *Alternaria dianthi* | | | 1 | | | | | | |
| | | | | | | | | | 20 |
| *Aspergillus fumigatus* | 300 | 300 | 193 | 268 | 289 | 295 | 300 | 226 | 300 |
| *Asper* | | | | | 1 | | 28 | | |
| *Aspergillus pennicilliodes* | 157 | 68 | 1 | 2 | | | | | 300 |
| | | | 2 | | | 1 | | | |
| Basidiomycetous fungus sp. 1 | | | 9 | | | | | | |
| Coelomycetous fungus sp. 1 | | | | | 18 | | | | |
| | | | | | | | 5 | 1 | |
| *Eurotium amstelodami* | 2 | 24 | 1 | | | | | | |
| *Eurotium* sp. 1 | | | 29 | 22 | 1 | 14 | | | |
| *Fusarium graminearum* | | | | 12 | | | | | |
| *Humicola* sp. 1 | 91 | 47 | | | | | | | |
| *Humicola* sp. 2 | 129 | 151 | | | | | | | |
| *Monilia* sp. 1 | | | | | | 6 | | | |
| *Mucor* sp. 1 | 39 | 33 | | | | | | 50 | 48 |
| *Mucor* sp. 2 | 1 | | | | | | | | |
| *i* | 129 | 98 | 72 | 32 | | | | | |
| *Paecilomyces* sp. 1 | | | | | | | 27 | | |
| *Penicillium* sp. 1 | | | | | | | 285 | | |
| *Penicillium* sp. 2 | | | | | | | 172 | | |
| *Penicillium* sp. 5 | | | | | | | | 118 | |
| *Penicillium* sp. 6 | | | | | | | | 20 | |
| *Penicillium* sp. 9 | | | | 6 | 3 | | | | |
| *Periconia* sp. 1 | | | 1 | | | | | | |
| | 64 | 22 | | | | | | | |
| *Rhizopus oryzae* | | | | 36 | 63 | 46 | 75 | 2 | |
| *Rhizopus* sp. 1 | | | | | | | | | 50 |
| Unknown sp. 1 | | | 4 | | | | | | |
| White mycelial fungus | | | | | | | 3 | 1 | |
| **Number of species (*S*)** | **9** | **8** | **11** | **7** | **6** | **6** | **8** | **7** | **5** |
| **Number of individuals (*N*)** | **912** | **743** | **320** | **378** | **375** | **365** | **895** | **418** | **718** |

**Table 2:** Fungal numbers at the surface of the bagasse pile at Stanger (Appendix B).

| Species | Sampling time (Days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **21** | **73** | **117** | **141** | **169** | **212** | **268** | **308** | **360** |
| *Acremonium* sp. 1 | 80 | 58 | | 8 | 12 | | | 25 | 17 |
| *Alternanria alternata* | | | 21 | | 45 | 54 | | 97 | |
| *Alternaria citri* | | | | 44 | 2 | | | | |
| *Alternaria longissima* | | 28 | | | 1 | 3 | | | |
| *Alternaria* sp. 1 | | | | | 165 | 84 | 42 | 8 | 36 |
| *Alternaria tennuissima* | | | 140 | 111 | 28 | 210 | 10 | 55 | |
| | | | | | 1 | | 2 | | |
| *Aspergillus niger* | | | | | | | 10 | | 32 |
| | | | | | 1 | | | | |
| *Aspergillus terreus* | 3 | | | | 1 | | 11 | | |
| *Aspergillus versicolor* | | 12 | 1 | 3 | | 5 | | | 21 |
| Basidiomycetous fungus sp. 1 | | | | | 7 | | | | 48 |
| *Botrytis cinerea* | | | | | 2 | | | | |
| | 221 | 126 | 200 | 227 | 282 | 261 | 23 | 190 | |
| *Cladosporium cucumerinum* | | 79 | 84 | 37 | 27 | 34 | | 29 | 166 |
| *Cladosporium sphaerospermum* | | 13 | 94 | 37 | | 106 | 19 | 187 | 5 |
| Coelomycetous fungus sp. 1 | | | | | | | | 9 | |
| *Epicoccum nigrum* | | | 5 | 15 | 1 | 2 | 4 | | 9 |
| | | | | | | | | | 20 |
| *Fusarium poae* | | | 1 | 5 | | | | 4 | |
| *Fusarium* sp. 1 | | | 7 | 9 | 23 | 8 | 11 | 73 | 23 |
| | | | 29 | 24 | | | | | |
| *Fusarium oxysporum* | 113 | 154 | | 12 | 7 | | | 21 | 2 |
| sp. 1 | 7 | 5 | | | | | | | |
| *Monilia* sp. 1 | | | 4 | 8 | 21 | | | | |
| *Mucor* sp. 1 | | | 56 | 33 | 9 | | 30 | 67 | |
| *Mucor* sp. 2 | | | | | | | 14 | | |
| *Nigrospora* sp. 1 | | | 1 | | | | | | |
| *Paecilomyces variotii* | | | 1 | 6 | 2 | 4 | 30 | | 11 |
| *Paecilomyces* sp. 1 | | | | | 24 | 25 | 3 | | |
| *Penicillium* sp. 1 | | | 28 | | | | 240 | 34 | |
| *Penicillium* sp. 2 | | | | | | | 98 | 50 | |
| *Penicillium* sp. 3 | | | 36 | | | | 58 | 45 | |
| *Penicillium* sp. 4 | | | 78 | | | | 1 | 17 | |
| *Penicillium* sp. 5 | | | 24 | | | | 8 | | |

**Table 2:** Continued

| Species | Sampling time (Days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **21** | **73** | **117** | **141** | **169** | **212** | **268** | **308** | **360** |
| *Penicillium* sp. 6 | | | 1 | | | | 6 | | |
| *Penicillium* sp. 7 | | | 8 | | | | | | |
| *Penicillium* sp. 8 | | | 40 | | | | | | |
| *Penicillium* sp. 9 | 28 | 46 | | 129 | 166 | 224 | | | 255 |
| | 96 | 92 | 254 | 199 | 61 | 245 | 136 | | |
| *Rhizopus oryzae* | | | 1 | | | | 55 | | 8 |
| *Rhizopus* sp. 1 | | | | | | | 4 | | |
| | | | 6 | 15 | 7 | 5 | | | |
| *Scopulariopsis* sp. 1 | | | 1 | | | | | | |
| *Sporotrichum* sp. 1 | | | | | | | 68 | | 89 |
| *ide* | | | 42 | 18 | | | 93 | | |
| *Trichoderma koningii* | 210 | 300 | | | 12 | | 23 | | 300 |
| *Trichoderma* sp. 1 | | | | | 139 | | 11 | | |
| Unknown sp. 1 | | | | | | | | 12 | |
| Yellow fungus | 71 | 12 | | | | | | | |
| White mycelial fungus | | | | | 1 | 8 | 9 | | 5 |
| **Number of species (*S*)** | **9** | **12** | **26** | **19** | **26** | **16** | **26** | **17** | **17** |
| **Number of individuals (*N*)** | **850** | **998** | **1280** | **1081** | **1216** | **1490** | **1019** | **1231** | **1407** |

The hyphomycete genus *Aspergillus*, occurs commonly in soil in warmer climates, in compost, decaying plant matter and stored grain (Domsch *et al.*, 1980) and it is, therefore, not surprising that *A. fumigatus* and *A. pennicillioides* were present on 92 % and 20 % of the bagasse pieces sampled from inside the pile, respectively (Table 1). *Aspergillus ochraceus* and *A. flavus* were found on sampled bagasse from the inner pile, and *A. niger, A. versicolor, A. terreus and A. fumigatus* were found in sampled bagasse from the surface. However, they were found in less than 10 % of the bagasse sampled. *Aspergillus fumigatus* is a thermotolerant fungus with a wide temperature range for growth (12 to 57°C), and optimum growth between 37°C and 43°C (Smith & Anderson, 1973). However, *A. fumigatus* is not limited to habitats with permanently high temperatures. Available data on soil investigations, indicate that

*A. fumigatus* prefers a pH in the range of 5 to 8.5 (Domsch *et al.*, 1980). The temperature and pH inside the bagasse pile at Stanger (Chapter 2), was within the range for optimal growth of this fungus, hence its high abundance (92 %). This fungus has previously been isolated from pines, barley, wheat, straw and grass compost, suggesting a high prevalence in lignocellulosic materials (Domsch *et al.*, 1980). *Aspergillus fumigatus* also produces various fibre-degrading enzymes, viz: 1,3-ß-glucanase (Flannigan & Sagoo, 1977); extracellular a- and ß-glucosidases, and xylanase (Flannigan & Sellars, 1977). *Aspergillus fumigatus* is known to grow well on glucose, cellulose and xylose media (Dickinson & Boardman, 1970). This fungus in large numbers can also be a health hazard and cause for concern, because it causes bagassosis, a condition characterized by fungal infections in the lungs (Wen-ching *et al.*, 1973; Moebius, 1966).

The second most abundant species belonged to the genus *Cladosporium*, that are amongst the most common air-borne fungi, and thus have a worldwide distribution (Harvey, 1967). In this study *C. cladosporioides, C. sphaerospermum* and *C. cucumerinum* were present on 57 %, 17 % and 17 % of the bagasse pieces sampled from surface of the bagasse pile, respectively (Table 2). *Cladosporium cladosporioides* is the most common species of its genus on plant material and in soil (Jensen, 1931). It has also been isolated from stored timber, wood pulp (Wang, 1965) and stored crops (Hubalek, 1974). *Cladosporium cladosporioides* was prevalent throughout bagasse storage and is known to have a high conidial production rate under moist conditions. However, this fungus still flourished on a bagasse pile with low moisture. The temperature and pH of the bagasse pile created optimal conditions (20 to 28°C; neutral pH) for the proliferation of *C. cladosporioides* (Chapter 2). Cellulolytic activity of this fungus is very low and this fungus has been known to grow on D-xylose (Domsch *et al.*, 1980).

Another frequently found species belonged to the genus *Trichoderma,* which includes mycoparasites and degraders of organic material that grow saprophytically on dying or dead plant material (Domsch *et al.*, 1980). In this study, *T. konongii* was present

on 21 % of bagasse sampled from the surface of the bagasse pile (Table 2). *Trichoderma koningii* has been known to proliferate on D-xylose, D-fructose, D-glucose and glycerol (Danielson & Davey, 1973), while the ability of this fungus to degrade cellulose with the production of $C_1$, $C_X$, ß-glucosidase and 1,4-ß-glucan cellobiohydrolase has been described (Fanelli & Cervone, 1977). According to Domsch *et al.* (1980), *T. konongii* occurs frequently on the surface layers of soils and generally in slightly acidic habitats with pH between 3.7 and 6.

The data collected during this study showed that *T. koningii* was more abundant at the beginning of the study when the pH levels were almost neutral (Chapter 2). Furthermore, this fungus normally becomes dominant during an advanced stage of decay (Domsch *et al.*, 1980), however, it was more abundant at the beginning of the present study when decomposition was in an early stage. The optimum temperature for the growth of *T. koningii* is between 26 and 32°C, and this temperature requirement was met by the conditions at the surface of the bagasse pile (Komatsu, 1976). *Trichoderma viride* was also found at the surface of the pile, however, in smaller numbers (Table 2).

*Penicillium* species are ubiquitous saprophytes with many fertile conidia dispersed through the atmosphere (Pitt, 1979). They have an important role as agents of decay, and in the natural processes of recycling of biological matter. In this study, nine *Penicillium* species were isolated and allocated numerals, accordingly to their sequence of isolation (Table 1 and 2). This is a diverse genus that contains morphologically similar species that are difficult to identify. According to Pitt (1979), there are between 150 to 300 species within the genus, with at least 1000 recognisably different phenotypes to be catalogued.

