Carbohydrate hydrolases and their role in bread production

A thesis submitted
for the degree of

Doctor of Philosophy

by

Jenny Higgins
B.Sc. (Hons)

School of Life Sciences and Technology
Victoria University
Australia
2005

Dedication

This work is dedicated to my mother and father

Declaration

I hereby declare that all work carried out in this project was performed while I was enrolled as a Ph.D. student in the School of Life Sciences and Technology, Victoria University of Technology, Werribee Campus. To the best of my knowledge, this work has not been submitted in whole or part for any other degree or diploma in any University and no material contained in this thesis has been previously written or published by another person, except where due reference is made in the text.

Jenny Higgins [redacted]

August 2003

Acknowledgments

I would like to express my sincere thanks to my supervisor, Dr Darryl Small for his guidance and encouragement throughout the study. His support during the preparation of this thesis is also gratefully appreciated. I would also like to thank the School of Life Sciences and Technology, Victoria University for giving me the opportunity to undertake the study.

To the laboratory staff, Stacey, Charmaine, Michael, Ira, Marjena and Vilnis, thankyou for your help and support throughout the study. I am particularly grateful to Vilnis for all his help with the HPLC phases of the study. Thanks also to Brain Hanrahan who shared his HPLC experience with me. I would like to thank the laboratory manager Dale Tomlinson for his quick attention to ordering specific chemicals required for the work and particularly for obtaining the appropriate flour when all seem lost.

Thanks to all those people working at Werribee, both academic and administrative as well as the library staff. Professor John Casey provided assistance with the DSC equipment and Dr Mrinal Bhave kindly loaned some relevant texts to me from her personal collection. I would also like to thank all the post graduate students at Werribee for helping to create an atmosphere of support and co-operation. Special thanks to Lan and Alexandra for their friendship and also to Robyn who has been a constant source of friendship and support throughout the study.

Finally I would like to express a very special thank you to my family: to my husband, Dennis and to my children, Melanie, Jessica and Tristan for their moral support, patience and understanding. Without them I would not have been able to complete this project and thesis. Also special thanks to my parents whose interest and enthusiasm were a constant source of inspiration.

Publications and presentations

Parts of the research reported in this thesis have been published and the details are as follows:

Journal article

Jennifer M. Higgins and Darryl M. Small. 2003. The effects of endo-β- xylanases on arabinoxylans during the production of white bread. Manuscript submitted to Cereal Chemistry – currently under review.

Conference presentations

J. M. Higgins and D.M. Small 1998. Carbohydrate hydrolases and their activities in breadmaking ingredients, poster paper presented at the Australian Institute of Food Science and Technology Conference held in Brisbane in September 1998.

J. M. Higgins and D.M. Small 1999. Carbohydrate hydrolases and their role in breadmaking, oral paper presented at the 49th Australian Cereal Chemistry Conference held in Melbourne in September 1999. In addition, this paper was included in the proceedings of the conference, pages 57-60, edited by J.F. Panozzo, M. Ratcliffe, M. Wootton, and CW Wrigley, and published by the Cereal Chemistry Division of the Royal Australian Chemical Institute, Melbourne, ISBN 0-909589-95-X.

J. M. Higgins and D.M. Small 1999. Carbohydrate hydrolases and their interactions as determinants of softness of baked products, poster paper presented at the Australian Institute of Food Science and Technology Conference held in Adelaide in September 1999.

J. M. Higgins and D.M. Small 1999. Carbohydrate hydrolases and their role in breadmaking, oral paper presented at the Annual Convention of the American Association of Cereal Chemists in Seattle, October 1999.

Abstract

From the completion of the baking process, loaf breads undergo changes which result in their appeal diminishing. Pentosans in wheat flour consist primarily of arabinoxylans with smaller levels of arabinogalactan often present. Commercial pentosanase preparations contain a range of xylanase and arabinase activities that are believed to act synergistically to produce the improving effects observed in breads when these preparations are added. The purpose of this study has been to investigate the significance of various carbohydrate hydrolases as anti-staling agents. Initially breads were treated with five purified endoxylanase preparations at a range of treatment levels to establish suitable levels of addition. Whilst it was found that changes at a molecular level impacted significantly on crumb and crust characteristics for some of the preparations, it appears that not all endoxylanases have beneficial effects when used alone in a basic bread formulation.

Initial baking trials showed that, whilst very low levels of endoxylanases enhanced loaf properties, increasing levels of endoxylanase addition impacted negatively on crumb and crust properties of baked breads. The results indicate that the crumb and crust characteristics of colour and softness can be altered, sometimes significantly, by the type of enzyme used as well as the level at which individual enzymes are added. The use of endoxylanases from *Trichoderma viride, Trichoderma longibrachiatum, Aspergillus niger* and *Humicola insolens,* all fungal endoxylanases produced dramatic changes in crust colour, crumb structure and the development of crumb stickiness at high treatment levels. At lower treatment levels significant differences were observed in the ability of the different preparations to produce breads with enhanced softness and that remained fresher during the shelf life trials. Not all the preparations produced significantly softer crumbs at the treatment levels used. It appeared that the enzymes differed in their substrate specificity. This finding was further supported when bread formulas were treated with composites of arabinofuranosidase and endoxylanases. The removal of arabinose units allowed greater access to the xylan backbone by the less effective endoxylanases resulting in a significant increase in crumb softness of the resulting loaves. The enhanced crumb softness found for some of the treatments used was not found to be due to a slowing of starch retrogradation. Thermal analysis of the

bread crumb using differential scanning calorimetry showed there was no difference between the level of starch retrogradation observed in the treated or untreated breads. Treated breads with a significantly softer crumb did not have a corresponding decrease in the level of starch retrogradation.

The overall conclusions of this investigation are that endoxylanase preparations from some, but not all fungal sources studied here can produce significant changes in the textural properties of white bread prepared under Australian bread making conditions. The presence of arabinofuranosidase can enhance the impact of those endoxylanases which alone do not slow the rate of staling of breads. When α-amylases from *Aspergillus oryzae* or *Bacillus licheniformis* were incorporated into dough formulations, softer crumb resulted. However only the *Bacillus* preparation enhanced the keeping characteristics of white bread.

Table of contents

List of tables

List of figures

Chapter 1

Introduction

1.1 Introduction

The art of bread making dates back thousands of years. Whilst there are no records of where and when bread originated the suggestions are that it was baked in Egypt as far back as 10,000 BC. Baked from emmer, an ancient variety of wheat, these breads were yeast leavened and baked in large clay pots (Howard 2001). Wheat was ground, formed into dough with water and allowed to ferment. Fermentation occurred naturally due to the presence of wild yeasts or by the addition of a small amount of previously fermented dough that contained viable yeast cells. Bread formulations have seen relatively little change over the centuries. Even today the only ingredients needed to produce good quality bread are flour, water, salt and yeast as shown by the famous French baguette (Stear 1990).

Today, bread manufacturers face the challenge of providing fresh high quality bread products on a daily basis. However wheat, the main ingredient from which bread is produced, can be highly variable in quality depending on the growing location, climate, harvesting, milling and storage conditions. Current automated production processes, necessary to enable high production demands to be met, have seen significantly decreased processing times become the standard. This also adds to the difficulties of producing a high quality product on a daily basis. In order to produce bread of consistently high quality under these conditions, a higher degree of dough tolerance and tolerance to ingredient variation is required. The use of commercial enzyme preparations as processing aids may impart the added tolerance called for in modern day bread manufacture (van Oort et al 1995).

Lifestyle changes have also led to increased pressure for the manufacturers of baked goods. For many of today's consumers time is at a premium. While current production methods produce loaf breads of high quality, there is a growing demand for breads that retain their freshness, and it's associated attributes for longer periods. In addition to this, today's health conscious consumer requires breads that are low in fat, high in fibre

and free of artificial additives. As consumer demand for 'natural' additives grows the use of enzymes to replace chemical agents has come under investigation. The commercial desirability, of a loaf with an extended shelf life and an increased volume, has seen investigations into the use of enzymes such as proteases, pentosanases and amylases in dough systems. When used at the correct levels these enzymes may allow more water to be retained in the dough. Loaves that have an increased volume and a softer crumb are produced and ultimately bread with extended shelf life is achieved. Increasingly bakeries are using commercial enzyme preparations in an attempt to slow the staling of baked goods while still retaining the aroma and flavour associated with the fresh product. But, while the improving effects of these preparations are apparent, the end products of these hydrolase enzymes and the effects at a molecular level are yet to be identified.

1.2 Bread production

A number of methods are available for bread production. Continuous bread making, the CBP, straight dough, no-time and chemical dough development and the sponge and dough method are just a few and these are briefly compared in Figure 1.1. Whilst differing in many ways, all the bread-making processes involve some form of mixing, fermenting, dividing, moulding, panning and baking to produce a light, well aerated bread with appetising flavour and aroma (Pomeranz 1987).

1.2.1 Continuous bread making process

Developed in 1953 the continuous bread making process is capable of preparing pan bread at a rate of 60 loaves per minute. In this method the dry dough ingredients are fed continuously to a mixer containing a ferment broth. The mass is passed to a dough pump and then to a dough developer where it is kneaded to optimum development. The dough is transferred to a divider that continuously divides and extrudes dough pieces of the desired weight and shape. The dough is extruded directly into baking pans, proofed

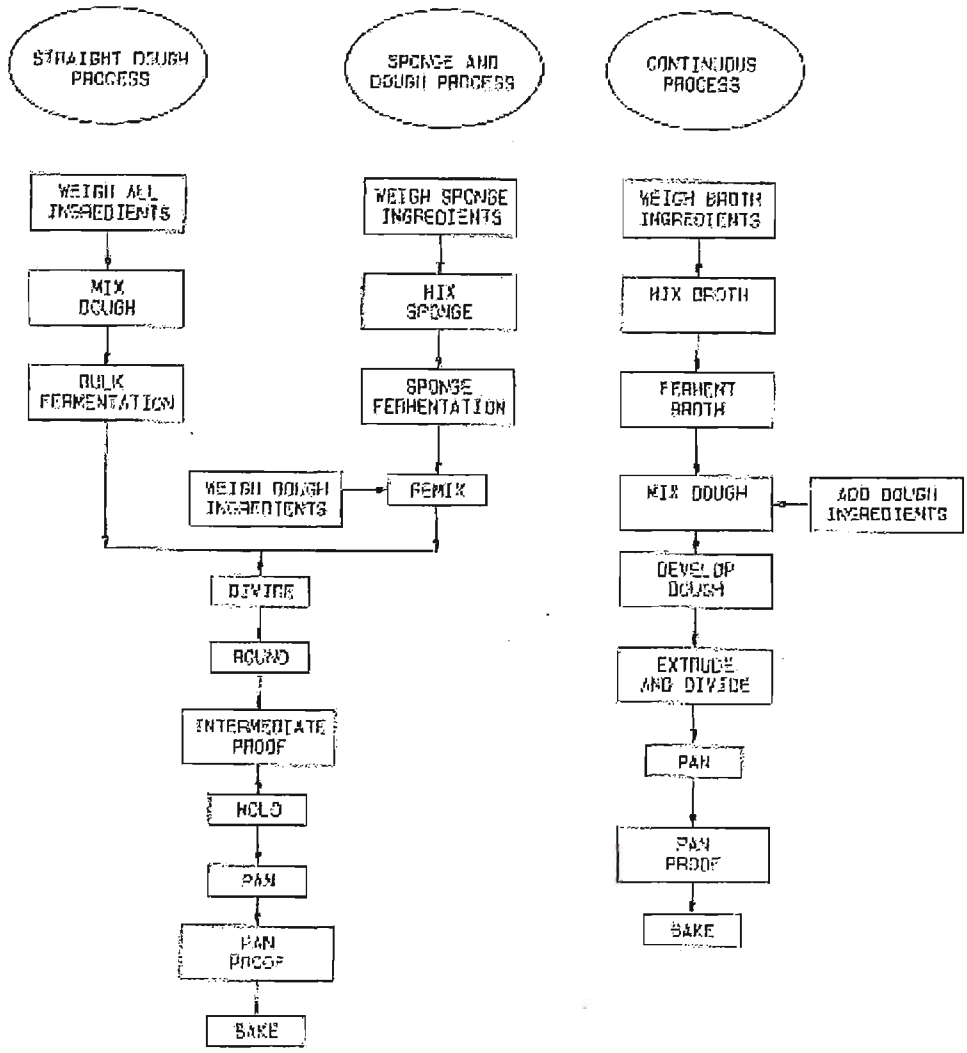


Figure 1.1 A schematic comparison of some commonly used breadmaking processes (Pyler 1988)

in the pan for a short period and baked. A bulk fermentation period is not required for this method because fermentable sugars and yeast in the preferment provide the necessary leavening power for the developed dough (Pomeranz 1987).

1.2.2 Sponge and dough method

This is a two-stage process. Initially a preferment of 50-70 percent of the total dough flour is fermented with the yeast in the sponge stage. During this period the yeast becomes fully active producing CO_2, ethanol and other flavour volatiles. The fermentation continues for 3-6 hrs during which time the gluten proteins undergo a

series of oxidation and reduction reactions culminating in sponge that has desirable elastic properties. The sponge is then combined with the remaining dough ingredients and mixed to develop the dough. The objective of the sponge stage is to bring about a uniform blending of the ingredients into a homogenous mass, completely hydrate the flour particles and form sufficient gluten to retain the CO_2 produced during this stage (Pyler 1988). Although the sponge and dough method requires less yeast and produces breads with improved grain and texture it has a long processing time. As much as seven hours is required to prepare loaves that are ready for baking.

1.2.3 The Chorleywood Bread Process (CBP)

This process develops the dough mechanically. By using high-speed mixers dough is developed to optimum in as little as five minutes (Matz 1992). High levels of oxidising and reducing agents are also required with the CBP but this is offset by the advantages which include less time required for bread production and increased product yield. Low protein flours also perform better when used for bread production with the CBP. One disadvantage of the CBP is that the high speeds involved in the process result in higher dough temperatures that may adversely affect yeast fermentation.

1.2.4 Straight dough bulk fermentation method

As with the rapid dough methods bread preparation using the straight dough bulk fermentation method involves mixing of all the ingredients to form an homogeneous dough. Unlike CBP the mixing is carried out using low speed mixers. A much longer fermentation period follows. A minimum of one hour of fermentation is required in a straight dough method.

1.2.5 Rapid dough methods

Whilst the sponge and dough method is a more traditional means of preparing bread, the extended production times make it less suitable for meeting the demands of todays market. Bread manufacturers have developed a number of methods to produce loaf bread of high quality yet require considerably shorter production times. Gas generation during bulk fermentation contributes little to the final product and most of the flavour volatiles are formed in the crust undergoing Maillard reactions during baking production methods have shortened the fermentation times to as little as 25min. Short time and no-time methods involve mixing all the ingredients together in a single step.

Initially the ingredients form a mass of wet clumps. As mixing continues the dough acquires elastic properties until it reaches optimum development. At this time it is smooth in appearance and has a dry surface. Short-time methods involve either the use of high-speed mixers to develop the dough rapidly or the use of improving agents with a reduced mixing time to develop the gluten matrix. One disadvantage of these rapid methods is that a higher level of yeast is required to allow sufficient gas to be produced in the shorter fermentation period. A slightly lower protein content is required for flours used in this process because stronger flours require longer fermentation periods to develop fully.

1.2.6 The rapid dough process

In a rapid chemical dough development all the dough ingredients required are mixed into a single batch. When water is added the ingredients become hydrated forming a wet sticky mass. The protein-starch bonds are weakened and as mixing continues the dough acquires the elastic characteristics associated with bread dough. The gluten forms a web of fibrils in which starch granules and gas bubbles are embedded. The starch molecules customise their shape to fit around the vacuoles influencing crumb grain and texture in the final product. Mixing continues until the dough reaches optimum development. It is now smooth and dry in appearance and will stretch readily without breaking. Underdeveloped doughs or green doughs will have insufficient elasticity while an over developed dough will tear when stretched. Bechtel et al (1978) using TEM found that the protein network in an overdeveloped dough was not continuous. The network was disrupted due to the protein forming thin strands that were easily torn. In the baking process, the final dough temperature required is around 22°C. At this temperature yeast fermentation will not proceed too rapidly which would otherwise result in a loss of dough stability (Kent and Evers 1993)

In the second stage of bread production the kneaded dough is left to ferment. The fermentation time will vary depending on the process but in Australian bakeries it can be as little as twenty-five minutes. During this time CO_2 is produced as a by-product of yeast metabolism, the gluten matrix is modified to ensure maximum retention of the CO_2 during baking and the number of yeast cells increases (Pomeranz 1987, Kent and Evers 1993). The gluten fibrils aggregate forming longer fibrils to produce elasticity in the dough.

At the completion of the fermentation period the dough is knocked back. This expels the CO_2 formed during fermentation. The bread is divided into dough pieces of the required weight. After dividing the dough has large areas of cut surface from which moisture and CO_2 can readily diffuse. The rounder helps overcome this problem. By forming the cut dough pieces into balls the surface of the dough dries slightly due to moisture loss and the presence of flour particles on the surface. The dried surface now forms a barrier to further loss of moisture and gas. In the final stage of processing the dough pieces are moulded into long cylindrical shapes ready for panning. A second resting period follows during which a fresh supply of CO_2 is evolved. The bread is now ready for baking in a hot oven for 45min.

When bread dough is placed in the oven a rapid expansion occurs due to an increase in the volume of the CO_2 present. This is known as oven-spring (Hoseney 1994). During this time yeast multiply and enzyme activity increases. When the crumb reaches temperatures, between 50-60°C, the yeast is inactivated and no more CO_2 is produced. As the temperature continues to rise the starch granules gelatinise, gluten proteins coagulate and enzyme activity ceases. At around 100°C the final loaf volume is set and water is lost as steam. While the crumb temperature does not exceed 100°C the temperature of the crust continues to rise. This is important for the development of the crust colour as dextrins are produced in the crust at temperatures of between 110-150°C (Pomeranz 1987). Starch decomposition and the formation of dextrins help create the shiny crust surface or crust bloom associated with bread that results in the continuing appeal of this product to consumers.

1.3 The Australian bread making industry

The Australian bread market has undergone enormous changes in the last 30 years. With the rise of small bakeries, hot bread shop franchises and supermarket in-store bakeries the market share of the large bakery companies has been whittled to around 65 percent. Hot bread shops now enjoy a 25 percent market share with the in-store and independent bakeries sharing 5 percent each of the remaining bread market (McKean 1999).

Bread consumption in Australia is relatively low, comprising around one third of total energy intake compared with countries such as the Far East where bread products account for up to 75 percent of the daily energy intake (Pomeranz 1987). Despite this sales of bread and bread products in Australia are worth around 2 billion dollars a year.

1.4 Breadmaking in Australia

The high dough temperatures inherent in the Chorleywood process make this method unsuitable for the hot Australian climate. Similarly the long preparation times and high production costs involved in the sponge and dough method make this process equally unsuitable for the bread industry in Australia. The method of choice for the major bread manufacturing companies including Sunicrust, Tip Top and Buttercup is the rapid dough method due to its decreased fermentation times. It uses carefully selected dough improvers to facilitate a satisfactory level of dough development in the shortened proofing period. Figure 1.2 shows a schematic diagram of a general bread-making process carried out in Australian bakeries.

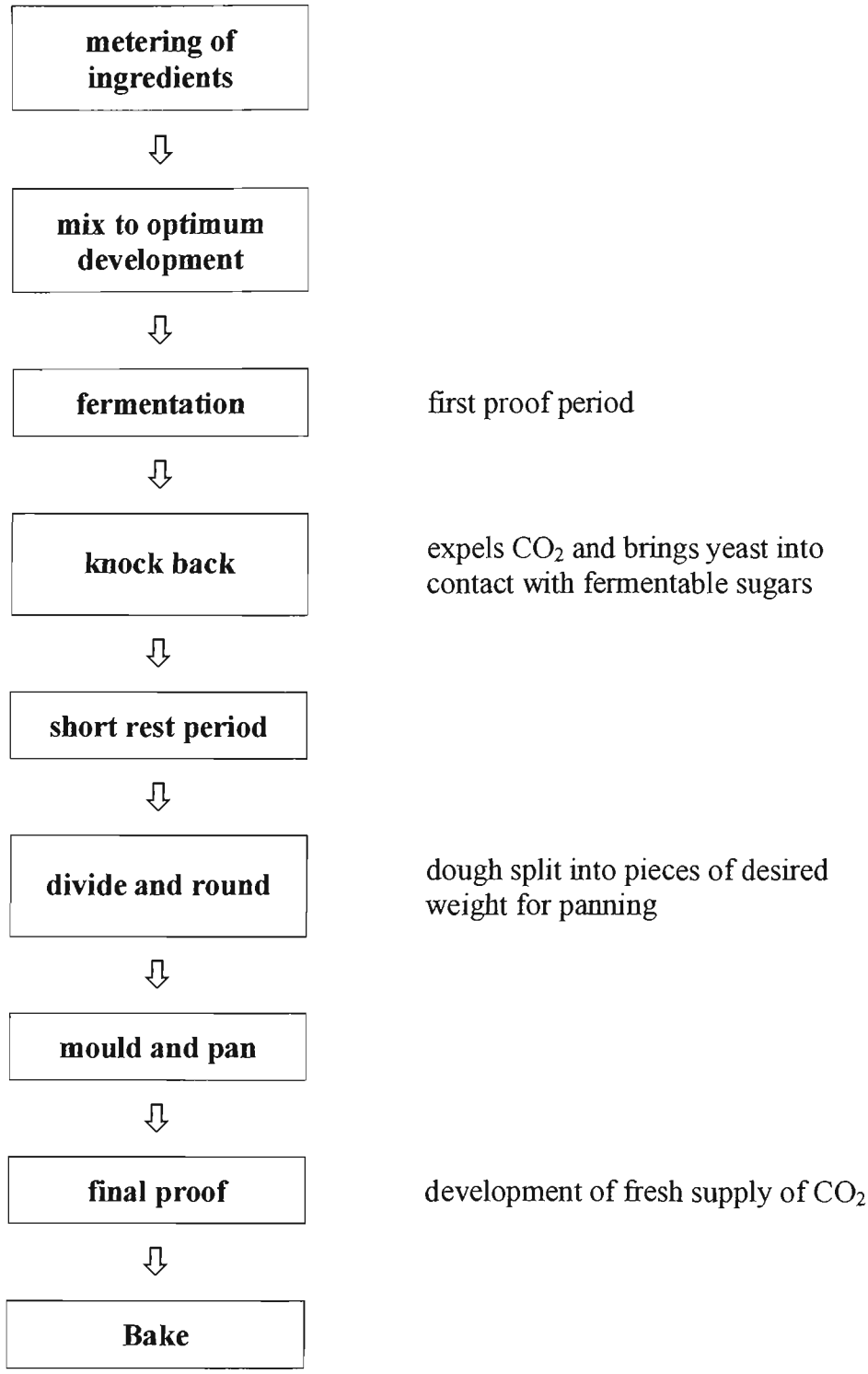


Figure 1.2 **Typical process used for breadmaking in Australia**
(Bread Research Institute of Australia 1989)

Chapter 2

Literature review

2.1 Major endosperm constituents and their role in the production of white bread

The term bread is used to describe a wide range of baked products that may differ dramatically in size, shape, texture and flavour. Regardless of the specific characteristics of the final product, bread has traditionally been prepared from flour milled from cereal wheat. Other cereals, pulses and even some legumes can be milled to produce flour. However it is the unique properties of wheat flour, in particular the gluten proteins that transform a wet mass of flour and water into a relatively uniform gelatinous dough mass that will become a yeast leavened loaf of bread (Cauvain and Young 1998).

2.1.1 Starch

When dough prepared from wheat flour is washed under a stream of water, three fractions are obtained. These are gluten, water-soluble components and starch. The major component of the wheat kernel is starch which accounts for up to 72 percent of the total wheat endosperm. Starch occurs in granules made up of two polysaccharides, the linear amylose fraction and the branched amylopectin. Both molecules consist of repeating glucose units joined by α-1,4-glucosidic bonds (Figure 2.1). The branch points in the amylopectin structure consist of glucose residues joined by an α-1,6-glucosidic bond.

During the proofing period of the baking process starch granules damaged in the milling process are degraded by amylase enzymes to provide energy in the form of maltose for the yeast. However, it is the undamaged starch granules that take up much of the water in the dough system. These granules continue to absorb water during baking until the point of gelatinisation when they split open and amylose molecules spill into the interstitial space. Upon cooling gelatinized amylose chains undergo retrogradation. Crystalline junctions form between the amylose chains resulting in a gradual firming of the crumb as water is excluded from the retrograded molecules (Whistler and Daniel

1985). In the early stages of cooling a certain level of retrogradation is necessary to allow bread to be sliced. Within several hours of removal from the oven all the amylose has irreversibly retrograded.

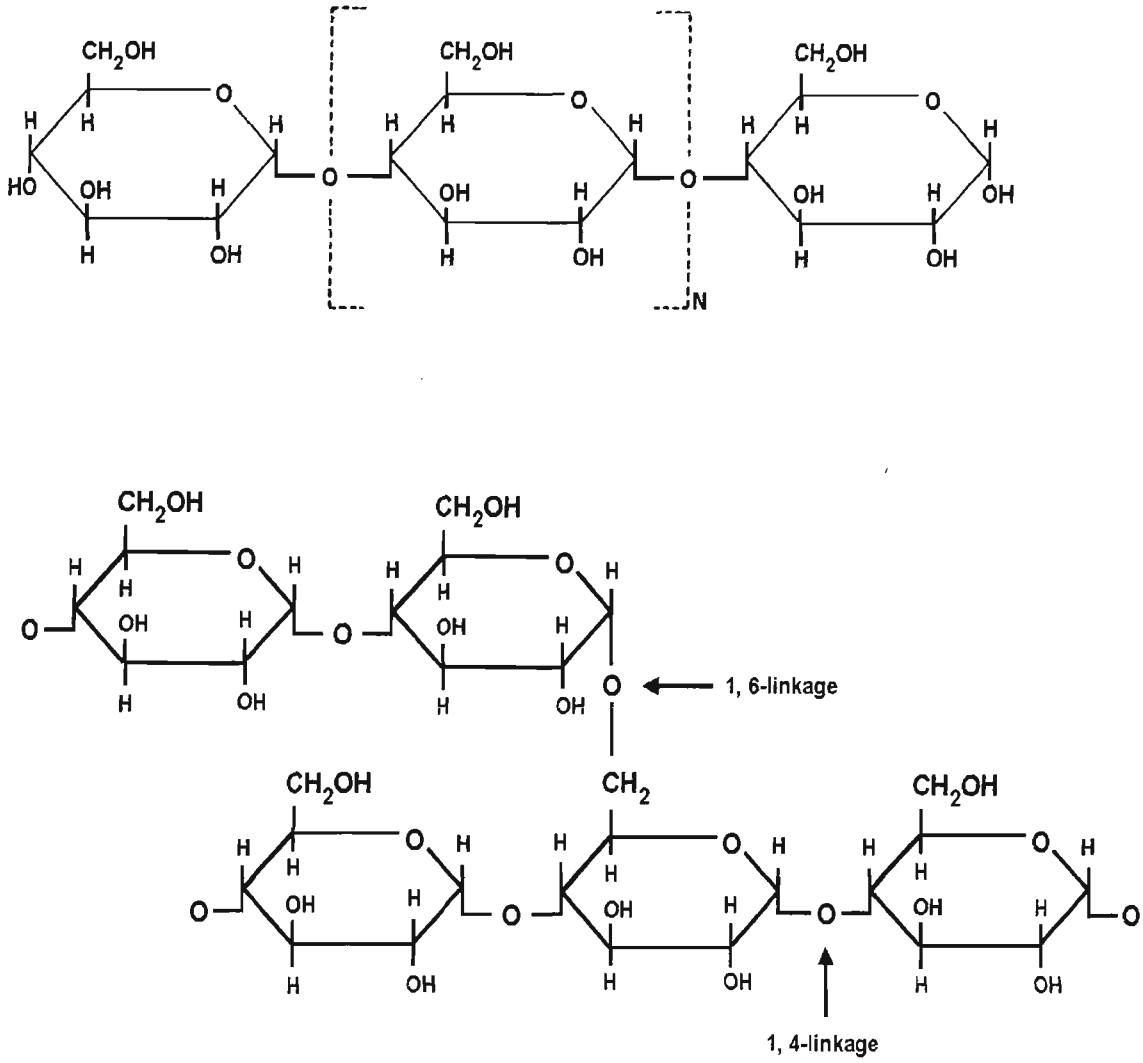


Figure 2.1 **The primary structural characteristics of starch molecules showing amylose (top) and amylopectin (lower)**
(Coultate 1996)

2.1.2 Gluten

Eliasson and Larsson (1993) described five classes of proteins in wheat endosperm, albumins, globulins, insoluble residues, glutenins and gliadins. It is the latter two, known collectively as gluten, which contribute to the viscoelastic properties observed in

bread dough. Gluten is the main storage protein in wheat flour accounting for 75-80 percent of total protein. Glutenins make up 40-50 percent of the total wheat protein. A model proposed in Blanshard et al (1988) describes glutenins as linear chains of polypeptide subunits joined head to tail by disulphide bonds. If tension is applied the chain of subunits can be stretched out of its natural configuration. When the tension is removed the chain recoils to its original arrangement. The elastic nature of glutenin is an important functional property of gluten contributing to dough strength and elasticity. Gliadins make up 30-35 percent to total wheat protein. They function as plasticisers that interact with the glutenin molecules to contribute extensibility to the bread dough. Gliadins are also responsible for controlling loaf volume and grain (Pomeranz 1987).

The coils of gluten proteins are held together by a variety of bonds including strong disulphide (-S-S-) bonds. During dough development the gluten molecules undergo significant changes. Severing of the disulphide bonds allows the gluten molecules to uncoil and rejoin at different positions. The gluten molecules become elongated and allow the linking of separate protein molecules together. Sulphydryl (-SH) groups are also present on the gluten proteins attached to the amino acid cysteine. Bonds form between these sulphydryl groups and the disulphide bonds. New inter- and intra-polypeptide bonds are formed that cause a relaxation of the dough by relieving stress caused during the mixing process (Kent and Evers, 1993). The gluten proteins are transformed from coarse gluten aggregates to a gluten film otherwise known as the bread dough (Karmel and Stauffer 1993). Optimum development of the gluten matrix is vital if expanding CO_2 produced by yeast fermentation is to be contained during baking. Soluble proteins are also believed to contribute to gas retention by forming a layer within the gas cells. This may in turn block pinholes in the cell walls preventing CO_2 from leaching out (Gan et al 1995).

2.1.3 Wheat flour pentosans

Wheat flour pentosans consist largely of arabinoxylans however a small amount of arabinogalactans may also be present. The arabinoxylans make up 3-5 percent of the total wheat endosperm and are considered the most important of the non-starch polysaccharides (van Oort et al 1995, Rouau et al 1993). They occur in two forms, water-soluble arabinoxylans (WSA) and the water-insoluble arabinoxylans (WISA) often referred to as hemicelluloses. The structure is similar for both forms and is shown

in Figure 2.2. Arabinoxylans consist of a xylose backbone made up of repeating D-xylopyranosyl units joined by a β-1,4 linkage. Branching L-arabinofuranosyl side chains are attached to the xylose chain at the C2 or C3 positions (Hoseney 1984, Kulp 1967). It is believed that the higher degree of branching observed on the WISA contributes to the poor solubility properties of these molecules (Eliasson and Larsson 1993).

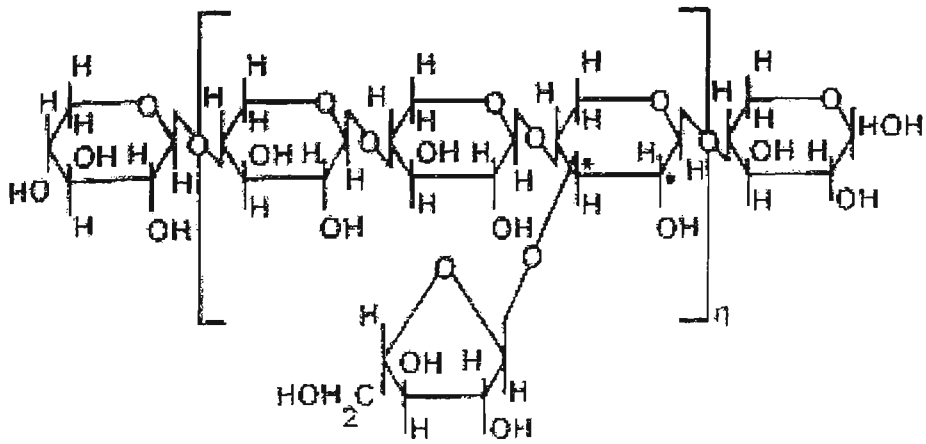


Figure 2.2 **The structure of arabinoxylan molecules found in wheat endosperm**
(Bushuk and Rasper 1994)

Pentosans have a high water binding capacity. It is estimated that arabinoxylan may bind 10-20 times its weight in water (van Oort et al 1995). Feruloyl groups are further esterified to some of the arabinan residues. These acids undergo oxidative gelation further increasing the water binding capacity of pentosans (D'Appolonia 1980, Yeh et al 1980). Arabinoxylans have a high affinity for water with isolates binding up to 10 times their own weight (van Oort et al 1995). In the presence of H_2O_2 WSA has been reported to undergo oxidative gelation due to the presence of feruloyl groups esterified to some arabinose residues (D'Appolonia 1980, Rouau 1993, Yeh et al 1980) further increasing the water binding capacity of the dough. This water is believed to then migrate to the starch molecules that have gelatinised during cooking to slow the rate of retrogradation (van Oort et al 1995).

WSA exist in flour in either the free form that is readily extracted by water, or in

association with protein and other polysaccharides. Pentosans influence dough consistency, crumb texture and elasticity, bread volume and symmetry, and the keeping properties of bread (D'Appolonia 1971, Stear 1990). Conflicting evidence exists as to the effects of the two forms on dough and bread properties. Work by Stear (1990), Kim and D'Appolonia (1977) and Kulp (1967) found that the insoluble hemicelluloses result in increased loaf volumes and are the most effective in stabilizing shelf life by controlling retrogradation of both the amylose and amylopectin fractions of the starch. This finding is supported by Blanshard et al (1988) who found that the addition of 2 percent of a WISA to a dough led to an increase in loaf volume of between 30-45 percent. Hoseney (1984) demonstrated that the addition of WSA had no effect on loaf volume while the addition of WISA resulted in loaves of reduced volume. Schiraldi et al (1996) found that added WSA increased starch retrogradation and produced a firmer crumb.

2.2 Ingredients of a typical white pan bread formulation

Four basic ingredients are required to produce white pan bread. These are yeast, high protein flour, salt and water. In addition to these basic ingredients a range of other ingredients and additives can be used including sugar, dough improvers and enzymes.

2.2.1 Flour

Most bread is produced from wheat flour. A suitable high quality bread-making flour will have gluten that is both sufficient in quantity and, when hydrated, is stable with good elasticity. A protein content of 11-13 percent is an important factor when selecting a flour for bread making. It should contain sufficient damaged starch to yield fermentable sugars to support yeast activity during the proofing stages. Low levels of mono-; and disaccharides totaling 0.8-1.6 percent and comprising sucrose 0.5-1.5 percent, glucose 0.03-0.1 percent, fructose 0-0.05 percent and maltose 0.02 percent are also necessary in a good bread making flour. The moisture content should not exceed 14 percent to permit safe storage in addition to satisfactory colour (Kent and Evers 1993, Hoseney 1994). The presence of native lipids also contributes to the suitability of flour for bread making. Studies carried out on the effects on bread quality prepared with untreated and defatted flours demonstrated that breads prepared from defatted flour had loaf volumes that were consistently lower than loaves prepared from untreated flour (Pomeranz 1987).

2.2.2 Yeast

Yeast facilitates dough aeration during the fermentation period. By metabolising maltose and other sugars produced by α-amylase a range of waste products arise including ethanol, acids and CO_2. A simplified equation that summarises the fermentation reaction is

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

In the production of bread *Saccharomyces cerevisiae* is the most commonly used yeast although some other yeasts or microorganisms with gas producing properties may be used for specialised breads. *S cerevisiae* used for baking has a high invertase content. Any sucrose in the dough is converted to glucose and fructose quickly and these in turn are utilised by the yeast to produce gas. Doughs containing high sugar levels may inhibit the action of the yeast. Such doughs require strains that produce lower levels of invertase.