*Alternaria tenuissima* and *A. alternata* were present on 21 % and 10 % of bagasse samples from the surface of the pile, respectively (Table 2). *Alternaria* species include a number of plant-parasites that are mostly host-specific and often seed-borne, with a few species being ubiquitous (Domsch *et al.*, 1980). *Alternaria alternata* is the

most common and cosmopolitan species occurring on many kinds of plant and other substrates while *A. tenuissima* is a secondary invader (Ellis, 1971). The optimum environment for conidium germination of *A. tenuissima* and *A. alternata* is at a temperature of between 25 and 28°C, and a pH between 4 and 5 (Domsch *et al.*, 1980). The bagasse environment at the surface provided suitable heat, but the pH was relatively alkaline (Chapter 2). These environmental conditions could have influenced the slow proliferation of *Alternaria* species at the surface of the pile.

Suitable carbon sources that are utilized by *A. alternata* include maltose, sucrose, raffinose, xylan and arabinoxylan (Baimataeva, 1970), while cellulose decomposition has been noted on a wide range of substrates (Lizak, 1975). An unknown *Alternaria* species was found on 13 % of the bagasse sampled at the surface of the pile (Table 2), however, identification to the species level was not possible. *Alternaria citri* and *A. longissima* were also present on the surface of bagasse, but in negligible numbers (Table 2), while *A. dianthi* was present minimally on bagasse sampled from the inner pile (Table 1).

In the Hyphomycetes genus, *Fusarium*, most species are soil fungi with cosmopolitan distributions, and many are active in the decomposition of cellulosic substrates such as bagasse (Domsch *et al.*, 1980). *Fusarium oxysporum* was present on 11 % of bagasse pieces sampled from the surface of the pile (Table 2). This fungus has a worldwide distribution as a soil saprophyte and occurs on numerous host plants. The optimal conditions for the growth of this fungus is at temperatures between 25 and 30°C, at pH 7.7, and at areas with relatively high moisture (Hejtmanek, 1960). Due to its high rate of cellulose decomposition (Domsch *et al.*, 1980), the presence of this fungus should be reduced for better bagasse preservation. *Fusarium graminearum*, *F. chlamydosporium* and *F. poae* were found in low numbers at the surface of the pile (Table 2).

Other fungal species that were present in lower numbers (<10 %), but which could be important degraders of bagasse fibre, included *Rhizopus oryzae*, *Humicola* sp., *Epicoccum nigrum* and *Paecilomyces variotii*. *Epicoccum* spp. are excellent xylan and

cellulose degraders, while *Rhizopus oryzae*, *Humicola* spp., and *Paecilomyces variotii* have been known to decompose cellulose (Domsch *et al.*, 1980). Most of the fungal species that have been isolated in this study have abilities according to literature, to degrade cellulose (Domsch *et al.*, 1980), and this could cause a major problem with bagasse storage at Stanger.

**Structural diversity**

**a) Species abundance**

The cumulative number of species increased steadily, both at the surface and inside the bagasse pile, over the 360 days of sampling (Figure 2). The final number of species at the surface and inside the pile amounted to 51 and 31, respectively. A steep increase was noted on the surface after 75 days, and continued increasing from 107 days. Due to the surface of the bagasse pile being exposed to contamination, the continued increase in the number of species at the surface towards the end of the 360 day sampling period, could indicate further increases in the number of species during continued storage.

If the storage time of bagasse inside the pile were to be increased to more than 360 days, the more stable species richness could reflect a stable population where no additional species were introduced. The stabilization of the cumulative number of species on the inside of the pile, indicates that fewer additional species would be introduced if the age of the bagasse pile were to be increased for more than 360 days (Figure 2).

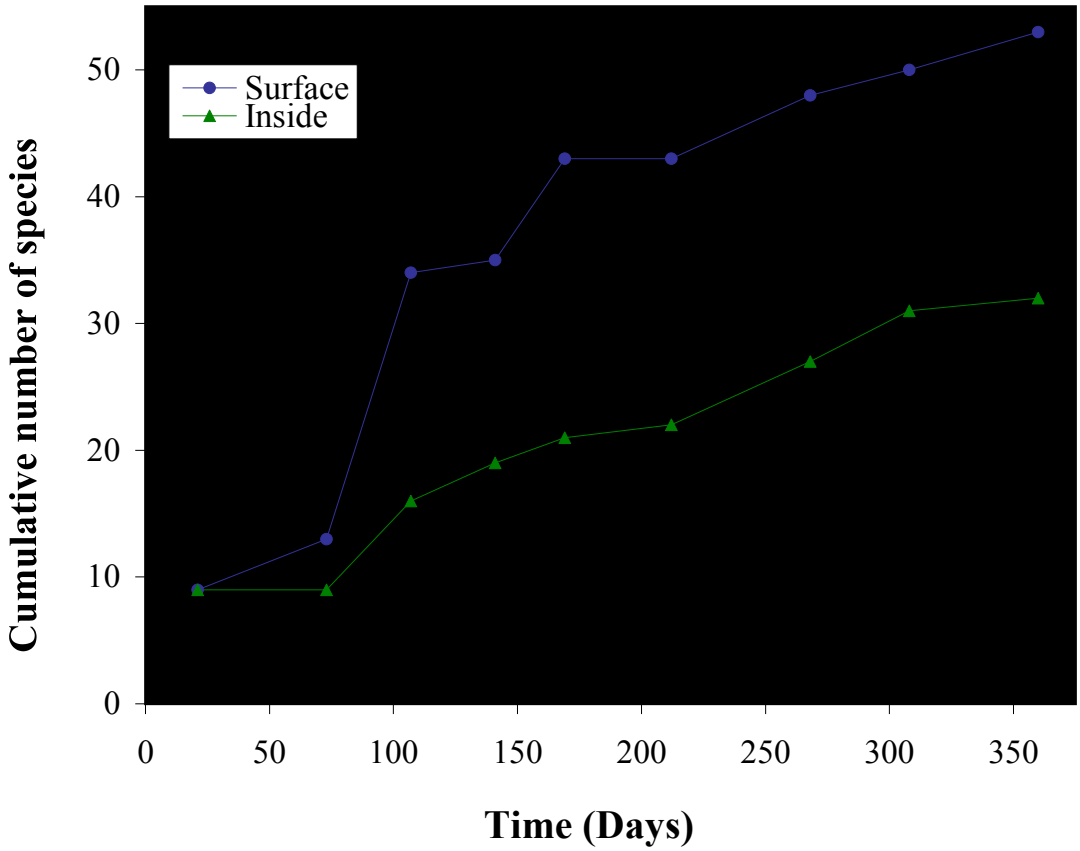


**Figure 2:** Cumulative number of species of fungi collected at the surface of the bagasse pile, and inside the pile, over the 360-day sampling period.

The $?^2$ test did not show a significant goodness of fit of ranked species abundance for any of the datasets to a distribution model. However, the individual curves when plotted, appeared to reflect certain models better than others (Table 3). The similarity of the observed data to some of the proposed models (Table 3) are illustrated in Figure 3, although no significant fit to any model was found. These models included the log normal distribution, log series, geometric series and the broken-stick distribution model.

**Table 3:** Apparent models most similar to distribution patterns for different samples from the surface and from the inner bagasse pile.

| Storage time (Days) | Surface | Inside |
|---|---|---|
| 21 | Log normal | Log normal |
| 73 | Log series | Log series |
| 107 | Broken stick | Geometric series |
| 141 | Broken stick | Geometric series |
| 169 | Broken stick | Geometric series |
| 212 | Broken stick | Geometric series |
| 268 | Broken stick | Broken stick |
| 308 | Log normal | Broken stick |
| 360 | Log normal | Broken stick |

According to Magurran (1988), the four models represent a progression ranging from an environment where fewer species are dominant on the available food-source (Geometric series distribution) to an environment where the species of intermediate abundance become more prevalent (Log series and Log normal distributions). The broken-stick model reflects an environment where many species are equally abundant. Employment of the broken-stick model would suggest that there is no single dominant species in the area studied.

The diversity of organisms at the surface and inside the pile, are reflected by a log normal distribution between one and 21 days followed by a log series distribution between 21 and 73 days (Table 3). According to Danoff-Burg (2003), a log-series distribution indicates a novel community where new species arrive, while few factors dominate the community. A log-normal distribution, furthermore, represents a large mature community where many factors dominate the community (Magurran, 1988). The results of the present study appears contrary to the literature. However, the samples collected after 21 days of storage, could have been contaminated by the bagasse remaining from the previous cutting season.

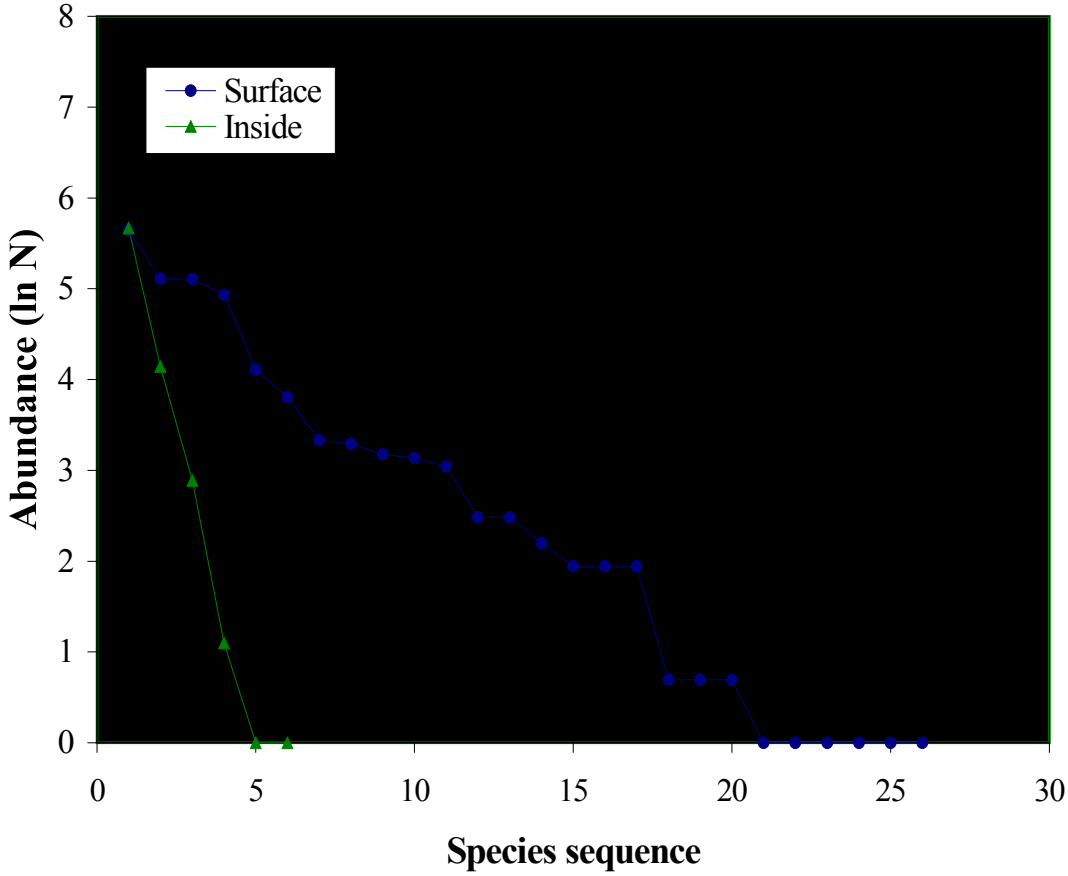


**Figure 3:** Ranked abundance plots reflecting distribution patterns most similar to the broken-stick model (Surface) and the geometric series model (Inside), after 169 days of storage.