Maltose is not readily usable by most yeast strains in the early stages of fermentation because the genes that encode for maltose transport (maltose permease) and hydrolysis (α- glucosidase) have not been induced (Wong 1995). Where a plain dough (no added sucrose) is to be produced the yeast strain must be capable of encoding the genes for maltase synthesis quickly because naturally occurring fermentable sugars are rapidly depleted by the yeast.

The result of yeast fermentation is a lowering of dough pH, a mellowing of the gluten matrix and the development of flavour volatiles. Over 150 flavour volatiles have been identified in the bread dough including alcohols, esters, aldehydes and ketones. Reactions between yeast proteins and amino acids during baking also contribute to flavour development. In addition to these changes is the formation of the characteristic sponge structure of the bread crumb due to the expansion of CO_2 during baking.

Selection of the appropriate yeast strain for baking is dependent on the formulation, the processing method to be used and the final product required. Yeasts used in the

production of sour doughs require a resistance to pH values of 4.7 and lower. The period in which the maltose enzymes are being synthesised, referred to as the maltose lag phase, is one of low gas production. Generally yeasts selected for production of no-time and rapid bread process will adapt quickly to the presence of maltose particularly when a straight dough is required.

Yeast is available in several forms. Compressed yeast involves filtering and extruding a yeast suspension to form a cake. The product has a water content of approximately 70 percent and requires refrigeration. Compressed yeast loses approximately 10 percent of its activity in four weeks at 4°C. This figure increases dramatically when stored at higher temperatures. Dry yeast can also be used for bread production. It can occur as regular active dry yeast (ADY) or instant active dry yeast. They are prepared from yeasts that exhibit resistance to dehydration. Regular ADY must be rehydrated prior to adding to the bread ingredients while instant ADY can be added directly to the bread formulation. Instant ADY was used for this study because of the shorter fermentation period used in the process.

2.2.3 Salt

Salt plays several roles in the development of bread dough. In addition to playing a vital role in flavour development, salt has important functional properties in dough development. It contributes to increased dough stability. By strengthening the gluten structure the gas holding capacity of the dough is increased. The action of yeast is inhibited however, in the presence of high salt levels, so care must taken not to exceed the appropriate level of addition (Stear 1990). The usual quantity of salt added to bread dough is 1.5-2 percent of flour weight, added as either an aqueous solution or as a dry solid (Kent and Evers 1993). Salt also aids in the formation of uniform brown crust.

2.2.4 Water

Water is a vital component in bread making. When water is added the dry flour components become hydrated. Blanshard et al (1988) describe this initial stage as a one-phase system. At about 30-35 percent water addition a second aqueous stage appears which allows the water-soluble albumins, globulins, water-soluble starch and pentosans to be dispersed throughout the dough (Tolstoguzov 1997). Water incorporated into the dough system is taken up by gelatinising starch molecules, the

gluten phase and the pentosans during baking. Modification of flour components such as pentosans allows higher levels of water to be incorporated into bread dough resulting in a softer crumb and extended shelf life.

2.3 Optional ingredients

2.3.1 Sugar

Sugar is added particularly when a shorter fermentation time is required. It provides an instant source of nutrition for yeast metabolism while the α-amylase is breaking down the starch granules damaged during the milling process.

2.3.2 Shortening

Lipid shortenings aid in the formation of a soft crumb and also contribute to extended shelf life. It is an essential ingredient in rapid dough methods and is added at a rate of 1-2 percent. Added lipids improve loaf volume, reduce crust toughness and produce a softer textured crumb that slices readily. Solid lipid shortening increases the amount of free lipid in the dough. Shortenings are believed to improve final loaf volume by helping to control the release of CO_2 in the dough during the early stages of baking (Pomeranz 1987). Blanshard et al (1988) demonstrated the ability of added shortenings to enhance loaf volume by preventing native lipids binding to the protein. This aided in stabilising the protein during baking. Added fats also contribute to extended shelf life by keeping the bread crumb softer and palatable for longer periods (Kent and Evers, 1993).

2.3.3 Dough improvers

Dough improvers are used in bread preparation to aid in the development of the gluten matrix. Improvers act by oxidising the cysteine sulphydryl groups in the wheat gluten. These groups no longer participate in exchange reactions with disulphide bonds causing a release in dough stresses and a reduction in extensibility. Generally, dough improvers contain oxidation and reduction agents, soy flour and malt flour. Malt flour serves as a source of α-amylase while the soy flour contributes lipoxygenases to the dough and these have a bleaching effect. β-Amylase is generally not added as part of a dough improver because it is already present in sufficient quantities in the wheat flour.

L-Ascorbic acid is an approved additive for use as a dough improver in Australia. No maximum level of addition has been set for ascorbic acid in Australia. Enzymes present in the flour oxidise ascorbic acid to dehydroascorbic acid (DHA) which is highly effective in increasing dough strength and enhancing loaf volume. Oxidising agents are particularly important in rapid mechanical bread production processes such as those used in Australia. L-Cysteine is another processing aid used to modify dough consistency. It is a highly reactive reducing amino acid that accelerates the disaggregation of disulphide bonds between proteins reducing the level of mechanical input required to achieve optimal dough development.

The high water activity of bread crumb makes it susceptible to microbial spoilage due to the action of yeasts and moulds. *Rhizopus* is a common mould associated with bread spoilage. To overcome this salts of fatty acids, particularly calcium propionate, are often added at levels of up to 300ppm (Stear 1990) as part of the dough improver.

2.3.4 Enzymes

Increasingly enzymes are being used as processing aids to optimise dough properties and enhance keeping quality of baked goods. The use of added enzymes produces a range of improving effects including an increase in the level of fermentable sugars by amylases, reduction in dough mixing time by proteases and an increase in dough consistency and machinability by amylases, proteases and pentosanases (Vinkx et al 1993)

In Australia, enzymes for commercial food applications are legally classified as processing aids in accordance with Codex principles. All sources used in this study are permitted for use in Australia.

2.4 Staling of white bread.

Bread and other baked goods are subject to an ongoing deterioration in quality that is directly related to storage time. Chemical and sensory changes include the development of a leathery crust, increased crumb firmness and crumbliness, and loss of taste and aroma attributes (Charalambous 1993, Fearn and Russell 1981, Sahlstrom and Brathen 1996, Schoch et al 1947). Changes in breads, cakes, doughnuts and other similar foods

that are not attributed to microbial spoilage are referred to as staling, a process that leads to a decrease in consumer acceptance of a product (Eliasson and Larsson 1993). Pomeranz et al (1971) demonstrated that crumb firming is a process that involves the redistribution of moisture within the loaf rather than moisture loss to the environment. It is in the control of this moisture migration that enzymes may have a role to play. Factors that contribute to bread staling include the processing methods used, the baking ingredients and biochemical components present in the flour.

Measuring bread staling can utilise a variety of subjective and objective analyses. Organoleptic methods involve using taste panels to assess subjectively the level of crumb firmness, loss of crust crispness and development of off flavours and odours associated with stale bread. Objective methods of measuring staling include X-ray diffraction patterns, differential scanning calorimetry (DSC) to measure the level of starch retrogradation, crumb compressibility, swelling capacity and absorptive capacity of the crumb as well as measuring the sedimentation rate of a slurry (Pomeranz 1987). This study utilised crumb compressibility measurements, level of starch retrogradation and sensory evaluation to measure staling of bread crumb.

2.4.1 Staling of the bread crust

Crust staling is attributed to absorption of moisture from both the atmosphere and from within the bread (Pomeranz 1987). During storage water migrates from the crumb near the outer surface to the crust. Eliasson and Larsson (1993) showed that the moisture content of bread crust stored for 100hrs increased from 15 to 28 percent with a corresponding decrease in the water content of the crumb closest to the crust margin from 45 to 32 percent. The increased moisture in the outermost layers results in the crisp brittle texture of fresh bread crust being lost as the crust becomes soft and leathery.

Furthermore, volatile flavour compounds formed by non-enzymic browning during baking are lost from the crust surface. A typical stale flavour arises in the loaf resulting in loss of consumer acceptance (Eliasson and Larsson 1993).

2.4.2 Staling of the bread crumb

Unlike the crust that stales as its water content increases, crumb staling or firming can occur without any significant change in water content. This is due to moisture

redistribution within the loaf rather than moisture loss as demonstrated by Pomeranz and Shellenberger (1971). Their work showed firm crumb to have the same moisture content as freshly baked bread.

Much of this water redistribution is attributed to the retrogradation of the starch molecules (Pomeranz 1991). The role of starch retrogradation is widely reported in the literature (Eliasson and Larsson 1993, Grossman and Debarber 1997, Kim and D'Appolonia 1977, Matz 1992, Pyler 1988, Russel 1983, Stear 1990, Zelenak and Hoseney 1986). A model describing the role of starch in staling and originally proposed by Schoch is presented in Figure 2.3. Retrogradation is an ongoing process, however, causing an ever increasing firming of the crumb until the loaf is perceived as stale (Whistler and Daniel 1985). Starch consists of two fractions: the linear amylose and branched amylopectin, and these undergo retrogradation at different rates. The amylose fraction crystallises rapidly upon removing the baked breads from the oven. Within several hours all of the gelatinised amylose molecules have retrograded in the cooled fresh bread (Kulp and Ponte 1981).

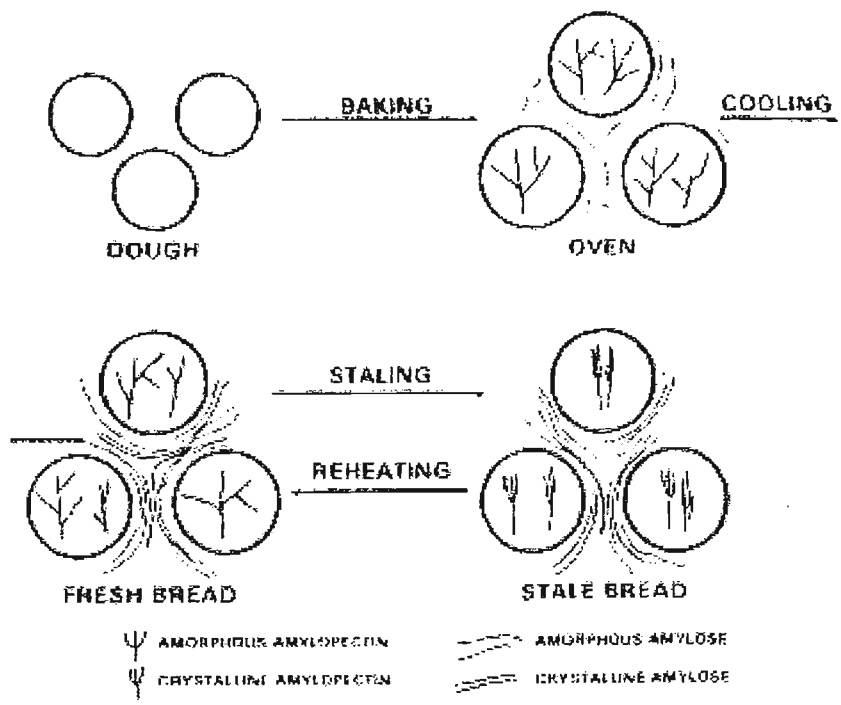


Figure 2.3 **A model of the changes to starch granules occurring during baking and staling in bread**
(Kulp and Ponte 1981, Schoch and French 1947)

The amylopectin fraction undergoes a slower form of retrogradation. As weak hydrogen bonds form between the outer branches, water is exluded. These bonds are relatively weak and are readily broken with gentle heating to around 70°C. This restores much of the original crumb softness and texture. Flavour compounds bound to the starch molecules are also released during the heating process thereby restoring the fresh aroma to the bread (Eliasson and Larsson 1993).

2.4.3 Interactions between gluten and starch and their effects on staling

The link between crumb firming and the formation of bonds between protein fibrils and amylose molecules leached out during baking is now being investigated. Martin et al (1991a and 1991b) have shown that dextrins with degrees of polymerisation between 4 and 9 are formed by the action of fungal and bacterial amylases during dough development and baking. These short dextrins are believed to interfere with the formation of the cross links thus slowing the rate of crumb firming. The migration of water from gluten proteins to the starch molecules may also affect the staling rate of bread (Pomeranz 1987).

2.4.4 Storage temperature and staling

The rate of staling is influenced by the temperature at which bread is stored. Upon completion of baking the crumb is an elastic gel with a temperature of around 98°C. Starch retrogradation will not occur until the crumb temperature falls below 60°C. Maintaining the storage temperature of bread above 60°C will therefore inhibit crumb firming. However, bread held at this temperature will not undergo sufficient starch retrogradation to produce a loaf which is palatable and therefore acceptable to the consumer (Stear 1990, Kim and D'Appolonia 1977).

At temperatures of between -7 and 60°C crumb firming is observed, the rate of its occurrence increasing as the temperature decreases. Staling rates are highest at refrigerator temperatures. Bread held at storage temperatures of below -7°C do not show any appreciable levels of crumb firming. Thawed breads will exhibit the same level of crumb firming as was present at the time of freezing (Stear 1990).

2.5 Enzymes and the role they play in bread production

2.5.1 Introduction

The unintentional use of enzymes in bread production has occurred for many centuries as enzymes occur naturally in wheat grains and the flour milled from them. The addition of malt and soy flour as ingredients of dough improvers adds a wide range of enzymes including carbohydrases and proteases, all of which contribute to overall loaf quality and stability. In recent times the use of cell wall degrading enzymes has increased (Poutanen 1997). These preparations affect functional properties of bread dough due to changes brought about at a molecular level. Table 2.1 provides an overview of the enzymes currently available to the bread industry and their main functions.

Table 2.1 Application of enzymes in breadmaking (van Oort et al 1995)

Key enzyme	Function
Protease	Mixing time
Peroxidase	Dough development
Xylanase	Proofing time, tolerance
Xylanase, amylase	Bread volume
Xylanase, peroxidase	Shape
Bacterial amylase	Anti-staling effects
Protease, peptidase	Flavour
Amylase, xylanase	Colour

2.5.2 Amylolytic Enzymes

This group of starch degrading enzymes plays an important role in the baking industry. When used as a flour supplement, amylases ensure a continuous supply of fermentable sugars for yeast growth and CO_2 production during dough preparation (Wong 1995). Amylases or glycosidases catalyse the hydrolysis of α-D-1,4 glucosidic linkages of starch. The two forms of amylases of particular interest to the food industry are the

α-; and β- amylases. α-Amylase (α-1,4-glucano hydrolase) is an *endo*-glycosidase that cleaves the α-1,4-glucosidic bond internally to yield a range of dextrins and oligosaccharides with the C1-OH in the alpha configuration. It has a MW in the 50 000 range and requires Ca^{2+} for stability and activity (Wong 1995). β-Amylases are a group of *exo*-acting enzymes that cleave maltosyl units from the non-reducing end of the substrate to yield maltose units with the C1-OH in the β configuration. Like the alpha enzyme this has a MW of around 50,000 but does not require the presence of Ca^{2+} for activity (Wong 1995). The combined action of both α- and β-amylase is required for the enzymolysis of starch. This produces a substantial increase in maltose that provides an energy source for the yeast as well as contributing to the formation of crust colour (Karmel and Stauffer 1993).

The three most common sources of amylase enzymes are cereal, usually added in the form of malt flour, fungal and bacterial. Malt flour is produced from germinating barley or wheat grains. During this process the level of α-amylase increases dramatically. Wheat flour can be supplemented with malt flour at the mill to ensure adequate levels of α-amylase for flours to be used for yeast-leavened bakery products (Asp et al, 1988. Fungal α-amylase is produced via the controlled fermentation of the mould *Aspergillus oryzae*. After extraction, the enzyme is partially purified and the level of activity is standardised. Some residual protease, pentosanase and oxidase activity is usually present in purified amylase preparations.

Bacterial α-amylase is sourced from *Bacillus subtilis.* As a bacterial preparation it is more tolerant of higher temperatures than fungal or cereal amylases thus some residual activity is often observed in baked breads during storage. Continued amylose hydrolysis can result in the development of gumminess of the crumb as the bread ages (Kulp 1967).

2.5.3 Amylase enzymes and bread staling

There is no significant effect on initial crumb softness of breads prepared using amylase preparations. The effect of these enzymes is seen as the bread crumb ages. Hebeda and Zobel (1996) demonstrated the ability of amylase preparation to extend shelf life of white bread by up to three days. The exact mechanism for the antistaling effect of

amylase on bread crumb is still unclear. The presence of low molecular weight saccharides that interact with water rather than other saccharide molecules or larger starch chains is the simplest theory offered to explain the action of amylase preparations (Axford et al 1968). However work carried out by Dragsdorf and Varriano-Marston (1980) demonstrated that breads baked with enzymes had a higher rate and degree of starch crystallization. At this time the relative significance of these mechanisms of staling and the role of starch-protein interactions described earlier, remain unclear.

2.5.4 Proteases and bread production

Proteolytic enzymes occur naturally in small amounts in wheat flour however, supplementation of doughs with commercial preparations can have dramatic effects on dough properties and the final product. Proteolytic enzymes can be of fungal, bacterial or cereal origin. Regardless of the source all proteases are inactivated by high temperatures encountered during baking thus limiting their activity to dough mixing and proofing periods (Karmel and Stauffer 1993). These enzymes can proceed by *endo-* or *exo*-splitting mechanisms, a factor which must be taken into consideration when selecting an enzyme preparation to produce a desired end result. Where a reduction in mixing time is required for example an *endo*-acting enzyme would be more suitable. The cleaving of internal peptide bonds by such enzymes has a more dramatic effect on dough rheological properties than the removal of terminal amino-acids by an *exo*-splitting enzyme (Karmel and Stauffer 1993). Commercial preparations generally contain a mixture of both forms of enzymes, the proportions of each differing between preparations as required. Dough strength is determined by the number of disulphide bridges present when the dough reaches optimum dough development. Enzyme modification of these disulphide bridges results in a "mellowed" dough that expands more readily during CO_2 production by the yeast (Eliasson and Larsson 1993).

2.5.5 Functional effects of proteolytic enzymes

The importance of proteases in the dough system becomes obvious when the functional effects of these enzymes are examined. Reductions in mixing time, improved machining characteristics; the reduction of dough tightness or buckiness; and increased extensibility to give improved gas holding properties and ultimately increased loaf volume are all possible when proteases are used at optimum levels. In addition to these features, loaves with improved symmetry and crumb softness are produced, the latter

being responsible for bread with increased shelf life properties (van Oort et al 1995, Karmel and Stauffer 1993).

2.5.6 Lipoxygenases

Wheat flour contains endogenous lipoxygenase activity that results in the oxidation of free unsaturated fatty acids. The addition of lipoxygenases results in the oxidation of many of complex lipids found in wheat flour. Typically lipoxygenases are added in the form of enzyme-active soy flour (Hebeda and Zobel 1996). In the bread dough these enzymes oxidize carotenoid endosperm pigments to aid in the formation of a white bread crumb that is highly desirable in loaf breads (Matz 1992). The addition of lipoxygenases also contributes to the formation of a soft bread crumb. The lipoxygenase-induced release of bound lipids may exert a change in conformation of the gluten proteins. The release of gluten-bound lipids may then provide additional free lipids for complexing with starch during baking to slow the rate of retrogradation (Hebeda and Zobel 1996).

2.5.7 Glucoamylases

These *exo*-acting enzymes cleave both amylose and amylopectin at the α-D-1-4- and α-D-1-6-glycosidic bonds. For bakery applications these are sourced from *Aspergillus* sp or *Rhizopus*. Residual activity of the enzyme is often found in other fungal preparations of amylase.

2.5.8 Pentosanases

During breadmaking modification of arabinoxylan may occur as a result of the action of enzymes broadly referred to as pentosanases. Of these, the enzyme most thoroughly researched is 1,4-β-D-Xylan xylanohydrolase (EC 3.2.1.8). This is an *endo*-hydrolase and is here referred to as endoxylanase. It appears that in wheat flour doughs this enzyme tends to solubilise the WISA fraction producing high molecular weight WSA. Work carried out by Courtin et al (1999) demonstrated the ability of endoxylanses to improve loaf volume by decreasing the amount of WISA in the dough and increasing the level of WSA. However it was also shown that extensive hydrolysis of the WISA and degradation of solubilised fractions decreased loaf volumes.

Enzymatic solubilisation of arabinoxylans requires the presence of endoxylanase. The

final level of solubilisation might be influenced by a number of factors including enzyme specificity, degree of access to the arabinoxylan chains and level of treatment (Rouau 1993). Recent studies have also indicated that the presence of endogenous endoxylanase inhibitors in wheat flour may impact on the degree of arabinoxylan solubilisation and degradation (Ingelbrecht et al 2000; Debyser et al 1999).

While commercial enzyme preparations have been shown to slow the firming of bread crumb (D'Appolonia and Morad 1981; Hoseney 1984; Kim and D'Appolonia 1977), the specific enzymes within these preparations and their action on the bread crumb are yet to be identified. Over a number of years the role of pentosans in the aging of bread has come under investigation (Krishnarau and Hoseney 1994, Kulp 1967, Kulp and Betchel 1968, Rouau 1993, Stacy 1964). Fungal and bacterial pentosanases are finding increasing acceptance in the baking industry as an aid to extending the shelf life of leavened bread. Endoxylanases are the most effective hemicullulases to be used in bread production. These enzymes appear to hydrolyse both water-soluble and insoluble arabinoxylans into relatively large oligomers which have an increased water binding capacity. Tests have shown that the use of highly purified endoxylanase preparations results in improved dough machinability, increased volume and improved appearance and colour in the final product. Excessive pentosan hydrolysis results in doughs which are slack and collapse over the sides of the baking pans. The use of endoxylanase preparations containing no xylosidase activity appears to overcome these problems (van Oort et al 1995).

Arabinofuranosidases and *endo*-arabinanase enzymes also aid in the hydrolysis of arabinoxylans. Arabinofuranosidases are *exo*-acting enzymes that hydrolyse terminal non-reducing residues from polysaccharides containing arabinoxylan. These enzymes are specific for the hydrolysis of (1→3) and (1→5)-α-arabinosyl linkages of arabinan. The likely role of this enzyme in decreasing the solubility of an arabinoxylan structure is shown in Figure 2.4. The other enzyme with arabinanase activity is the *endo*-(1→5)-α-L-arabinanase, an enzyme that cleaves arabinose chains in an *endo*- fashion.

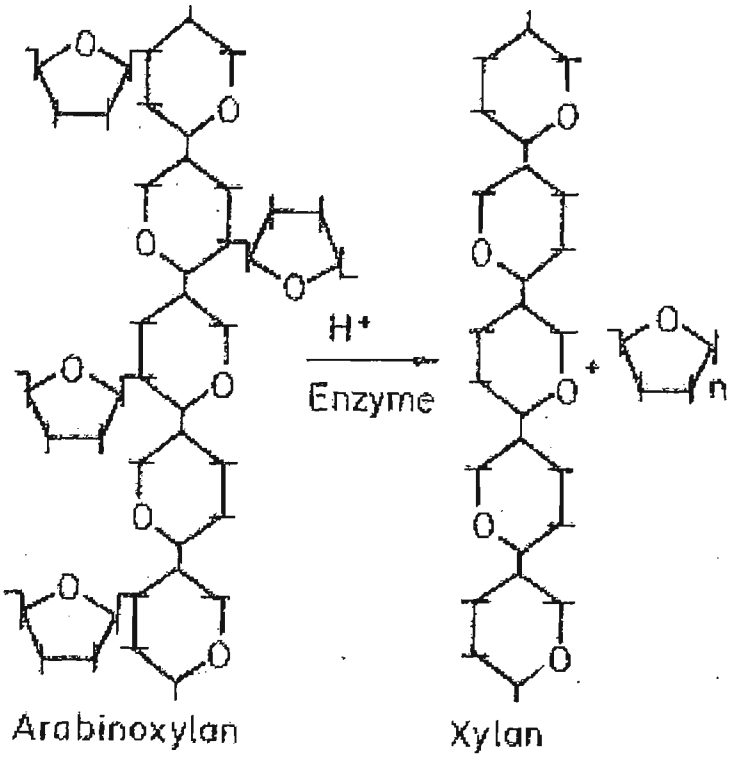


Figure 2.4 **The mode of action of arabinosidase in removal of arabinose side chains from arabinoxylans of wheat flour**
(Mueser and Sukow 1988)

The ability of enzyme additives to retard staling may also be dependent on their source. In general, it has been found that bacterial enzymes have a higher thermal stability than those of cereal and fungal origins. In traditional bread making processes, enzymes from bacterial sources including *B subtilis* and *B licheniformis* have proven unsuccessful (Godfrey and West 1996). Due to the long fermentation periods inherent in traditional bread making processes, bacterial enzymes produce excessive levels of hydrolysis resulting in sticky doughs and crumb. However, in modern commercial bread production the use of carefully controlled doses of these enzymes may provide a chemical free alternative for the production of bread with improved keeping properties. Studies by Asp et al (1985) found that in addition to remaining active during the baking process, some bacterial enzymes remain active after baking and during the subsequent storage of the product. If these enzyme do survive the baking process they may have a greater impact on the keeping qualities of baked goods.

2.6 Aims of this study

A range of highly purified enzyme preparations have recently become available to the baking industry. The use of α-amylases to slow retrogradation and pentosanases to hydrolyse arabinoxylans is well known. However the end products of these hydrolysis processes and the effects of these simpler carbohydrates on crumb firming are yet to be determined.

The current study set out to examine the effects of endoxylanase and arabinfuranosidase using a strong bread making flour milled from Australian wheat. Specifically, the aim of this project is to investigate the effects of a number of newly available, highly purified enzyme preparations, on the carbohydrate properties of bread during baking, cooling and storage. The sensory attributes of treated and untreated breads, as perceived by the consumer, will also be investigated.

The individual aims of this project have been to:

1) Establish and validate procedures for the measurement of carbohydrates in flour, bread doughs and baked products. Carbohydrates to be measured include glucose, maltose, xylose and arabinose.

2) Identify the products of starch and pentosan hydrolysis during bread production and storage.

3) To evaluate the addition of amylase and xylanase preparations from fungal, bacterial and cereal sources in a series of baking trials. Evaluation was based on the ability of the enzyme to enhance crumb and crust properties and extend shelf life of the baked product.

4) To identify the effects of amylase and xylanase preparations on the rate of starch retrogradation as bread ages.

5) To investigate the possible synergistic effects of arabinofuranosidase and xylanase on bread production.

6) To evaluate and compare bacterial and fungal enzyme preparations in relation to thermal stabilities and their effect on the properties of loaf breads during storage.

7) To evaluate the addition of these enzymes in other baked products in terms of specific mechanisms of the particular enzymes and their interactions with the products during processing and storage.

Chapter 3

Materials and methods

3.1 Materials

3.1.1 Consumable materials

Maximus strong bakers flour having a protein of 12.1% w/w (expressed as N×5.7 and on a wet basis (wb)) and moisture 13.5% was obtained from Weston Milling, Melbourne, Victoria. Fermipan rapid dried yeast, lard, cooking salt, sugar, eggs (61g), butter and skim milk powder were obtained locally from a commercial outlet. An ascorbic acid based dough improver was obtained from Bunge and was used for all phases of this study. Five highly purified endoxylanase preparations were Megazyme International products obtained from Deltagen Australia. They were identified as M1 (from *Trichoderma viride*, 157 U/mg); M2 and M3 (from *T longibrachiatum*, 159 U/mg and 274U/mg respectively); M4 (from *A niger*, 220 U/mg); and M5 (from *Humicola insolens*, 200 U/mg)). α-L-Arabinofuranosidase (from *A niger*, 200U/mL) used in the composite pentosanases studies was a Megazyme product from Deltagen Australia. α-Amylases sourced from *A oryzae* and *B licheniformis* containing 67 U/mg and 1000U/mL and 54 U/mg and 3000 U/mL respectively were also Megazyme products obtained from Deltagen Australia. Megazyme substrates used to assay the flour, dough improver and enzyme preparations for the presence of xylanase, mannanase and amylase were Xylazyme AX test tablets, beta-Mannazyme test tablets and Amylazyme test tablets from Deltagen Australia. *p*-Nitrophenyl-arabinofuranoside used to measure arabinofuranosidase activity was from Sigma. DSC pans (50μL) and lids (50μL) were obtained from Perkin Elmer.

3.1.2 Equipment

Panasonic Bread Bakers

Varian HPLC equipped with RI detector for sugar analysis

Varian HPLC equipped with UV detector for pentosan analysis

Instron Universal Testing Machine

Differential Scanning Calorimeter (Perkin Elmer DSC7)

Tecator Kjeltec System (Model 1015)

Pharmacia Biotech LKB Novaspec II spectrophotometer

Kenwood Mixer

Water bath

Biohit Proline Automatic Pipette 5-50μL and 1000μL

C-18 reverse phase organic acid column

Guard column

C18-ODS reverse phase column

3.2 Methods

3.2.1 Proximate analyses

All procedures were performed in triplicate and the mean results presented here. Proximate analyses were carried out according to AACC procedures for moisture (method 44-40) and crude protein (method 46-12). Loaf volume was assessed using the official testing method described by the Royal Australian Chemical Institute, Cereal Chemistry Division (RACI) (RACI 1995 method 07-01).

The moisture content was determined by the vacuum oven method AACC 44-40. A sample (2g) of each loaf of bread was weighed into moisture dishes that had been dried and cooled to room temperature previously in a desiccator. The dishes were partially covered and dried in a vacuum oven at 70°C under vacuum to constant weight (approximately 12hrs). Lids were secured and dishes cooled to room temperature in a desiccator prior to weighing. Each control and experimental loaf was measured in duplicate.

For protein content a Tecator Kjeltec System consisting of the digester (Model 1015 for 20 tubes) and the distillation unit (Model 1002) including an alkali tank were used. Samples (2 g) were weighed onto Whatman filter paper (No 1) and the folded sample digested with concentrated sulphuric acid to convert the organic nitrogen to ammonium ions. Under distillation the liberated ammonia was released by the addition of 4 M NaOH solution and trapped into 4% boric acid. The excess boric acid solution was titrated against 0.1 M standard HCl solution. The protein content was calculated as N×5.7.

3.2.2 Enzyme assays

Enzyme preparations were diluted and assayed to measure activity levels of both the principle enzyme and any contaminating enzyme activity as listed by the manufacturer. All assays were carried out in triplicate and a series of blanks was also run in conjunction with each assay. Dilutions of each preparation are described. To assay the flour and dough improver a sample (1g) of each was dissolved in sodium acetate extraction buffer (25mM, pH 4.6). The samples were extracted for 30min at room temperature stirring constantly. The resulting mixture was filtered using Whatman No 1 filter paper and an aliquot (0.5mL) of each diluted in sodium acetate buffer (25mM pH 4.5) to give a final dilution of 1:10. The samples were assayed for the presence of endoxylanase, arabinofuranosidase and α-amylase as described below.

3.2.3 Assay of endoxylanase activity

Substrate

This assay utilized Xylazyme AX Test Tablets, a Megazyme product obtained from Deltagen Australia. The tablets contain dyed cross-linked wheat arabinoxylan as the active ingredient that made the tablets a more suitable alternative for the measurement than wheat flour arabinoxylan.

Dilution of enzyme solutions

Using an automatic pipette (1000μL) 0.5mL of enzyme was added to 4.5mL of sodium acetate dilution buffer (25mM, pH 4.7) for *A niger,* or sodium phosphate buffer (25mM, pH 6.0) for *H insolens* and *T longibrachiatum spp.* The mixtures were vortexed thoroughly. A series of further dilutions were carried out by dispensing an aliquot (0.5mL) of each dilution to a further 4.5mL of dilution buffer to a final dilution of 1:10 000.

Assay procedure

Aliquots (0.5mL) of suitably diluted enzyme preparation in sodium acetate buffer (25mM, pH 4.7) or sodium phosphate buffer (25mM, pH 6.0) were pre-equilibrated at 40°C for 5min in glass test tubes. The reaction was initiated by the addition of a Xylanase AX test tablet without stirring. The reaction was terminated after 10min by the addition of 10.0mL of Trizma base solution (2%w/v) or 10.0mL or tri-sodium phosphate solution (2%w/v) with vigorous stirring on a vortex mixer. The tubes were

left to equilibrate to room temperature and the slurry stirred again. The slurry was filtered using Whatman No. 1 (9cm) filter paper. Absorbance of the filtrate was measured using a Pharmacia Biotech LKB Novaspec II spectrophotometer at wavelength 590nm. Blanks were also prepared by adding the appropriate stopping reagent (Trizma base or tri-sodium phosphate) to the enzyme solution before the addition of the Xylazyme AX tablet and leaving the slurry at room temperature. Xylanase activity was calculated by reference to the appropriate standard curve supplied by the manufacturer (Appendix 1) to convert absorbance (590nm) to milli-Somogyi Units of activity per assay and then calculated as follows

Units/mL of original preparation

= milli-Units (per assay i.e. per 0.5mL) × 1/1000 × 2 × Dilution

where:

1/1000 = conversion from milli-Units to Units

2 = conversion of volume (0.5mL) to 1.0mL of extract

Dilution = final dilution factor of the initial solution.

The flour and dough improvers were also assayed for activity levels as described with the following changes. Samples (1g) of each were dissolved in 100mL of water and extracted for 30 min stirring constantly. Aliquots (0.5mL) of each were then diluted and assayed as previously described. Enzyme levels were determined using the following equation.

Units/gram of original preparation

= milli-Units (per assay i.e. per 0.5mL) × 1/1000 × 2 × 100 × Dilution

where:

1/1000 = conversion from milli-Units to Units

2 = conversion of volume (0.5mL) to 1.0mL of extract

100 = initial extraction volume (i.e. 100mL per g of solid)

Dilution = final dilution factor of the initial solution

3.2.4 Mannanase assay

To measure the levels of contaminating mannanase in the endoxylanase preparations a solid substrate beta-Mannazyme (a Megazyme product obtained from Deltagen Australia) was used. This substrate contains Azurine cross-linked-galactomannan and is used to assay for the presence of endo- 1,4-β-D-mannanase.

Dilution of enzyme

Aliquots (0.5mL) of enzyme were added to 4.5mL of sodium acetate dilution buffer (25mM, pH 4.7) for *A niger*, or sodium phosphate buffer (25mM, pH 6.0) for *H insolens* and *T longibrachiatum sp.* The mixtures were vortexed thoroughly. A series of further dilutions were carried out by dispensing an aliquot (0.5mL) of each dilution to a further 4.5mL of dilution buffer to a final dilution of 1:1000.

Assay procedure

Aliquots of suitably diluted enzyme solution were equilibrated in a water bath for 10min at 40°C. The reaction was initiated by the addition of a beta-Mannazyme tablet without stirring. The reaction was continued for a period of 10min after which it was terminated by the addition of 10mL of Trizma base (2% w/v) or tri-sodium phosphate solution (2% w/v). Tubes were equilibrated to room temperature and filtered using Whatman No 1 filter paper. Absorbance of the resulting supernatant was measured at 590nm.

Units/mL of original preparation:

= milli-Units (per assay i.e. per 0.5mL) × 1/1000 × 2 × Dilution

where:

1/1000 = conversion from milli-Units to Units

2 = conversion of volume (0.5mL) to 1.0mL of extract

Dilution = final dilution factor of the initial solution.

The flour and dough improvers were also assayed for activity levels as described with the following changes. Samples (1g) of each were dissolved in 100mL of water and extracted for 30 min stirring constantly. Aliquots (0.5mL) of each were then diluted and

assayed as previously described. Enzyme levels were determined using the following equation.

Units/gram of original preparation

= milli-Units (per assay i.e. per 0.5mL) × 1/1000 × 2 × 100 × Dilution

where:

1/1000 = conversion from milli-Units to Units

2 = conversion of volume (0.5mL) to 1.0mL of extract

100 = initial extraction volume (i.e. 100mL per g of solid)

Dilution = final dilution factor of the entail solution.

Flour and dough improvers assayed for the presence of endoxylanase used the equation above with the following changes

3.2.5 Arabinofuranosidase assay

For this assay the substrate *p*-nitrophenyl-arabinofuranoside (obtained from Sigma) was used to measure the level of arabinofuranosidase in the enzyme preparation (Corder and Henry 1989). The substrate was prepared by dissolving in extraction buffer at a rate of 2.5g/mL. In addition to measuring enzyme activity in the arabinofuranosidase preparation dilutions of the five endoxylanases listed above were carried out to measure the level of arabinofuranosidase listed as a contaminant in these preparations.

Dilution of enzyme solution

Dilutions of arabinofuranosidase were carried out using sodium acetate buffer (50mM, pH 5.0). Each endoxylanase was diluted as previously described using sodium acetate dilution buffer (50mM, pH 4.5). The dilution procedure for all the enzyme preparations was as follows. An aliquot (0.5mL) of the enzyme preparation was diluted in 4.5mL of sodium acetate dilution buffer or sodium phosphate dilution buffer. The solution was mixed thoroughly by vortex and a series of further dilutions (0.5mL sample in 4.5mL dilution buffer) until a final dilution of 1:10 000 and 1:100 for arabinofuranosidase and endoxylanase preparations respectively.