Southwood (1978) suggested that the geometric-series distribution represents a situation of maximum niche pre-emption while the broken-stick model reflects minimal niche sequestration with resources equally distributed. The broken-stick model is the biologically realistic expression of a uniform distribution (Magurran, 1988). The present study showed that the surface of the bagasse pile started showing minimal niche pre-emption between 107 days and 308 days (Table 3), suggesting a uniform distribution of species at the surface of the bagasse pile during this time. Populations at the surface of the pile exhibited characteristics of the log-normal distribution during the last 50 days of this study. This log-normal distribution indicates that the community at the surface is mature and that more factors started to dominate the community (Magurran, 1988).

The inside of the bagasse pile, however, shows a situation of maximal niche pre-emption (Geometric series) from 107 days, continuing until a pile age of 268 days (Table 3). This model is characteristic of a few organisms being abundant, while the rest are fairly uncommon. This distribution also suggests a species-poor environment that is either due to the harsher environment, or that the inner pile is still in the early stages of succession. After 268 days, until completion of this one-year study, the inner pile started adhering to the characteristics portrayed by the broken stick model. This trend would suggest that at a later stage of succession, well established species became equally abundant and that these species were better adapted to the environment inside the pile. This theory is supported by the cumulative number of species that stabilizes towards the end of the study (Figure 2).

**b) a-diversity**

The Shannon diversity index (*H'*) indicated that the highest fungal diversity was found at the surface of the bagasse pile, when compared to the inside of the bagasse pile, throughout the 360 day storage period (Figure 4). The largest number of fungal species was found at the surface of the bagasse pile, possibly due to the more exposed surface area that is open to contamination, but also due to the more favourable environment for growth at the surface of the bagasse pile (Chapter 2). It, therefore, seems that the lower temperature (16 to 34.5°C) and the lower moisture (28 to 43 %) at the surface of the pile (Chapter 2), accounts for the higher number of fungal species.

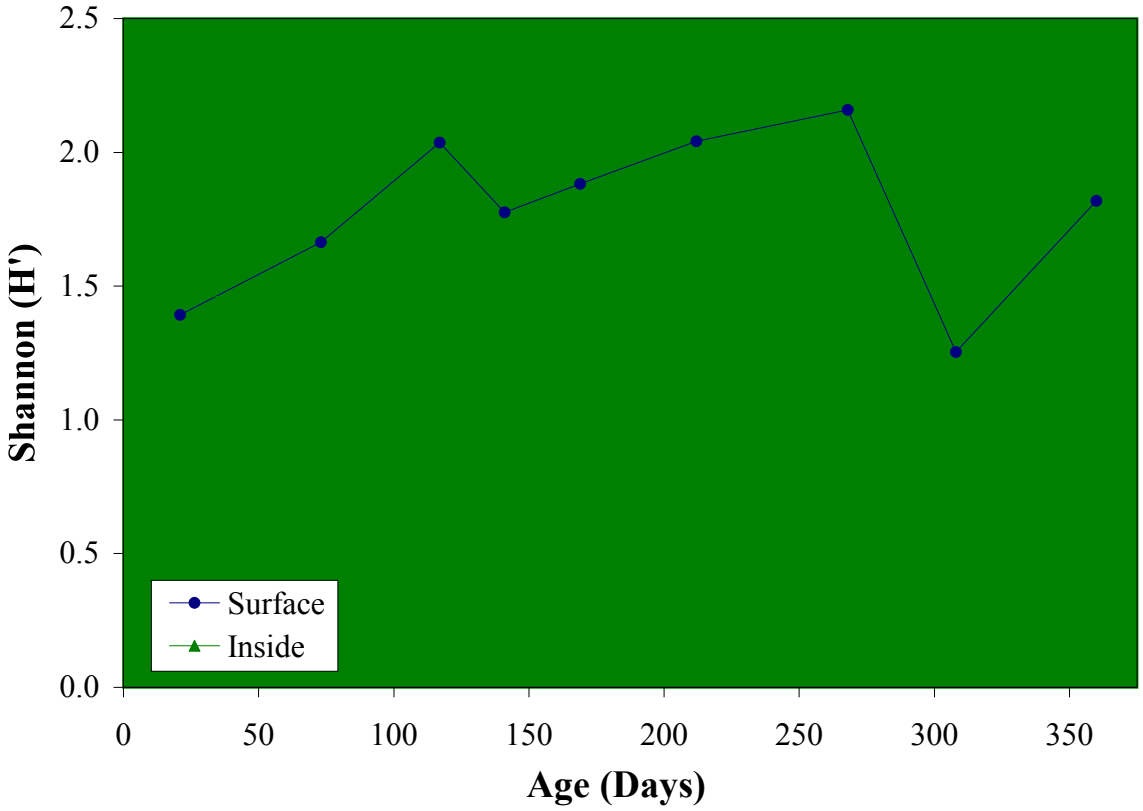


**Figure 4:** The relationship between the Shannon index and storage time at the surface and inside the bagasse pile, over the 360-day storage period (Appendix C 1).

Changes in the Shannon index reflect changes in the number of species and in their proportional abundances (Magurran, 1988), and the Shannon index values changed constantly, both at the surface and inside the bagasse pile (Figure 4). Diversity at the surface increased from 1.39 to 2.16 until 268 days when 26 species were counted (Table 2). The increased diversity reflected by an increase in the number of species by 17 (Table 2), could possibly be attributed to an overall increase in temperature and moisture content at the surface of the pile (Chapter 2). The increase in temperature at the surface of the pile could account for the optimum proliferation of mesophilic fungi. After 268 days of storage until the end of this study, the Shannon index decreased overall from 2.16 to 1.82, reflecting a reduction in the number of species from 26 to 17 species (Table 2). The environment at the surface of the pile thus changed and became more restrictive as the moisture content and the pH decreased (Chapter 2).

Diversity inside the bagasse pile decreased from 1.52 to 0.62 until 117 days, and then remained relatively constant until 212 days of storage (Figure 4). The changes in diversity until 117 days of storage were apparently brought about by the decrease in the number of individuals isolated, by as much as 65 % (from 912 to 320) after 117 days (Table 1). This decrease in the number of individuals could be due to a decrease in temperature between 21 and 73 days, followed by an increase between 73 and 212 days (Chapter 2). The environment within the bagasse pile possibly determined that thermophilic fungi will proliferate more than mesophilic fungi due to the higher temperatures (between 25.7 and 44.67°C). The changes in diversity (H') inside the pile generally reflect proliferation of thermophiles that replaced the mesophilic fungi that were initially present. Fungi present inside the bagasse pile showed an overall increase in diversity after 212 days until the end of the study, whilst the moisture content also increased significantly (16 %) over this period (Chapter 2). There was a large number of *Pennicillium* spp. found during these surveys, that contributed to the higher number of species, therefore, accounting for the increase in species diversity.

The Berger-Parker indices confirmed that the highest degree of dominance was present inside the bagasse pile, compared to the surface of the bagasse pile (Figure 5). This result was expected because the environment within the bagasse pile, being harsher, is less suited to a diverse population. Dominance at the surface of the bagasse pile showed no significant changes throughout the course of this study, and remained relatively lower than the inner pile. The lower dominance on the surface reflects the greater number of species occurring in an environment suitable to the growth of most fungi (Chapter 2). The dominance in the inner pile displayed a maximum increase over the first 212 days of storage, followed by a decrease to the end of the storage period.

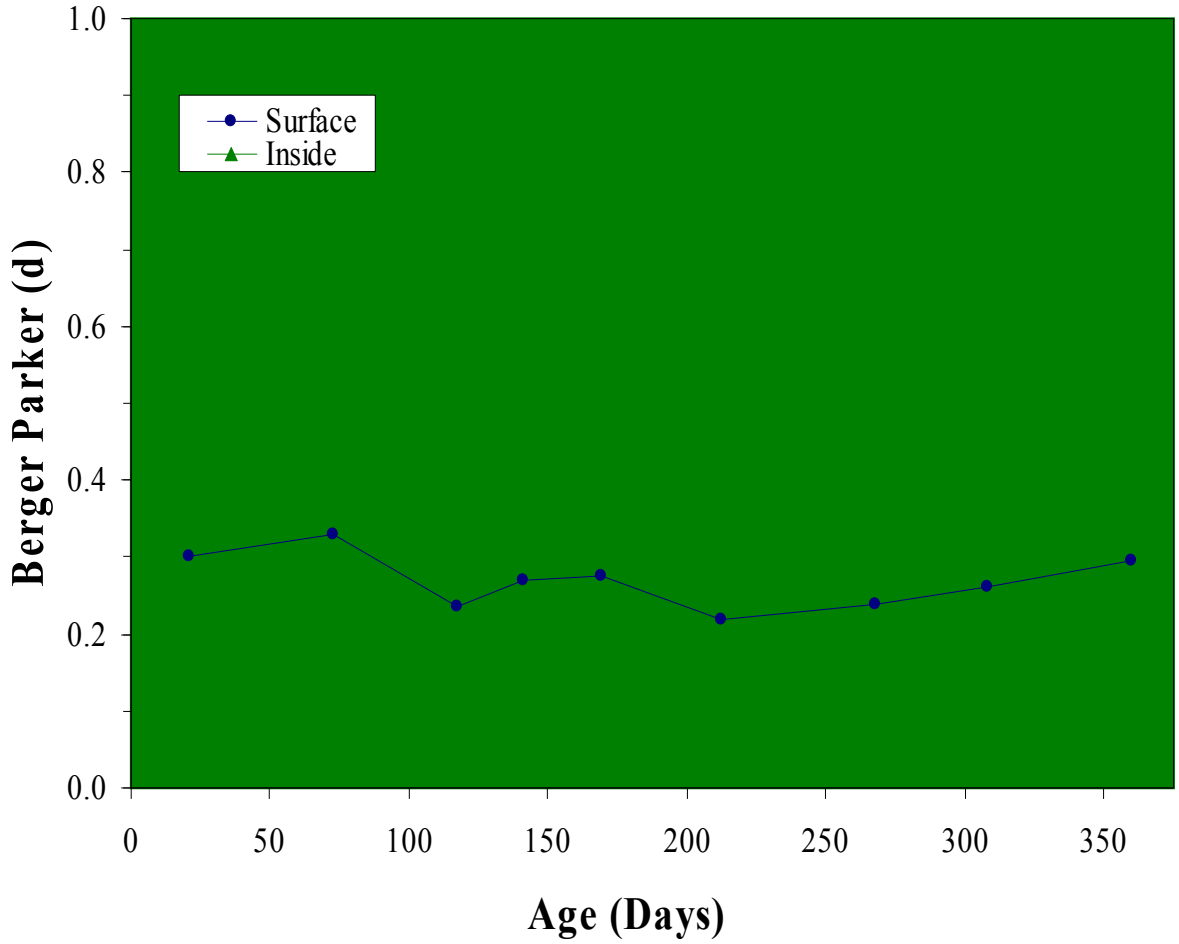


**Figure 5:** The Berger-Parker index plotted against time showing the difference in dominance at the surface and inside the bagasse pile, over the 360-day storage period (Appendix C 2).

The increase in dominance followed by the decrease towards the end of the storage period, seems to support the niche pre-emption (Geometric-series model) exhibited inside the bagasse pile between 117 and 212 days of storage, followed by lower pre-emption (Broken-stick model) until completion of this study. Fewer species, such as *A. fumigatus*, dominated the inner pile in higher numbers until sampling at 212 days, while the changing environment created conditions for more species to start proliferating, thus bringing about a decrease in dominance (Table 1). The temperature in the pile showed significant increases until 212 days (approximately 9°C) and it, therefore, seemed that the temperature within the bagasse pile was the driving force behind the development of the community, hence the dominance by a small number of fungal species.

**c) ß-diversity**

The ß-diversity of the surface and inside the bagasse pile was compared by using Sorenson's measure, because this index incorporates the similarity of samples or sites using standard ecological techniques of ordination and classification (Magurran, 1988). Four distinct clusters were noted sharing similarity of 32 %, 38 %, 48 % and 42 %, respectively (Figure 6).