Assay procedure

Arabinofuranosidase activity was assayed as described by Corder and Henry (1989). Each enzyme preparation was diluted in sodium acetate buffer (50nM, pH 5.0) as previously described. Aliquots (0.2mL) of suitably diluted enzyme were pre-equilibrated in water bath at 40°C for 10min. The substrate was also pre-equilibrated alongside the samples. The reaction was initiated by the addition aliquots of substrate (0.2mL) to the samples. The reaction was continued for 50min at 40°C. Reactions were terminated by the addition of Trizma base (1% w/v). Tubes were equilibrated to room temperature and the absorbance of the solutions read at 400nm.

3.2.6 α-Amylase assay

In this section α-amylase activity from three sources were measured. Endogenous α-amylase activity in the flour and dough improver were determined in addition to the activity levels present in the α-amylases from *A oryzae* and *B licheniformis*. Three different extraction/dilution buffers were required. The flour and dough improver were measured for the presence of cereal amylase using a sodium maleate buffer (0.1M, pH 6.0); *A oryzae* used a sodium acetate buffer (0.1M, pH 4.0) for fungal amylase activity; and *B licheniformis* required a MOPS buffer (0.1M, pH 7.0).

Extraction and dilution of enzyme preparations

The flour and dough improver were extracted and diluted as described previously using sodium acetate buffer extraction and dilution buffer. Dilution of the enzyme preparations was carried out as previously described (i.e. 0.5mL aliquots of sample were diluted in 4.5mL of appropriate dilution buffer to a final dilution of 1:10 000 and 1:100 000 for the fungal and bacterial preparations respectively).

Assay procedure

Aliquots (1.0mL) of suitably diluted enzyme solution were equilibrated in a water bath at 40°C for 10min. The reaction was initiated by the addition of an Amylazyme tablet to each tube without stirring. After 10min the reaction was terminated by the addition of 10mL Trizma base (2% w/v). Tubes were equilibrated to room temperature and filtered using Whatman No1 filter paper. The absorbance of the remaining supernatant was read at 590nm. Blanks were carried out for each set of determinations by adding

the appropriate stopping reagent to the tubes prior to the addition of the enzyme solutions.

α-Amylase activity was then determined by reference to the appropriate standard curve (Appendix 2) to convert absorbance (590nm) to milli-Ceralpha Units of activity per assay (i.e. per 1.0mL) and then calculated as follows:

Units/gram or mL

=milli-Units per assay × 1/1000 × 100 × Dilution

where:

1/1000 = conversion from milli-Units to Units

100 = initial extraction volume (for flour and dough improver)

Dilution = final dilution factor of the initial solution

3.2.7 Baking Procedures

A series of baking trials was carried out in order to determine an appropriate level of addition for each enzyme preparation. Laboratory scale trials utilised a set of 4 Panasonic Bread Bakery units – Model SD-BT10P. The bread formulation was based on a commercial baking formulation consisting of 100 parts flour, 1.5 parts rapid instant dried bakers yeast, 2 parts sugar, 1.6 parts salt, 1 part dough improver, 2 part shortening and 65 parts water. Validation of the units was carried out as part of an earlier study. Three loaves of bread were prepared with each machine. Loaf volume, compression and colour parameters were recorded and analysed to determine the reproducibility of each unit as well as comparability between the units. In an earlier study (Higgins and Small 1996) the bread units were validated to determine reproducibility. Five loaves of bread were prepared for each unit. Each loaf was assessed for subjective properties as listed below. They were also assessed objectively for crumb and crust colour and crumb compressibility. Statistical analysis of this data showed no significant differences between the units to produce comparable loaves of bread.

The Bread machines were used on a 3-hour rapid bake cycle. This consisted of a 15 min kneading stage, 90 min fermentation, 10 min knockback and knead, 20 min second

proof and 45 min baking. In all trials breads were prepared in triplicate for each treatment, each trial was run twice and means values for each parameter are reported throughout.

In preliminary trials each endoxylanase, arabinofuranosidase and α-amylase was added to bread formulations supplemented at rates of 5, 10 and 50μL per dough. Each loaf was assessed for colour and textural characteristics to determine suitable treatment levels for subsequent work. In the following sections breads were treated with 5μL of the xylanase and arabinosidase preparations and 10μL of the α-amylase preparations based on suitable treatment levels established in the preliminary trials.

3.2.8 Objective assessment of crumb and crust colour

Colour analyses were carried out using the Minolta Chroma Meter model CR 300 (Minolta Camera Co Ltd, Japan). This was set to read in the Hunterlab colour space (L*, a* and b*) (Hutchings 1999) and used following the RACI standard procedure (RACI 1995, method 09-02). Fifteen readings for each were taken for each colour parameter (i.e. crust and crumb colour) per loaf of bread and the averages reported. Any apparent stickiness of the crumb was also noted.

3.2.9 Instron analysis of initial crumb firmness and increasing firmness in aging bread

Crumb firmness was measured using AACC method 74-09. An Instron Universal Testing Machine (UTM) fitted with a 5kN load cell fitted with a 36mm diameter indenter with blunted edges to remove sharpness was used. Breads were sliced to 25mm thickness using an electric knife and a knife guide to ensure uniformity of thickness. The upper crosshead was positioned so that the plunger was located 1mm above the sample prior to compressing. The lower cross head limit was set at 40% or 10mm compression depth. The crosshead speed was 100mm/min. Samples were centered under the plunger taking care to avoid any areas of irregular crumb formation that were not representative of the bread crumb. In particular care was taken to avoid compressions taken too close to the crust margin which gave a higher reading than those taken closer to the centre of the loaves. Compression values were measured at 25% compression with the curves extending beyond 25% to 40%. A total of 6 compressions were taken for each loaf of bread and the mean reported.

3.2.10 Estimation of loaf volume

Prior to slicing, the loaf volumes were determined for the treated and untreated breads. Volumes were determined using the RACI standard method 07-01. This method involved measuring the circumferences of the loaves lengthways and breadthways, adding the two measurements together and ascertaining from the graph in Appendix 3, the volume value corresponding to this total. A maximum of 30 points is allotted for volume with a score of less than 19 considered as commercially unacceptable.

3.2.11 Determination of total and water-soluble pentosan content

To measure total water-soluble pentosan content of the flour samples were subjected to two different extraction methods (Wootton et al., 1995). In the first method duplicate flour samples (1g) were extracted in 80%v/v ethanol while incubating at 40°C for 1 hour. The solutions were centrifuged and the supernatant, termed water-soluble arabinoxylan (WSA) retained for hydrolysis. In the second method duplicate samples (1g) were extracted with 80%v/v ethanol and incubating 85°C for 1hr. After centrifuging absolute ethanol was added to the supernatant to precipitate the pentosans. The pellet obtained after centrifuging was dissolved in distilled water and set aside for the hydrolysis. This sample was called hot water soluble arabinoxylans (HWSA). A further flour sample (5g) was hydrolysed for total pentosan (TP) content without the initial extraction procedure.

3.2.12 Hydrolysis of pentosans by distillation

The extract and a flour sample were analysed by a distillation procedure (Wootton et al 1995). All samples were placed in round-bottomed flasks and 100mL of dilute HCl (12% by mass) added to each flask. Anti bumping granules were added and the temperature of the heating mantle adjusted to distil approximately 30mL every 20min. For each 30mL of distillate collected a further 30mL of acid was added to the flask through a dropping funnel. Distillation was continued until 360mL of distillate had been collected. Each distillate was transferred to a 500mL volumetric flask and diluted to 500mL with deionized. Using 100mL volumetric flasks a 1:10 dilution of each sample was prepared and 1mL of each was filtered into HPLC vials using 0.45 micron filter. Solutions were stored at 4°C until ready for analysis.

Preparation of furfural standards

Furfural (density = 1.16g/mL) was obtained from Sigma and stored at 4°C. Standards were prepared by weighing 50mg of standard Furfural into a 100mL volumetric flask and diluting with de-ionized water to give a 500ppm standard solution. An aliquot (10mL) of the stock solution was diluted to 100mL to give a 50ppm standard. This solution was then used to prepare a range of standards for 0.5, 1, 2.5, 5, 7.5, 10, 15, 25 and 50ppm.

Another set of standards was prepared using 5-hydroxymethyl-2-furfuraldehye (HMF) also from Sigma. A stock solution (500ppm) was prepared by diluting 250mg HMF in 500mL deionised water. An aliquot (10mL) of the stock solution was diluted to 100mL in a volumetric flask go give a 50ppm standard solution. Further standards were again prepared for the range 0.5-50ppm.

3.2.13 HPLC analysis of total and water-soluble pentosan content of flour samples

Samples were analysed using a Varian HPLC equipped with a UV detector wavelength set at 284nm following the procedure of Wootton et al (1995). A reverse phase column type C-18 ODS (250×4.6mm) was used in conjunction with an isocratic methanol/water (10:90) mobile phase. A flow-rate of 1.5mL/min. and a run time of 10min produced clear well separated peaks for the flour samples and standards. All samples and standards were run in triplicate and the data analysed using Microsoft Excel software.

3.2.14 HPLC analysis of xylose and arabinose profile of breads

Extraction of WSA was carried out using the protocol of Cleemput et al. (1995). Initially 3 loaves of bread were prepared for each enzyme preparation. The weights were recorded and the loaves combined for the extraction process. To extract the WSA the loaves were combined with water and incubated for 30min/30°C with occasional stirring. The slurry was centrifuged at 6000×*g* for 15min at 4°C. The supernatant obtained was heated to 50°C. An aliquot (1.0mL) of α-amylase from *A oryzae* was added and the solution incubated for 2 hrs to hydrolyse residual starch. The solution was cooled to room temperature and centrifuged at 6000×*g* for 15min at 4°C. Arabinoxylan was precipitated by the stepwise addition of absolute ethanol to a final volume of 65%v/v. The solution was held overnight at 4°C to precipitate the WSA.

The precipitate was recovered by centrifuging and redissolved in 500mL ddH_2O. Absolute ethanol was added stepwise to a final volume of 60%v/v and the solution held overnight to precipitate WSA. The final precipitate was washed twice with 100mL ethanol 90%v/v and once with 100mL acetone over period of 120min stirring constantly and filtering between washes with Whatman No1. filter paper. The final precipitate was termed total water –soluble arabinoxylan (TWSA). Samples of each TWSA fraction were hydrolysed using 0.1M sulphuric acid, cooled and filtered prior to analysis. Samples were analysed using a Varian HPLC equipped with a Varian RI detector. A C-18 reversed-phase organic acid column and guard column were used. Good separations of the reference sugars were achieved when pure standards were used and also in extracts of flour and breads.

3.2.15 Fractionation of TWSA

Extracted TWSA was fractionated using absolute ethanol to give final fractions soluble at 0-30%, 30-50% and 50-65% ethanol. Fractions were designated as Flour 0-30%, Con 0-30%, M1 0-30% and so on depending on the preparation. A fraction of the remaining supernatant was also taken for analysis and termed fraction '65+'. A complete list of sample designations is presented in Table 3.1.

Table 3.1 Designations of samples based on treatments used and solubility of fractions

Fraction Treatment	Soluble in 0-30% ethanol	Soluble in 30-50% ethanol	Soluble in 50-65% ethanol	Remaining supernatant
Flour	Flour 0-30	Flour 30-50	Flour 50-65	Flour 65+
Control	Con 0-30	Con 30-50	Con 50-65	Con 65+
M1	M1 0-30	M1 30-50	M1 50-65	M1 65+
M2	M2 0-30	M2 30-50	M2 50-65	M2 65+
M3	M3 0-30	M3 30-50	M3 50-65	M3 65+
M4	M4 0-30	M4 30-50	M4 50-65	M4 65+
M5	M5 0-30	M5 30-50	M5 50-65	M5 65+

***see Table 4.1 for details of treatments used**

3.2.16 Hydrolysis of TWSA fractions

Hydrolysis of TWSA fractions was achieved using the method of Michniewicz et al (1990). Samples (approximately 50mg of solid and 0.1mL supernatant) of each TWSA fraction were hydrolysed in 5mL, 0.1M sulphuric acid in a boiling water bath for 2hrs. The samples were cooled to room temperature and centrifuged. The supernatants were further filtered through 0.45 micron filters into HPLC vials prior to injecting.

3.2.17 Preparation of standard curves for HPLC analysis of WSA

Standard curves were prepared for xylose, xylobiose, xylotriose and arabinose. To make up standard curves a 1% stock solution of each pentose sugar was prepared by dissolving 0.25g of the appropriate carbohydrate in 25mL 0.1M H_2SO_4. Aliquots (10mL) of each stock solution were placed in the water bath alongside the samples for the same time duration. After allowing the standards to come to room temperature a series of standards covering the range 0.02-1.0% was prepared by diluting the appropriate volumes of stock solution with 0.1M H_2SO_4

3.2.18 HPLC analysis of WSA and standards

Samples were analysed using a Varian HPLC equipped with a Varian Refractive Index detector (273nm). An Aminex HPX 87H ion exclusion column and guard column were used. The column was operated at 65°C with a flow rate of 0.6mL/min using 5mM H_2SO_4 as the mobile phase and a run time of 30 min.

3.2.19 Extraction of mono-, and disaccharides for HPLC analysis

To investigate the effects of added α-amylase at a molecular level treated and untreated breads were extracted using the method of Potus et al. (1994) with the following changes. Duplicates of each bread (5g) were extracted using 80%ethanol (v/v) under reflux. The extracts were dried under vacuum to a final volume of 2.5mL and the remaining extract made up to 10mL with milli-Q water. Samples were filtered using 0.45 micron filters and stored at –18°C.

3.2.20 Preparation of glucose, sucrose, maltose, fructose and maltotriose for HPLC analysis

Stock solutions (1%) were prepared by accurately weighing 1g of each carbohydrate into a 100mL volumetric flasks and making up with milli-Q water. The stock solutions were used to prepare standards covering the range 0.02-1.0%.

3.2.21 HPLC analysis of mono-, di-; and trisaccharide contents of flour and bread samples

Analysis was carried out using a C-18 reverse phase organic acid column heated to 60°C with 5mm H_2SO_4 as the mobile phase. Standard solutions of glucose, fructose, sucrose, maltose and maltotriose were used to prepare standard curves in the range of 0.02.-1.0%. Calibration curves were prepared using Microsoft Excel software. The concentration of each sugar was calculated and finally used to determine the content of each sugar present in bread, expressed in units of g/100g.

3.2.22 DSC analysis

DSC analysis was carried out using a Perkin Elmer DSC-7 equipped with a Thermal analysis controller TAC 7/DX. Samples were prepared by weighing approximately 50mg of each bread sample into 50µL DSC pans followed by sealing of the pans. Water was used as the reference. Initially pans were heated across a range of 25-105°C to establish a thermogram for untreated bread. Further samples were then heated across the range of 25°C to 95°C and the staling endotherms recorded using the Perkin Elmer DSC software package.

3.2.23 Sensory evaluation

In this section of work a sensory evaluation was carried out to determine if differences detected in crumb softness using the Instron UTM could also be detected by a group of panelists. The evaluation used only those breads treated with the 5 xylanase preparations. An untrained panel of 24 was selected to simulate typical consumers who might purchase bread from a supermarket. Panelists were representative of a wide cross section of society and coming from many and varied cultural backgrounds. A brief survey was conducted to ascertain panelist preferences for different types of bread and bread sources.

Panelists were presented with six bread samples containing a control sample and one sample each representing each of the treatments used. Panelists were asked firstly to compress the samples gently and record their findings based on the perceived crumb softness on a scale of 1-5 with 1 representing the firmest crumb. In the second stage panelists were asked to bite into each sample and again record the perceived softness of each sample. In addition to rating the samples according to softness panelists were also asked to comment on any other aspects of the samples they felt were relevant. To avoid influencing the panel the breads were given identification numbers and set up in random order.

3.2.24 The application of fungal β-xylanases in other baked goods

Yeast leavened doughnuts were selected for this section of the study because they utilize a similar formulation to that of the breads and also have a fermentation period. The formulation consisted of a combination of bakers flour (35%) and cake flour (65%), sugar, yeast, salt, low fat milk powder and egg. To make up the ingredients were added to a mixing bowl and kneaded on low speed for 5 min. The dough was covered and left to ferment at room temperature for 45 min. After knocking back the dough was sheeted to 1cm thick and cut into rounds. The rounds were allowed to proof for a further 15 min before frying at 285°C in a commercial cooking fat. Care was taken to maintain a constant temperature for the frying fat and that each doughnut was cooked for a constant time frame of 3 min per side for each doughnut.

3.2.25 Statistical analysis of experimental data

All statistical analyses unless otherwise stated were carried out using Sigmastat software package version 2.03. One-way ANOVA was used to determine statistical significance. Pairwise Multiple Comparison Procedures used the Tukey Test.

Chapter 4

Results and discussion – the impact of enzyme preparations on product characteristics

4.1 The effects of different treatment levels of purified pentosanases on baked breads

The staling of bread is a major practical challenge facing the baking industry. Despite various hypotheses developed over the years the chemical basis of staling remains to be fully elucidated. New microbial enzyme preparations have become available in the baking industry in recent years some of which appear to aid in producing soft crumb texture and enhanced keeping quality of loaf breads. These commercial preparations contain a range of enzyme activities including various carbohydrases and proteases. The action of these carbohydrases at a molecular level is still unclear. In this section of the study a range of highly purified endoxylanase and arabinofuranosidase preparations were added at varying levels to a series of baked loaves. Changes in texture and colour parameters were measured. These data were then used to determine an appropriate level of addition for each preparation to allow breads to be treated in subsequent phases of this study.

4.2 Measurement of the level of enzyme activity in the enzyme preparations

A series of five endoxylanase preparations from fungal sources and two α-amylase preparations, one fungal and one bacterial, was used in this section of the study. Table 4.1 lists the enzymes used, their sources, pH and temperature optima and specific activity on the appropriate substrate.

Initially each preparation was measured for levels of enzyme activity as well as the presence of any contaminating enzymes. Flour and ascorbic acid dough improver were also measured for the presence of endoxylanase and α-amylase activities. Using protocols and enzyme preparations obtained from Megazyme International, suitably diluted enzymes were pre-equilibrated in a water bath at 40°C. Substrates were added and the reactions continued for 10min. The reactions were terminated by the addition of an appropriate stopping reagent. The solutions were filtered and absorbances measured

Table 4.1 Details of enzymes used in this study

Enzyme	Designated abbreviation	Source	Specific activity U/mg	Substrate	pH optimum	Temperature optimum (°C)
β-D-xylanase	M1	*Trichoderma viride*	157	Wheat flour arabinoxylan	4.5	40
β-D-xylanase	M2	*Trichoderma longibrachiatum*	159	Wheat flour arabinoxylan	4.5	40
β-D-xylanase	M3	*Trichoderma longibrachiatum*	274	Wheat flour arabinoxylan	6.0	40
β-D-xylanase	M4	*Aspergillus niger*	220	Wheat flour arabinoxylan	4.5	40
β-D-xylanase	M5	*Humicola insolens*	200	Wheat flour arabinoxylan	6.0	40
α-amylase	α-A(F)	*Aspergillus oryzae*	66.9	Soluble starch	4.5	40
α-amylase	α-A(B)	*Bacillus licheniformis*	53.7	Soluble starch	7.2	40

Note: Information here is that supplied by the manufacturer and obtained from their website (Megazyme International 2002)

at wavelengths appropriate to the substrate being used for assay. Endoxylanase activities were determined by reference to a standard curve supplied by the manufacturer of the substrate (Appendix 1).

The results are presented in Table 4.2. These show that some of the preparations contain enzyme activity at levels lower than those described in the technical data supplied by the manufacturer. Both preparations M1 and M5 demonstrated ~25 percent loss of activity. Preparations M2 and M3 had a loss of activity of 10 and 5 percent respectively while the preparation M4 showed no measurable loss of enzyme activity.

As care was taken to ensure the enzyme preparations were stored correctly the loss of activity may have been due to incorrect handling during transport or natural decline in activity during storage. As neither the shelf life nor the production date were supplied it was not possible to determine the exact cause for the apparent decrease in activity.

Technical data supplied by the manufacturer indicated all of the endoxylanase preparations to have residual activities of α-L-arabinofuranosidase, β-D-xylopyranosidase and β-D-mannanase at levels of <0.02 percent. Using the appropriate substrate and assay procedures each of the contaminant activities was tested and the results are presented in Table 4.2. In each case these were not found to be present at measurable levels.

4.2.1 Investigation of the effects of endoxylanase at different treatment levels on baked breads

A series of baking trials was carried out in order to determine an appropriate level of addition for each enzyme preparation. Laboratory scale trials utilised a set of Panasonic Bread Bakery units – Model SD-BT10P. The bread formulation was based on a commercial baking formulation consisting as described in Section 3.2.7

For the experimental loaves five sets of breads were prepared in triplicate and each set treated with a different enzyme preparation. Breads were treated with individual enzymes added at the levels of 5, 10 and 50μL for each preparation. A series of loaves was also prepared in triplicate using only the basic bread formula. These breads were used as a comparison for the treated loaves.

Table 4.2 Levels of hemicellulase activities of selected ingredients and commercial enzyme preparations

	endo xylanase assayed (U/mL)	Specified value* (U/mL)	β-D-mannanase (U/mL)	α-L-arabino furanosidase	β-xylosidase (U/mL)
Flour	0.038	-	ND	ND	ND
Dough improver	0.062	-	ND	ND	ND
M1	730	1000	ND	ND	ND
M2	360	400	ND	ND	ND
M3	1900	2000	ND	ND	ND
M4	1000	1000	ND	ND	ND
M5	220	300	ND	ND	ND

One unit of enzyme activity is defined as the amount of enzyme required to produce one μmole of product per minute under defined assay conditions.

M1 – xylanase from *T viride*
M2 – xylanase from *T longibrachiatum*
M3 – xylanase from *T longibrachiatum*
M4 – xylanase from *A niger*
M5 – xylanase from *H insolens*
* provided by Megazyme
- no comparable literature values available
ND – not found at detectable levels

The freshly baked loaves were assessed objectively for crumb and crust colour using the Minolta Chroma Meter. Crumb firmness was assessed using the Instron Universal Testing Machine (UTM) and AACC method 74-09 as previously described. Baked loaves were also assessed visually for any observable changes in crumb and crust characteristics between the treated loaves and untreated loaves.

4.2.2 Visual observations

Whilst flavour and aroma are important factors in consumer acceptance of baked products the general appearance of a food also greatly influences the overall impression and acceptability of the food to the consumer. In order to evaluate the appearance of the control and treated breads it was necessary to evaluate a range of factors including overall loaf symmetry, loaf volume, crumb and crust colour and textural properties including crumb softness. Visual differences and similarities observed in the treated and untreated breads at the different treatment levels are presented in Figures 4.1 – 4.10.

4.2.2.1 Subjective assessment of crust colour and loaf symmetry

In the first stage of loaf assessment all control and treated loaves were assessed for crust and crumb characteristics. Loaves were assessed prior to slicing for overall appearance, loaf symmetry, crust bloom and degree of crust crispness. Measurements for calculation of loaf volume scores were also recorded prior to slicing. The interior surface of the bread crumb was assessed for colour, texture and structure. Any apparent stickiness of the crumb was also noted.

The control loaf prepared using only the basic bread formulation had loaf symmetry consistent with consumer perceptions. Crust colour was uniform without being too dark or too light and crust bloom, observed as the level of shine or glossiness of the crust was high. The degree of crust crispness was in keeping with the consumer perception for this feature. The experimental loaves were also inspected prior to slicing for overall loaf symmetry, uniformity of crust colour and degree of crust bloom. Of the treated loaves those to which 5μL of each preparation was added exhibited uniform loaf symmetry comparable to that of the control breads. Crust colour was even for each of the experimental breads at the lower treatment level and crust bloom was good. Breads baked using 10μL of each preparation were also found to be visually comparable to the control loaves. Crust colour and bloom were uniform and loaf symmetry was again

acceptable. There was no observable difference in crust bloom for breads treated with 5 and 10μL of each preparation compared with the control breads. However, when breads were treated with 50μL of each of the endoxylanase preparations the resulting loaves exhibited a perceivable loss of crust bloom. Loss of bloom was observed as a reduction in the degree of glossiness of the crust surface. The results of this section of the work are presented in Figures 4.1 – 4.5.

No observable difference was found between the crispness of the crust for all breads prepared with the different preparations when added at levels of 5 and 10μL. For breads treated with 50μL crusts deterioration in crispness for each of the preparations used was observed. This deterioration was perceived as the development of a softer crumb that exhibited leathery properties more consistent with bread that had been allowed to age. A thickening of the crumb crust margin in these breads was also evident at the highest level of enzyme addition for each of the preparations used.

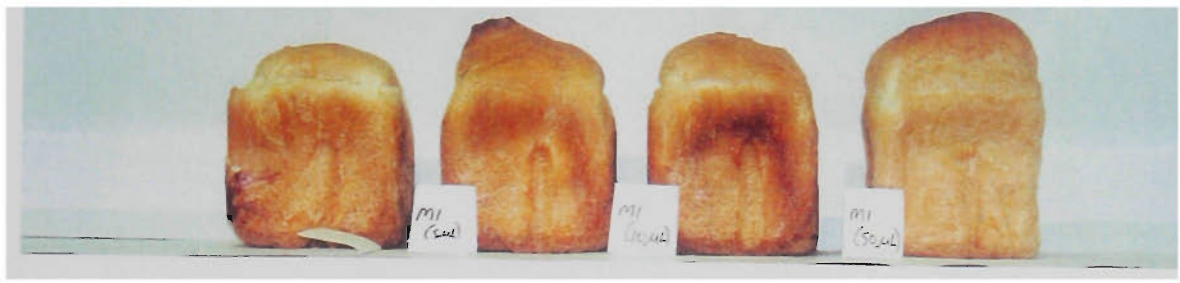


Figure 4.1 **Experimental breads (from left to right) treated with endoxylanase from preparation M1 (*T viride)* at 0, 5, 10 and 50μL treatment levels**

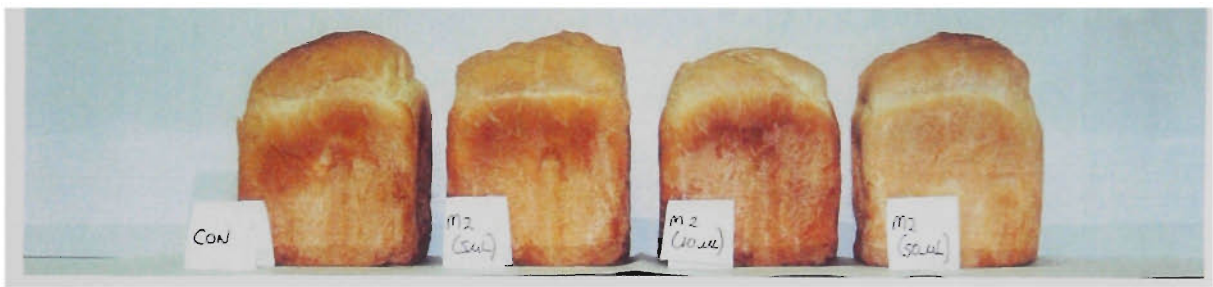


Figure 4.2 **Experimental breads (from left to right) treated with endoxylanase from preparation M2 (*T longibrachiatum)* at 0, 5, 10 and 50μL treatment levels**

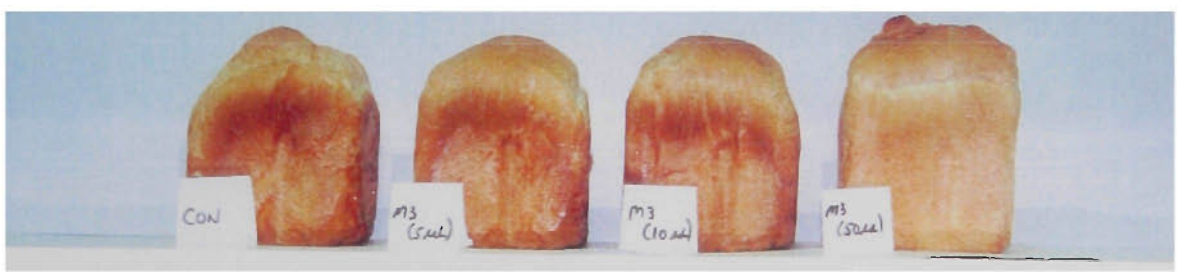


Figure 4.3 **Experimental breads (from left to right) treated with endoxylanase from preparation M3 (*T longibrachiatum)* at 0, 5, 10 and 50μL treatment levels**

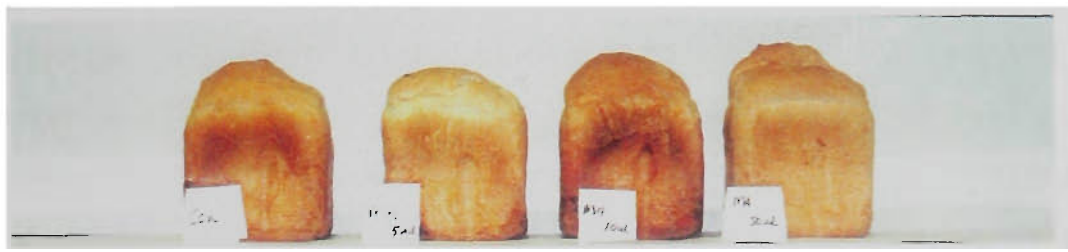


Figure 4.4 **Experimental breads (from left to right) treated with endoxylanase from preparation M4 (*A niger)* at 0, 5, 10 and 50μL treatment levels**

Figure 4.5 **Experimental breads (from left to right) treated with endoxylanase from preparation M5 (*H insolens)* at 0, 5, 10 and 50μL treatment levels**

4.2.2.2 Subjective assessment of crumb structure and colour

In the next stage of assessment the breads were carefully cut into slices each 2.5cm in thickness. Examination of the individual slices revealed the bread crumb of the control loaves to be of uniform colour throughout the loaf. Crumb structure of the three control loaves showed the air cells to be of consistent size and evenly distributed throughout the bread crumb of each slice. There was no evidence of crumb stickiness or gumminess in these breads. The treated loaves were also sliced into 2.5cm slices. Inspection of the cut surfaces showed variation in crumb structures for the different preparations used. Differences were observed as variation in size and distribution of the air cells and lack of uniformity of crumb colour. The visual impact of endoxylanase treatments on the structure and colour of bread crumb are presented in Figures 4.6 – 4.10.

The first series of breads were prepared with the preparation designated M1 sourced from *T viride* (Figure 4.6). Breads prepared with this preparation at a level of 5 and 10μL had larger, evenly distributed air cells. This structure was consistent throughout the loaves. Crumb colour of these loaves was also uniform at the lower levels of enzyme addition. When 50μL of the enzyme was added there was an observable loss of crumb structure. Air cells became larger and less regularly distributed throughout the loaf. The formation of large vacuoles at the crumb-crust margin was apparent at the highest treatment level. Crumb colour was also affected at higher enzyme additions. Loss of uniformity was observed as the development of areas of discolouration in the crumb surface. A closer inspection showed these areas to be slightly gummy.

Breads prepared with the enzyme preparation designated M2 (from *T longibrachiatum)* are shown in Figure 4.7. These loaves exhibited bread crumb that were uniform in both structure and colour at the 5μL level of addition. The size of the air cells was smaller than that observed for preparation M1 but distribution was regular throughout the loaves. Crumb structure was still regular for loaves prepared with 10μL but a slight loss of uniformity of colour was apparent at this level of treatment. This deterioration in colour was observed as the development of discolored areas similar to those observed at the highest treatment level with preparation M1, again appearing at irregular intervals throughout the crumb. When enzyme was added at a level of 50μL this grainy appearance was even more apparent. The crumb structure had also deteriorated with the

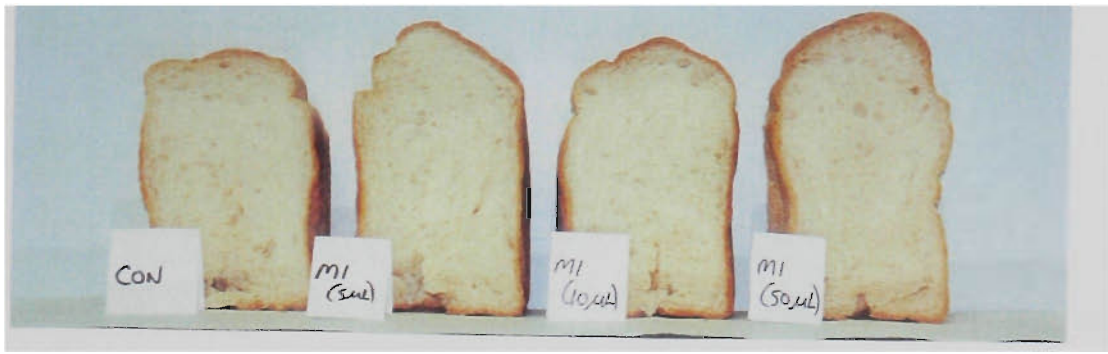


Figure 4.6 **Control and experimental breads (from left to right) treated with endoxylanase from preparation M1 (*T viride)* at 5, 10 and 50µL treatment levels**

Figure 4.7 **Control and experimental breads (from left to right) treated with endoxylanase from preparation M2 (*T longibrachiatum)* at 5, 10 and 50µL treatment levels**

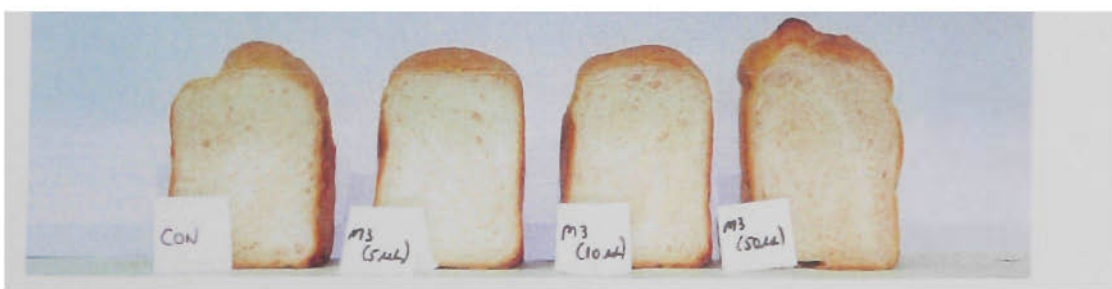


Figure 4.8 **Control and experimental breads (from left to right) treated with endoxylanase from preparation M3 (*T longibrachiatum)* at 5, 10 and 50µL treatment levels**

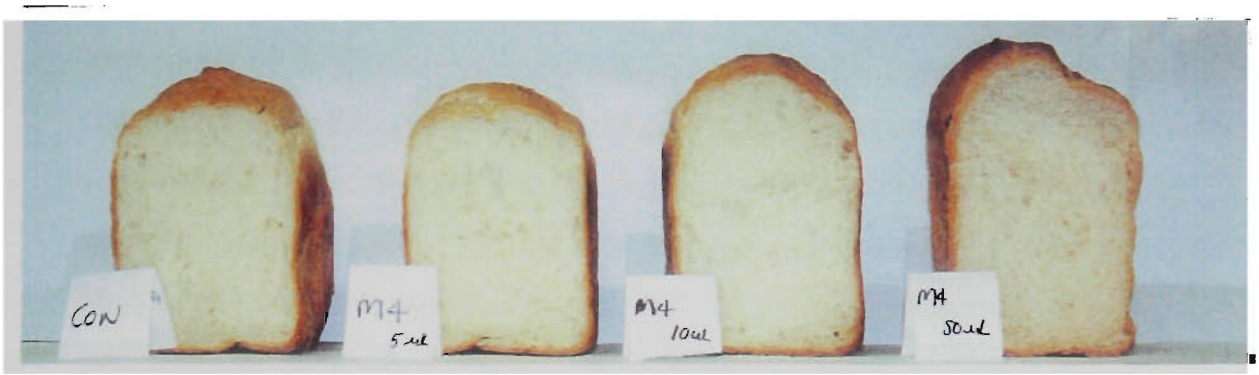


Figure 4.9 **Control and experimental breads (from left to right) treated with endoxylanase from preparation M4 (*A niger)* at 5, 10 and 50μL treatment levels**

Figure 4.10 **Control and experimental breads (from left to right) treated with endoxylanase from preparation M5 (*H insolens)* at 5, 10 and 50μL treatment levels**

development of irregular air cells throughout the loaf. Breads treated at this level also exhibited noticeable stickiness.

The next series of breads were treated with the enzyme preparation designated M3 also sourced from *T longibrachiatum*. As with the previous two treatments the breads containing 5μL of the preparation had crumb structures that were uniform in both colour and structure. There was no noticeable difference observed for these characteristics when enzyme was added at 10μL. Crumb properties again showed deterioration at the 50μL level of addition with the development of a grainy appearance and loss of uniformity of the crumb structure. There was also a noticeable degree of stickiness in these breads.

When the breads were treated with 5μL of the preparation designated M4 the crumb exhibited areas of discolouration even at the lower level of addition (Figure 4.9). The crumb structure was denser than that of the control loaf with smaller air cells, and having a more uniform distribution. The crumb was also gummy to touch at this level of addition. The degree of deterioration increased as the level of treatment increased with the breads treated with 50μL being totally unacceptable in terms of colour, texture and degree of stickiness. Van Oort et al 1995, suggest that excessive breakdown of arabinoxylans results in a decreased water binding capacity of these molecules leading to doughs that are slack and bread crumb that is sticky. The enzyme preparation used in this section of the study was sourced from *A niger*. The results of the baking trials using *A niger* indicate that a higher level of arabinoxylan degradation is occurring than when other treatments were used.

The final set of loaves was treated with the enzyme preparation M5 sourced from *H insolens*. The results are shown in Figure 4.10. When treated with 5 and 10μL the bread crumb characteristics were uniform for both structure and colour. There was no apparent crumb stickiness at the lower levels of addition. When endoxylanase was added at 50μL the crumb developed the same grainy appearance observed in the other experimental loaves. Crumb stickiness was also apparent at the higher treatment level.

4.2.3 Objective assessment of crumb and crust colour using the Minolta Chroma Meter

The colour of baked bread serves as a primary indicator of quality and degree of baking. A consumer's psychological perception of how a given type of bread should appear in terms of its colour attributes plays a major role in the purchase of a given loaf of bread.

In order to determine if endoxylanases affected crumb and crust colour in the treated loaves a Minolta Chroma Meter was used. This is a tristimulus instrument that utilises a series of filters to simulate the standard functions of the eye giving values for colour in terms of X,Y,Z, or L, a, b. The Hunter L, a, b colour system was selected as the opponent-colour dimensions correspond most closely to the actual visual signals transmitted to the brain from the eye (Hutchings 1999). The Hunter colour components are described in Figure 4.11. The system measures the L* value for black to white along a vertical axis, with 100 being white and 0 being black. The 'b' value for blue to yellow (from front to back) and the 'a'value for red to green (from right to left).

In the context of the current investigation, the measurements of importance were the L* and 'b' values. By taking the average of 15 readings for each colour parameter per loaf of bread it was possible to measure variations in the degree of darkness observed in the crust and the level of whiteness (L* value) of the crumb for each of the breads. The results of this work are presented in Figures 4.12 and 4.13.

4.2.4 Crust appearance

When endoxylanase from preparations M1 and M2 were added to the basic bread formulations the resulting breads had crusts that were significantly darker than the control loaves for all the treatment levels applied ($p<0.05$). At 50μL, these treatments also produced significantly darker crusts than the M3, M4 and M5 treatments ($p<0.05$). Treatments M3, M4 and M5 produced significantly darker crusts than the untreated breads at the 10μL treatment level ($p<0.05$).

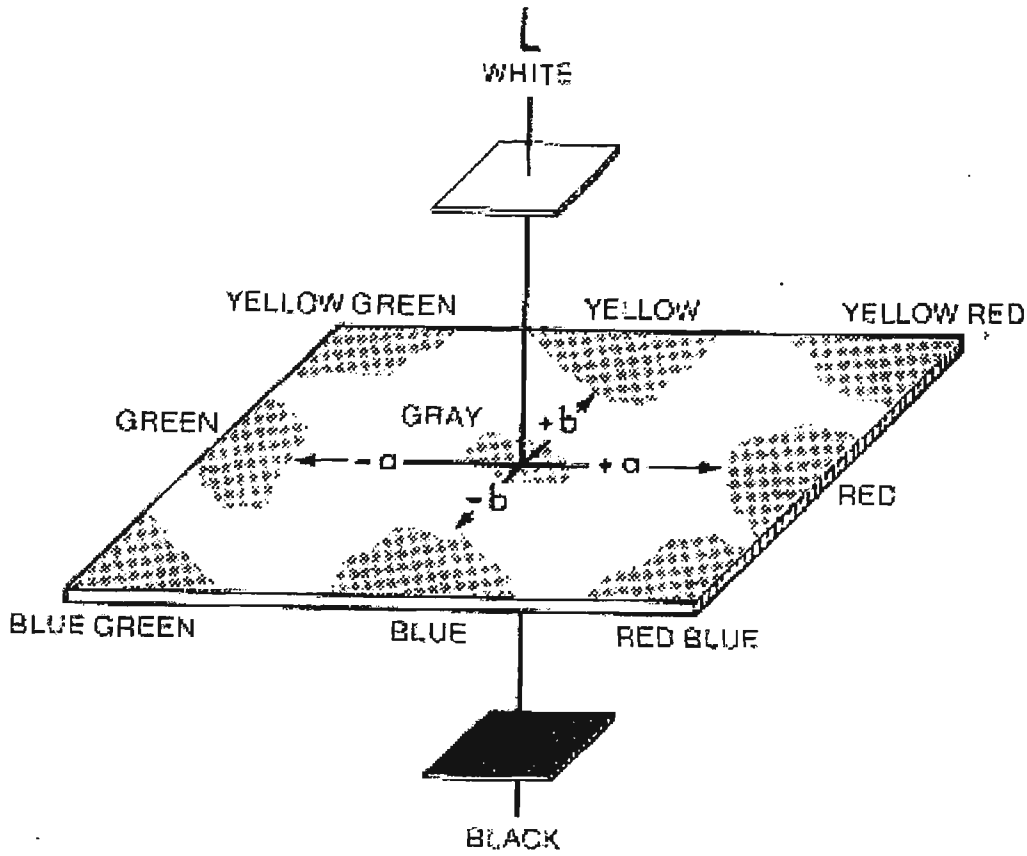
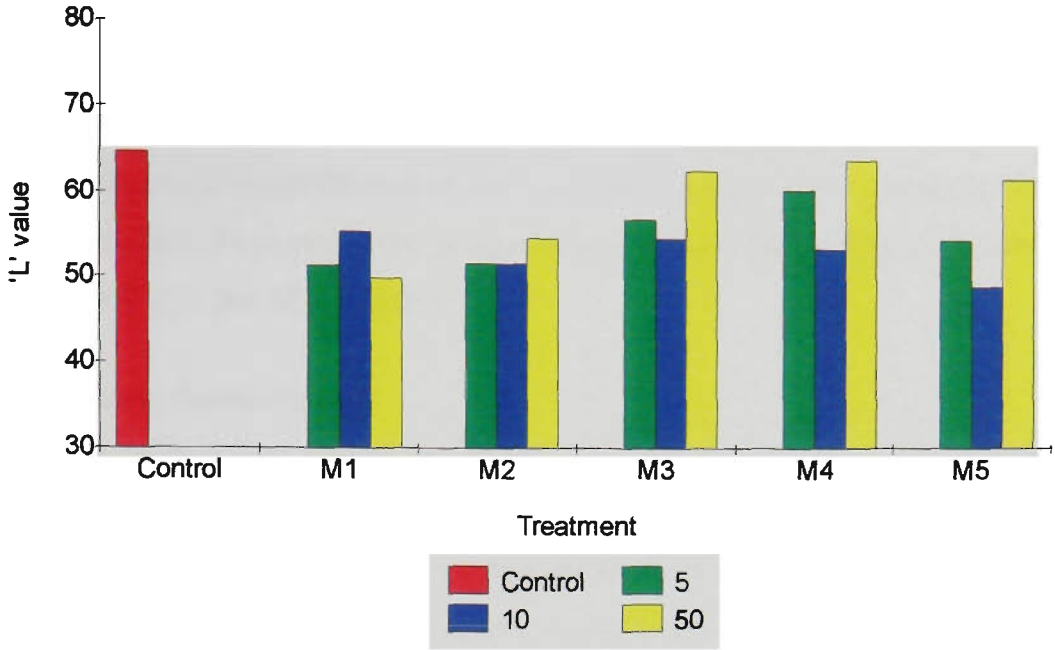


Figure 4.11 The colour components measured in the Hunter system and designated as L, a and b
Source: Minolta (publication year unknown)

Breads to which the preparation M3 was added had crusts that were darker at the treatment level of 5 and10μL ($p<0.05$). A significantly darker crust ($p<0.05$) was observed when 10μL of preparation M4 was added to the bread formulation. When the enzyme was added at 5μL and 50μL no significant difference was observed between the crusts of the and untreated loaves. The 10μL treatment also had crusts that were significantly darker than the 5 and 50μL treatment levels ($p<0.05$).



+

Figure 4.12 **Variations in crust colour development by the different preparations across a range of treatment levels. (Statistical data in Appendix 4.1)**

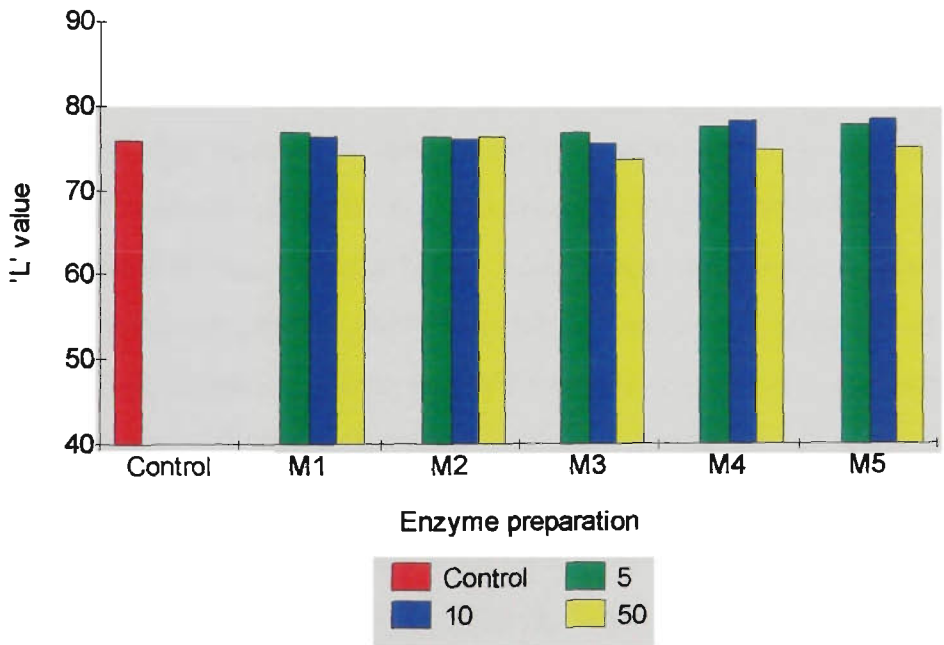


Figure 4.13 **Variations in the degree of whiteness produced in bread crumb by the different endoxylanases at the different treatment levels. (Statistical data in Appendix 4.2)**

Breads treated with the preparation M5 had bread crusts that were significantly darker at 5μL than the control and M4 ($p<0.05$). At 10μL this preparation produced significantly darker crusts than the control and those treated with M1 and M3 ($p<0.05$). At 50μL there was no significant difference in the untreated crust and that to which M5 was added. However, the crusts of this preparation were significantly lighter than those prepared with M1 and M2 ($p<0.05$).

4.2.5 Crumb characteristics

The results of the colour assessment (Figure 4.13) showed some variation in crumb whiteness when different enzyme preparations were added to the bread formulation. The M1 treatment did not produce any significant difference in crumb whiteness at any of the treatment levels compared with the control loaves. However, the 50μL treatment did result in crumb that was significantly darker than the untreated loaves ($p<0.05$). This finding may have been due to the less developed crumb in the higher treatment levels as noted in the subjective assessment.

No differences were found for the M2 treatment at any of the treatment levels compared with the control bread. However this treatment produced crumb that was significantly darker than those treated with M5 at each treatment level ($p<0.05$). This treatment also resulted in a significantly less white crumb than preparations M1 and M3 at 50μL ($p<0.05$). No significant difference was found between crumbs of untreated breads and those to which M3 was added at 10μL. This treatment did produce crumbs that were significantly whiter than M5 and M4 ($p<0.05$). The other treatment levels did not produce any difference in crumb whiteness compared with the control breads or the other treatments. When added at 10μL the M4 preparation produced significantly whiter crumb compared with the untreated loaves ($p<0.05$). A similar finding was observed between the M4 loaves and those to which M1 and M2 were added. The crumbs of the latter breads were significantly less white than the M4 breads ($p<0.5$). Breads treated with the M5 preparation had crumbs that were significantly whiter than the untreated loaves at the 5 and 10μL treatment levels ($p<0.05$). Further work is needed to identify what is happening to the flour pentosans when xylanase from this organism is added to a basic bread formulation.

4.2.6 Loaf volume

Prior to slicing the loaf volumes were determined for the treated and untreated breads. Volumes were determined using the RACI standard method 07-01. The results of this work are presented in Figure 4.14.

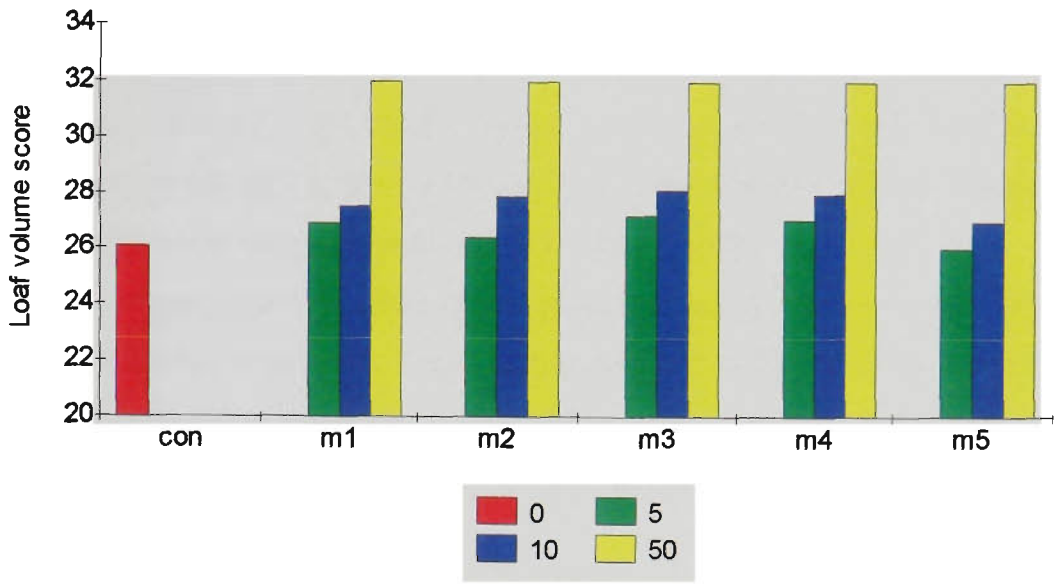


Figure 4.14 Loaf volume of treated and untreated loaves. (Statistical data in Appendix 4.3)

No significant difference was observed between breads treated with preparations M2 and M5 at the 5μL level. The other treatments all resulted in loaf volumes significantly greater than the control breads ($p<0.05$). Breads, to which 5μL of preparation M5 was added, were slightly smaller than the control breads but again this was not significant. Higher levels of enzyme addition resulted in breads with loaf volumes that increased as the level of enzyme increased. All the endoxylanase preparations contributed to loaf volumes that were significantly larger than the control loaves when added at 10μL and 50μL ($p<0.05$). The addition of endoxylanases at 50μL level resulted in baked breads with scores for loaf volume in excess of 32. This finding was observed for each of the preparations used. However, the deterioration in crumb structure, texture and colour observed for the highest treatment level for each preparation make the use of higher treatment levels non-viable despite the commercial appeal of bread with a greater loaf volume.

4.2.7 Compression analysis of bread crumb

To measure crumb softness an Instron Universal Testing Machine (UTM) was used AACC method 74-09 (Section 3.2.9).

The compression data showed that only the preparations M1, M2 and M3 produced crumbs that were significantly softer than the control breads (Figure 4.15). The crumbs of these loaves were significantly softer ($p<0.05$) when the enzyme preparations were added at a level of 5μL. When added at 10μL, breads treated with M1, M2, M4 and M5 had crumb that was significantly softer than the control breads ($p<0.05$). When added at 50μL all the preparations produced softer crumb than the control ($p<0.05$). With the exception of preparation M3, each of the treatments used at 50μL had crumb that was softer than the 5μL treatments. However, the visual observations for these breads showed that the poorly developed crumb structure would not make these loaves acceptable to the consumer despite the softer crumb.

Investigation of the source of each enzyme preparation showed preparations M1, M2 and M3 were all sourced from *Trichoderma sp.* Preparations M4 and M5 were sourced from *A niger* and *H insolens* respectively. These findings suggest that the source of enzyme and its temperature and pH optimum as well as the level of treatment have a role to play in the development of textural properties of white bread crumb.

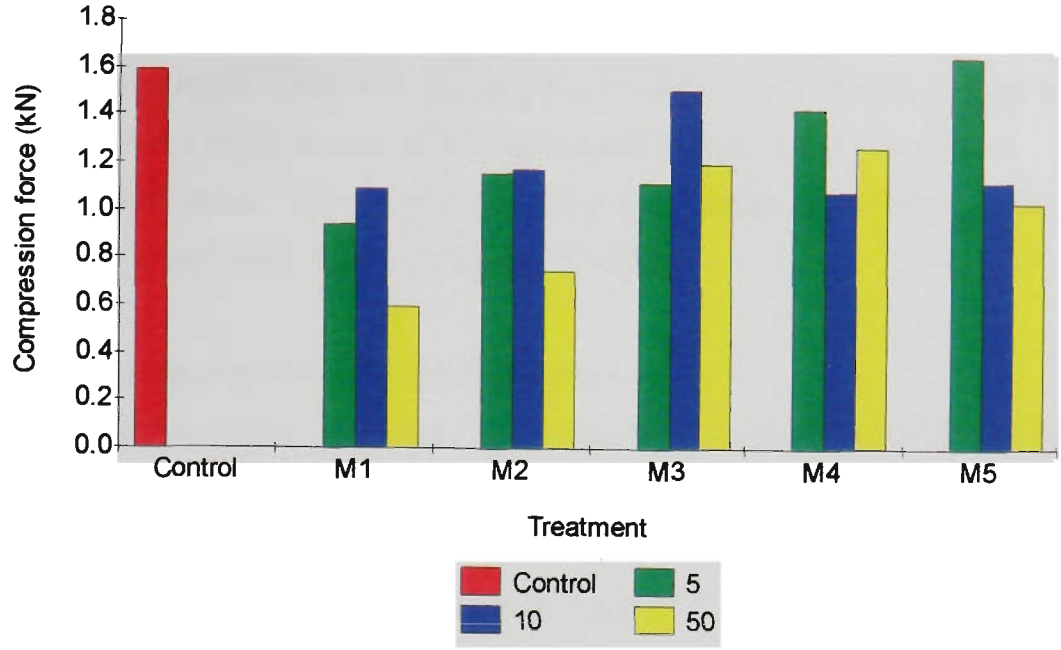


Figure 4.15 Crumb softness of controls and breads incorporating endoxylanases measured using Instron UTM. (Statistical data in Appendix 4.4)

4.3 Baking trials to investigate treatment levels of α-L-*endo*-arabinanase in baked breads

In the next section of the study endoxylanase was replaced in the basic bread formulation by a purified α-L-*endo*-arabinanase sourced from *A niger*. The specific activity for this enzyme was 8.0 U/mg under defined conditions (40°C, pH 4.0 on CM-linear 1,5-α-L-arabinan). Breads were prepared in triplicate using the same treatment level as that used for breads treated with endoxylanase enzyme preparation. It should be noted here that the specific activities for these preparations are different to those of the xylanase preparations. The treatment levels have remained the same to minimize confusion that may have resulted had a different set of treatments been established. Subjective and objective assessments were carried out for crumb and crust colour, loaf volume and crumb softness.

4.3.1 Visual observations

Inspection of the baked breads showed that all the treatment levels produced loaves that were uniform in overall loaf symmetry with a uniform crust colour. There was no apparent loss of crust bloom however a lighter crust colour was evident for those breads to which 50μL of the preparation was added.

No observable differences were observed for the crumb colour or structure for any of the treatment levels compared with the control breads. The formation of large air cells observed in the breads treated with endoxylanases was not observed in these experimental breads. The use of *endo*-arabinanase did however result in the formation of a sticky crumb even at the lowest level of addition.

4.3.2 Objective analysis of crumb and crust colour

Crumb and crust colour parameters were measured using the Minolta Chroma Meter and the Hunter Lab colour scale. Crust and crumb were measured for brightness with a higher L* value demonstrating a brighter or lighter crust or crumb. The results for colour analysis are presented in Figures 4.16 and 4.17 (Statistical data in Appendices 4.5 and 4.6). Significantly darker crust ($p<0.05$) was observed in breads treated with 5µL of the enzyme preparation compared with the control breads. This treatment level also resulted in significantly darker crusts than those produced with the 50µL treatment ($p<0.05$). No significant differences were observed for crust colour when breads were treated with 10 µL compared with the control breads while the 50µL treatment had crusts that were significantly lighter than the control breads ($p<0.05$).

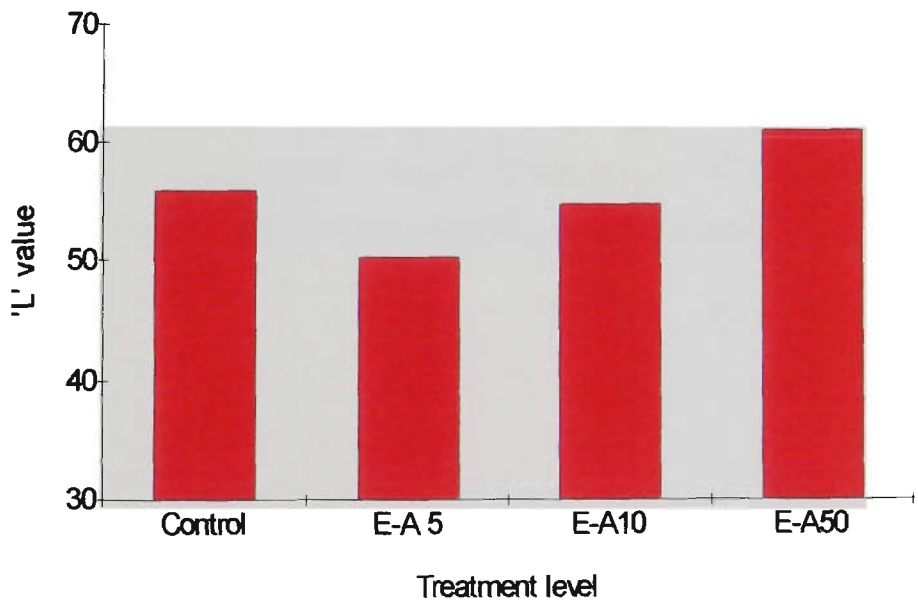


Figure 4.16 Analysis of crust colour characteristics of bread treated with *endo*-arabinanase at 5, 10 and 50µL treatment levels (E-A 5, E-A 10 and E-A 50)

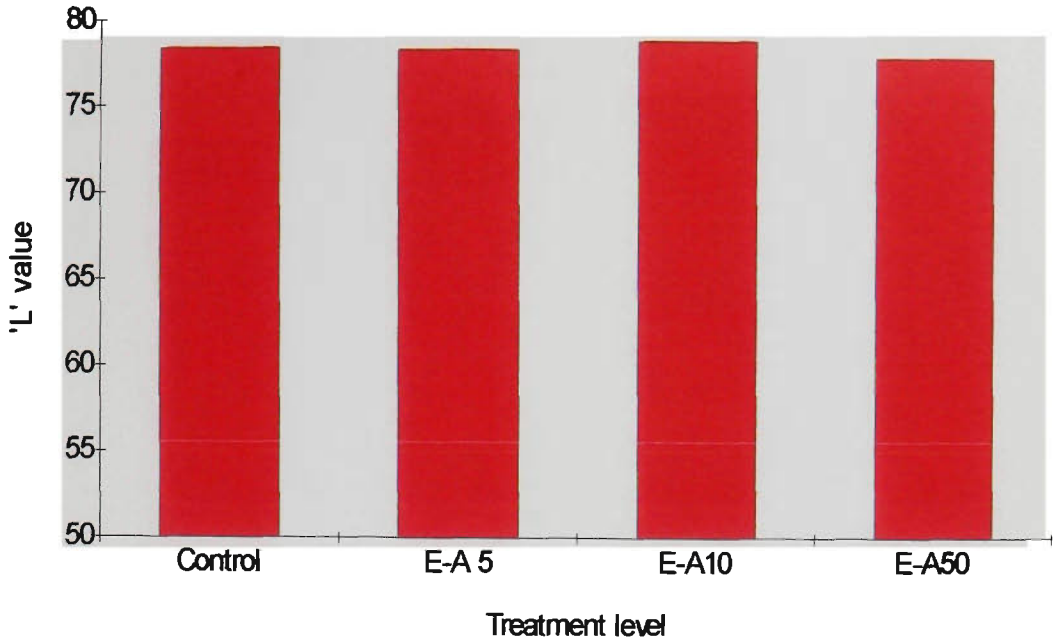


Figure 4.17 Analysis of crumb colour characteristics of bread treated with *endo*-arabinanase at 5, 10 and 50µL treatment levels (E-A 5, E-A 10 and E-A 50)

No significant differences in crumb whiteness were observed in the breads treated breads treated with *endo*-arabinanase in the range of 5-50µL.

4.3.3 Analysis of crumb softness

The results of the compression measurements are given in Figure 4.18. They indicate that the use of this particular arabinase preparation resulted in the development of a firmer crumb in the baked breads. Analysis of variance showed that the crumb of the treated loaves was significantly firmer than the untreated breads ($p<0.05$; Appendix 4.7).

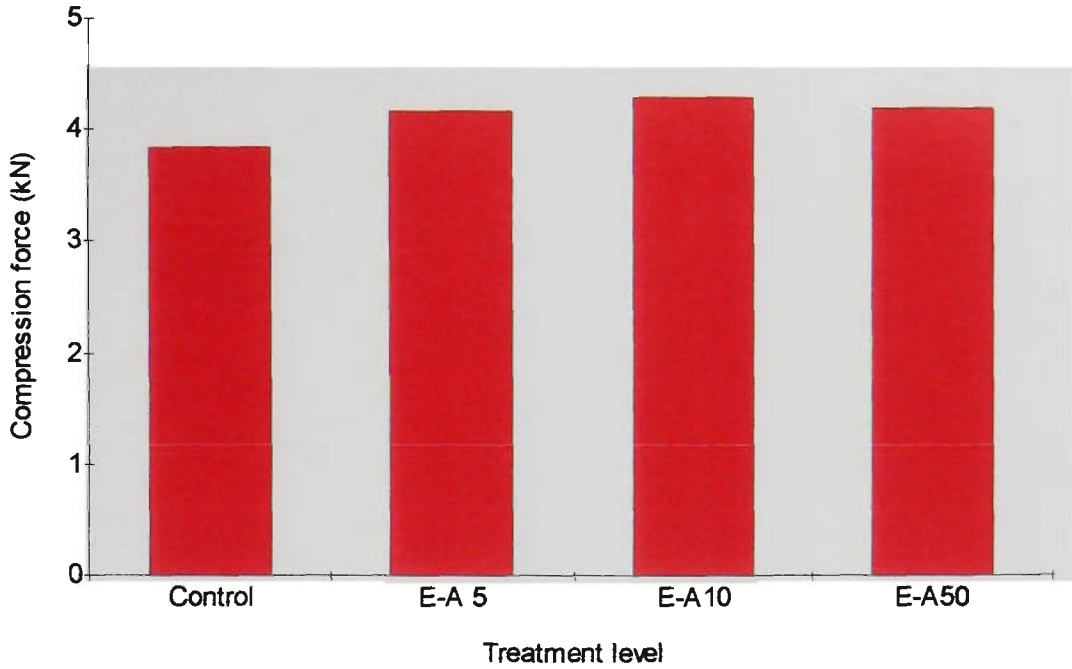


Figure 4.18 **Compression data obtained for control breads and breads to which *endo*-arabinanase was added at treatment levels of 5, 10 and 50μL (E-A 5, E-A 10 and E-A 50)**

4.3.4 Loaf volume

The effects of *endo*-arabinanase on loaf volume are presented in Figure 4.19. The results show no significant change in loaf volume between the control and the 5μL treatment (statistical data is presented in Appendix 4.8). The 10μL treatment was significantly smaller than the control and the 5μL treatment ($p<0.05$), whilst the 50μL treatment was significantly larger than the control and the other two treatment levels ($p<0.05$).

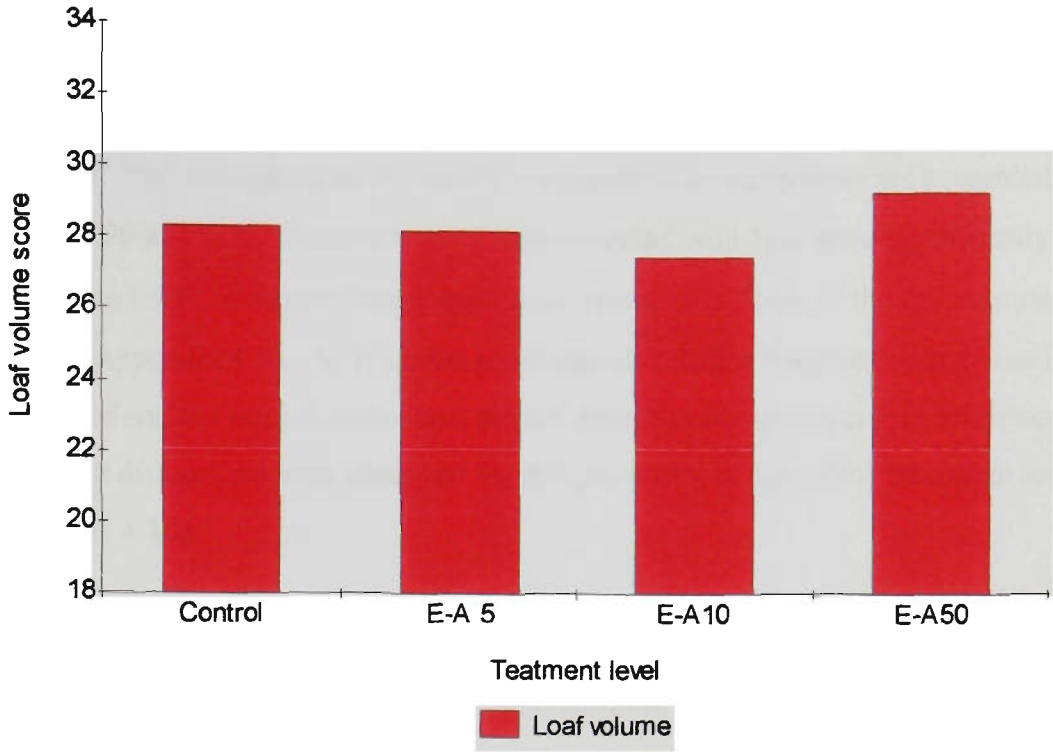


Figure 4.19 **Loaf volumes obtained for control breads and breads to which *endo*-arabinanase was added at treatment levels of 5, 10 and 50µL (E-A 5, E-A 10 and E-A 50)**

4.4 Baking trials to investigate changes produced in baked breads using different treatment levels of α-L-arabinofuranosidase with the basic bread formulation

In the final part of this phase of the study an arabinofuranosidase also sourced from *A niger* was utilised in the bread formula. The enzyme was an α-L-arabinofuranosidase with a specific activity of 1.3 U/mg (40°C pH 4.0 on wheat arabinoxylan, 10mg/mL).

4.4.1 Visual observations

Inspection of loaves prior to slicing showed breads with added arabinofuranosidase in the range of 5 and 10µL had crusts that were visibly darker than the control breads. These breads were also visibly darker than breads treated at the higher level of enzyme addition. No visible differences were observed in the crumb structure for any of the treatment levels with this preparation. The large gas cells observed in breads treated with the higher levels of endoxylanases were not evident when breads were treated with arabinofuranosidases. The larger loaf volumes observed with the higher xylanase treatments were not observed in breads treated with arabinofuranosidases.

4.4.2 Assessment of crust and crumb colour

Changes in crust colour were measured using the Minolta Chroma Meter as previously described. The average of all the results are reported here and these are presented in Figures 4.20 and 4.21. Bread crusts of loaves treated with 5μL were significantly darker than both the control loaves and those treated with 50μL of the preparations ($p<0.05$, Appendix 4.9). At 10μL the crust was also darker than the control breads. The addition of *endo*-arabinofuranosidase did not appear to impact on crumb brightness. No significant differences were observed for this parameter at any of the treatment levels (Appendix 4.10).

4.4.3 Analysis of crumb softness

The effect of arabinofuranosidase on crumb softness was measured as previously described. Measurable differences in crumb softness were reported for the different treatment levels (Figure 4.22; statistical data is presented in Appendix 4.11). All treatments resulted in breads being produced that had softer crumb than the control breads however these differences were not found to be significant.

4.4.4 Loaf volume

Slightly smaller loaf volumes were observed for breads treated separately with 5 and 10μL of arabinofuranosidase (Figure 4.23). These differences were not significant. Breads to which 50μL of the enzyme was added had a significantly larger loaf volume than the control breads ($p<0.05$; Appendix 12). The loaf characteristics are shown in Figures 4.24 –4.27. Two possible explanations of these findings are the release of arabinose residues from the xylose backbone impacting on solubility and water binding properties or the release of arabinose allowing endogenous endoxylanases greater access to the xylose backbone of the chains.