The first cluster (A) incorporated the first six samples (21 to 212 days) collected at the surface of the bagasse pile in Stanger while Cluster C incorporated the first six samples (21 to 212 days) collected from inside the bagasse pile (Figure 6). Cluster D included the two samples (268 days) collected from both the surface and the inner pile, while the last two samples of the ageing bagasse pile collected from the surface and the inner pile after 268 days. The last two samples (308 days and 360 days) of the ageing bagasse pile collected at both the surface and inside the pile, were grouped in Cluster B.

These results suggest that up until 212 days of storage, the fungal populations isolated from the surface differed from the the populations inside the pile. The bagasse pile after 268 days had similar populations inside and at the surface but a low similarity to the populations existing prior (Figure 6). The results further illustrate that towards the end of the study (308 to 360 days of storage), the fungal communities inside the pile were similar to the communities on the surface of the pile and different from any communities existing before. Similar communities could indicate similarity in their respective environments and examination of the environmental parameters showed that the pH towards the end of the storage period, was similar at the surface and inside the bagasse pile (Chapter 2), therefore, pH appears to be a strong driver of microbial diversity in the bagasse pile studied here.

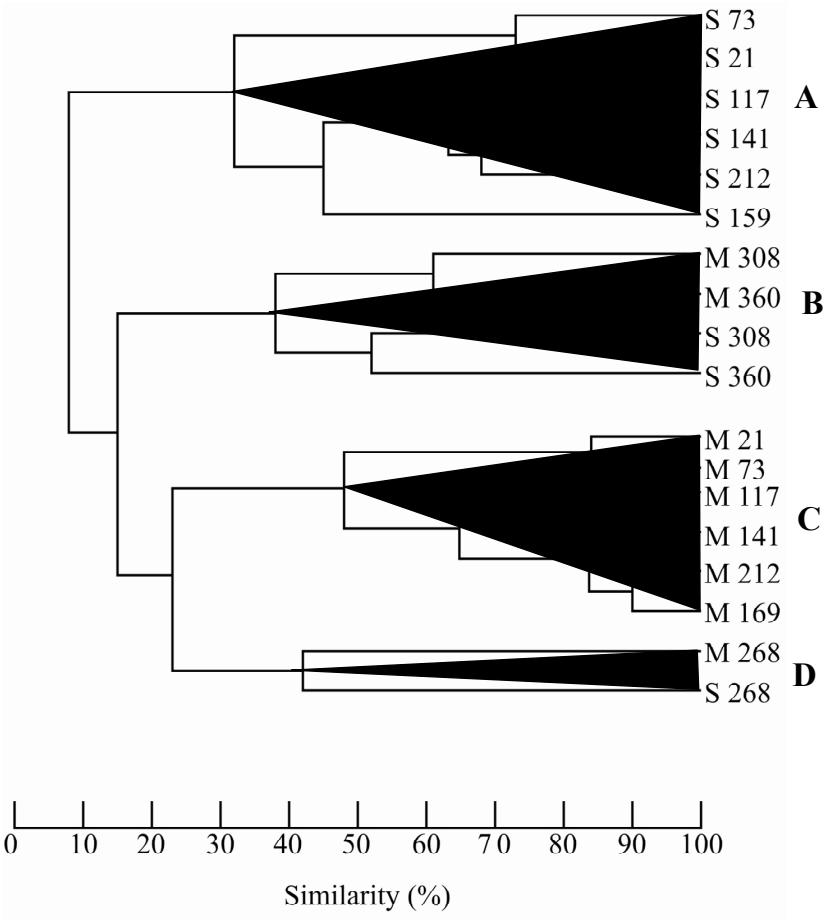


**Figure 6:** Similarity between different samples collected at the surface and inside the bagasse pile. Dendogram was obtained with UPGMA of Sorenson's index. A, B, C and D represent clusters of similar communities.

* S 21 to S 360: Samples of bagasse collected at the surface of the bagasse pile at the different time intervals.

** M 21 to M360: Samples of bagasse collected at the surface of the bagasse pile at the different time intervals.

## CONCLUSIONS

A comprehensive understanding of the fungal populations existing on the surface and inside a bagasse pile is very important in being able to estimate the decay of bagasse in long-term storage. A study by Ramaswamy *et al.* (1989) concluded that fungi can cause up to an 8 % reduction in yield, and this is a huge cause for concern regarding fibre properties for the use of bagasse in the pulp and paper industry in South Africa. The present study was carried out at intervals throughout one cutting season and has monitored the bagasse storage pile at the Sappi Stanger mill for a period of 360 days. To my knowledge, a study of this magnitude has not been published, which makes the results all the more important to the pulp and paper industry.

A total of 51 and 31 fungal species were isolated from the surface and the inner bagasse pile, respectively. This suggests a higher activity of fungal communities on the surface of the bagasse, and it could be expected that the surface of the pile experienced more decay. It was also found that the surface of the pile could experience further increases in the number of species if storage were to continue over 360 days, while the inner pile would maintain a more stable population and the number of species would not increase further. The communities on the surface of the pile was also found to be more diverse and displayed lower dominance than the inner pile, possibly due to the more adequate environment that was more exposed to contamination. The temperature on the surface and inside the pile seemed to play a pivotal role in the determination of the most abundant species. The inner pile however, supported fewer species of fungi that could not be replaced because of the environment being less exposed, and the temperature promoting the growth of only thermophillic fungi.

As the bagasse pile aged, the fungal populations inside the bagasse pile became similar to the populations at the surface of the pile. The most probable change in the environment that could have been responsible for the similarities between the inside and

the surface of the pile towards the end of storage, was the decrease in pH inside the pile (Chapter 2).

The emerging distribution patterns regarding the surveys did not have a significant fit to any of the previously discussed models, but were validated to some degree as the more diverse community at the surface displayed strong characteristics of the broken-stick model, suggesting a larger number of species controlling the niche space. Also, this community progressed to a mature community, as displayed by the log normal distribution for the surface towards the end of storage. The inner pile, having a higher dominance, displayed characteristics of the geometric-series model, where fewer species dominated the niche. However, towards the end of storage, the community inside the pile showed similarity to the surface of the pile, and characteristics of the broken-stick model became more apparent.

This study has demonstrated the characteristics and the nature of fungal populations at the surface and inside the bagasse pile in Stanger. The biodiversity indices have provided qualitative and quantitative data regarding the storage of bagasse for extended periods of time, and have aided the understanding of microbial interactions in a bagasse pile. These results demonstrate that lower microbial numbers were brought about by the harsher environment inside the bagasse pile, and that better preservation of fibre can be expected inside the bagasse pile.

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# CHAPTER 4

# PRODUCTION OF HOLO-CELLULOLYTIC ENZYMES IN STORED BAGASSE

**ABSTRACT**

In order to better understand the degradation of fibre that occurs in a bagasse pile, and in addition to understanding the environment and the microbial populations, the activities of the cellulose and hemicellulose-degrading enzymes need to be studied. Cellulose and hemicellose are the main components of bagasse fibre that is used by the pulp and paper industry, and the degradation of these components account for the losses of fibre before pulping. The aim of this study was to quantify the activities of cellulases and xylanases produced in bagasse. It was determined that these enzymes were present at both the surface and the inner pile. However, these enzymes were found to be more active inside the bagasse pile. The higher enzyme activity inside the bagasse pile suggested that more degradation of fibre could occur inside the bagasse pile. This indicates that enzyme activity is a more realistic reflection of fibre damage than fungal enumeration. It can also be concluded that fungal numbers are not necessarily a reliable indication of biomass or enzyme concentrations in a bagasse pile.

## INTRODUCTION

Bagasse is a fibrous material that is stored for periods of up to six months or longer (Rangamannar *et al.*, 1993; Granick, 1979), but its industrial value decreases as the storage time increases. According to Atchison (1987), losses of atleast 10 % can be expected, where half of this amount is caused by losses from the deterioration of bagasse fibre by cellulolytic microorganisms. These losses can reach between 20 to 30 % if proper storage practices are not put in place. The Ritter process was developed to provide an environment that would prevent the activity of cellulolytic microorganisms, thereby assisting in the preservation of bagasse with minimal fibre decay (Atchison, 1987; Wang & Tao, 1978; Salaber & Maza, 1971). Bagasse fibre contains about 75 % hydrolysable cellulose and hemicellulose, thus making it an ideal substrate for celluloytic fungi (Duenas *et al.*, 1995). When different lignocellulosic substrates were studied for the production of cellulases, it was discovered that bagasse supported high activities of cellulases (Ojumu *et al.*, 2003; Guitierrez-Correa & Tengerdy, 1997; Duenas *et al.*, 1995).

Cellulose is the major carbohydrate that is synthesized by plants and its degradation by microorganisms represent an important part of the carbon cycle within the biosphere (Beguin & Aubert, 1994). This carbohydrate is a linear polymer made of glucose sub-units that are linked by ß-1,4 bonds and forms chains with numerous inter-molecular and intra-molecular hydrogen bonds that account for the formation of rigid, insoluble microfibrils (Ghose, 1987). These chains are oriented in parallel to form highly-ordered crystalline domains, that are interspersed by more disordered amorphous regions (Beguin & Aubert, 1994). The crystalline microfibrils are usually embedded in a matrix of hemicellulose and lignin and are highly resistant to enzymatic hydrolysis (Beguin & Aubert, 1994). The characterization of cellulases is problematic, due to the natural substrate, cellulose, being both insoluble and structurally variable, where organisms often produce many enzymes with different specificities that act in synergy in order to degrade cellulose (Sarikaya & Ladisch, 1997; Ghose, 1987). Ghose (1987) described a number of assays that are available to measure cellulase activities, including the cellobiase assay, carboxymethyl cellulase assay and the filter paper assay. However,

the substrates used in the filter paper assay is more applicable to the pulp and paper industry. By using this substrate, the activity of cellulases on bagasse fibre is more closely related to process results and, therefore, more applicable to this study.

Hemicellulose, closely linked to cellulose and a component of the cell-wall structure, is composed of a complex of carbohydrate monomers, with xylans and glucomannans as the major components (Beguin & Aubert, 1994). The xylan backbone carries acetyl, methyl-glucuronyl and arabinofuranosyl side chains in differing proportions. The degradation of hemicellulose is performed by an array of enzymes, that hydrolyse the xylan or glucomannan backbone and the various bonds linking the side chains. Xylanases such as endo-1,4-ß-D-xylan xylanohydrolase, thus play an important role in the break-down of xylan, which is a supportive and recalcitrant component in the cellulose structure (Sjöström, 1993). Various xylanase assays were tested on the basis of production of reducing sugars from polymeric 4-*O*-glucuronoxylan and considerable variation of the results was reported (Bailey *et al*., 1992). Even when the procedural details of differing assays were similar, the use of different xylan substrates led to poor comparability. The method described by Bailey *et al*. (1992), was recommended for most fungal xylanases and, therefore, chosen together with birchwood-xylan as the substrate for the assay of xylanases produced on bagasse.

The fibrous component of bagasse needs to be preserved for higher pulp quality and, therefore, cellulose and hemicellulose degradation needs to be at a minimum. Fungal enumeration and the structural diversity of fungi in a bagasse pile, aid the understanding of degradation of bagasse fibre in storage. The fungal numbers does not give an indication of the biomass present at the surface or inside the bagasse pile and, therefore, the enzyme concentrations need to be studied. Knowledge of the activities of the lignocellulosic enzymes produced, could aid in a better understanding of the biomass in a bagasse pile and suitable recommendations can be made to minimize the effects of decay. The primary objective of this study was to determine the activities of cellulases and xylanases on stored bagasse, in order to improve understanding of fibre degradation in a bagasse pile. Due to the preservation of bagasse fibre being the primary concern of

this study, the cellulases and xylanases produced on stored bagasse were not characterized, but used for an overall estimation of their effect on bagasse fibre at the surface and inside the bagasse pile.