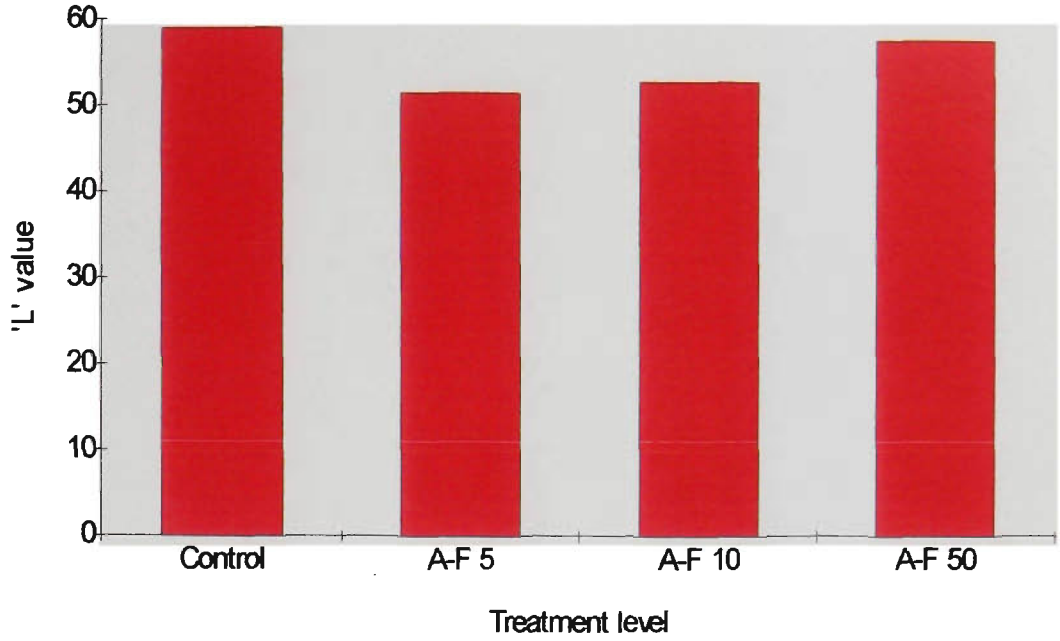


Figure 4.20 **Variations crust colour of bread treated with arabinofuranosidase at the 5, 10 and 50µL levels (A-F 5, A-F 10 and A-F50). Statistical data is presented in Appendix 9**

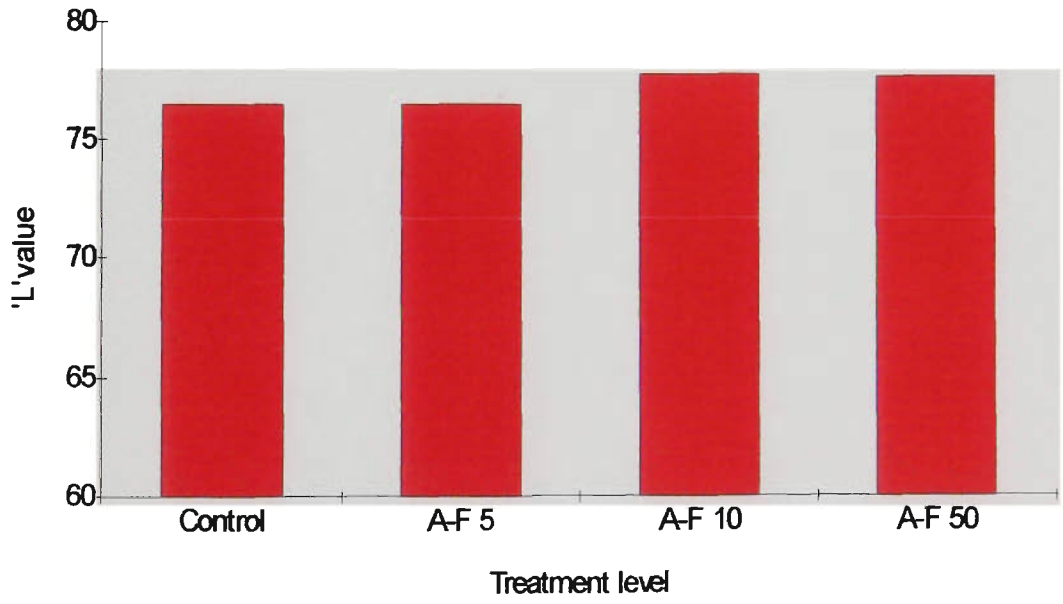


Figure 4.21 **Variations in crumb brightness of bread treated with arabinofuranosidase at the 5, 10 and 50µL levels (A-F 5, A-F 10 and A-F50). Statistical data is presented in Appendix 10**

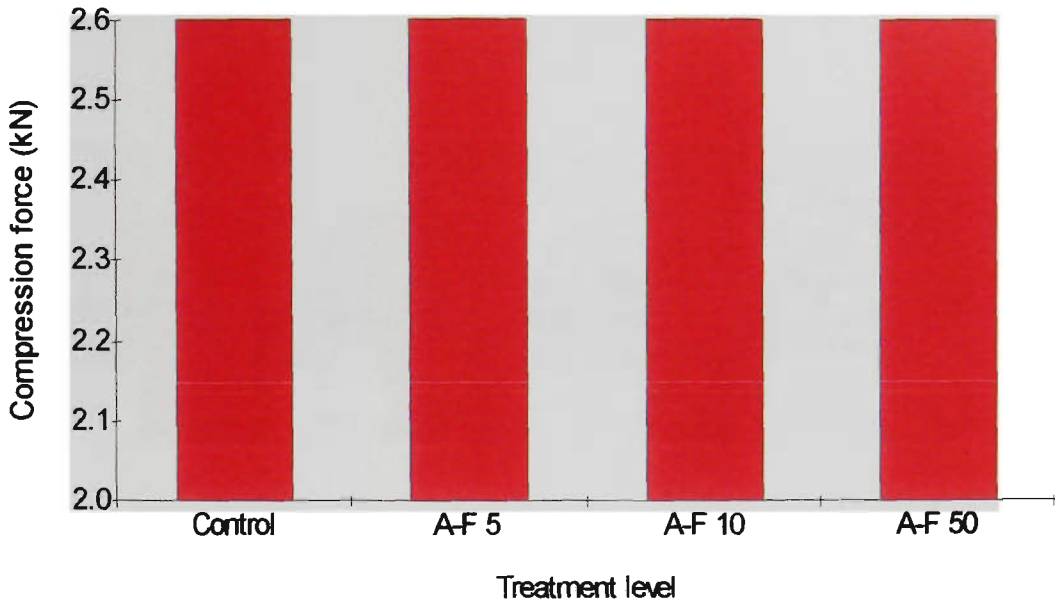


Figure 4.22 **Crumb softness of bread treated with arabinofuranosidase at the 5, 10 and 50µL levels (A-F 5, A-F 10 and A-F50)**

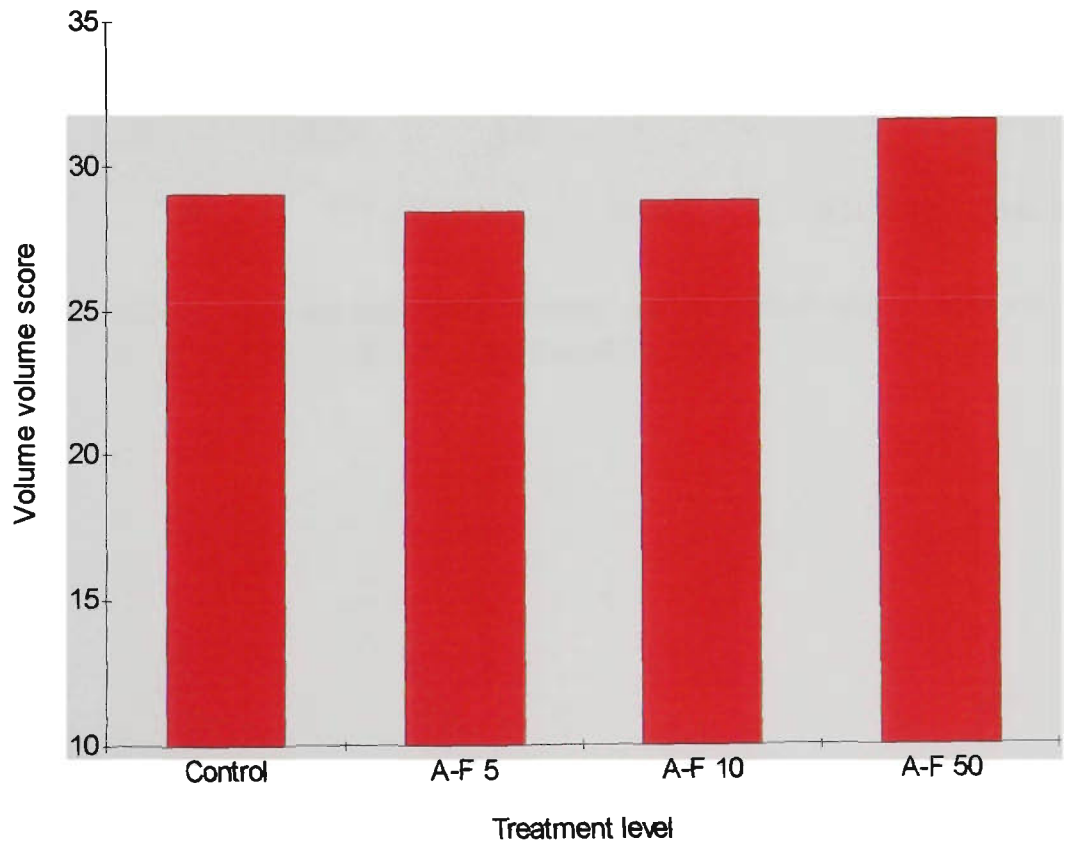


Figure 4.23 **Loaf volume scores for control loaves and breads treated with arabinofuranosidase at 5, 10 and 50µL (A-F 5, A-F 10 and A-F50)**

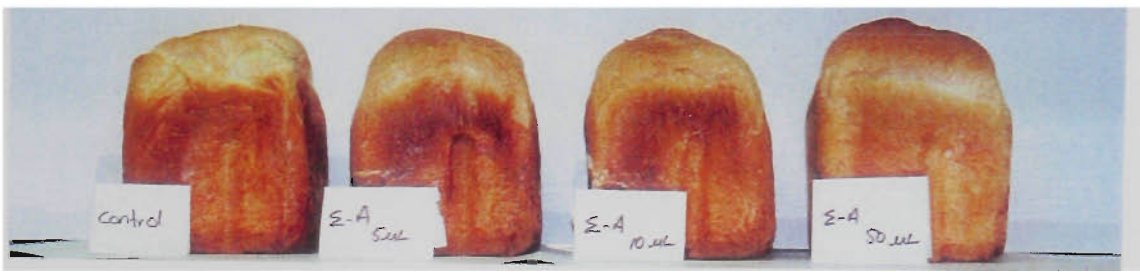


Figure 4.24 **External appearance of breads with added *endo*-arabinanase at 5, 10 and 50µL (EA 5, EA 10 and EA 50)**

Figure 4.25 **Interior crumb structure of breads with added *endo*-arabinanase at 5, 10 and 50µL (EA 5, EA 10 and EA 50)**

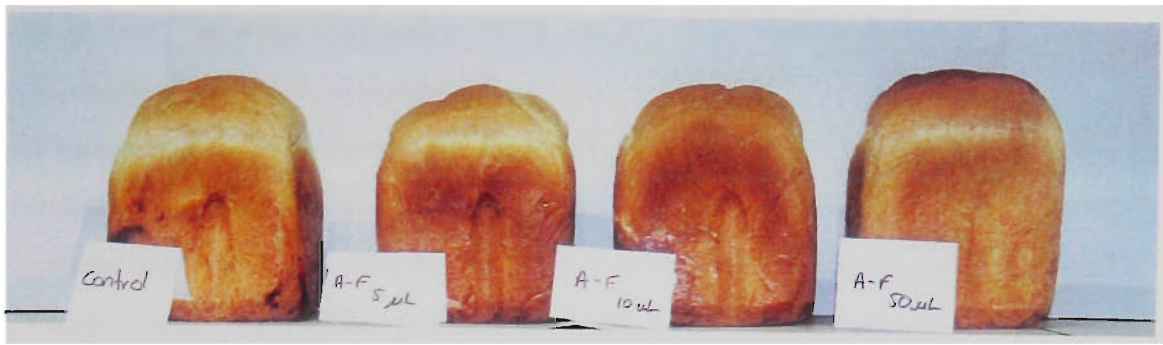


Figure 4.26 **External loaf symmetry of breads with added arabinofuranosidase at 5, 10 and 50µL (A-F 5, A-F 10 and A-F50)**

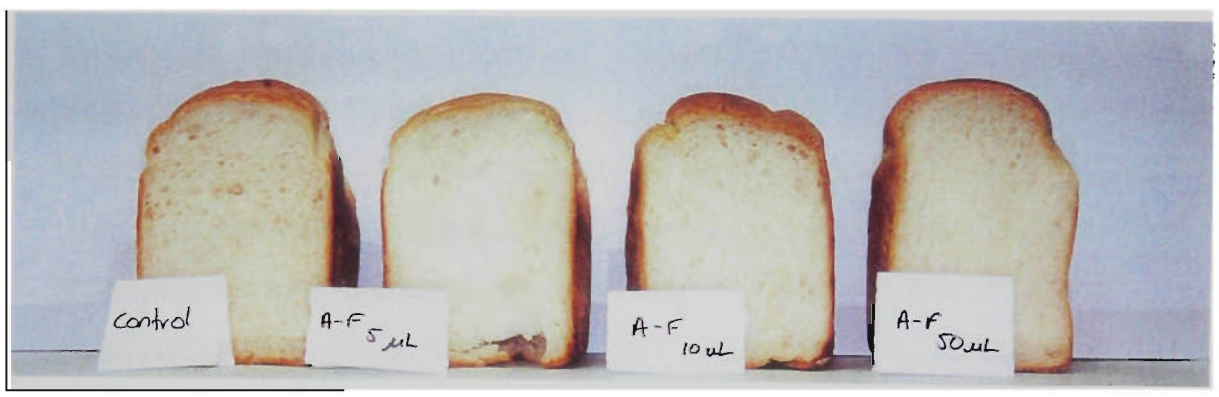


Figure 4.27 **Internal crumb structure of breads with added arabinofuranosidase at 5, 10 and 50µL (A-F 5, A-F 10 and A-F50)**

4.5 Discussion

The ability of enzymes with xylanase activity to affect the properties of bread crumb has been demonstrated in a number of studies (Bonnin et al 1998, Cleemput et al 1995, Cleemput et al 1997, Collar et al 2000, Jimenez and Martinez-Anaya 2000, Laurikainen et al 1998, Martinez-Anaya and Jimenez 1998). However many of these studies have focused on the effects of endoxylanases with purified WSA and water-insoluble arabinoxylan (WISA) (Jorgensen et al 1999). In addition to this the studies have utilised a range of European wheats with varying bread making capabilities. The current study set out to examine the effects of endoxylanase and arabinfuranosidase using a strong bread making flour milled from Australian wheat.

The experimental data presented here has demonstrated the ability of endoxylanase preparations to bring about measurable differences in crumb and crust characteristics of baked breads when used at very low levels. By adding each preparation at increasingly higher levels it was possible to establish a treatment level at which an unacceptably high level of penotosan degradation was occurring. This was determined by the development of stickiness in the crumb accompanied by the development of discoloured areas in the crumb structure and loss of crust colour, bloom and crispness. Crumb stickiness was first evident to varying degrees across the range of treatments when the different endoxylanase preparations were added at 10μL suggesting an undesirably high level of pentosan hydrolysis was occurring. The degree of stickiness observed increased when the level of enzyme addition was increased to 50μL. This finding is consistent with those of van Oort et al (1995) who found that excessive pentosan degradation led to the development of a sticky bread crumb evident in the baked product when excessive pentosan degradation has occurred. The development of an increasingly sticky crumb in the experimental breads in this study suggests that the wheat flour pentosans are being hydrolysed past the point of optimum hydrolysis. However whether deterioration in the crumb structure observed at the higher treatment levels was due to increased solubilisation of WISA or hydrolysis of the WSA fraction or a combination of both requires further investigation.

Other factors that suggest excessive arabinoxylan solubilisation or degradation is occurring at the higher treatment levels include the loss of uniform crumb colour and the loss of uniformity of crumb structure. The development of large irregular air cells

particularly at the crumb crust margin requires further investigation. It may be due to a weakening of the gluten matrix but further analysis of the dough properties is required to identify exactly what caused this to occur. The findings of the current study support this finding. A steady increase in loaf volume was observed as the level of enzyme treatment increased. Work by Gan et al (1995) indicates that interfacial films at the gas/liquid interface of fermenting doughs are involved in gas retention. Components responsible for this stabilisation effect are believed to include polar lipids, proteins and possibly pentosans. The increase in loaf volumes and development of an irregular gas vacuole pattern at the higher addition levels used in this study suggest that the excessive degradation of arabinoxylans may have had a destabilising effect on the gluten matrix. CO_2 produced during fermentation is not being retained within the gluten matrix. van Oort et al (1995) suggested that excessive pentosan breakdown leads to a destabilising of the gluten matrix. CO_2 produced during proofing is not held within the gluten structure leading to the loss of uniformity of crumb structure observed in this study. The development of irregular air cells also contributed to loss of loaf symmetry. These factors in addition to loss of crust bloom at the higher treatment levels also detracted from the visual appeal of these loaves.

The formation of significantly lighter crusts in the loaves treated with 50µL of the preparations M4 and M5 was unexpected. The primary factor involved in the development of crust colour is the non-oxidative browning known as the Maillard reaction in addition to caramelisation. Maillard browning involves a series of complex reactions resulting in the formation of brown pigments known as melanoidans. The Maillard reaction is not well defined (Whistler and Daniel 1985) however the minimum requirements for it to occur are an amino-bearing compound, a reducing sugar and water. An intermediate moisture level and a pH of 4-7 produces changes in colour, flavour and nutritional value as lysine is destroyed (Kroh 1994, O'Brien and Morrisey 1989). There is little reported in the literature in relation to the effect of pentosan hydrolysis and crust colour however the findings of this study indicate that excessive degradation of arabinoxylan has a negative impact on the development of the crust properties of colour, bloom and crispness. More work is required in this area to determine the exact relationship between colour development and arabinoxylan hydrolysis. The lack of crust colour combined with the loss of both loaf symmetry and

crust crispness characteristic of fresh bread resulted in breads with a decreased loss of consumer acceptance for breads treated with 50μL of each preparation.

The data obtained for the compression analysis demonstrated the ability of endoxylanase preparations to influence crumb firmness in baked bread. The firmer crumb observed at the higher treatment levels was attributed to the increasing levels of crumb stickiness observed in these breads. It is believed that the presence of crumb stickiness enhanced the forces of attraction between individual crumb particles thus increasing the degree of force required to compress the crumb.

The endoxylanase preparations used in this study were obtained from several fungal sources. The results indicate that enzymes from different sources and with different specific activities produce measurable changes in the properties of bread. In addition to this it appears that enzymes sourced from the same fungal species but with differing properties also produce a variety of changes in white bread. The use of preparations M2 and M3, both sourced from *T longibrachiatum* (pH optima 4.5 and 6.0 respectively) resulted in baked breads with differing crumb and crust properties. Whilst the use of preparation M2 and M3 resulted in breads that exhibited no significant difference in crumb colour compared with the control, breads treated with M2 had crusts that were significantly darker than the control loaves at all the treatment levels. The M1, M2 and M3 treatments also produced significantly softer crumb at the lower treatment level, a finding not observed for the M4 and M5 treatments. Harado et al (2000) demonstrated the ability of endoxylanase to improve crumb softness in rapid bread making methods. The data obtained for crumb colour showed only the addition of M5 produced changes in colour significantly different from the control breads. These differences may have been in part due to the higher level of crumb deterioration observed at all the treatment levels when M5 was used. The results of the current study have demonstrated a connection between the enzyme source in addition to the individual enzyme characteristics such as pH and temperature optima and specific activity and their ability to influence the quality of the final product.

The data presented in this section of the study suggests that the source of the enzyme preparation is responsible for changes observed in the textural parameters of the bread crumb. However the results of the data obtained for the enzyme assays must also be

considered. Significant losses in enzyme activity were observed for preparations M1 and M5 ($p<0.05$). Although M1 appeared to have 25% loss of activity when added to a bread formulation the resulting loaves still exhibited significantly softer crumb compared with both the controls and the M5 breads. Whether this particular enzyme preparation would enhance crumb softness at full activity is speculative. The differences observed in crumb and crust characteristics for the treatments used are therefore based on enzyme activity as measured in this experiment.

Consumer acceptance of loaf bread is governed by its sensory attributes. Crust gloss, colour and loaf volume and symmetry are important factors that are at once obvious to the consumer (Fance 1991). If even one of these features is perceived to be of an inferior standard the bread will not be purchased. In carrying out the above study the most important factor considered was whether or not breads baked with the different endoxylanase treatments would be acceptable to the consumer. Based on the experimental data obtained in this section of the work it was determined that the level of endoxylanase addition required to repeatedly produce breads that would meet consumer expectations was 5μL.

The use of *endo*-arabinanase and arabinofuranosidase preparations, both sourced from *A niger* resulted in breads being produced that were quite different to those obtained using the endoxylanase preparations. The excessive loaf volumes observed in breads treated with 50μL of the endoxylanases were not reported in breads treated with a higher level of either the arabinofuranosidase or the *endo*-arabinanase preparations. Deterioration in crumb structure and crumb colour was also reduced when these enzyme preparations were added to a basic bread formulation. Arabinofuranosidases are *exo*-acting enzymes that cleave the terminal non-reducing residues from polysaccharides containing arabinose (van Oort et al 1995). This study showed that breads treated with *endo*-arabinanase had crumbs that were significantly firmer than the untreated loaves whilst no significant difference was found between the control loaves and those with added arabinofuranosidase. By comparison breads with added endoxylanase from preparations M1, M2 and M3 had significantly softer bread crumb than the untreated loaves. These findings suggest that the hydrolysis of the arabinose residues alone does not result in a softening of bread crumb and may in fact have an adverse effect on crumb softness.

Development of crust colour was affected by the treatment level when breads were treated with both endo-arabinanase and arabinofuranosidase preparations. As with the xylanase treatments, darker crusts were observed with the lower treatment levels but lighter crusts were produced when breads were treated with the higher levels of each enzyme. It appears that the excessive arabinose hydrolysis also impacts on the formation of crust colour. The results of this study have shown that both xylanase and arabinase enzymes when used at higher levels appear to inhibit the development of crust colour. This finding may be due to an excessive hydrolysis causing a reduction in the amount of reducing sugars available to take part in the Maillard reaction.

4.6 Summary

In this phase of the study individual endoxylanase, *endo*-arabinanase and arabinofuranosidase enzymes were added to a basic bread formulation at varying levels. The results indicate that the crumb and crust characteristics of colour and softness can be altered significantly by the type of enzyme used as well as the level at which individual enzymes are added. For the remainder of the study a universal treatment level of 5μL of each of the xylanases per dough was used. Whilst recognizing that the actual level of enzyme in each preparation varied it was felt that a basic dosage rate was more appropriate to work with since this is how the preparations would be applied in commercial practice.

Chapter 5

Results and discussion – The effects of a range of fungal endoxylanases on the properties of white loaf breads

5.1 Introduction

Wheat flour pentosans have important functional properties in dough development. Pentosans influence loaf volume and crumb and crust characteristics of the bread (Westerlund et al 1990). Modification of the arabinoxylan fraction of wheat flour has been shown to influence the textural characteristics of white bread. Courtin et al (1999) demonstrated through fractionation and reconstitution experiments the ability of endoxylanases to impact on the level of WISA resulting in enhanced loaf volumes. While commercial enzyme preparations containing a range of enzyme activities have been shown to slow the firming of bread crumb (D'Appolonia and Morad 1981, Hoseney 1984, Kim and D'Appolonia 1977) the specific enzymes within these preparations and their action at a molecular level are yet to be identified. In addition, much of the research involving purified enzymes demonstrating pentosanase activity has been carried out using an enzyme specific substrate (Jorgensen et al., 1999). This phase of the study utilized a range of highly purified endoxylanase preparations in a series of baking trials carried out under Australian conditions to examine the effects of individual enzymes during baking. Factors assessed include loaf volume, moisture content of fresh and aged breads, crumb and crust colour as breads aged, initial crumb softness and rate of crumb firming as breads aged.

5.2 Experimental loaves using previously established enzyme treatment levels

5.2.1 Baking studies conducted to determine the effects of purified endoxylanases on freshly baked bread

A series of 5 loaves of bread was baked for each preparation using Panasonic bread machines used in the previous section of the study. Breads were prepared on a 3-hr rapid bake cycle. At the completion of baking breads were assessed subjectively for appearance, variation in loaf volume, crumb softness, moisture content and crumb and crust colour characteristics. Uncut breads were assessed visually for loaf symmetry,

and overall crust colour and bloom. Crusts colour was also assessed using the Minolta Chroma Meter. The loaves were cut into 2.5cm slices taking care not to tear the crumb. Bread crumb was assessed for colour using the Minolta Chroma Meter as well as texture and structure.

5.2.2 Moisture analysis

Moisture content was measured for freshly baked breads and loaves that had been aged for 72hrs. The results are presented in Figure 5.1. Although the treated breads appeared to have higher moisture contents than the control loaves, the differences were not statistically significant ($p>0.05$). The use of endoxylanases contained in preparations M1, M2, M4 and M5 resulted in baked loaves with average moisture contents of 45.5%, 45%, 44.5% and 44.5% respectively. Use of the preparation M3 resulted in breads that had the lowest moisture content of the treated loaves. An average of 44% moisture was measured for these loaves. The untreated loaves by comparison had an average moisture content of 43%. These findings are consistent with those of van Eijk and Hille (1996) who found that the use of enzymes does not produce significant changes in moisture content of freshly baked breads.

Significant moisture loss occurred in the crumb of breads that had been aged for 72hrs ($p<0.05$). Both the treated and untreated breads had significantly lower moisture contents than the freshly baked breads. Moistures for breads designated Control, M1, M2 M3, M4 and M5 were 35%, 35%, 35.5%, 35%, 37% and 37% respectively. This finding is contrary to those of other researchers who have found no significant difference moisture loss between fresh and aged breads (Pomeranz and Shellberger 1971). During storage moisture migrates from the crumb to the bread crust resulting in a loss of crumb moisture and an associated increase in moisture content of the crust. Removing the crust appears to result in no significant loss of crumb moisture during aging. In addition to this the ability of endoxylanases to retard staling by influencing the distribution of water in the crumb has been widely reported (Hilhorst et al 1999, Holas and Tippler 1978, Hoseney 1984, van Oort et al 1995). To investigate the ability of modified arabinoxylans to influence moisture redistribution in the crumb the current study stored the loaves in plastic bags with the crust intact. The significant moisture losses observed for both the control and the treated breads suggests that endoxylanase

modification of arabinoxylan by the preparations utilized in the current study does not influence the movement of moisture during storage.

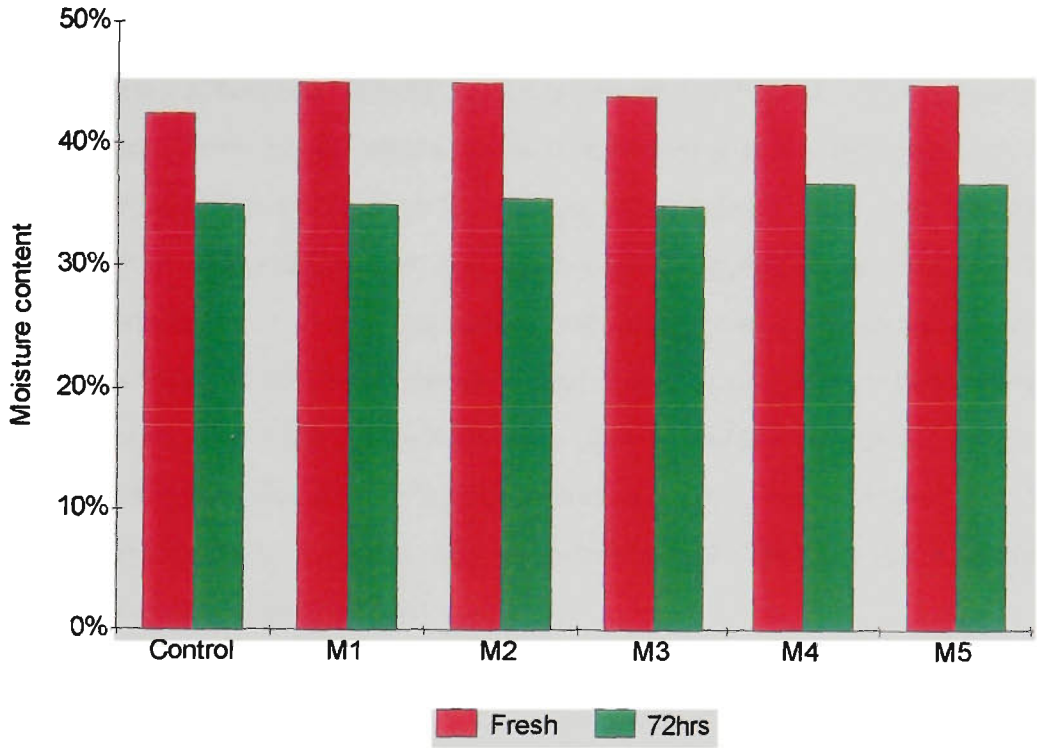


Figure 5.1 **Moisture content of fresh and aged breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5). Statistics are presented in Appendix 5.1**

5.2.3 The effects of endoxylanases on loaf volume

At the completion of baking loaves were removed from the baking pans and allowed to cool at room temperature for a period of 90min. Loaf volumes were determined using the RACI standard method 07-01 as previously described.

The results of this section are presented in Figure 5.2. The volumes of each of the breads were found to be commercially acceptable in that they were all well over the standard score of 19. Some variation in volume was observed for the treated loaves when compared with the control as well as between the breads baked using the different preparations. Breads to treated with preparations M1, M3 and M4 were significantly larger than the control breads ($p<0.05$; Appendix 5.2). The mean loaf volume scores for these breads were 26.6, 26.5, and 27.1 respectively. Breads baked with the addition

of the enzyme preparation designated M5 had loaf volumes slightly less than those of the controls. The average loaf score for these breads was 26.0 compared with a 26.1 loaf score for the controls. The use of commercial preparations containing a range of side activities has been shown to produce breads with increased loaf volumes (Kulp and Betchel 1968). The findings of the current study indicate that modification of the arabinoxylan fraction may directly impact on the increased loaf volume observed in the other studies. However, the extent to which these preparations may impact on loaf volume may also depend on other factors such as the type of wheat used and the sources of the enzyme. Other researchers have used softer European wheat flours, while the strong Australian flour used in the current study, may have impacted on the ability of some of the preparations to produce modified arabinoxylans that enhance loaf volume of white bread. The results of the current study were obtained when individual preparations were added directly to a bread formulation. Enzyme activity may have been affected by factors including access to substrate and the presence of naturally occurring endoxylanase inhibitors.

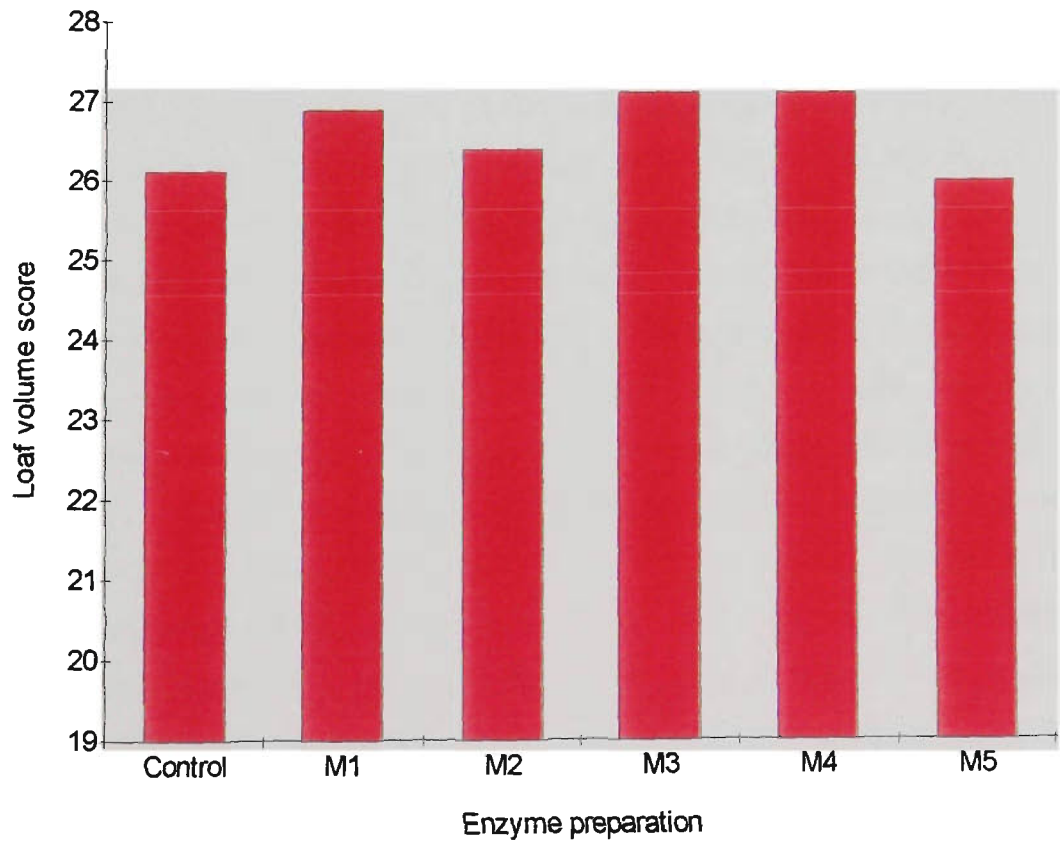


Figure 5.2 **Loaf volume scores for treated and untreated breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

5.2.4 Subjective and objective analysis of crumb and crust properties of control and treated breads

In this stage of the study, the treated loaves were assessed for crumb and crust characteristics to determine if endoxylanases from different sources produced variations in these properties. Crumb and crust characteristics of the control loaves were measured for comparison with the different treatments. The results for this section are presented in Figures 5.3 and 5.4. Beginning with overall loaf symmetry, the treated loaves were generally found to have loaf symmetry comparable to those of the control breads. Slight lack of uniformity observed for some of the preparations, was attributed to experimental deviation, since similar deformations were occasionally observed in the control loaves throughout the course of the study. The control loaves in this section were found to be of uniform structure, loaf symmetry, colour and crust bloom. The experimental loaves were also inspected prior to slicing for overall loaf symmetry, uniformity of crust colour and degree of crust bloom. The experimental loaves exhibited uniform loaf symmetry and crust colour comparable to that of the control breads. Measurements for calculation of loaf volume scores were recorded prior to slicing.

After slicing examination of the individual slices revealed the bread crumb of the control loaves to be of uniform colour throughout the loaf. Crumb structure of the three control loaves showed an even distribution of gas cells appearing in each slice. Inspection of the cut surfaces of the treated breads showed little variation in crumb structures for the different preparations used. In the first series of breads, those baked using the preparation designated M1 sourced from *T viride* the crumb structure had an even distribution of gas cells. When compared with the other treatments the M1 preparation resulted in the formation of slightly larger gas cells with a corresponding thinner cell wall. Crumb colour of these loaves was also uniform. Breads to which the enzyme preparation designated M2 *(*from *T longibrachiatum)* was added, exhibited bread crumb that was also uniform in both structure and colour at the 5μL level of addition. The size of the gas cells was smaller than that observed for preparation M1 but distribution was regular throughout the loaves.

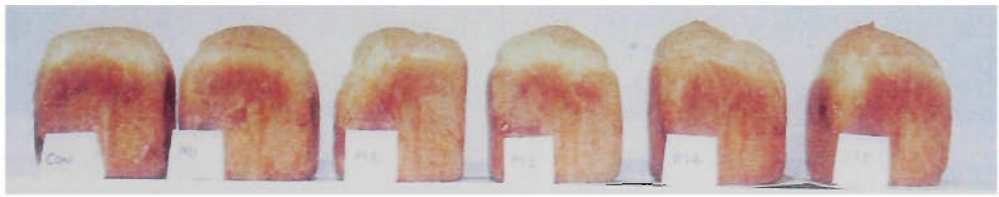


Figure 5.3 **Loaf colour and symmetry of control breads and loaves treated with endoxylanases *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

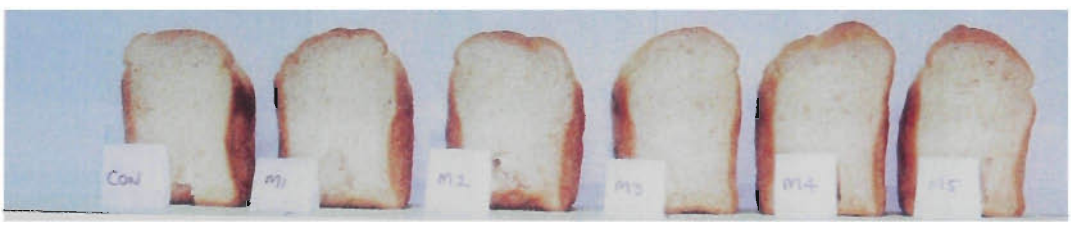


Figure 5.4 **Crumb colour and structure of control breads and loaves baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

Similar findings were observed for breads to which enzymes M3 and M5 were added. These preparations were sourced from *T longibrachiatum* and *H insolens* respectively. When the breads were treated with 5µL of the preparation designated M4 the crumb exhibited a grainy appearance even at the lower level of addition. The crumb structure was denser than that of the control loaf however there was an even dispersion of gas cells throughout the crumb. Crumb stickiness was also evident at this level of addition

Differences in crumb and crust colour were measured objectively using the Minolta Chroma Meter and results for 'L' values are presented in Figure 5.5. Significant differences were found for crust darkness. For each of the preparations M1, M2, M3 and M4 were significantly darker than the control loaves and those treated with M5

(p<0.05; Appendix 5.3). No significant difference was observed between the control loaves and those treated with M5.

Data obtained for crumb whiteness showed no significant changes in crumb whiteness for any of treatments used compared with the controls.

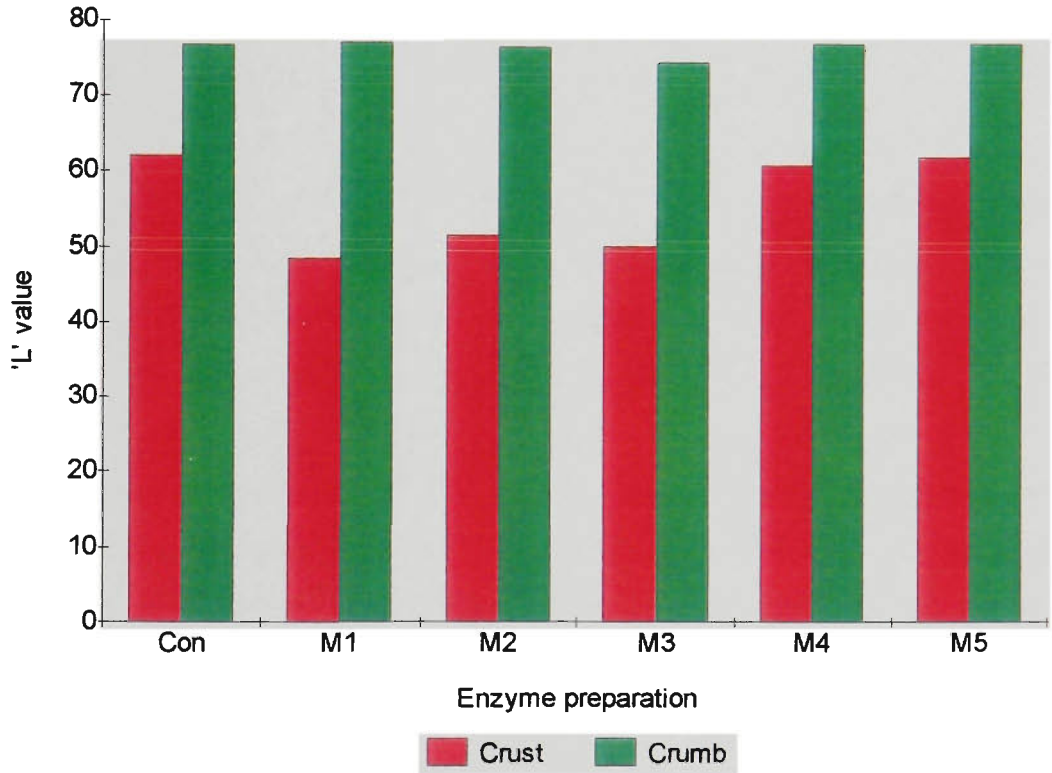


Figure 5.5 Degree of crumb whiteness and crust darkness of breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)

5.2.5 Crumb softness of freshly baked breads

Crumb firmness or softness is an important sensory attribute of bread. Whilst the "squeeze test" carried out by the consumer gives a sufficiently sound indication as to the apparent freshness of a loaf of bread it is a difficult factor to quantify in the laboratory. To overcome this the use of instruments including the Universal testing machine (UTM) and the Baker Compressimeter that measure the amount of force required to compress a slice of bread have been used to give an objective measurement crumb firmness and

hence loaf freshness. Details of this procedure are described in Chapter 3 (Section 3.2.9).

After baking breads were cooled and sliced for further analysis. The freshly baked loaves prepared using the different preparations were all found to have crumbs that were measurably softer than the control loaves (Figure 5.6). However, only preparations M1, M2 and M3 produced crumbs that were significantly softer than the untreated breads ($p<0.05$; Appendix 5.4). Breads to which endoxylanase from M1 had been added were also significantly softer than those baked using preparations M4 and M5 ($p<0.05$). Of the treated loaves those breads prepared with the addition of preparation M4 had the firmest crumb. These breads were also found to have areas of crumb that were slightly underbaked as demonstrated by the presence of doughy patches in the cut crumb. A lack of crumb elasticity due to an underdeveloped alveoli network in addition to the presence of crumb stickiness was believed to contribute to the firmer crumb observed in the fresh loaves treated with preparation M4. A softer crumb was observed for breads baked with the preparations M2 and M3, while breads prepared with the addition of preparation M5 had a crumb that was not significantly softer than those baked with M4. The firmer crumb observed in the breads prepared using the M4 preparation was attributed to the doughy patches observed. The underbaked areas of these breads lacked the springiness associated with the bread crumb of the sufficiently baked loaves that contained the other endoxylanase preparations.

5.2.6 Influence of dough pH on the action of endoxylanases

During the dough mixing stage samples (2g) were taken after mixing, at the completion of the first and second proofs and after baking for measurement of pH. The results of these analyses are presented in Table 5.1. The dough pH was measured to determine if the dough pH was impacting on the enzyme preparations used. The optimum pH for each of the enzymes is given in Table 5.1. A drop in pH of up to 0.5 was observed in the untreated loaves as well as the M1, M2, M4 and M5. Use of preparation M3 had an increase in pH after the second proof.

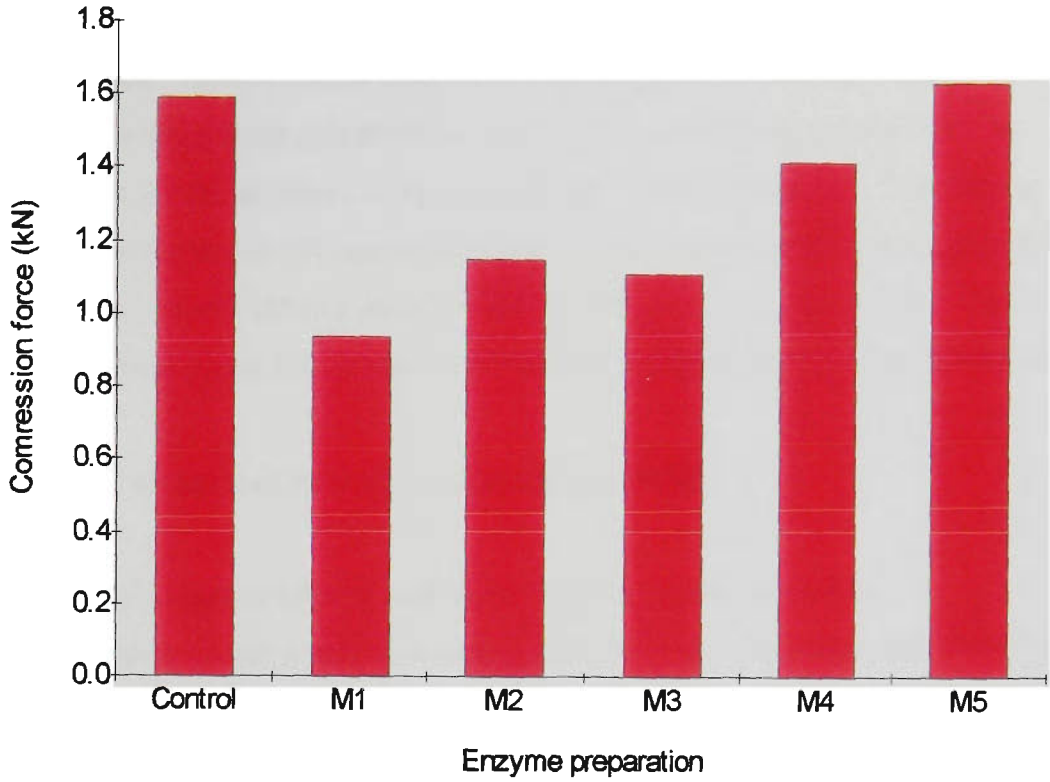


Figure 5.6 **Crumb softness of treated and untreated breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

Table 5.1 **pH of treated and untreated bread dough at four stages during processing**

	Control	M1	M2	M3	M4	M5
1	4.98	4.95	4.94	5.09	5.44	5.41
2	4.92	4.80	4.84	5.19	5.24	5.26
3	4.46	4.48	5.09	4.57	4.64	4.64
4	4.4	4.40	4.49	5.33	5.28	5.29
Optima		4.5	4.4	6.0	4.5	6.0

Note – Stages of processing are 1) At the completion of mixing; 2) After the first proof; 3) After the second proof 4) At the completion of baking

The pH optima, as reported by the supplier, for preparations M1, M2 and M4 are 4.5, 4.4 and 4.5 respectively while preparations M3 and M5 had pH optima 6.0. Breads treated with preparations M1 and M2 had crumb that was significantly softer than the control breads. The pH values measured in the dough at the various stage of development when these preparations were used do not vary as greatly from the optimum pH for the enzymes compared with the other preparations. This finding suggests that the dough pH may be impacting on the ability of the endoxylanases from preparations M3 and M5 to hydrolyse arabinoxylans in the dough system. The firmer crumb observed in the M4 breads was attributed to other factors as already discussed.

5.3 HPLC analysis of treated and untreated breads

5.3.1 Determination of total and water-soluble pentosan profiles

The total water-soluble pentosan content of the flour was measured using the method of Wootton et al 1995. Water- soluble pentosans were extracted using two different methods. The first method involved extracting pentosans in 80%v/v ethanol while incubating at 40°C. The supernatant obtained, termed water-soluble arabinoxylans (WSA) was retained for hydrolysis. In the second method pentosans were extracted with 80%v/v ethanol and incubating at 85°C for 1hr. After centrifugation, absolute ethanol was added to the supernatant to precipitate the pentosans. The pellet obtained after centrifuging was dissolved in distilled water and set aside for hydrolysis. This sample was designated as HWSA. A flour sample was also extracted in order to facilitate measurement of TP by hydrolysis with HCl in the distillation step without an intermediate extraction step.

Duplicate extract and flour samples were hydrolysed by distillation with 12% HCl. Samples were analysed using a Varian HPLC equipped with a UV detector. A C18-ODS reverse phase column was used with a methanol/water (10:90) isocratic mobile phase. 5-Hydroxymethyl-2-furaldehyde and furfural standard curves were used to determine the pentosan content of the flour. The average total pentosan content of the flour was found to be 2.5%. This figure is consistent with literature values for white flour which indicate that the pentosan contents are in the range of 2-4% (Meuser and Suckow 1988). The WSA content of the flour was 0.35% and the HWSA content was found to be 0.65%. Literature values for pentosans of wheat flour suggest that they

consist of approximately 2/3 water-soluble and 1/3 water-insoluble pentosans. The results of this analysis show that the hot water extraction resulted in a higher extraction rate of the water-soluble pentosans. For further calculations however the figures obtained for the first extraction method were used because the next stage of the study involved extracting arabinoxylans at 30°C.

5.3.2 Analysis of xylose and arabinose profile of breads using HPLC

Extraction of WSA was carried out using the protocol of Cleemput et al (1995). Initially 3 loaves of bread were prepared for each enzyme preparation. The WSA was extracted in water and the incubated with α-amylase from *A oryzae* to hydrolyse residual starch. Arabinoxylan was precipitated with ethanol to a final volume of 65%v/v. The final precipitate, termed TWSA was fractionated using absolute ethanol to give final fractions soluble at 0-30%, 30-50% and 50-65% ethanol. Fractions were designated as Flour 0-30%, Con 0-30%, M1 0-30% and so on depending on the preparation. A fraction of the remaining supernatant was also taken for analysis. Pentosan recovery rates are given in table 5.2. Analysis of the recoveries for each preparation showed an increase in WSA extracted from bread to which endoxylanase preparations M2, M3, M4 and M5 were added. Breads treated with preparation M1 had less WSA than the control loaves. Inherent losses during extraction and fractionation may account for this finding since breads treated with M1 and aged for 72 hrs had a higher level of WSA than the control breads. These losses also made it impossible to determine whether or not more WSA was being released during baking.

Samples of each TWSA fraction were hydrolysed using 0.1M sulphuric acid, cooled and filtered prior to analysis. Good separations of the reference sugars were achieved when pure standards were used and also in extracts of flour and breads. Some examples of the chromatographs obtained are presented in Figures 5.7 to 5.10.

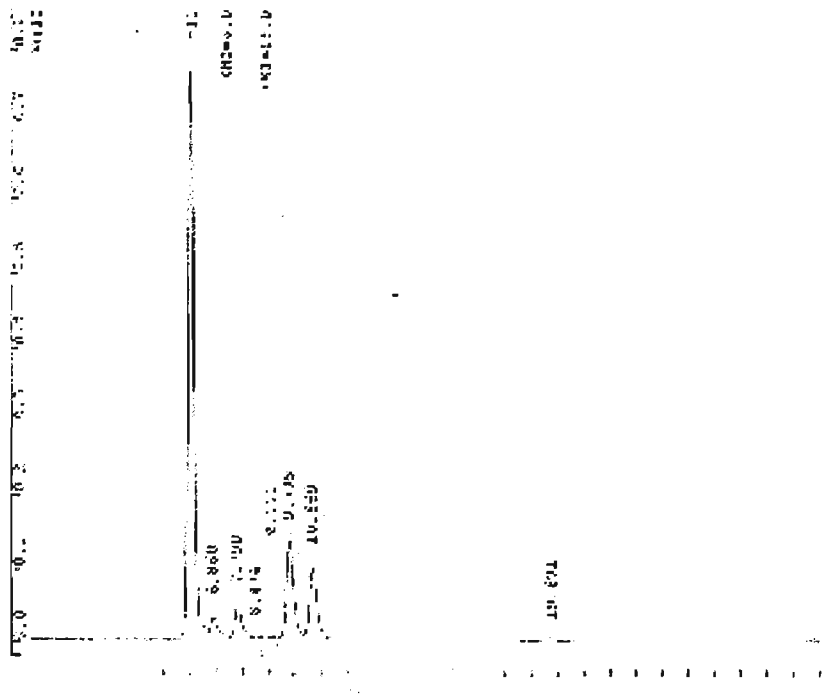


Figure 5.7 **Chromatogram obtained for HPLC analysis of flour pentosan fraction soluble in 0-30%v/v ethanol**

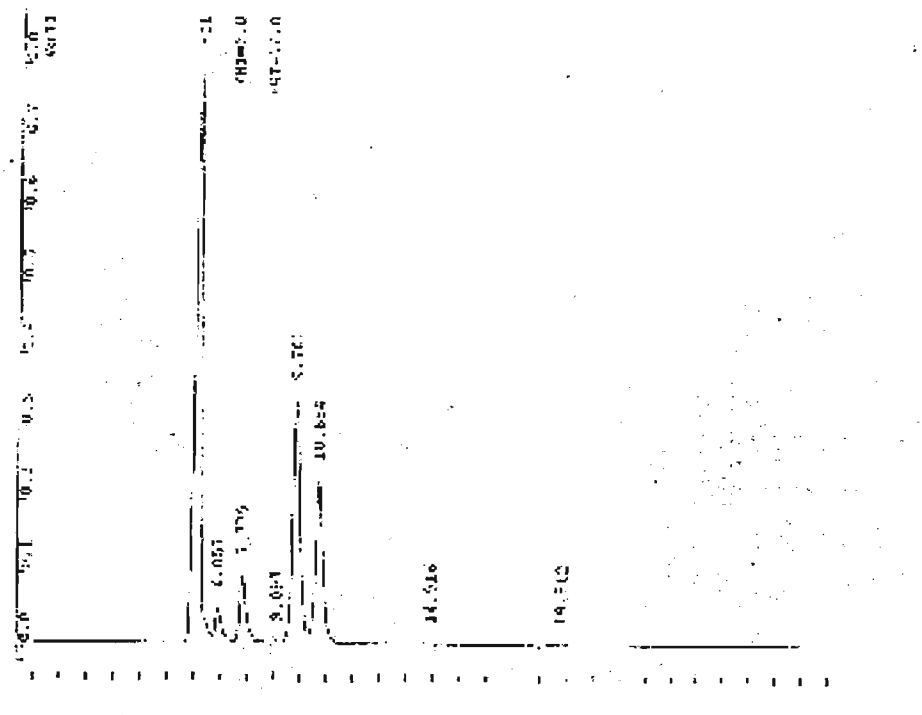


Figure 5.8 **Chromatogram obtained for HPLC analysis of flour pentosan fraction soluble a 30-50%v/v ethanol**

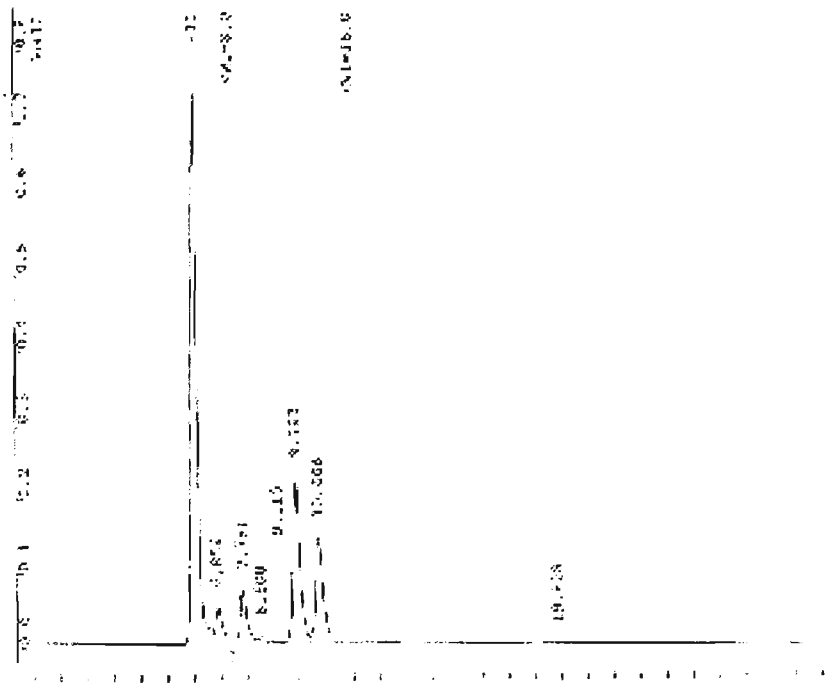


Figure 5.9 **Chromatogram obtained for HPLC analysis of pentosan fraction soluble in 0-30%v/v ethanol extracted from the untreated breads**

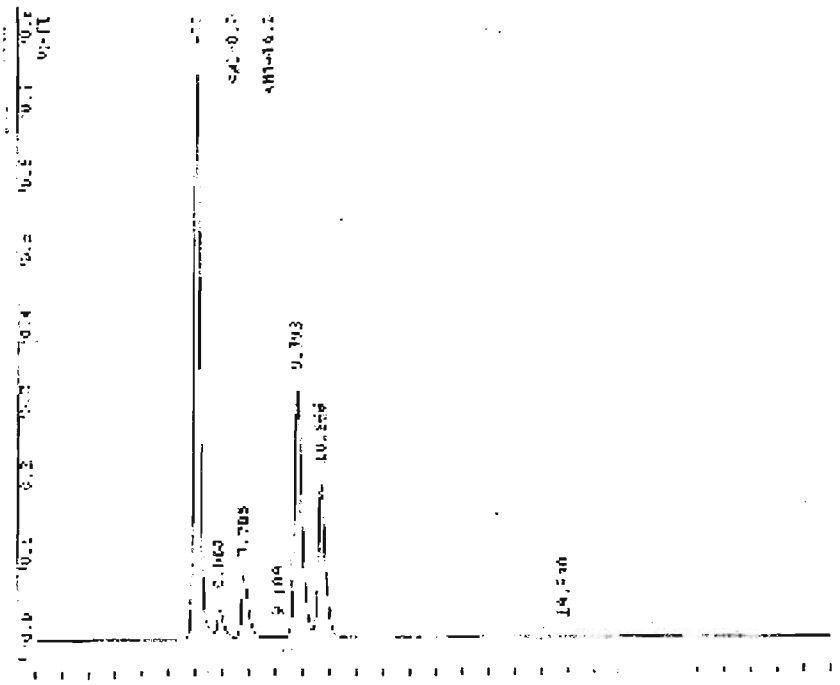


Figure 5.10 **Chromatogram obtained for HPLC analysis of pentosan fraction soluble in 30-50%v/v ethanol extracted from the untreated breads**

The peak area counts were used to plot standard curves using Microsoft Excel. A regression line fitted to data showed all standard curves to have correlation values greater than 0.98. Using the standard curves for each standard and the peak area counts of the samples the concentrations of xylose, xylobiose, xylotriose and arabinose were calculated. Small peaks corresponding to xylotetraose were also observed at levels of less than 0.002% and so were not included in the data. Finally the concentration values were used to calculate the total amount of each carbohydrate present per kg of flour.

The results of the HPLC analyses are presented in Table 5.3. Use of the different endoxylanase preparations in bread making resulted in a range of pentosan profiles being produced all of which differed from the control to varying degrees. Results are presented for pentosan fractions soluble at 0-30% ethanol and 30-50% ethanol. The results are based on breads prepared using 1 kg of flour and adjusted to a dry weight basis. In addition to xylose and arabinose small amounts of xylobiose and xylotriose were also obtained. These were not found to be present at significant levels and were assumed to be small amounts of xylose di-; and trisaccharides that failed to breakdown completely in the hydrolysis step. For the purpose of this study xylobiose and xylotriose have been included in the xylose results. Fractions obtained using 65%v/v ethanol yielded insufficient amounts of pentosan material to allow further analysis and so are not reported here. Fractions designated 65+ were also found to contain no arabinoxylan. In addition, galactose was not found at detectable levels. This finding is consistent with those of Cleemput et al (1993) who found that ethanol precipitation of arabinoxylan fractions to 65%v/v did not yield appreciable amounts of D-galactose.

Breads prepared using the basic bread formulation without any additional endoxylanase preparations had almost twice as much of the smaller or more soluble xylan molecules. Breads to which endoxylanases from M1 and M2 were added had a similar xylose profile with each having a higher level of the more soluble xylan molecules. Breads baked using the preparation M3 had almost three times the amount of xylose sugar soluble at 30-50%. Breads prepared using endoxylanases from M4 and M5 were found to have higher levels of the larger or less soluble xylan molecules, those soluble in 0-30% ethanol.

Table 5.2 **Recoveries of pentosans extracted from freshly baked breads using the basic bread formulation and breads treated with endoxylanase preparations**

	Weight (g/kg flour)	Recovery per step (%)	Overall recovery (%)
Untreated bread			
Total measured	2.91	-	-
Total extracted	1.65	56	-
Fraction 0-30%	0.84	32	29
Fraction 30-50%	0.53	27	18
Fraction 50-65%	0.06	3.7	2.1
Preparation M1			
Total measured	2.91	-	-
Total extracted	1.51	51	-
Fraction 0-30%	0.45	29	15
Fraction 30-50%	0.4	27	14
Preparation M2			
Total measured	2.91	-	-
Total extracted	1.58	54	-
Fraction 0-30%	0.44	28	15
Fraction 30-50%	0.45	29	15
Preparation M3			
Total measured	2.91	-	-
Total extracted	1.78	61	-
Fraction 0-30%	0.30	17	10
Fraction 30-50%	0.74	42	25
Fraction 50-65%	0.07	4	2.4
Preparation M4			
Total measured	2.91	-	-
Total extracted	1.77	61	-
Fraction 0-30%	0.75	42	26
Fraction 30-50%	0.39	22	13
Fraction 50-65 %	0.05	3	1.7
Preparation M5			
Total measured	2.91	-	-
Total extracted	1.70	58	-
Fraction 0-30%	0.81	48	28
Fraction 30-50%	0.54	32	19
Fraction 50-65 %	0.09	5	3.1

Table 5.3 Individual pentose sugars of bread and flour (g/kg)

Fraction	Xylose	Arabinose	Xyl:Ara
Flour			
0-30	0.11	0.05	2.2
30-50	0.33	0.11	2.3
Control			
0-30	0.18	0.10	1.8
30-50	0.35	0.22	1.9
M1			
0-30	0.20	0.10	2.2
30-50	0.27	0.12	1.9
M2			
0-30	0.23	0.11	2.1
30-50	0.27	0.13	2.0
M3			
0-30	0.12	0.06	2.2
30-50	0.36	0.20	1.9
M4			
0-30	0.44	0.30	2.0
30-50	0.26	0.14	2.0
M5			
0-30	0.41	0.21	2.0
30-50	0.32	0.17	1.9

A similar pattern emerged for the arabinose data. Again the controls and experimental loaves treated with preparations M1, M2 and M3 had higher levels of the more soluble arabinose molecules while breads treated with preparations M4 and M5 had more of the less soluble arabinose molecules. Statistical analysis of the data was not possible because the inherent product losses at each stage of the procedure made it impossible to accurately compare the raw data. To overcome this problem the ratio of sugars at the different solubility levels was determined. This allowed a direct comparison of the data. The last column of Table 5.3 shows the ratio of xylose to arabinose sugars. Statistical

analysis of this data showed no significant difference for the ratios obtained using the different preparations.

The ratio of xylose fractions is given in Table 5.4. Ratios show very different hydrolysis profiles for the different preparations used in this study. Analysis of the ratio of xylose sugars at the two solubility levels found that all the preparations except that designated M2, M4 and M5 had ratios that were significantly greater than that of the control breads. Significant difference was also observed between the experimental loaves. Breads prepared with preparation M4 and M5 had xylose ratios significantly different to those prepared with preparation M3 ($p<0.05$; Appendix 5.5).

Table 5.4 Ratio of xylose sugars for the different solubility levels

Treatment	Xyl 0-30%:Xyl 30-50
Control	0.32
M1	0.81[a b]
M2	0.85[a b]
M3	0.4[b]
M4	1.91[a b]
M5	1.3[a b]

a denotes xylose ratios of experimental breads significantly different to the control
b denotes xylose ratios of treated breads significantly different from other treated breads

5.4 Baking studies of the impact of endoxylanases on breads aged for 72 hrs

The effects of endoxylanases on the staling patterns of white bread were investigated. Compression measurements, colour parameters and were used to assess the application of these enzymes in the extension of shelf life of bread.

5.4.1 Changes in crumb colour in aged breads

Crumb colour data are presented in Figure 5.11. Minor variations were observed in the crumb whiteness as the bread aged. Breads treated with preparation M1 were significantly whiter than the M4 and M5 breads after 72hrs ($p<0.05$; Appendix 5.6). However there was no significant difference in crumb whiteness between the treated and untreated.

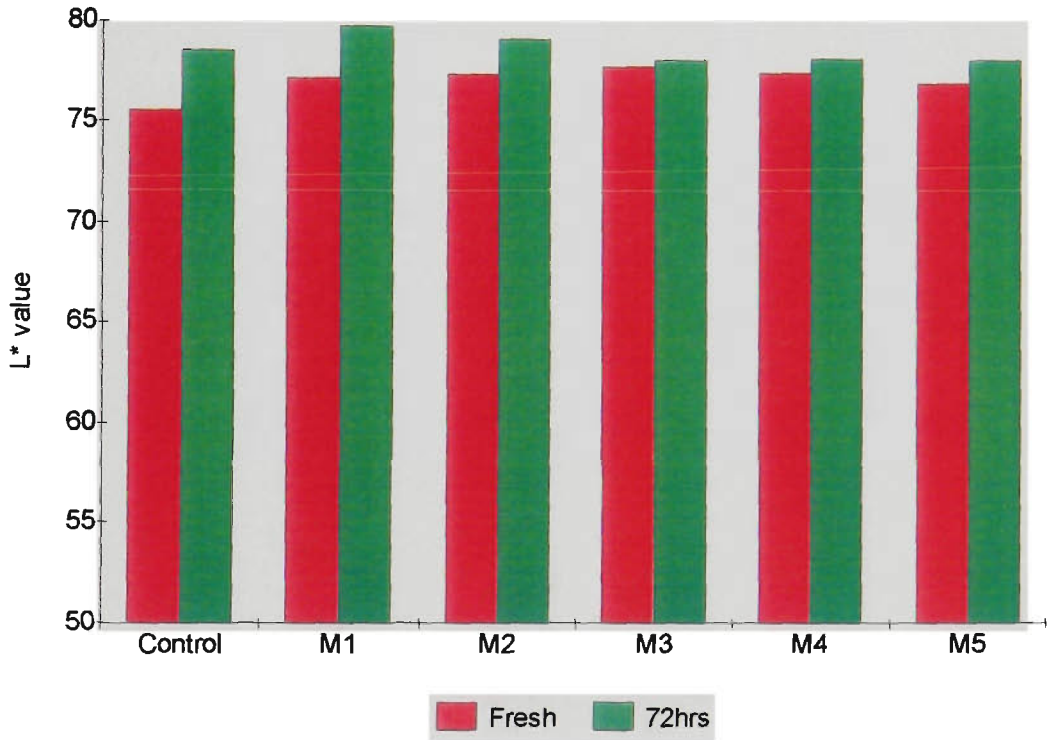


Figure 5.11 Crumb whiteness of fresh breads and aged breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)

Whilst bread crumb of the aged loaves did not appear visibly brighter a yellow tinge that had developed during aging was visible. This was observed for both the treated and untreated breads. Significant differences in crumb yellowing were detected using the Minolta Chroma Meter. The data (Figure 5.12) shows that bread crumb of the untreated breads was significantly more yellow than that of breads treated with M1, M2 and M3 ($p<0.05$; Appendix 5.7). No significant variation was observed between the different preparations and their ability to reduce yellowing over the aging period. Breads baked with the addition of endoxylanase from preparation M1 had a significantly more yellow

crumb in the aged bread compared with the fresh loaves ($p<0.05$). The use of endoxylanases from preparations M1, M2 and M3 also resulted in significantly less yellow crumb after aging ($p<0.05$) compared with the control. The development of a yellow crumb is a highly undesirable property in bread crumb. It results in reduced consumer acceptance, hence the ability of xylanases to reduce crumb yellowing, combined with improved stale retarding abilities may help extend the shelf life of loaf breads.

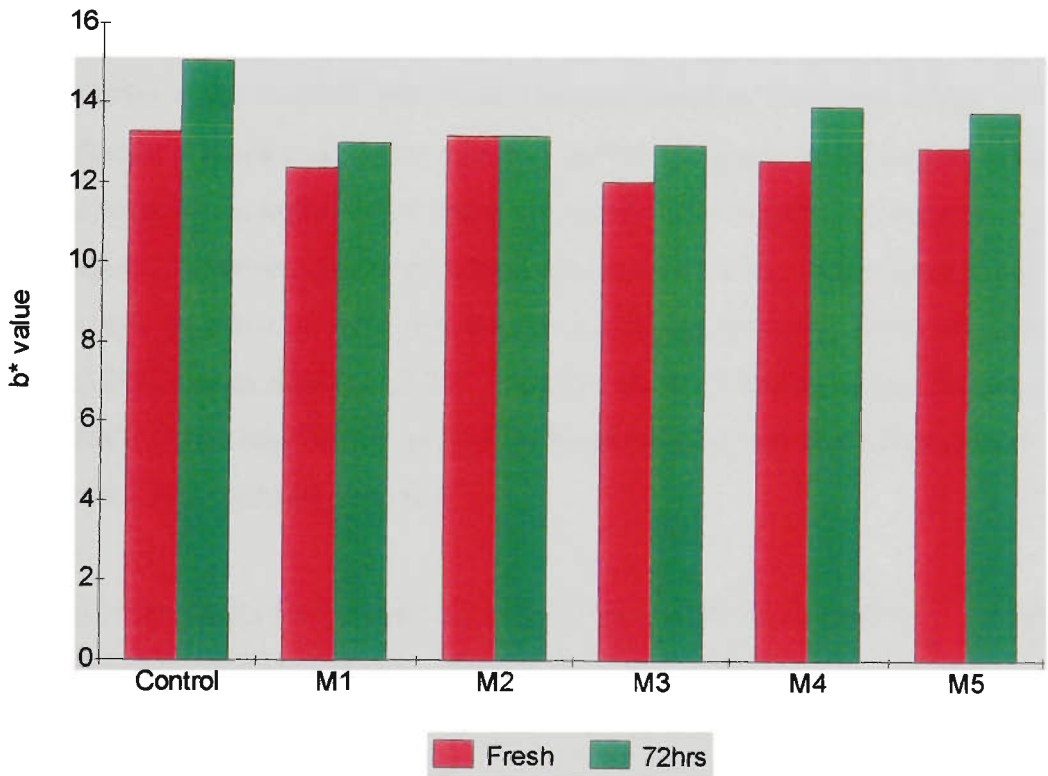


Figure 5.12 Formation of yellowness in crumb as bread ages breads baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)

5.4.2 Staling studies of breads prepared using purified endoxylanases

In order to assess the effects of endoxylanases on crumb firmness of breads a series of treated loaves was freshly baked over a period of 72hrs. Control loaves were also prepared to correspond with each day of the study. On the first day control and treated breads were prepared and stored in plastic bags at room temperature. These loaves corresponded to breads aged for 72hrs. The procedure was repeated on the second day and the breads stored and labelled as 48hrs loaves. On the next two consecutive days

the procedure was again repeated producing loaves that corresponded to breads aged for 24hrs and freshly baked loaves respectively. At the completion of the time frame compression measurements allowed the staling patterns of treated and untreated loaves to be assessed. The procedure was repeated to produce duplicate studies for the staling assessment. The average of these results is given Figure 5.13. The statistical data is presented in Appendix 5.8

The ability of endoxylanases from different fungal sources to impact on crumb softness in fresh bread has already been demonstrated in the current study (Figure 4.4.3). Further variation was observed between the ability of the different preparations to produce softer crumb in the freshly baked loaves as well as the ability of the preparations to maintain the softer crumb as the breads aged. Breads baked using only the basic bread formulation, were found initially to have the firmest crumb in the freshly baked breads. However after being allowed to age for 24 and 48 hrs these breads were found to have crumbs that were significantly softer than loaves to which preparations M4 and M5 had been added ($p<0.05$). Between 48 and 72hrs however, the control breads firmed more rapidly than any of the breads treated with the endoxylanase preparations during the same period.

As noted in the earlier Instron analysis only the use of endoxylanases from preparations M1, M2 and M3 produced bread crumbs in the freshly baked loaves that were significantly softer than the control breads. The staling data presented in Figure 5.13 shows that all three preparations had a positive effect on crumb firming throughout the study. These treatments resulted in bread crumb that was significantly softer than the control breads after 72hrs ($p<0.05$). In addition to this M2 produced a more consistent level of crumb firming throughout the trial. The M1 loaves exhibited a small increase in the firming rate between 24 and 48hrs, and a more rapid increase in firming in the final 24hrs of the trial. At the 48hr stage the loaves treated with M1 were significantly softer than the controls ($p<0.05$). These breads also had crumbs that were significantly softer than breads treated with preparation M5 after 72hrs ($p<0.05$).

The graph showed that the use of endoxylanases M4 and M5 resulted in no significant difference in crumb softness than the controls in both the fresh breads and those treated for 72hrs. However, after 24hrs both preparations had crumbs that were significantly

firmer than the control breads for the same period ($p<0.05$). The increase in crumb firmness for these two preparations was even further increased in breads aged for 48 hrs. At the completion of the staling study no significant difference was found in crumb firmness between the control loaves and those treated with preparations M4 and M5.

In the breads treated with M3, the fresh loaves and those aged for 72hrs had significantly softer crumb that the control breads ($p<0.05$). Although preparations M2 and M3 are obtained from the fungal species *T longibrachiatum*, they differ in their pH optima (4.5 and 6.0 respectively). The data obtained in this section suggest that the pH optima of a particular preparation may impact on its ability to retard crumb firming as bread ages.

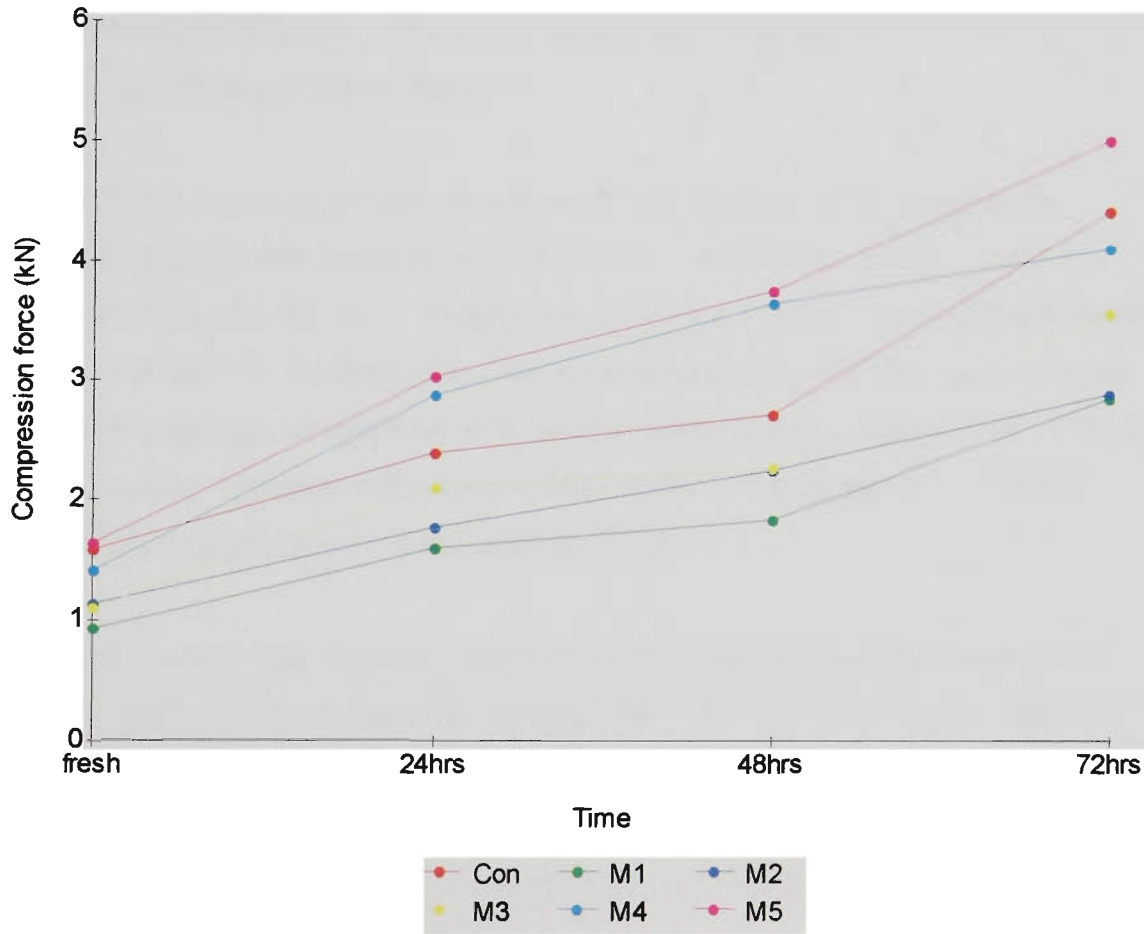


Figure 5.13 Staling profiles of treated and untreated breads aged over a period of 72hrs baked using purified endoxylanases from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)

The data presented here has shown that the use of endoxylanases from different sources in bread making can produce significant changes in the compression profile of the bread crumb as it ages. Endoxylanases found in preparation M1 were sourced form *T viride*, M2 came from *T longibrachiatum* as mentioned and preparations M4 and M5 were from *A niger* and *H insolens* respectively. It appears from the data that whilst initially producing a softer crumb endoxylanase from *T viride* was unable maintain a regular staling profile as the bread aged. The use of endoxylanases from *A niger* and *H insolens* did not result in significantly softer crumb at any stage of the staling study.