## MATERIALS AND METHODS

### Site selection

This study was conducted on a part of the bagasse pile at the Sappi Stanger mill as decribed previously (Chapter 3). Bagasse from six areas were selected for sampling; three at the surface and three for sampling 50 cm into the pile during the May to December 2004, and the May to December 2005 cutting seasons. A total of nine surveys were conducted as discussed previously (Chapter 3).

### Sampling and statistical analysis

The samples were collected and transported in the same way as described for the determination of moisture content (Chapter 2). A portion of the samples was used to measure moisture content, while the rest of the bagasse was used in the enzyme assays. A factorial experiment was conducted, with the position in the pile and storage time as factors. The data was analysed using one-way analysis of variance (ANOVA), and the means of the different factors were compared using Tukey's test (Winer, 1971) at a 95 % confidence level.

### Cellulase assay

The sample for the cellulase assay was prepared by suspending 5.0 g (bone dry equivalent) of dried bagasse in 100 ml Na-Citrate buffer (pH 4.8). The sample was homogenised in a kitchen blender, centrifuged (5000x*g*) and then filtered to remove the solids. The cellulase activity was determined according to the filter paper assay for saccharifying cellulase as described by Ghose (1987). The batches for the cellulase assay of each survey included the test sample (Whatman No. 1 filter-paper strips as the substrate, 0.5 ml sample filtrate and 1.0 ml of 0.05 M Na-citrate buffer); an enzyme blank

(1.0 ml of 0.05 M Na-citrate buffer and 0.5 ml sample filtrate); a reagent blank (1.0 ml of 0.05 M Na-citrate buffer) and a set of four glucose calibration standards, with concentrations ranging from 2.0 to 6.7 mg/ml. The reaction mixtures were heated to 50°C in a water bath, after which the filter paper strips were added to the test samples and the reagent blanks. The reaction mixtures were then incubated at 50°C for 60 minutes in a water bath.

Dinitrosalicylic acid (3 ml) was added to each of the reaction mixtures and vortexed before boiling for five minutes in a water bath, after which 20 ml of distilled water was added (Bailey *et al*., 1992). The reactions were then mixed by inverting and left on the bench at room temperature for 20 minutes. The absorbance of the reaction mixtures was determined spectrophotometrically at 540 nm at room temperature. A standard curve of absorbance was plotted against glucose concentration.

The absorbance values of the samples and standards were expressed as the difference in absorbance (?□Abs) between the test samples and the enzyme blanks. These values were used to read the glucose concentration (µ□mol/ml) from the standard curve and calculate the cellulase activity. Enzyme activity was expressed in filter paper units (FPU) where one unit of activity is defined as the amount of enzyme producing 1 µ□mol of glucose in one minute.

**Xylanase assay**

The sample for the xylanase assay was prepared by suspending 5.0 g (bone-dry equivalent) of bagasse in 100 ml Na-citrate buffer (pH 6.5), homogenizing it and obtaining a filtrate as above. The xylanase activity was determined according to a Dinitrosalicylic acid-stopping method as described by Bailey *et al*. (1992). This method is based on the production of reducing sugars from polymeric 4–*O*–methyl glucuronoxylan. The substrate consisting of 4 –*O*– glucuronoxylan from 1 % birchwood (pH 5.3) was prepared in a 0.05 M Na-citrate buffer, homogenized at 60°C in a kitchen blender, and then heated to boiling point with stirring. The mixture was cooled, covered

and left overnight with continued stirring. The mixture was then made up to 100 ml with Na-citrate buffer and aliquots of 25 ml were stored at -20°C until further use.

The batches for the xylanase assay for each survey included a test sample, reagent blank and enzyme blank, each containing the 1.8 ml xylan substrate. Each survey also included a set of four xylose calibration standards with concentrations ranging from 2.0 to 10.0 μmol/ml. The reactions were then heated in a water bath to 50°C before the sample filtrate (200 μl) was added to the test sample. The test sample was then incubated at 50°C in a water bath for five minutes, after which 3 ml of Dinitrosalicylic acid (DNS) was added. DNS (3 ml) was added to each of the calibration standards, enzyme blanks and reagent blanks, and the batches were incubated at 50°C in a water bath for five minutes.

The samples, standards and blanks were then each mixed and boiled in a water bath for five minutes, and then cooled in an ice bath. The absorbances of the reaction mixtures were determined spectrophotometrically at 540 nm at room temperature. The absorbance values of the samples and standards were expressed as the difference in absorbance (Δ Abs) between the samples and the blanks. These values were used to calculate the xylose concentration ( mol/ml) from the linear equation obtained from the xylose standard curve, and to calculate the xylanase activity. Enzyme activity was expressed in Xylanase Units (XU), where one unit of activity is defined as the amount of enzyme producing 1 mol of xylose in one minute.

## RESULTS AND DISCUSSION

### Cellulase activity

Cellulase activity was higher inside the bagasse pile than outside, throughout the bagasse storage period (Figure 1). The activity of cellulase also increased throughout the duration of this study, both at the surface and inside the bagasse pile. The higher cellulase activity inside the pile could possibly be attributed to the large numbers of

*Aspergillus fumigatus* that was isolated from the inner pile (Chapter 3). This fungus is noted for its ability to produce a number of cellulose degrading enzymes such as 1,3-ß-glucanase; extracellular a- and ß-glucosidases (Flannigan & Sagoo, 1977; Flannigan & Sellars, 1977). *A. fumigatus* has also produced high levels of Carboxymethylcellulase in some studies, and this fungus could, therefore, be an important source of cellulases inside the pile. Another fungus that was isolated from inside the pile with high cellulolytic capabilities, is *Epicoccum nigrum* (Domsch *et al*., 1980).

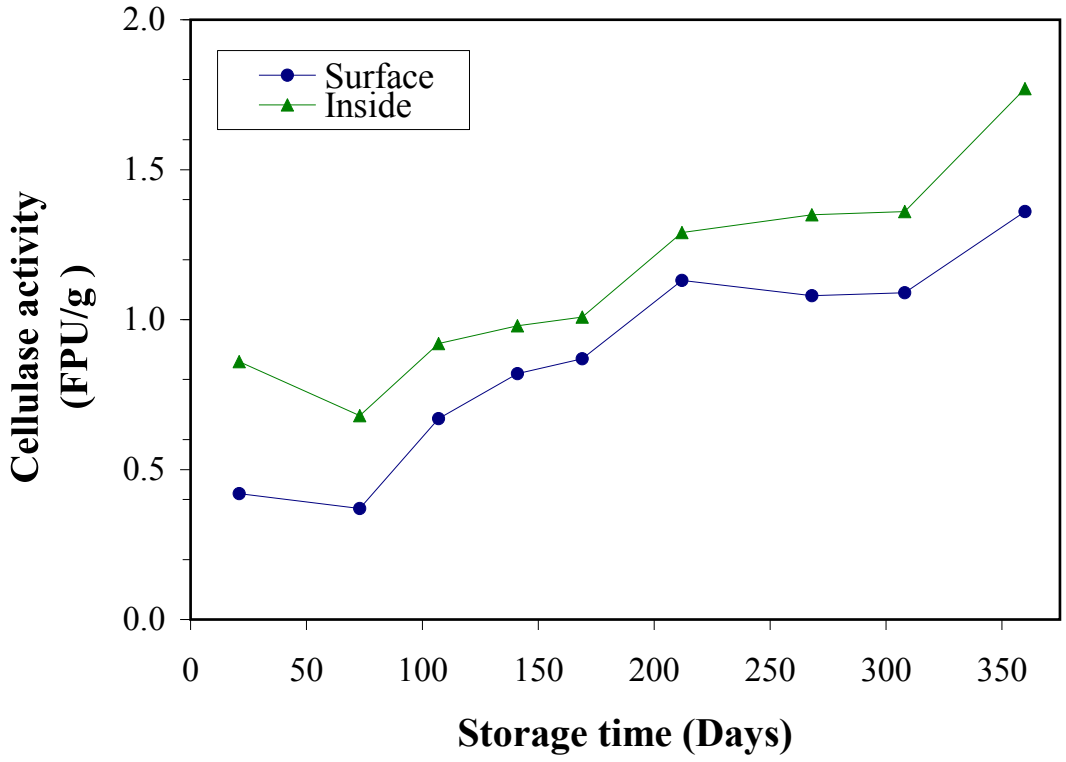


**Figure 1:** Activity of cellulase extracted from bagasse at intervals during the ageing of the bagasse pile at the Stanger paper mill (Appendix D 1).

Although the surface of the pile contained less cellulase activity, the possibility of cellulose degradation is present. Known fungal degraders of cellulose that were present at the surface of the bagasse pile included *Trichoderma koningii*, *Alternaria alternata* and *Fusarium oxysporum* (Chapter 3). The continued increase in cellulase activity suggests that the cellulose-degrading fungi better established themselves and their biomass

increased proportionally (Chapter 3). The continued increase further suggests that the environment inside the bagasse pile was conducive to the proliferation of thermo-tolerant fungi that have cellulolytic capabilities. A second theory that supports the continued increase in cellulase activity both at the surface and inside the pile, relates to decreased free sugars as the pile aged. As the primary colonising fungi metabolized the residual sugars in the earlier stages of storage, numbers of secondary colonizers started to increase and with only polymeric carbohydrates remaining, cellulase activity increased (Frankland, 1992).

**Xylanase activity**

The xylanase activities, as in the case of cellulase from the bagasse pile at Stanger, were higher inside the bagasse pile throughout the 360-day storage period of bagasse, and increased over time (Figure 2). The same fungal species responsible for cellulose degradation in bagasse, are also known for their abilities to degrade xylan (Domsch *et al.*, 1980). An example of a highly frequent fungus inside the pile, *A. fumigatus*, could also account for the higher rate of xylanase activity. This fungus has been described to have high activity on xylan-rich substrates (Dickinson & Boardman, 1970). Important degraders of hemicellulose at the surface could be *T. koningii*, *A. alternata* and *F. oxysporum* (Chapter 3).

The higher rates of enzyme activity within the bagasse pile (Figure 1 and 2) suggests that there is more degradation to holocellulose inside the bagasse pile, compared to the surface of the bagasse pile. The inner pile accounts for a larger portion of the bagasse pile and, therefore, decay in this area can be of critical importance.

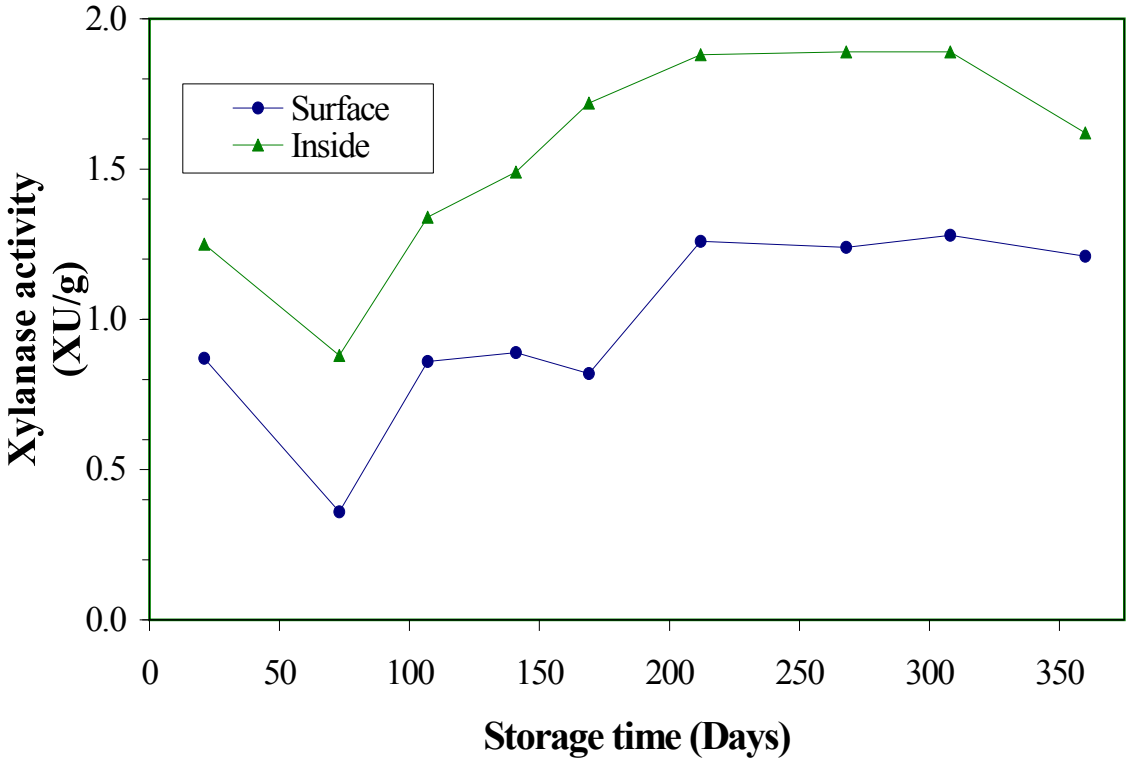


**Figure 2:** Activity of xylanase extracted from bagasse at intervals during the ageing of the bagasse pile at the Stanger paper mill (Appendix D 2).