5.5 HPLC analysis of breads aged for 72hrs

A change at a molecular level in pentosan profile as the breads aged was also investigated. Breads were prepared and allowed to age for a period of 72hrs. Extraction and fractionation of the arabinoxylans was by the method of Cleemput et al (1995) as already described in Chapter 3.

Total pentosan recoveries of fresh breads and breads aged for 72 hrs are given in Table 5.5. Significantly higher levels of WSA were observed in both the treated and untreated breads after 72 hrs. A comparison of the total WSA for fresh and aged breads is given in Table 5.6. Analysis of the data showed a significantly higher level of water extractable pentosans were present in the breads to which preparations M2, M3, M4 and M5 were added. Increases in the water-soluble arabinoxylan extracted from breads using these preparations were in the range of 17-24%.

Endoxylanases are effective pentosanases hydrolyzing both water-soluble and water insoluble arabinoxylans during bread making (van Oort et al 1995). Flour contains low levels of endogenous enzymes that exhibit xylanase activity. Between 15-20% of water insoluble pentosans are solubilised due to the actions of these endogenous enzymes. However, this figure can increase to an estimated 40-65% solubilisation when endoxylanase preparations are added to a bread formulation (van Oort et al 1995). Initially the results of this study suggest residual enzyme activity may be occurring in bread crumb as it ages. However, since each of the enzymes used is inactivated at temperatures reached during baking, a more probable explanation is that chemical and physical changes occurring in the bread crumb render the WSA and WISA more readily extractable. The significantly higher level of pentosan material extracted from some of

the treated loaves suggests that the addition of specific endoxylanases enhances the hydrolysis of water insoluble arabinoxylans as bread ages ($p<0.05$; Appendix 5.10). In addition to this the breads aged for 72 hrs were found to have higher levels of the smaller or most soluble fraction of pentosans, those soluble in 50-65% ethanol v/v.

HPLC analyses of the individual fractions are given in Tables 5.7 and 5.8. The data showed that separation of the pentosans into individual fractions produced similar results to those observed in the fresh breads with the exception of the control breads that had almost equal amounts of xylose sugars in the 0-30% and 30-50% solubility range. The same trend seen in the fresh breads was also evident in the aged loaves. Breads treated with preparations M1, M2 and M3 had higher levels of the more soluble xylose, that soluble in 30-50% ethanol. The remaining breads, those treated with preparations M4 and M5 had more of the larger arabinoxylan molecules. In addition to this use of the preparations M2 and M4 resulted in the formation of the most soluble pentosan molecules, those soluble in 50-65% ethanol.

Table 5.5 **Recoveries of pentosans extracted from breads baked using the basic bread formulation and added endoxylanases and aged for 72 hrs**

	Weight (g/kg flour)	Recovery per step (%)	Overall recovery (%)
Untreated breads			
Total measured	2.91		
Total extracted	1.85	64	
Fraction 0-30%	0.72	40	25
Fraction 30-50%	0.51	28	18
Preparation M1			
Total measured	2.91	-	-
Total extracted	1.98	68	
Fraction 0-30%	0.39	44	13
Fraction 30-50%	0.40	45	14
Preparation M2			
Total measured	2.91	-	-
Total extracted	2.57	88	
Fraction 0-30%	0.66	26	23
Fraction 30-50%	0.82	32	28
Preparation M3			
Total measured	2.91	-	-
Total extracted	2.35	81	64
Fraction 0-30%	2.02	86	-
Fraction 30-50%	0.08	3.4	2.8
Preparation M4			
Total measured	2.16	-	-
Total extracted	2.54	87	
Fraction 0-30%	1.15	45	40
Fraction 30-50%	0.379	15	13
Fraction 50-65 %	0.281	11	9.6
Preparation M5			
Total measured	2.91	-	-
Total extracted	2.53	87	
Fraction 0-30%	0.70	28	24
Fraction 30-50%	0.73	29	25

Table 5.6 Comparison of total pentosan recoveries for fresh breads and those aged for 72 hrs

	Fresh bread		Bread aged for 72 hrs	
	Pentosan extracted g/kg flour	**% Recovery**	**Pentosan extracted g/kg flour**	**% Recovery**
Control	1.65	56	1.85	64
M1	1.51	51	1.98	68[a]
M2	1.58	54	2.57	88[a]
M3	1.78	61	2.35	81[a]
M4	1.77	61	2.54	87[a]
M5	1.70	58	2.53	87[a]

[a] denotes percentages that are significantly different to the corresponding fresh breads ($p<0.05$; Appendix 49).

Table 5.7 **Contents of xylose and arabinose monomers after extraction and analysis by HPLC for breads aged for 72 hrs**

Fraction		Xylose	Arabinose	Xyl:Ara
Control				
	0-30	0.30	0.01	30
	30-50	0.30	0.24	1.25
M1				
	0-30	0.14	0.07	0.22
	30-50	0.18	0.83	0.22
M2				
	0-30	0.23	0.01	23
	30-50	0.46	0.24	1.9
	50-65	0.21	0.04	5.3
M3				
	0-30	0.38	0.20	1.9
	50-65	0.54	0.30	1.8
M4				
	0-30	0.47	0.22	2.1
	30-50	0.22	0.11	2.0
	50-65	0.14	0.08	1.8
M5				
	0-30	0.47	0.19	2.5
	30-50	0.18	0.01	18

Table 5.8 Ratios of the solubility fraction of xylose and arabinose

Sample	Xylose 0-30:30-50	Arabinose 0-30:30-50
Control		
0-30 - 30-50%	0.99	0.05
M1		
0-30 – 30-50%	0.78	0.08
M2		
0-30 – 30-50%	0.5	0.4
30-50–50-65%	2.1	6.0
0-30 – 50-65%	1.1	2.5
M3		
0-30 – 50-65%	0.7	0.7
M4		
0-30- 30-50%	0.21	2.0
30-50- 50-65%	1.6	1.4
0-30 - 50-65%	0.34	2.8
M5		
0-30 – 50-65%	2.6	19

5.6 Sensory evaluation of baked loaves

In this section of work a sensory evaluation was carried out to determine if differences detected in crumb softness using the Instron UTM could also be detected by a group of panelists. An untrained panel was selected to simulate normal consumers. Panelists were representative of a wide cross section of society coming from many and varied cultural backgrounds. Cultures from which panelists were selected included Australian, Indian, Turkish, German, Sri Lankan, Vietnamese, American and Italian. A brief survey was also conducted to ascertain panelist preferences for different types of bread and bread sources. The results of this survey are given in Table 5.9. Generally most of the panelists selected white bread as their first preference with average bread consumption being 1-2 slices per day.

Panelists were presented with six bread samples containing a control sample and one sample for each of the treatments used. Panelists were asked to firstly to compress the samples gently and then to bite into each sample. They recorded their findings based on the perceived crumb softness on a scale of 1-5 with 1 representing the firmest crumb. The results of this evaluation appear in Table 5.11. In addition to scaling the samples according to softness panelists were also asked to comment on any other aspects of the samples they felt relevant. To avoid influencing the panel the breads were given identification numbers and set up in random order. A listing of the identification numbers is given in Table 5.10.

Table 5.9 Survey results

Attribute	Proportion preferred (%)
Bread Type	
White bread	78
Wholemeal bread	11
Multigrain bread	5
Flat bread	-
Specialty bread (vogel, soy & linseed etc.)	-
Rye bread	5
Source	
Hot bread bakery (Brumbies, Bakers delight)	60
Commercial bakery (Sunicrust, Buttercup)	10
Home made	30
Amount consumed per day	
< 1slice	
1-2 slices	43
3-4 slices	27
4+ slices	30

Table 5.10 Treated and untreated breads and their identification numbers

Treatment	Identification number
Control	S617
M1	S435
M2	S291
M3	S784
M4	S329
M5	S187

Table 5.11 Results of sensory analysis of bread samples

Sample name	Sample I.D.	Bite test	Squeeze test
Control	S617	4.15	4.11
M1	S435	3.65	3.56
M2	S291	3.85	3.87
M3	S784	3.30	3.32
M4	S329	3.81	3.52
M5	S187	3.70	3.64

The results indicate that for both of the tests carried out by the panelists the control loaves were perceived to have the softest crumb. However when the data was analysed further these differences were not found to be significant. This finding was supported by comments made by a number of panelists that the differences observed between crumb softness of the samples were at best very small. Other panelists noted that it was difficult to detect any differences between the different samples. One of the aims of this study was to determine whether or not the addition of purified endoxylanases to a commercial bread formulation could produce significant changes in bread crumb that would be perceived by the consumer. The results of the sensory evaluation show that despite a significant reduction in crumb firmness measured in compression tests for some endoxylanases, these changes were not as obvious when subjected to sensory analysis.

Another common comment from panelists was that there was no detectable difference in flavour between the samples presented. Flavour and aroma are highly regarded attributes of bread (Martinez-Anaya 1996). The subtle flavour of bread is made up of many components that act synergistically relative to their proportions (Coffman 1965). These components arise in the bread from fermentative and thermal reactions during dough proofing and baking. The use of enzymes to replace other improving agents in bread making may indirectly influence bread flavour by modifying molecular components that take place in fermentation and baking processes. The ability of lipases, proteases and amylases to contribute to the formation of flavour and aroma compounds has been reported (Baker et al 1953, Maga 1974, Pomeranz and Finney 1975). The

results of the current study suggest that increased levels of WISA and WSA degradation components do not impact on bread flavour to any significant degree however the effects of these compounds on the formation of flavour and aroma volatiles requires further investigation.

5.7 The application of fungal endoxylanases in yeast leavened doughnuts

Having demonstrated the ability of certain endoxylanases to contribute to enhanced crumb softness and keeping properties in baked breads the final stage in this section of the study was to examine the effects these enzyme preparations have on crumb and crust characteristics of other baked goods. Yeast leavened doughnuts were selected for this study because they utilize a similar formulation to that of the breads and also have a fermentation period. The formulation is given in Chapter 3, section 3.2.24.

Visual observations of the untreated doughnuts showed a crumb structure that was dense and compact with a regular dispersion of gas cells (Figure 5.14). When each of the xylanase preparations was added to the basic formulation the resulting final products all exhibited crumbs that were slightly undercooked in the centre although the cooked part of the crumb was comparable to the untreated doughnuts. To investigate this further another series of treated doughs were prepared and the frying time increased to 5min per side. Visual inspection of the doughs again showed crumbs that were slightly undercooked in the centre. It was not considered practical to further increase the frying time at this point because the resulting darker crusts were approaching an unacceptable colour. The doughnuts were analysed for crumb softness using the Instron UTM and the result of this analysis are presented in Figure 5.15.

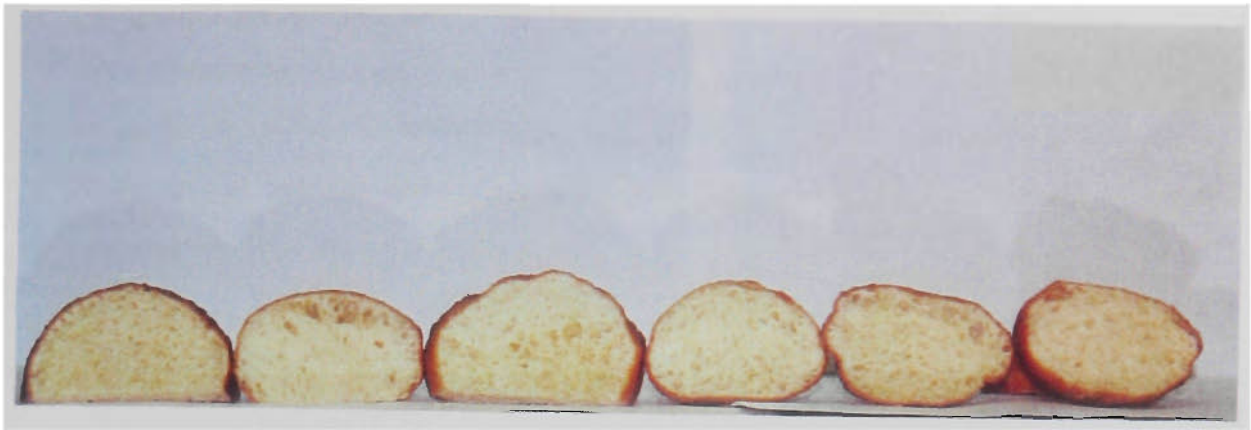


Figure 5.14 **Treated and untreated doughnuts prepared using endoxylanase preparations from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

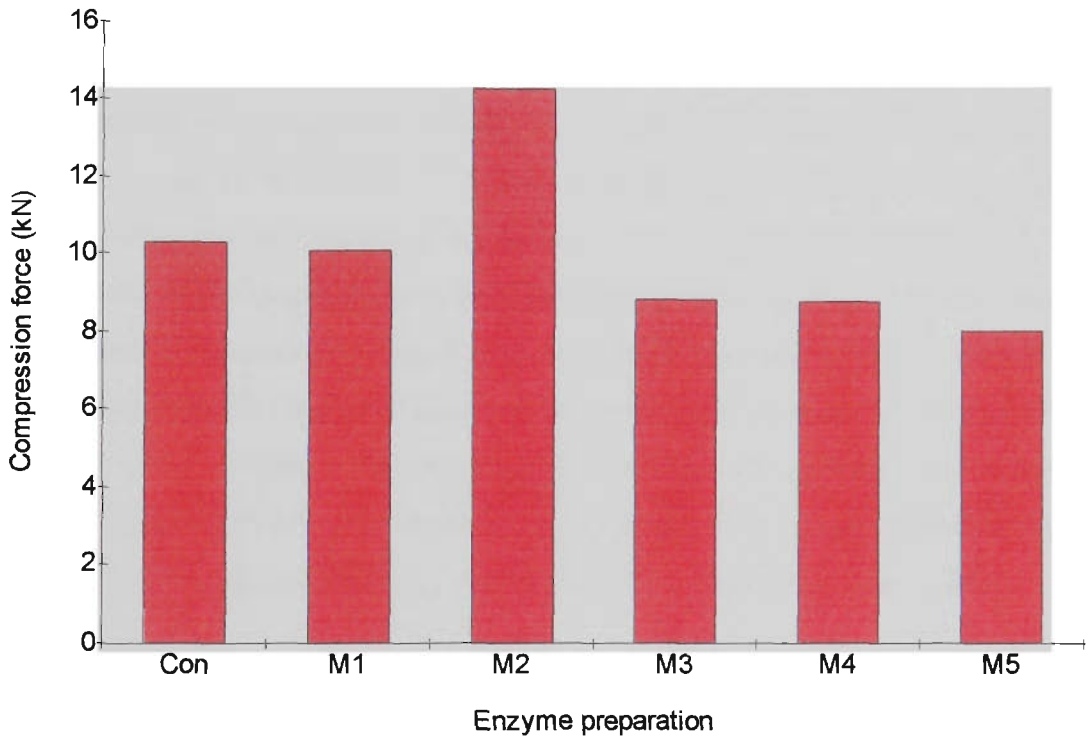


Figure 5.15 **Compression analysis of doughnuts treated with endoxylanase preparations from *T viride* (M1), *T longibrachiatum* (M2 and M3), *A niger* (M4) and *H insolens* (M5)**

No significant differences in crumb softness were observed between the untreated doughnuts and those to which the preparations M1, M3 and M4 were added. Doughnuts treated with preparation M5 were found to have a significantly softer crumb than the controls ($p<0.05$; Appendix 5.9)). Those to which preparation M2 was added had a crumb that was significantly firmer that the control doughnuts as well as those treated with the other preparations ($p<0.05$).

5.8 Discussion

The addition of pentosans, both the water-soluble and water insoluble fraction to bread formulations has been extensively investigated often with varied and conflicting results (Hoseney et al 1984, Kim and D'Appolonia 1977, Kulp 1967, Schiraldi et al 1996, Stear 1990). During breadmaking modification of arabinoxylans may occur as a result of the action of enzymes broadly referred to as pentosanases. Of these, the enzyme most thoroughly researched is 1,4-β-D-Xylan xylanohydrolase (EC 3.2.1.8). This is an endohydrolase and is here referred to as endoxylanase. It appears that in wheat flour doughs this enzyme tends to solubilise the WISA fraction producing high molecular weight WSA. Work carried out by Courtin et al (1999) demonstrated the ability of endoxylanses to improve loaf volume by decreasing the amount of WISA in the dough and increasing the level of WSA. However it was also shown that extensive hydrolysis of the WISA and degradation of solubilised fractions decreased loaf volumes. Enzymatic solubilisation of arabinoxylans requires the presence of endoxylanase. The final level of solubilisation might be influenced by a number of factors including enzyme specificity, degree of access to the arabinoxylan chains and level of treatment (Rouau 1993). Recent studies have also indicated that the presence of endogenous endoxylanase inhibitors in wheat flour may impact on the degree of arabinoxylan solubilisation and degradation (Debyser et al 1999, Ingelbrecht et al 2000).

In the current study a range of highly purified endoxylanase preparations has been used in a series of baking trials. The aim was two-fold. Initially the study investigated and compared the impact of a range of highly purified endoxylanases on wheat pentosan structure. These changes were then related to textural properties of freshly baked breads. In the second stage of the study changes in loaf properties during aging were investigated

The use of endoxylanases at the addition levels used in this study did not impact on the final loaf volume of baked breads (Figure 5.2). The slightly larger volumes observed for the treated loaves were not significant. This finding was contrary to those of van Eijk and Hille (1996) who found that bread volume was enhanced by the action of endoxylanases. The use of commercial enzyme preparations containing a range of side activities has been shown to produce breads with increased loaf volumes (Karmel and Stauffer 1993, van Oort et al 1995). Work carried out by Courtin et al (1999) involved fractionating wheat flour into gluten, prime starch, a squeegee fraction containing most of the water insoluble arabinoxylan and a water extractable pentosan fraction. The latter two fractions were treated with endoxylanases and all the fractions reconstituted for bread making. The results showed that endoxylanases exert a positive effect on loaf volume by lowering the concentration of water insoluble arabinoxylans thus increasing the level of soluble pentosans. It was also found that significant breakdown of solubilised arabinoxylans had a negative impact on loaf volume. The researchers suggested that the ratio of solubilising to degrading activity contributed to the volume increasing effects of endoxylanases. The current study involved the addition of endoxylanase preparations directly to the bread formulation to simulate commercial practices. The results suggest that the action of endoxylanases on water-soluble and insoluble arabinoxylans alone in the dough system is not sufficient to contribute to the enhanced loaf volumes observed by other researchers. A number of possibilities may account for this finding. Differences in flour strength, ability of enzymes to access the arabinoxylan and reduced mixing and proofing periods inherent in rapid dough methods may all have affected enzyme activity in this work. The action of endoxylanases in a commercial breadmaking application and the specific interaction between the products of arabinoxylan hydrolysis and other dough components that may enhance loaf volume require further investigation.

Significant moisture loss was observed in the breadcrumb (Figure 5.1) together with a corresponding increase in crust moisture evidenced by the development of a soft and leathery crust. As bread ages the moisture holding capacity of the starch granules decreases (Kulp and Ponte 1981). Water molecules are strongly attracted to gluten and some other crumb components thereby increasing crumb firmness. van Eijk and Hille (1996) suggest that starch crystallization and crumb firming are dependent on the

amount of free water present and that even a small change in this water content may affect crumb softness. Zeleznak and Hoseney (1986) showed that the presence of free water in bread during aging rather than that present at gelatinisation was a controlling factor in retrogradation. The results of the moisture data for this study clearly demonstrate that whilst the amount of free water present in fresh breads may impact on initial crumb softness the final moisture content does not play a significant role in the staling of bread crumb.

In relation to the development of crust colour a wide level of variation was observed. Use of all of the preparations except M5 resulted in significantly darker crust being recorded compared with the control breads. Crust browning is due to a complex series of reactions that occur during baking. At temperatures of around 100°C sugar/protein interactions produce melanoidin compounds in a process known as Maillard browning. As the baking temperature exceeds 150°C the crust acquired a brown colouration due to caramellisation. Dextrins from starch degradation contribute to the formation of crust aroma at this temperature due to the formation of volatile carbonyl compounds (Stear 1990). To date little work has been carried out into the effects of arabinoxylan hydrolysis and the formation of crust colour. Around 2-3% residual sugars are required for caramelisation to occur.

The ability of endoxylanases to hydrolyse water soluble pentosans has been clearly shown. The increased level of the smaller or most soluble xylose arabinoxylans, those soluble at 50-65% v/v ethanol in the aged breads indicates that theses molecules are continuing to be broken down during aging. This is most likely due to the presence of residual enzyme activity.

Analysis of the ratios of the xylose fractions (Table 5.4) shows that breads with xylose ratios approaching one, those made with preparations M1 and M2, had crumbs that were significantly softer than that of the control loaves. Breads that had xylose ratios well in excess of one, those baked with preparations M4 and M5, had crumbs that were not significantly softer than the control breads. Breads made with preparation M3 had a xylose ratio similar to that of the control breads and were also found to have a crumb that was significantly softer than the control. Preparations M4 and M5 were obtained

from different sources to those of M1, M2 and M3. The latter were all sourced from *Trichoderma sp.* while preparations M4 and M5 were sourced from *A niger* and *H insolens* respectively.

Compression measurements carried using the Instron UTM were not consistent with the findings of the sensory panel. Panelists were unable to identify any significant variation in crumb texture between any of the loaves. Most of the panelists found the control breads to have the softest crumb with the breads having the added preparation M1 being the next softest. A study carried out by Brady and Mayer (1985) measured correlations between sensory and instrumental methods when assessing bread texture. The study showed only weak correlations to exist between sensory and compression data. They suggested that bread texture may alter when chewed due to the presence of moisture and temperature in the mouth making it difficult to compare a sample that has been chewed to one that has been compressed by a flat metal press. Thus the test conditions of the samples for the sensory and compression analyses are not identical. A similar explanation may also explain the differences observed when the panelists compressed the samples. Body temperature combined with the amount of force applied by each individual would also alter the test conditions to which the breads are being subjected. In addition to this the fact that the panel was untrained may have also influenced the result.

Finally the addition of endoxylanases to other baked goods was investigated. The results showed that the positive impact observed in white bread did not translate to other baked goods, in particular, doughnuts. The doughy crumb texture observed in the doughnuts suggests that increasing the level of WISA in conjunction with hydrolysis of WSA can impact negatively under different formulation and processing conditions.

Whether the results observed for this section of the study were the result of an inappropriately high dosage, shorter kneading time leading to an underdeveloped gluten network or due to the presence of one or more ingredients not used in the bread making trials requires further study.

In conclusion the current study has demonstrated the ability of endoxylanases from different fungal sources to impact on white bread produced under Australian bread making conditions. The degree of impact was influenced by several factors including dough pH, treatment level and pH optima of the different endoxylanases. Correlations were observed between the dough pH and crumb softness with softer crumb being observed in breads treated with endoxylanases whose pH optima approached that of the bread dough prior to baking.

Chapter 6

Results and discussion – Changes in crust and crumb characteristics when purified endoxylanase and arabinanase are added to a basic bread formulation

6.1 Introduction

Arabinoxylans are large molecules with relative molecular weights of up to 500,000. The structure consists of repeating units of β-D-xylopyranosyl units to which single α-L-arabinofuranosyl units are substituted at the C-2 or C-3 position (Hoseney,1994). Ferulic acid residues are further esterified to some arabinose units where they contribute to the oxidative gelation of pentosans. Acetyl groups, glucuronopyranosyl residues and their methyl esters can also be found substituted to the xylose backbone (van Oort et al 1995). The complete hydrolysis of these large molecules requires the use of a number of hydrolytic enzymes including xylanase, xylofuranosidase, arabinofuranosidase and ferulic acid esterase (Saha 2000). In the study so far the effects of individual endoxylanases have been studied on bread characteristics of crust colour, loaf volume and crumb structure and colour. Significant differences have been observed for a number of these parameters when purified endoxylanases from a number of fungal sources were added. It was found that the source of the enzyme as well as individual differences such as pH optima and activity level results in significant differences in crumb and crust characteristics. In the next part of the current study the effects of combined α-L-*endo*-arabinofuranosidase and endoxylanase preparations on bread properties were investigated. The structure of arabinoxylan suggests that α-L-*endo*-arabinanase does not have a role to play in the hydrolytic break down of arabinoxylan. However since much of the research available on the structure of arabinoxylan is based on European flours the possible role of *endo*-arabinanase in the hydrolysis of arabinoxylan in Australian wheat flour was investigated in this section of the study.

6.2 Effects of *endo*-arabinanase and α-L-arabinofuranosidase on crumb and crust characteristics of white bread

In order to establish what if any changes were attributable to the combination of these pentosanases the effects, if any, of adding *endo*-arabinanase and α-L-arabinofuranosidase only to a bread formulation were explored. Breads were prepared in triplicate using the basic bread formulation described in Chapter 3. Based on baking studies reported in chapter 4 treatments consisted of adding 5μL of either an *endo*-arabinanase preparation sourced from *A niger* or an *endo*-arabinofuranosidase also from *A niger* to the bread formulation. The data obtained from this section of the study in conjunction with data obtained when endoxylanase were incorporated into a bread formulation in the previous chapter provided a sound basis against which the composite formulations could be compared.

6.2.1 Effects of *endo*-arabinanase and α-L-arabinofuranosidase on loaf volume

Breads treated with *endo*-arabinanase and arabinofuranosidase at a level of 5μL, all had volumes greater than the commercially acceptable minimum value of 19. However no significant differences were found between the loaf volumes of the treated and control breads (Appendix 6.1). The results of this section of the study are presented in Figure 6.1.

The addition of *endo*-arabinanase and α-L-arabinofuranosidase to the bread formulation (Figure 6.2) resulted in the development of bread crusts that were significantly darker than that of the untreated breads($p<0.05$; Appendix 6.2). When crumb brightness was examined no visible or measurable difference was found to exist for either treatment compared with the control breads (Appendix 6.3). The results are given in Figure 6.3.

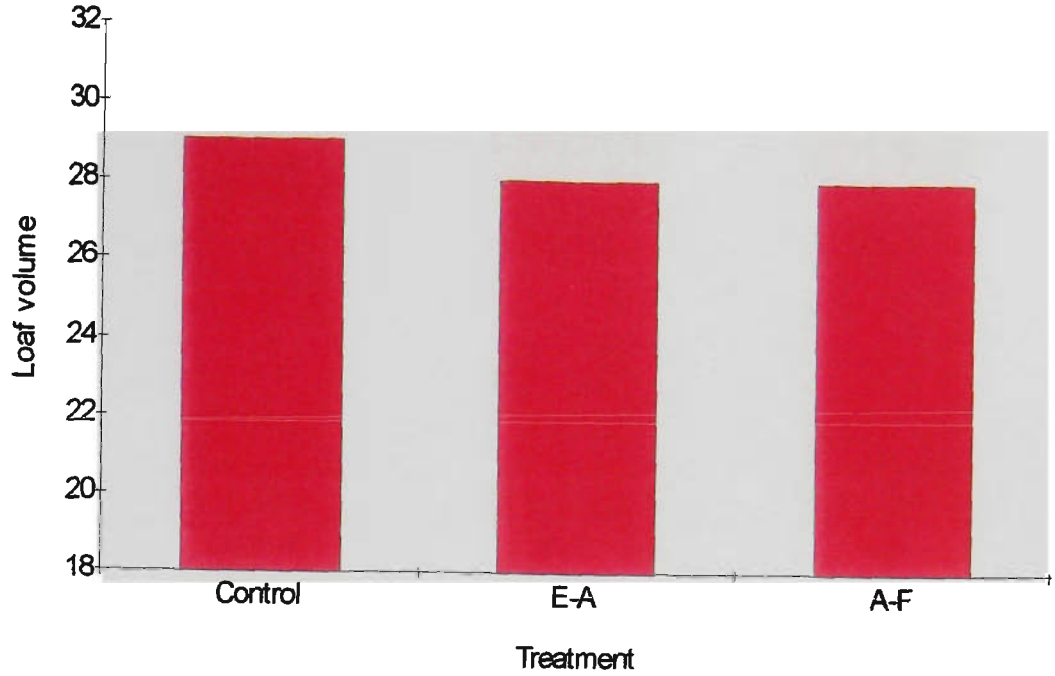


Figure 6.1 **Final loaf volume scores of breads treated with *endo*-arabinanase (E-A) and α-L-arabinofuranosidase (A-F)**

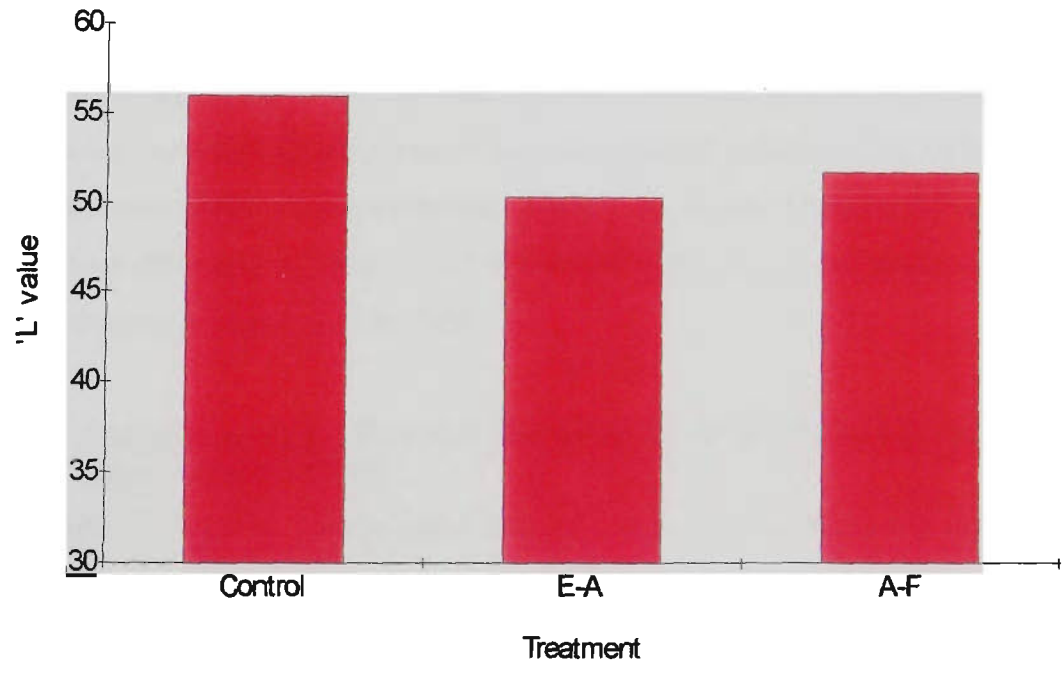


Figure 6.2 **Evaluation of crust colour of breads treated with *endo*-arabinanase (E-A) and α-L-arabinofuranosidase (A-F)**

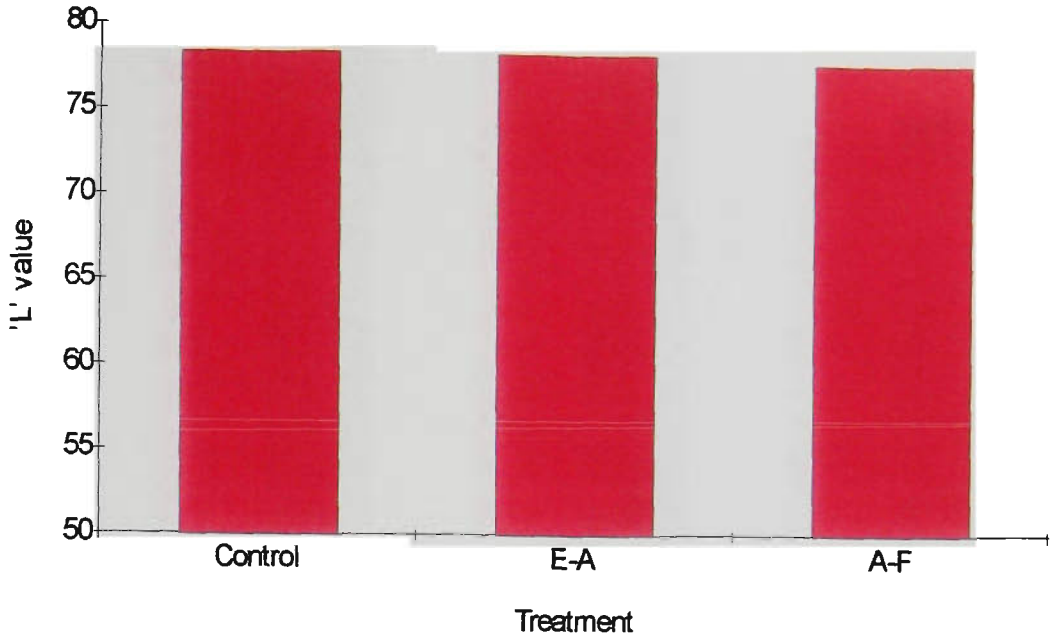


Figure 6.3 **Evaluation of crumb brightness for breads treated with *endo*-arabinanase (E-A) and α-L-arabinofuranosidase (A-F)**

Finally the development of a yellow crumb during aging was investigated. The results are presented in Figure 6.4. In the freshly baked breads those treated with arabinofuranosidase were significantly less yellow than the control breads ($p<0.05$; Appendix 6.4). In the breads that had aged for 72 hrs the same treatment resulted in breads that were significantly less yellow than both the control breads and those treated with the *endo*-arabinanase preparation. The use of *endo*-arabinanase did not produce a significant difference in the fresh bread but after 72hrs was significantly less yellow than the control breads aged for 72hrs.