## CONCLUSION

Bagasse is a lignocellulosic material upon which fungi and other microorganisms can proliferate (Ramaswamy *et al.*, 1989; Rangamannar *et al.*, 1993). Atchison (1987) has made mention of the relatively high losses of bagasse due to the damage caused to the fibre by deleterious microorganisms, mainly fungi. Considerable effort has been made in developing bagasse as a non-wood fibre for the pulp and paper industry, however, the seasonality of this material requires that bagasse be stored for extended periods, making bagasse susceptible to microbial attack (Atchinson, 1987; Venter, 1978; Salaber & Maza, 1971; Marshall 1938). The present study has quantified the activities of cellulase and xylanase that can be responsible for degradation of this fibrous material.

Both cellulase and xylanase activities were noted to be higher on the inside of the bagasse pile at Stanger. The activities were also noted to continue increasing over time, throughout storage. These results suggest that there is more decay to fibre inside the bagasse pile, than on the surface. In Chapter 3, fungal populations were enumerated and the structural diversity was evaluated. It was found that the surface of the pile, which was more exposed to contamination, had a higher number of species and individuals than the inside of the bagasse pile. These data suggested that the surface would have more fibre decay than the inside pile. However, the present study has proved that the inside of the bagasse pile contained higher activities of holo-cellulose degrading enzymes, which suggests more damage to fibre on the inside of the pile. It seems that the environment inside the bagasse pile was more restrictive to fungal diversity, however, these fungal populations have the potential to bring about more degradation to the bagasse fibre. The extent of fibre loss can only be compared by pulping of representative samples and should be considered for future trials.

This study has indicated that enzyme activity can be a more appropriate estimation of the actual damage occurring inside or on the surface of a bagasse pile, than microbial enumeration, since fungal numbers do not necessarily reflect biomass or enzyme production. This study has facilitated a more holistic understanding of the bagasse pile that can be functional in managing the storage of bagasse to preserve fibre for pulping.

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# CONCLUSIONS

Sugar-cane bagasse is well established as a valuable source of fibre for paper-making (Atchinson, 1987; Venter, 1978; Salaber and Maza, 1971; Marshall 1938), and has been proved to have suitable physical, optical and surface properties for paper (Viswanathan, 1998). Storage of bagasse is a problematic issue for the pulp and paper industry, where microbial degradation, biochemical deterioration and the danger of fire are constant threats causing losses of bagasse fibre (Hurter, 1991; Atchison, 1971). Fungi, in particular, contribute largely to the deterioration of bagasse in storage (Ramaswamy *et al.*, 1989). The high residual sugar content, the presence of water solubles, the heterogeneity of the tissues and a vast exposed surface area makes bagasse an ideal substrate for these deleterious microorganisms (Ramaswamy *et al*., 1989; Cusi, 1979).

In the present study, it was observed that the inside of the bagasse pile displayed higher temperatures and moisture content, but more acidic pH levels, than the surface of the pile. The temperature was found to be 15°C and 5°C lower than the ideal for preservation at the surface and inside the bagasse pile, respectively (Rangamannar *et al*., 1993). The pH at both the surface and inside the pile, were up to 2.0 units too high, while the moisture was 30 % lower than ideal conditions for the control of fungal growth (Wang & Tao, 1978; Pelczar *et al.*, 1977; Moebius, 1966). The prevailing environmental conditions favoured fungal proliferation, and essentially bagasse deterioration.

When the fungal species on bagasse were enumerated, the cumulative number of species reflected more species at the surface of the pile, than inside the pile. Also indicated, was the trend for the surface to be inhabited by an increasing number of species, while the inside of the pile remained relatively stable over the storage period. The populations at the surface were also found to be more diverse and less dominant than populations from inside the pile. The dominant fungus found inside the pile was *Aspergillus fumigatus* (92 %) with smaller numbers of *A. pennicillioides* (20 %). *Cladosporium cladosporioides*, *Phoma sorghina* and *Penicillium* spp. were present in the highest numbers at the surface of the pile. Many species of lignocellulosic fungi

(Domsch *et al*., 1980) were observed and it appeared that the higher temperature measured inside the pile, were influential in establishing conditions for specialised fungi and thus accounting for the higher dominance on the inside.

The ß-diversity index (Sorenson's measure) indicated that, although the populations on the surface and inside the bagasse pile were very different at the beginning of storage, they became similar towards the end of storage. This information was supported by the abundance models, where the inner pile displayed characteristics of minimal niche pre-emption followed by equal sharing of resources. The surface populations, on the other hand, displayed maximal niche occupation followed by the establishment of a mature community. It can be concluded that the microbial populations on a bagasse pile have established themselves and would most probably continue proliferating on the bagasse pile and causing decay. It was also thought that since the surface displayed more species and a higher diversity, that there would be more degradation in this region of the pile.

However, it was found that the cellulase and xylanase activities were higher inside the bagasse pile, than on the surface. Therefore, it could be expected that there would be more degradation to fibre inside the bagasse pile. This could possibly reflect the ability of the dominant fungus inside the pile (*A. fumigatus*) to produce lignocellulolytic enzymes. The activities of cellulases and xylanases was lower on the surface, but still present at high levels to cause significant degradation of holocellulose at the surface as well.

This study has indicated that bagasse at Stanger was stored under ideal conditions for fungal development and for lignocellulosic degradation. In order to improve the preservation of bagasse, lactobacilli can be added and temperature, pH and moisture must be managed more efficiently. The excess leached water should be recirculated to the pile in order to maintain lower pH levels and decrease aeration. Compaction with specialized machinery can also be used to reduce aeration. In order to reduce the surface area, the pile can also be stacked higher.

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# APPENDICES

## APPENDIX A: ENVIRONMENTAL CONDITIONS

**Appendix A.1:** Temperatures of each replicate at the surface and inside the bagasse pile recorded over the 360-day storage period.

| Storage time (Days) | Surface Temperature (°C) | | | | Inside Temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 25.4 | 25.2 | 26.3 | 25.6 | 31 | 30 | 33.5 | 31.5 |
| **73** | 15.4 | 16.7 | 17.7 | 16.6 | 23.9 | 26.6 | 26.6 | 25.7 |
| **117** | 24 | 23 | 21 | 22.7 | 37.6 | 38 | 40 | 38.5 |
| **141** | 27.6 | 26.4 | 28.3 | 27.4 | 39.7 | 39.4 | 41.8 | 40.3 |
| **169** | 30.4 | 31.7 | 32.4 | 31.5 | 41.6 | 40.8 | 42.3 | 41.6 |
| **212** | 34.6 | 34.9 | 34.2 | 34.6 | 44.9 | 44.6 | 44.5 | 44.7 |
| **268** | 32.8 | 33.5 | 33 | 33.1 | 43.4 | 42.5 | 41.3 | 42.4 |
| **308** | 32.8 | 33.6 | 33.9 | 33.4 | 42.7 | 43.9 | 43.8 | 43.5 |
| **360** | 33.8 | 33.1 | 33.6 | 33.5 | 44.6 | 43.8 | 43.4 | 43.9 |

**Appendix A.2:** pH readings for each replicate at the surface and inside the bagasse pile recorded over the 360-day storage period.

| Storage time (Days) | Surface pH | | | | Inside pH | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 6.9 | 7 | 7 | 7 | 6.1 | 6.1 | 6 | 6.1 |
| **73** | 7 | 7.1 | 7.1 | 7.1 | 6.3 | 6.1 | 6.1 | 6.2 |
| **117** | 7 | 6.9 | 6.8 | 6.9 | 5.9 | 6.1 | 5.8 | 5.9 |
| **141** | 6.9 | 6.8 | 6.9 | 6.9 | 5.9 | 5.7 | 5.7 | 5.8 |
| **169** | 6.8 | 6.6 | 7.1 | 6.8 | 5.4 | 5.5 | 5.7 | 5.5 |
| **212** | 6.9 | 6.8 | 6.7 | 6.8 | 5.5 | 5.2 | 5.7 | 5.5 |
| **268** | 6.8 | 6.9 | 7 | 6.9 | 5.5 | 5.3 | 5.3 | 5.4 |
| **308** | 6.7 | 6.5 | 6.6 | 6.6 | 5.4 | 5.5 | 5.3 | 5.4 |
| **360** | 5.6 | 5.4 | 5.5 | 5.5 | 5.7 | 5.4 | 5.3 | 5.5 |

**Appendix A.3:** The moisture content recorded for each replicate at the surface and inside the bagasse pile throughout the 360-day storage period.

| Storage time (Days) | Surface Moisture Content (%) | | | | Inside Moisture Content (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 28 | 32 | 32 | 30.7 | 40 | 46 | 45 | 43.7 |
| **73** | 32 | 38 | 31 | 33.7 | 44 | 41 | 48 | 44.3 |
| **117** | 40 | 36 | 32 | 36 | 47 | 48 | 53 | 49.3 |
| **141** | 33 | 36 | 38 | 35.7 | 53 | 51 | 49 | 51 |
| **169** | 31 | 32 | 29 | 30.7 | 56 | 51 | 53 | 53.3 |
| **212** | 32 | 27 | 28 | 29 | 58 | 52 | 54 | 54.7 |
| **268** | 36 | 55 | 42 | 44.3 | 64 | 76 | 73 | 71 |
| **308** | 39 | 42 | 35 | 38.7 | 59 | 68 | 72 | 66.3 |
| **360** | 43 | 45 | 42 | 43.3 | 58 | 62 | 69 | 63 |

## APPENDIX B : FUNGAL ENUMERATION

**Appendix B 1:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 21 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | 44 | 24 | 12 | 80 | | | | |
| *Aspergillus fumigatus* | | | | | 100 | 100 | 100 | 300 |
| *Aspergillus pennicilliodes* | | | | | 52 | 55 | 50 | 157 |
| *Aspergillus terreus* | 2 | 1 | | 3 | | | | |
| *Cladosporium cladosporioides* | 90 | 86 | 45 | 221 | | | | |
| *Eurotium amstelodami* | | | | | | 2 | | 2 |
| *Fusarium oxysporum* | 50 | 18 | 45 | 113 | | | | |
| *Humicola* sp. 1 | 4 | | 3 | 7 | 46 | 45 | | 91 |
| *Humicola* sp. 2 | | | | | 54 | 25 | 50 | 129 |
| *Mucor* sp. 1 | | | | | 24 | | 15 | 39 |
| *Mucor* sp. 2 | | | | | | | 1 | 1 |
| *Paecilomyces variotii* | | | | | 46 | 54 | 29 | 129 |
| *Penicillium* sp. 9 | 10 | 15 | 3 | 28 | | | | |
| *Phoma sorghina* | 52 | | 44 | 96 | 26 | 25 | 13 | 64 |
| *Trichoderma koningii* | 100 | 100 | 10 | 210 | | | | |
| Yellow fungus | 46 | | 25 | 71 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 2:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 73 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | 26 | | 32 | 58 | | | | |
| *Alternaria longissima* | 12 | 10 | 8 | 30 | | | | |
| *Aspergillus fumigatus* | | | | | 100 | 100 | 100 | 300 |
| *Aspergillus pennicilliodes* | | | | | 25 | 30 | 13 | 68 |
| *Aspergillus versicolor* | | 12 | | 12 | | | | |
| *Cladosporium cladosporioides* | 42 | 48 | 36 | 126 | | | | |
| *Cladosporium cucumerinum* | 26 | 28 | 25 | 79 | | | | |
| *Cladosporium sphaerospermum* | 13 | | | 13 | | | | |
| *Eurotium amstelodami* | | | | | 3 | 15 | 6 | 24 |
| *Fusarium oxysporum* | 50 | 48 | 56 | 154 | | | | |
| *Humicola* sp. 1 | 1 | 3 | 1 | 5 | 25 | 22 | | 47 |
| *Humicola* sp. 2 | | | | | 59 | 38 | 54 | 151 |
| *Mucor* sp. 1 | | | | | 22 | | 11 | 33 |
| *Paecilomyces variotii* | | | | | 38 | 31 | 29 | 98 |
| *Penicillium* sp. 9 | 21 | 14 | 11 | 46 | | | | |
| *Phoma sorghina* | 58 | | 34 | 92 | 21 | | 1 | 22 |
| *Trichoderma koningii* | 100 | 100 | 100 | 300 | | | | |
| Yellow fungus | 12 | | | 12 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 3:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 117 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | | | | | 2 | | 5 | 7 |
| *Alternanria alternata* | 17 | 1 | 3 | 21 | | | | |
| *Alternaria dianthi* | | | | | | 1 | | 1 |
| *Alternaria tennuissima* | 84 | 11 | 45 | 140 | | | | |
| *Aspergillus fumigatus* | | | | | 72 | 82 | 39 | 193 |
| *Aspergillus pennicilliodes* | | | | | 1 | | | 1 |
| *Aspergillus versicolor* | 1 | | | 1 | | | | |
| *Aureobasidium pullulans* | | | | | 2 | | | 2 |
| Basidiomycetous fungus sp. 1 | | | | | | 9 | | 9 |
| *Cladosporium cladosporioides* | 71 | 64 | 65 | 200 | | | | |
| *Cladosporium cucumerinum* | | 55 | 29 | 84 | | | | |
| *Cladosporium sphaerospermum* | 89 | 5 | | 94 | | | | |
| *Epicoccum nigrum* | 4 | | 1 | 5 | | | | |
| *Eurotium amstelodami* | | | | | | | 1 | 1 |
| *Eurotium* sp. 1 | | | | | 11 | 14 | 4 | 29 |
| *Fusareum poae* | 1 | | | 1 | | | | |
| *Fusarium* sp. 1 | 3 | 4 | | 7 | | | | |
| *Fusarium graminearum* | 21 | 3 | 5 | 29 | | | | |
| *Monilia* sp. 1 | | 2 | 2 | 4 | | | | |
| *Mucor* sp. 1 | 19 | 37 | | 56 | | | | |
| *Nigrospora* sp. 1 | | | 1 | 1 | | | | |
| *Paecilomyces variotii* | | | 1 | 1 | 2 | 16 | 54 | 72 |
| *Penicillium* sp. 1 | | 28 | | 28 | | | | |
| *Penicillium* sp. 3 | | 36 | | 36 | | | | |
| *Penicillium* sp. 4 | | 78 | | 78 | | | | |
| *Penicillium* sp. 5 | | 24 | | 24 | | | | |
| *Penicillium* sp. 6 | | | 1 | 1 | | | | |
| *Penicillium* sp. 7 | | | 8 | 8 | | | | |
| *Penicillium* sp. 8 | | | 40 | 40 | | | | |
| *Periconia* sp. 1 | | | | | 1 | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 3:** Continued

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Phoma sorghina* | 100 | 64 | 90 | 254 | | | | |
| *Rhizopus oryzae* | | | 1 | 1 | | | | |
| *Rhodotorula* sp. 1 | | 2 | 4 | 6 | | | | |
| *Scopulariopsis* sp. 1 | | 1 | | 1 | | | | |
| *Trichoderma viride* | 19 | 7 | 16 | 42 | | | | |
| Unknown 1 | | | | | 2 | 1 | 1 | 4 |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 4**: Fungal numbers for each replicate at the surface and inside the bagasse pile after 141 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | 2 | 1 | 5 | 8 | | | | |
| *Alternaria citri* | 27 | 9 | 8 | 44 | | | | |
| *Alternaria tennuissima* | 91 | 11 | 9 | 111 | | | | |
| *Aspergillus fumigatus* | | | | | 81 | 97 | 90 | 268 |
| *Aspergillus pennicilliodes* | | | | | | 2 | | 2 |
| *Aspergillus versicolor* | 2 | 1 | | 3 | | | | |
| *Cladosporium cladosporioides* | 82 | 76 | 69 | 227 | | | | |
| *Cladosporium cucumerinum* | | | 37 | 37 | | | | |
| *Cladosporium sphaerospermum* | 35 | | 2 | 37 | | | | |
| *Epicoccum nigrum* | 2 | 5 | 8 | 15 | | | | |
| *Eurotium* sp. 1 | | | | | 12 | 7 | 3 | 23 |
| *Fusarium poae* | 5 | | | 5 | | | | |
| *Fusarium* sp. 1 | 4 | 5 | | 9 | | | | |
| *Fusarium graminearum* | | 17 | 7 | 24 | 12 | | | 12 |
| *Fusarium oxysporum* | | 12 | | 12 | | | | |
| *Monilia* sp. 1 | | 5 | 3 | 8 | | | | |
| *Mucor* sp. 1 | 12 | | 21 | 33 | | | | |
| *Paecilomyces variotii* | | | 6 | 6 | 8 | 18 | 6 | 32 |
| *Pennicillium* sp. 9 | 65 | 15 | 49 | 129 | 3 | 2 | 1 | 6 |
| *Phoma sorghina* | 65 | 54 | 80 | 199 | | | | |
| *Rhizopus oryzae* | | | | | 24 | 3 | 9 | 36 |
| *Rhodotorula* sp. 1 | 4 | 5 | 6 | 15 | | | | |
| *Trichoderma viride* | | 18 | | 18 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 5:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 169 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Total | Rep 1 | Rep 2 | Rep 3 | Total |
| *Acremonium* sp. 1 | | 1 | 11 | 12 | | | | |
| *Alternanria alternata* | 45 | | | 45 | | | | |
| *Alternaria citri* | 2 | | | 2 | | | | |
| *Alternaria longissima* | 1 | | | 1 | | | | |
| *Alternaria* sp. 1 | 59 | 66 | 40 | 165 | | | | |
| *Alternaria tennuissima* | 7 | 13 | 8 | 28 | | | | |
| *Aspergillus fumigatus* | 1 | | | 1 | 100 | 90 | 99 | 289 |
| *Aspergillus ochraceus* | 1 | | | 1 | 1 | | | 1 |
| *Aspergillus terreus* | | | 1 | 1 | | | | |
| Basidiomycetous fungus sp. 1 | | 7 | | 7 | | | | |
| *Botrytis cinerea* | 1 | 1 | | 2 | | | | |
| *Cladosporium cladosporioides* | 99 | 94 | 89 | 282 | | | | |
| *Cladosporium cucumerinum* | | 25 | 2 | 27 | | | | |
| Coelomycetous fungus sp. 1 | | | | | 2 | 8 | 8 | 18 |
| *Epicoccum nigrum* | 1 | | | 1 | | | | |
| *Eurotium* sp. 1 | | | | | | | 1 | 1 |
| *Fusarium* sp. 1 | 2 | 2 | 19 | 23 | | | | |
| *Fusarium oxysporum* | | 7 | | 7 | | | | |
| *Monilia* sp. 1 | 15 | 6 | | 21 | | | | |
| *Mucor* sp. 1 | 9 | | | 9 | | | | |
| *Paecilomyces variotii* | | | 2 | 2 | | | | |
| *Paecilomyces* sp. 1 | | 15 | 9 | 24 | | | | |
| *Penicillium* sp. 9 | 73 | 71 | 22 | 166 | 1 | 1 | 1 | 3 |
| *Phoma sorghina* | 41 | 15 | 5 | 61 | | | | |
| *Rhizopus oryzae* | | | | | 49 | 6 | 8 | 63 |
| *Rhodotorula* sp. 1 | 3 | 1 | 3 | 7 | | | | |
| *Trichoderma koningii* | 12 | | | 12 | | | | |
| *Trichoderma* sp. 1 | | 76 | 63 | 139 | | | | |
| White mycelial fungus | 1 | | | 1 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 6:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 212 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | | | | | 2 | 1 | | 3 |
| *Alternanria alternata* | 28 | | 26 | 54 | | | | |
| *Alternaria longissima* | 2 | | 1 | 3 | | | | |
| *Alternaria* sp. 1 | 45 | 5 | 34 | 84 | | | | |
| *Alternaria tennuissima* | 77 | 45 | 88 | 210 | | | | |
| *Aspergillus fumigatus* | | | | | 100 | 95 | 100 | 295 |
| *Aspergillus versicolor* | 3 | | 2 | 5 | | | | |
| *Aureobasidium pullulans* | | | | | | | 1 | 1 |
| *Cladosporium cladosporioides* | 87 | 89 | 85 | 261 | | | | |
| *Cladosporium cucumerinum* | 4 | 18 | 12 | 34 | | | | |
| *Cladosporium sphaerospermum* | 82 | | 24 | 106 | | | | |
| *Epicoccum nigrum* | 1 | | 1 | 2 | | | | |
| *Eurotium* sp. 1 | | | | | | 8 | 6 | 14 |
| *Fusarium* sp. 1 | 6 | | 2 | 8 | | | | |
| *Monilia* sp. 1 | | | | | 3 | 1 | 2 | 6 |
| *Paecilomyces variotii* | 1 | | 3 | 4 | | | | |
| *Paecilomyces* sp. 1 | 1 | 17 | 7 | 25 | | | | |
| *Penicillium* sp. 9 | 78 | 82 | 64 | 224 | | | | |
| *Phoma sorghina* | 89 | 62 | 94 | 245 | | | | |
| *Rhizopus oryzae* | | | | | 5 | 16 | 25 | 36 |
| *Rhodotorula* sp. 1 | 3 | 1 | 1 | 5 | | | | |
| White mycelial fungus | 1 | 5 | 2 | 8 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 7:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 268 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Alternaria* sp. 1 | 8 | 25 | 9 | 42 | | | | |
| *Alternaria tennuissima* | | 2 | 8 | 10 | | | | |
| *Aspergillus fumigatus* | 2 | | | 2 | 100 | 100 | 100 | 300 |
| *Aspergillus niger* | | 10 | | 10 | | | | 0 |
| *Aspergillus ochraceus* | | | | | 9 | 15 | 4 | 28 |
| *Aspergillus terreus* | 10 | 1 | | 11 | | | | |
| *Cladosporium cladosporioides* | 7 | 16 | | 23 | | | | |
| *Cladosporium sphaerospermum* | | 19 | | 19 | | | | |
| *Epicoccum nigrum* | 4 | | | 4 | | 3 | 2 | 5 |
| *Fusarium* sp. 1 | 3 | 2 | 6 | 11 | | | | |
| *Mucor* sp. 1 | 1 | 12 | 17 | 30 | | | | |
| *Mucor* sp. 2 | | 8 | 6 | 14 | | | | |
| *Paecilomyces variotii* | 14 | 10 | 6 | 30 | | | | |
| *Paecilomyces* sp. 1 | 1 | 2 | | 3 | 6 | 8 | 13 | 27 |
| *Penicillium* sp. 1 | 95 | 50 | 95 | 240 | 95 | 90 | 100 | 285 |
| *Penicillium* sp. 2 | 15 | 14 | 69 | 98 | 40 | 56 | 76 | 172 |
| *Penicillium* sp. 3 | 3 | 41 | 14 | 58 | | | | |
| *Penicillium* sp. 4 | 1 | | | 1 | | | | |
| *Penicillium* sp. 5 | 2 | 6 | | 8 | | | | |
| *Penicillium* sp. 6 | 6 | | | 6 | | | | |
| *Phoma sorghina* | 50 | 30 | 56 | 136 | | | | |
| *Rhizopus oryzae* | 15 | 20 | 20 | 55 | 45 | 21 | 8 | 74 |
| *Rhizopus* sp. 1 | 2 | 2 | | 4 | | | | |
| *Sporotrichum* sp. 1 | 54 | | 14 | 68 | | | | |
| *Trichoderma viride* | | 45 | 48 | 93 | | | | |
| *Trichoderma koningii* | 13 | 10 | | 23 | | | | |
| *Trichoderma* sp. 1 | 2 | 4 | 5 | 11 | | | | |
| White mycelial fungus | | 2 | 7 | 9 | 2 | 1 | | 3 |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 8:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 308 days of storage at Stanger.

| Species | Pile surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | 21 | 2 | 1 | 24 | | | | |
| *Alternanria alternata* | 47 | 23 | 27 | 97 | | | | |
| *Alternaria* sp. 1 | 4 | 1 | 3 | 8 | | | | |
| *Alternaria tennuissima* | 14 | 28 | 13 | 55 | | | | |
| *Aspergillus fumigatus* | | | | | 86 | 72 | 68 | 226 |
| *Cladosporium cladosporioides* | 76 | 67 | 47 | 190 | | | | |
| *Cladosporium cucumerinum* | 22 | | 7 | 29 | | | | |
| *Cladosporium sphaerospermum* | 57 | 61 | 69 | 187 | | | | |
| Coelomycetous fungus sp. 1 | 7 | 1 | 1 | 9 | | | | |
| *Epicoccum nigrum* | | | | | | 1 | | 1 |
| *Fusareum poae* | 3 | 1 | | 4 | | | | |
| *Fusarium* sp. 1 | 42 | 23 | 8 | 73 | | | | |
| *Fusarium oxysporum* | 12 | | 9 | 21 | | | | |
| *Mucor* sp. 1 | 46 | 21 | | 67 | 32 | 16 | 2 | 50 |
| *Penicillium* sp. 1 | 26 | 8 | | 34 | | | | |
| *Penicillium* sp. 2 | 38 | | 12 | 50 | | | | |
| *Penicillium* sp. 3 | 24 | 21 | | 45 | | | | |
| *Penicillium* sp. 4 | 12 | 3 | 2 | 17 | | | | |
| *Penicillium* sp. 5 | | | | | 39 | 46 | 33 | 118 |
| *Penicillium* sp. 6 | | | | | 11 | 2 | 7 | 20 |
| *Rhizopus oryzae* | | | | | 1 | 1 | | 2 |
| Unknown 1 | 12 | | | 12 | | | | |
| White mycelial fungus | | | | | | 1 | | 1 |

[a] Incubated at 25°C
[b] Incubated at 45°C

**Appendix B 9:** Fungal numbers for each replicate at the surface and inside the bagasse pile after 360 days of storage at Stanger.

| Species | Pile Surface [a] | | | | Inside pile [b] | | | |
|---|---|---|---|---|---|---|---|---|
| | **Rep 1** | **Rep 2** | **Rep 3** | **Total** | **Rep 1** | **Rep 2** | **Rep 3** | **Total** |
| *Acremonium* sp. 1 | 4 | 8 | 5 | 17 | | | | |
| *Alternaria* sp. 1 | 8 | 12 | 16 | 36 | | | | |
| *Aspergillus flavus* | | | | | 10 | 7 | 3 | 20 |
| *Aspergillus fumigatus* | | | | | 100 | 100 | 100 | 300 |
| *Aspergillus niger* | 15 | 1 | 16 | 32 | | | | |
| *Aspergillus pennicilliodes* | | | | | 100 | 100 | 100 | 300 |
| *Aspergillus versicolor* | 6 | | 15 | 21 | | | | |
| Basidiomycetous fungus sp. 1 | 22 | 11 | 15 | 48 | | | | |
| *Cladosporium cucumerinum* | 80 | | 86 | 166 | | | | |
| *Cladosporium sphaerospermum* | | | 5 | 5 | | | | |
| *Epicoccum nigrum* | 2 | | 7 | 9 | | | | |
| *Fusarium chlamydosporium* | 3 | 12 | 5 | 20 | | | | |
| *Fusarium* sp. 1 | 4 | 5 | 14 | 23 | | | | |
| *Fusarium oxysporum* | | 2 | | 2 | | | | |
| *Mucor* sp. 1 | | | | | 15 | 11 | 22 | 48 |
| *Paecilomyces variotii* | 4 | 7 | | 11 | | | | |
| *Penicillium* sp. 9 | 88 | 85 | 82 | 255 | | | | |
| *Rhizopus oryzae* | 5 | | 3 | 8 | | | | |
| *Rhizopus* sp. 1 | | | | | 14 | 21 | 15 | 50 |
| *Staphylotrichum* sp. 1 | 26 | 25 | 38 | 89 | | | | |
| *Trichoderma koningii* | 100 | 100 | 100 | 300 | | | | |
| White mycelial fungus | 4 | | 1 | 5 | | | | |

[a] Incubated at 25°C
[b] Incubated at 45°C

## APPENDIX C: BIODIVERSITY

**Appendix C 1**: The Shannon indices (*H'*) calculated for each replicate at the surface and inside the bagasse pile throughout the 360-day storage period.

| Storage time (Days) | Shannon index (Surface) | | | | Shannon index (Inside) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 1.868 | 1.126 | 1.182 | 1.392 | 1.73 | 1.512 | 1.309 | 1.517 |
| **73** | 1.975 | 1.397 | 1.62 | 1.664 | 1.554 | 1.249 | 1.098 | 1.3 |
| **117** | 2.011 | 2.351 | 1.748 | 2.037 | 0.545 | 0.688 | 0.63 | 0.621 |
| **141** | 1.984 | 1.526 | 1.819 | 1.776 | 0.82 | 0.645 | 0.537 | 0.667 |
| **169** | 1.987 | 2.072 | 1.586 | 1.882 | 0.662 | 0.493 | 0.533 | 0.563 |
| **212** | 2.333 | 1.596 | 2.194 | 2.041 | 0.465 | 0.58 | 0.626 | 0.557 |
| **268** | 1.927 | 2.372 | 2.177 | 2.159 | 1.342 | 1.368 | 1.273 | 1.328 |
| **308** | 2.809 | 1.601 | 1.285 | 1.898 | 0.876 | 0.76 | 0.606 | 0.747 |
| **360** | 1.91 | 1.407 | 2.139 | 1.819 | 1.028 | 1.02 | 1.015 | 1.021 |

**Appendix C 2:** The Berger-Parker index (*d*) calculated for each replicate at the surface and inside the bagasse pile throughout the 360-day storage period.

| Storage time (Days) | Berger-Parker index (Surface) | | | | Berger-Parker index (Inside) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 0.251 | 0.41 | 0.241 | 0.301 | 0.287 | 0.327 | 0.388 | 0.334 |
| **73** | 0.277 | 0.38 | 0.33 | 0.329 | 0.341 | 0.424 | 0.467 | 0.411 |
| **117** | 0.233 | 0.185 | 0.288 | 0.235 | 0.277 | 0.38 | 0.33 | 0.329 |
| **141** | 0.23 | 0.325 | 0.258 | 0.271 | 0.579 | 0.752 | 0.826 | 0.719 |
| **169** | 0.265 | 0.235 | 0.325 | 0.275 | 0.654 | 0.857 | 0.846 | 0.786 |
| **212** | 0.175 | 0.275 | 0.211 | 0.22 | 0.909 | 0.785 | 0.746 | 0.813 |
| **268** | 0.308 | 0.151 | 0.25 | 0.236 | 0.337 | 0.34 | 0.33 | 0.336 |
| **308** | 0.164 | 0.258 | 0.347 | 0.256 | 0.509 | 0.518 | 0.618 | 0.548 |
| **360** | 0.27 | 0.373 | 0.245 | 0.296 | 0.418 | 0.418 | 0.417 | 0.418 |

## APPENDIX D: ENZYME ACTIVITIES

**Appendix D 1:** Cellulase Activity (FPU/g) from each replicate at the surface and inside the bagasse pile recorded over the 360-day storage period.

| Storage time (Days) | Cellulase activity (Surface) | | | | Cellulase activity (Inside) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 0.31 | 0.38 | 0.56 | 0.42 | 0.89 | 0.78 | 0.91 | 0.86 |
| **73** | 0.42 | 0.21 | 0.48 | 0.37 | 0.62 | 0.68 | 0.73 | 0.68 |
| **117** | 0.54 | 0.59 | 0.88 | 0.67 | 0.87 | 0.85 | 1.04 | 0.92 |
| **141** | 0.79 | 0.81 | 0.86 | 0.82 | 0.92 | 0.96 | 0.06 | 0.65 |
| **169** | 0.83 | 0.79 | 0.99 | 0.87 | 0.97 | 0.99 | 1.07 | 1.01 |
| **212** | 1.05 | 1.21 | 1.13 | 1.13 | 1.45 | 1.27 | 1.15 | 1.29 |
| **268** | 1.06 | 1.01 | 1.18 | 1.08 | 1.05 | 1.39 | 1.6 | 1.35 |
| **308** | 1.11 | 0.92 | 1.25 | 1.09 | 1.38 | 1.33 | 1.37 | 1.36 |
| **360** | 1.41 | 1.38 | 1.29 | 1.36 | 1.73 | 1.76 | 1.82 | 1.77 |

**Appendix D 2:** Xylanase Activity (XU/g)from each replicate at the surface and inside the bagasse pile recorded over the 360-day storage period.

| Storage time (Days) | Xylanase activity (Surface) | | | | Xylanase activity (Inside) | | | |
|---|---|---|---|---|---|---|---|---|
| | Rep 1 | Rep 2 | Rep 3 | Mean | Rep 1 | Rep 2 | Rep 3 | Mean |
| **21** | 0.92 | 0.88 | 0.81 | 0.87 | 1.15 | 1.25 | 1.36 | 1.25 |
| **73** | 0.41 | 0.32 | 0.36 | 0.36 | 0.87 | 0.92 | 0.84 | 0.88 |
| **117** | 0.89 | 0.86 | 0.83 | 0.86 | 1.28 | 1.38 | 1.36 | 1.34 |
| **141** | 0.87 | 0.85 | 0.95 | 0.89 | 1.41 | 1.44 | 1.62 | 1.49 |
| **169** | 0.79 | 0.81 | 0.84 | 0.81 | 1.92 | 1.79 | 1.45 | 1.72 |
| **212** | 1.14 | 1.31 | 1.33 | 1.26 | 1.64 | 1.91 | 2.09 | 1.88 |
| **268** | 1.21 | 1.29 | 1.22 | 1.24 | 1.86 | 1.88 | 1.94 | 1.89 |
| **308** | 1.27 | 1.24 | 1.32 | 1.28 | 1.88 | 1.92 | 1.88 | 1.89 |
| **360** | 1.25 | 1.21 | 1.18 | 1.21 | 1.61 | 1.54 | 1.71 | 1.62 |