6.2.2 Moisture content of treated and untreated breads following baking and also after storage for 72hrs

The addition of arabinofuranosidase and *endo*-arabinanase to the bread formulation resulted in significantly more water being retained in the crumb during aging ($p<0.05$; Appendix 6.5). A possible explanation for this finding is that the use of both *endo*-arabinanase and arabinofuranosidase may result in some debranching of some of the arabinose residues thus increasing the water binding capacity of the arabinoxylans. The results of the moisture analyses are presented in Figure 6.5

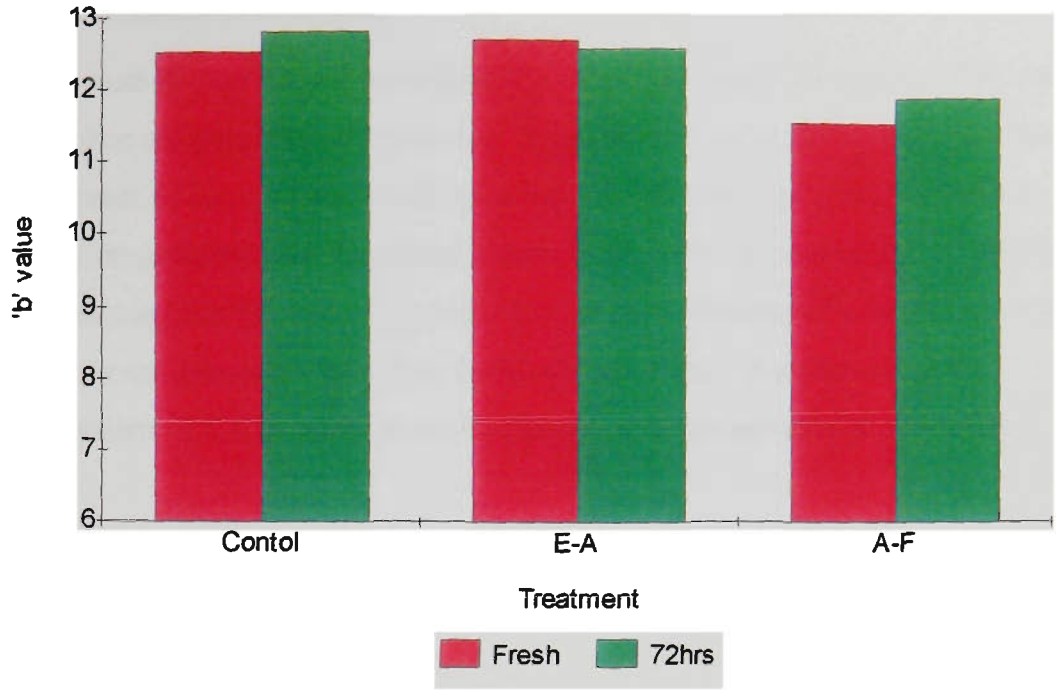


Figure 6.4 **Evaluation of the development of yellow crumb as bread ages for breads treated with 5µL *endo*-arabinanase (E-A) and 5 µL α-L-arabinofuranosidase (A-F)**

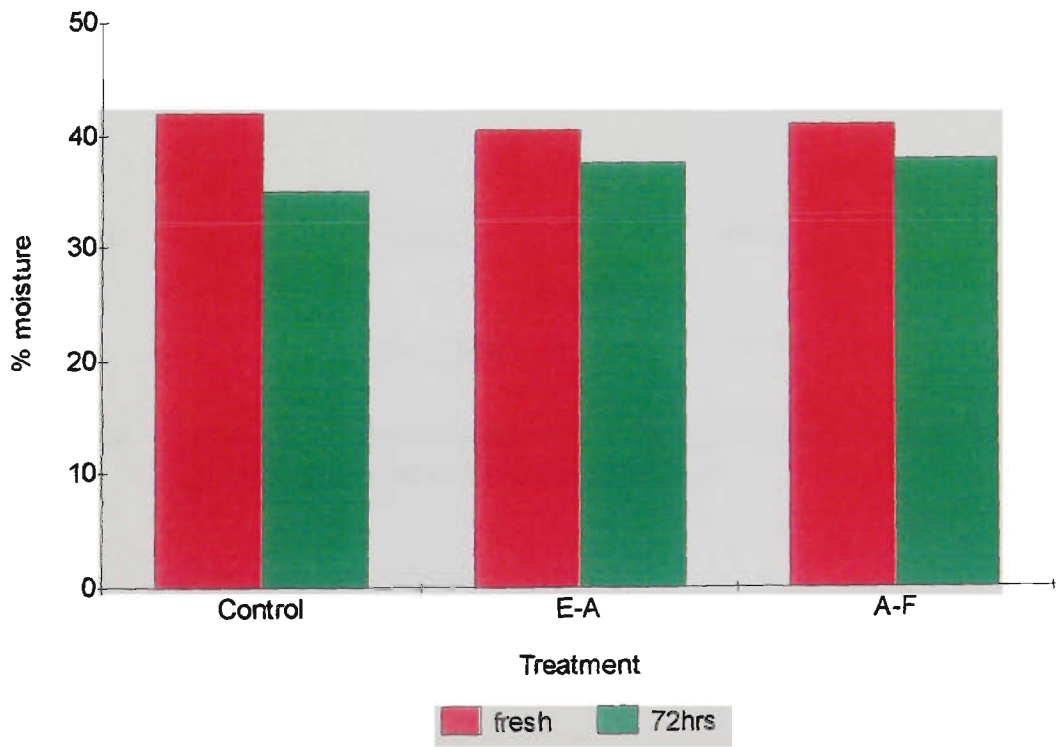


Figure 6.5 **Moisture analysis of fresh and aged breads treated with 5µL *endo*-arabinanase (E-A) and 5µL α-L-arabinofuranosidase (A-F)**

6.2.3 Staling analysis of breads treated with *endo*-arabinanase and α-L-arabinofuranosidase

Staling studies were carried out using the method previously described. The results of this section are presented in Figure 6.6. No significant differences were observed in the staling rates of control breads and those treated with either the *endo*-arabinanase or the arabinofuranosidase although breads treated with arabinofuranosidase had the least firm crumb throughout the study (Appendix 6.6). These findings indicate that the release of arabinose residues from the xylose backbone alone did not contribute to the development of softer crumb under standard test conditions used in this study.

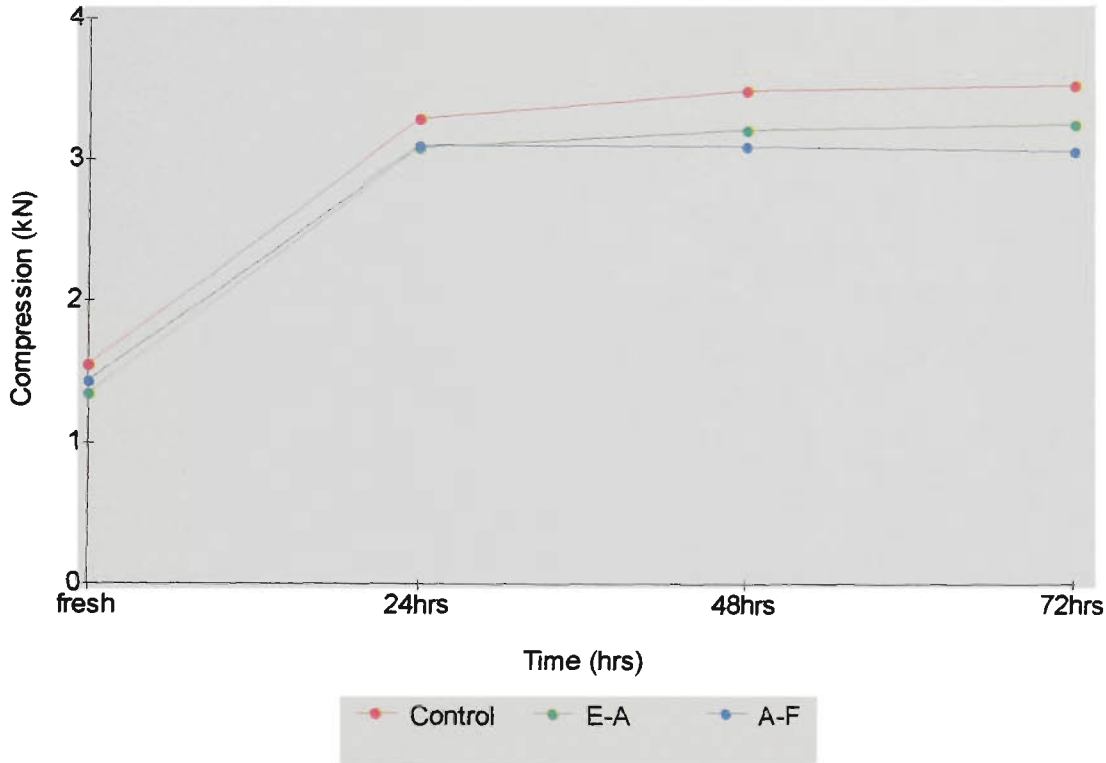


Figure 6.6 Analysis of the staling profile of breads treated with *endo*-arabinanase and arabinofuranosidase

6.3 The synergistic effects of enzymes with endoxylanase and arabinanase activity

In this section of the study the five endoxylanases studied in the previous section were used as composites of a pentosanase preparation made up of either one part xylanase and one part *endo*-arabinanase or one part endoxylanse and one part α-L-arabinofuranosidase. A complete list of treatments and treatment codes accorded each treatment appears in Table 6.1. Analyses, included the assessment of crumb and crust

colour, texture, structure, volume and moisture content. Breads were baked in triplicate for each of the treatments listed. Control loaves were also prepared with each series of experimental loaves. At the completion of the baking stage a cooling period of 90 mins was allowed prior to assessing.

Significant variations were observed between the loaves for the different combinations of endoxylanase and *endo*-arabinanase and endoxylanase and α-L-arabinofuranosidase and their ability to influence crumb and crust characteristics, colour properties, loaf volume and crumb softness. Figures 6.7 to 6.16 demonstrate visible differences observed in crumb and crust colour and loaf structure for the various enzyme combinations.

Table 6.1 **Treatments applied to breads to investigate composite endoxylanase and α-L-arabinofuranosidase combinations on crumb and crust properties**

Treatment	Endoxylanase (μL)	*endo*-Arabinanase (μL)	α-L-arabino-furanosidase (μL)
Control	-	-	-
M1	5	-	-
M1 E-A	5	5	-
M1 A-F	5	-	5
M2	5	-	-
M2 E-A	5	5	-
M2 A-F	5	-	5
M3	5	-	-
M3 E-A	5	5	-
M3 A-F	5	-	5
M4	5	-	-
M4 E-A	5	5	-
M4 A-F	5	-	5
M5	5	-	-
M5 E-A	5	5	-
M5 A-F	5	-	5

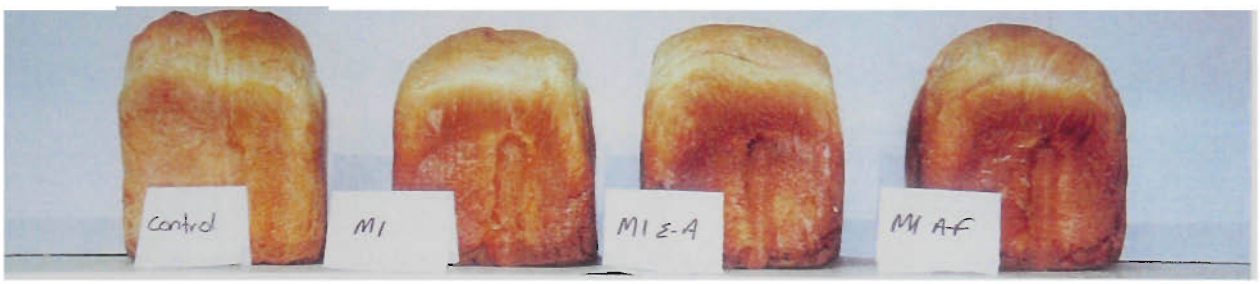


Figure 6.7 **Loaf characteristics observed when bread doughs were treated with endoxylanase preparation M1 as well as composites of M1 and either *endo*-arabinanase (M1E-A) or arabinofuranosidase (M1A-F)**

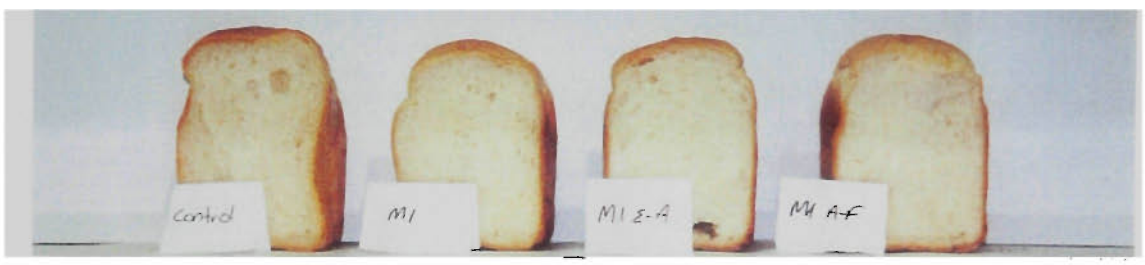


Figure 6.8 **Crumb characteristics observed when bread doughs were treated with endoxylanase preparation M1 as well as composites of M1 and either *endo*-arabinanase (M1E-A) or arabinofuranosidase (M1A-F)**

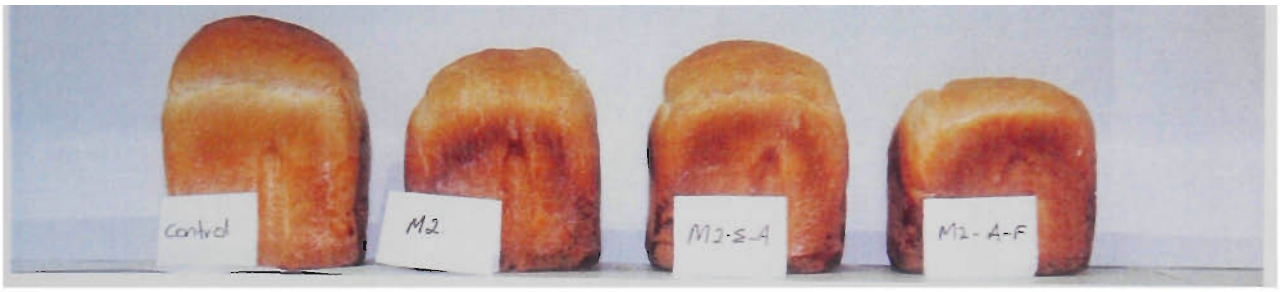


Figure 6.9 **Loaf characteristics observed when bread doughs were treated with endoxylanase preparation M2 as well as composites of M2 and either *endo*-arabinanase (M2E-A) or arabinofuranosidase (M2A-F)**

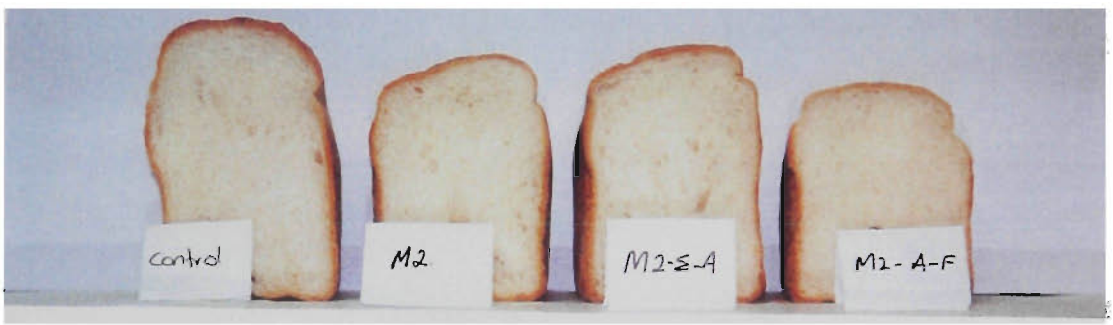


Figure 6.10 **Crumb characteristics observed when bread doughs were treated with endoxylanase preparation M2 as well as composites of M2 and either *endo*-arabinanase (M2E-A) or arabinofuranosidase (M2A-F)**

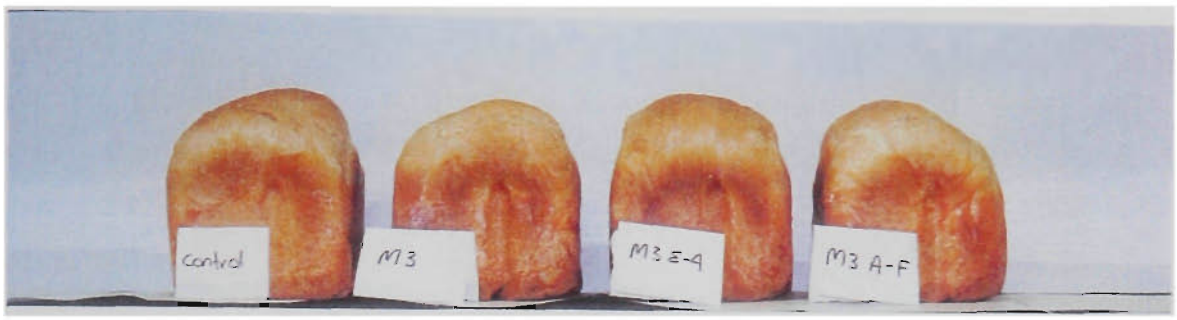


Figure 6.11 **Loaf characteristics observed when bread doughs were treated with endoxylanase preparation M3 as well as composites of M3 and either *endo*-arabinanase (M3E-A) or arabinofuranosidase (M3A-F)**

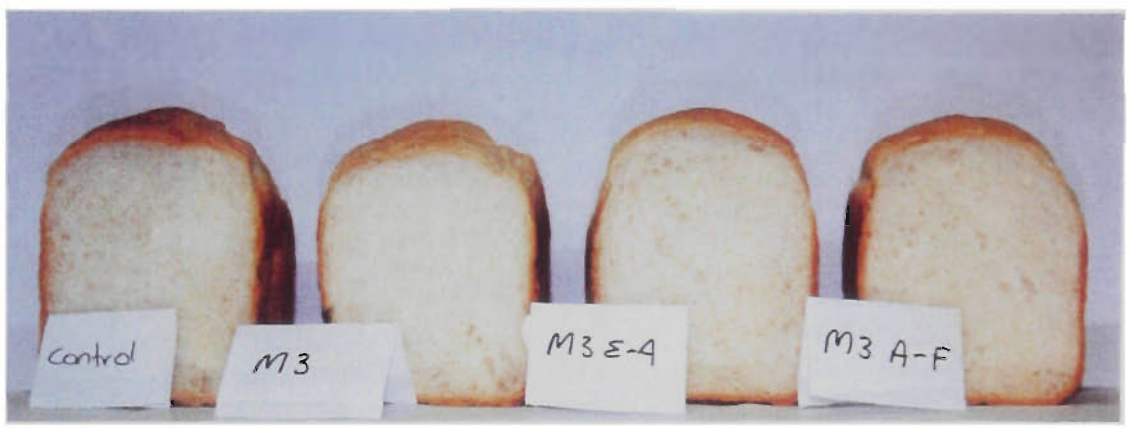


Figure 6.12 **Crumb characteristics observed when bread doughs were treated with endoxylanase preparation M3 as well as composites of M3 and either *endo*-arabinanase (M3E-A) or arabinofuranosidase (M3A-F)**

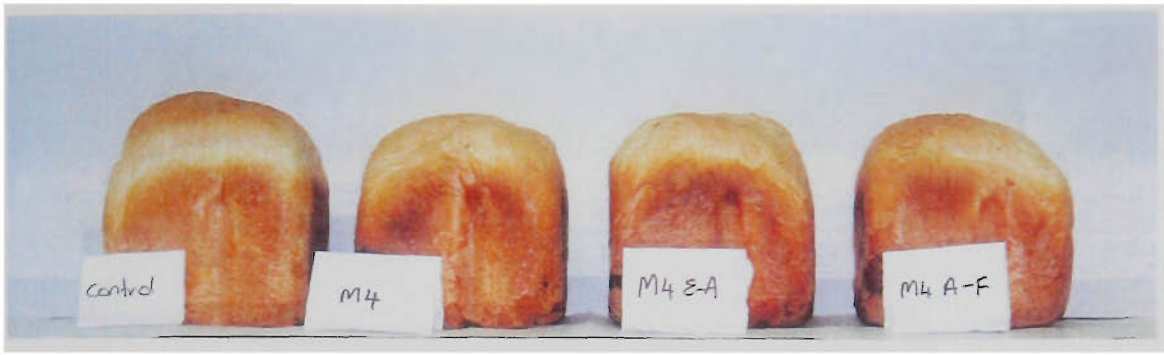


Figure 6.13 **Loaf characteristics observed when bread doughs were treated with endoxylanase preparation M4 as well as composites of M4 and either *endo*-arabinanase (M4E-A) or arabinofuranosidase (M4A-F)**

Figure 6.14 **Crumb characteristics observed when bread doughs were treated with endoxylanase preparation M4 as well as composites of M4 and either *endo*-arabinanase (M4E-A) or arabinofuranosidase (M4A-F)**

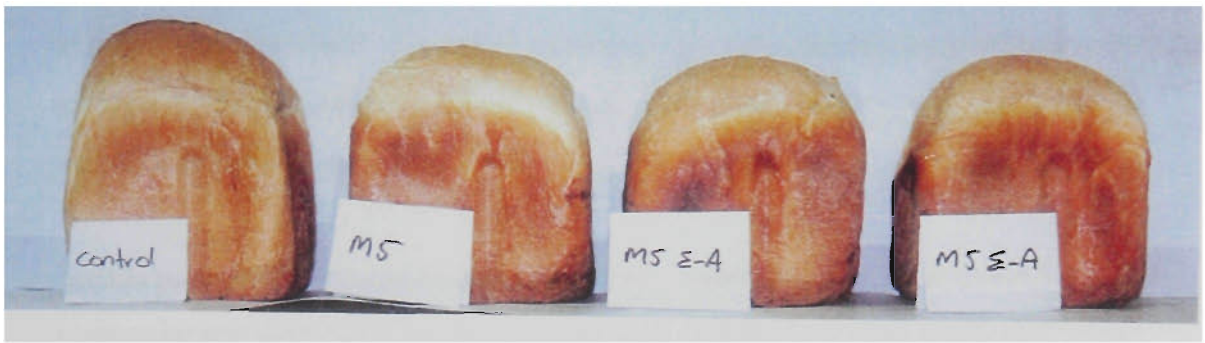


Figure 6.15 **Loaf characteristics observed when bread doughs were treated with endoxylanase preparation M5 as well as composites of M5 and either *endo*-arabinanase (M5E-A) or arabinofuranosidase (M5A-F)**

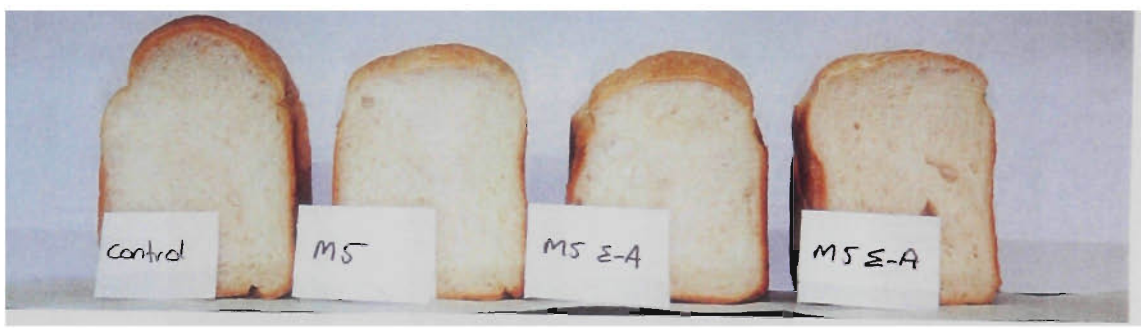


Figure 6.16 **Crumb characteristics observed when bread doughs were treated with endoxylanase preparation M5 as well as composites of M5 and either *endo*-arabinanase (M5E-A) or arabinofuranosidase (M5A-F)**

6.3.1 Impact of endoxylanase in combination with either *endo*-arabinanase or α-L-arabinofuranosidase on crumb and crust colour

The properties of crumb and crust colour were examined using a Minolta Chroma Meter as previously described. A total of 5 readings for each property were taken for each loaf giving a total of 15 readings for crumb and crust colour respectively. The mean 'L' values are given Figures 6.17 to 6.21.

Breads prepared with a combination of endoxylanase from preparations M1 and the composite preparations of M1E-A and M1A-F (Figure 6.17) had crusts that were significantly darker than the control breads ($p<0.05$; Appendix 6.7). No significant difference in crust colour was observed amongst any of the treated loaves. None of the treatment combinations resulted in significant differences in crumb brightness.

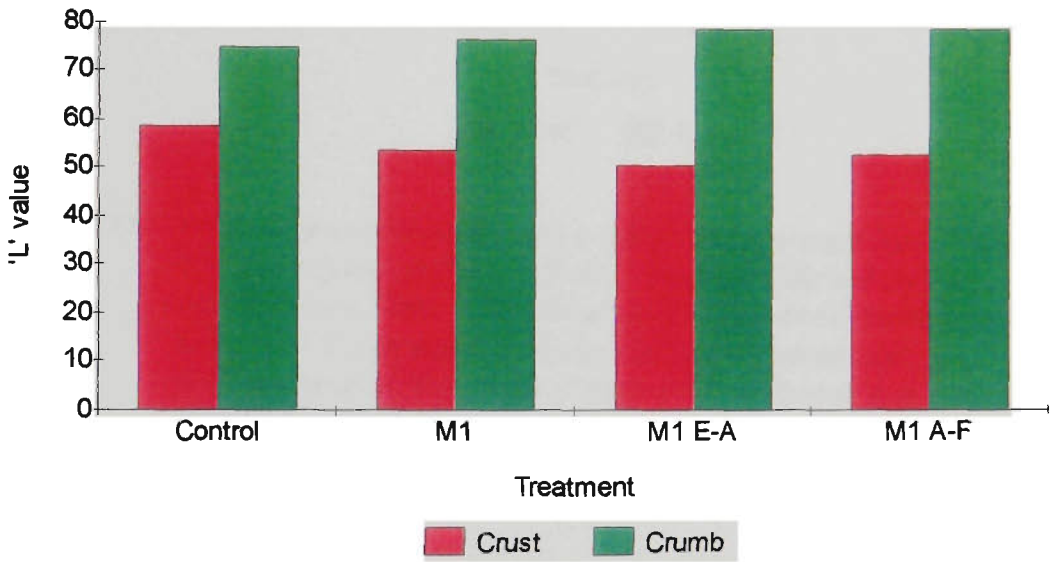


Figure 6.17 Impact on crumb and crust colour when pentosanase composites comprising endoxylanase from *T viride* (M1); endoxylanase from *T viride* and *endo*-arabinanase from *A niger* (M1 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M1 A-F) were added to a basic bread formulation

The addition of endoxylanase from *T longibrachiatum* (preparation M2) alone and in combination with arabinofuranosidase (M2A-F) resulted in breads that had crusts that were significantly darker than the control breads ($p<0.05$, Appendix 6.8). The results are presented in Figure 6.18. No differences were observed in crust colour between any of the treated breads.

No significant differences were observed in crumb colour for any of the treated breads.

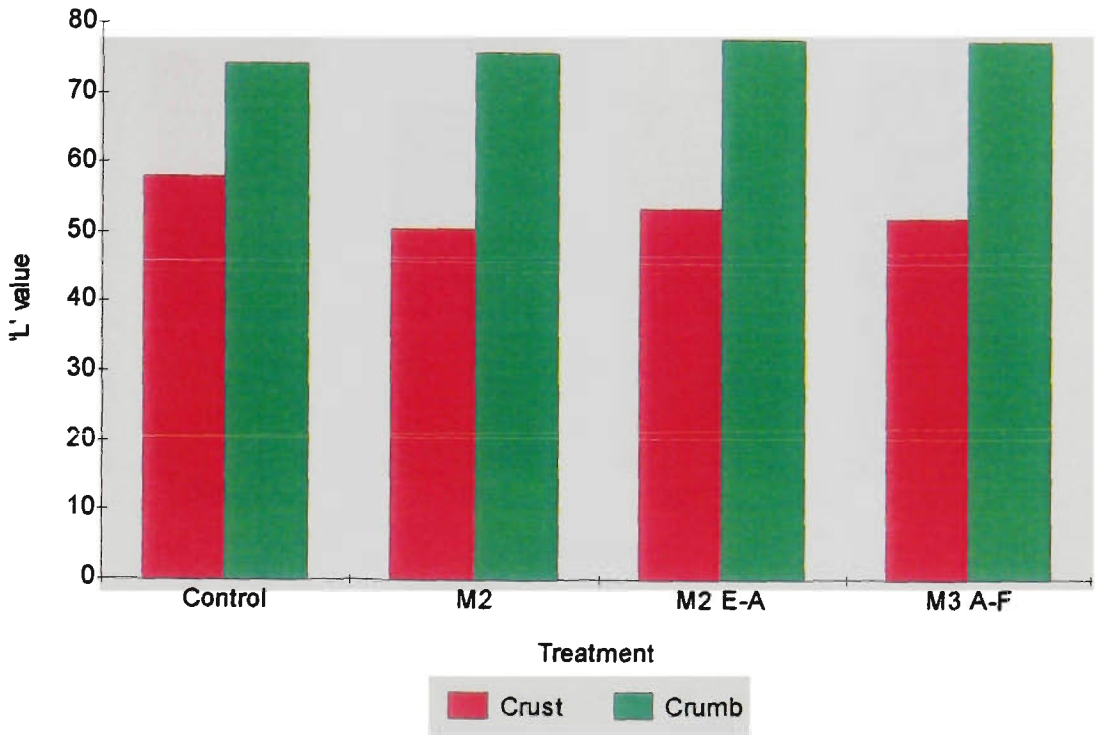


Figure 6.18 **Impact on crumb and crust colour when pentosanase composites comprising endoxylanase from *T longibrachiatum* (M2); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M2 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M2 A-F) were added to a basic bread formulation**

In the next series of breads, endoxylanase from preparation M3, also sourced from *T longibrachiatum*, were added singly and in conjunction the E-A and A-F preparations (Figure 6.19). Significantly darker crusts were observed for the M3 and M3E-A composite were used ($p<0.05$; Appendix 6.9). The latter also produced a significantly darker crust than the M3A-F preparation. No significant differences were observed in crumb brightness for any of the treatments used.

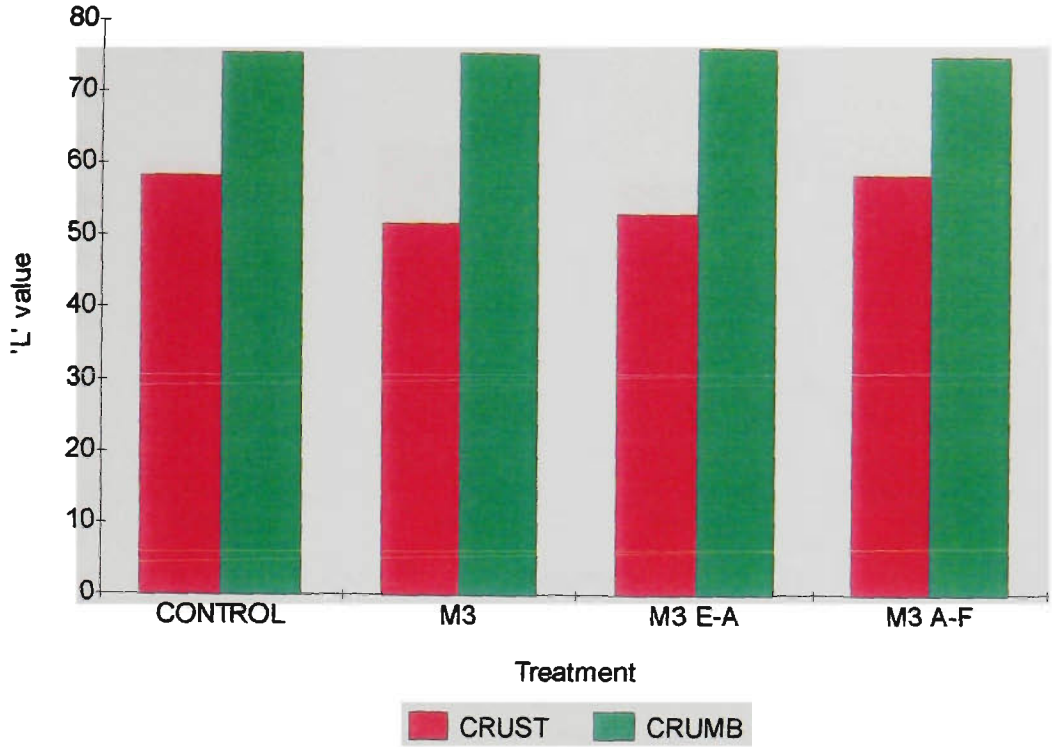


Figure 6.19 **Impact on crumb and crust colour when pentosanase composites comprising endoxylanase from *T longibrachiatum* (M3); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M3 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M3 A-F) were added to a basic bread formulation**

When bread formulations were supplemented with endoxylanase from M4 (from *A niger*) and M4 A-F and M4 A-F also from *A niger*, no significant differences were observed in the development of crust colour (Appendix 6.10). The crusts of these loaves were visibly darker than the untreated loaves (Figure 6.20). No significant differences were observed in the crumb whiteness for any of the experimental loaves of M4 and its composites.

In the final series of experimental breads treated with endoxylanase M5 (from *H insolens*) was added in combination with *endo*-arabinanase and arabinofuranosidase (Figure 6.21). Again crumb colour remained unaffected by the different treatments used. In addition to this the composite treatments did not impact on the crust colour to any significant degree (Appendix 6.11).

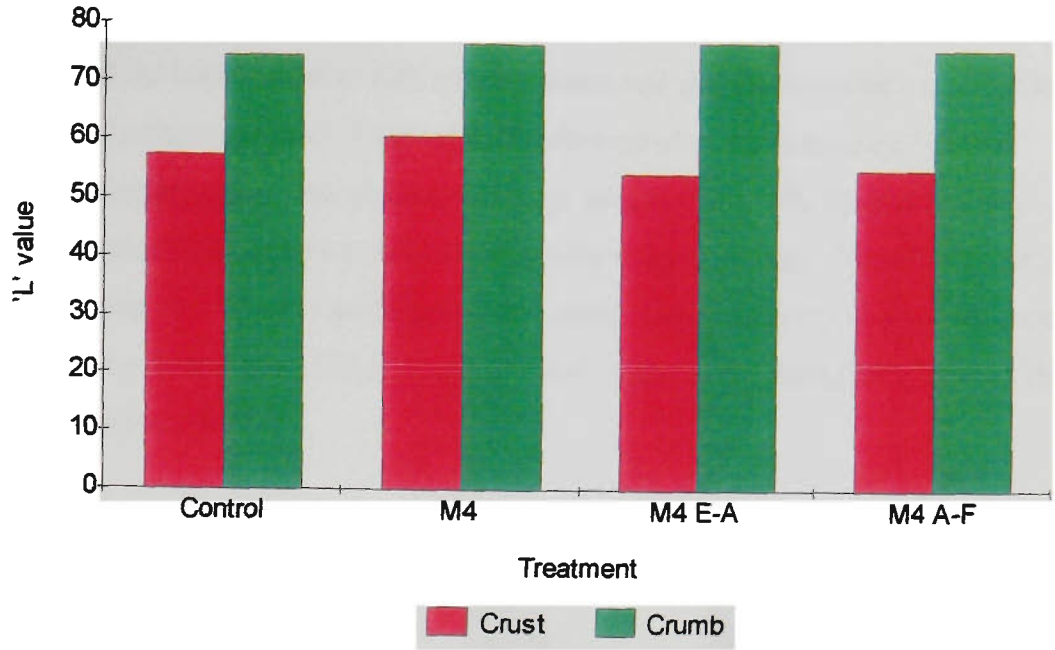


Figure 6.20 **Impact on crumb and crust colour when pentosanase composites comprising endoxylanase from *T longibrachiatum* (M4); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M4 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M4 A-F) were added to a basic bread formulation**

6.3.2 Loaf volume

To establish the effects of endoxylanase and *endo*-arabinanase and endoxylanase and α-L-arabinofuranosidase combinations on loaf volume of pan bread all the treated and untreated loaves were measured and the score for loaf volume ascertained as described previously. The results of the loaf volume analysis are presented in Figures 6.22-6.26. Each of the treated breads had loaf volumes that were commercially acceptable in that each bread had a loaf volume greater than 19. However variations were observed in the extent to which loaf volume was enhanced between the different enzyme combinations utilised. The statistics for the loaf volumes for all the preparations are presented in Appendix 6.12.

Breads treated with M1 E-A and M1 A-F were significantly larger than the control loaves (p0.05). These breads were significantly larger than the than those treated with the endoxylanase preparation only (p<0.05). The larger gas cells observed in breads

treated with preparation M1 in earlier parts of the study were also noted in the breads treated with the pentosanase combinations already mentioned. However a loss of regularity in the structure of the gas cell and hence the overall crumb structure was observed for breads treated with endoxylanase and arabinofuranosidase. This loss of crumb structure was also observed in breads treated with preparation M5 and arabinofuranosidase. The presence of large irregular gas cells throughout the loaf crumb contributed to an overall loss of quality of these breads. In addition to this the breads treated with *endo*-arabinanase and arabinofuranosidase and endoxylanases from *H insolens* (preparation M5 E-A and M5 A-F) were significantly smaller than the control breads ($p<0.05$).

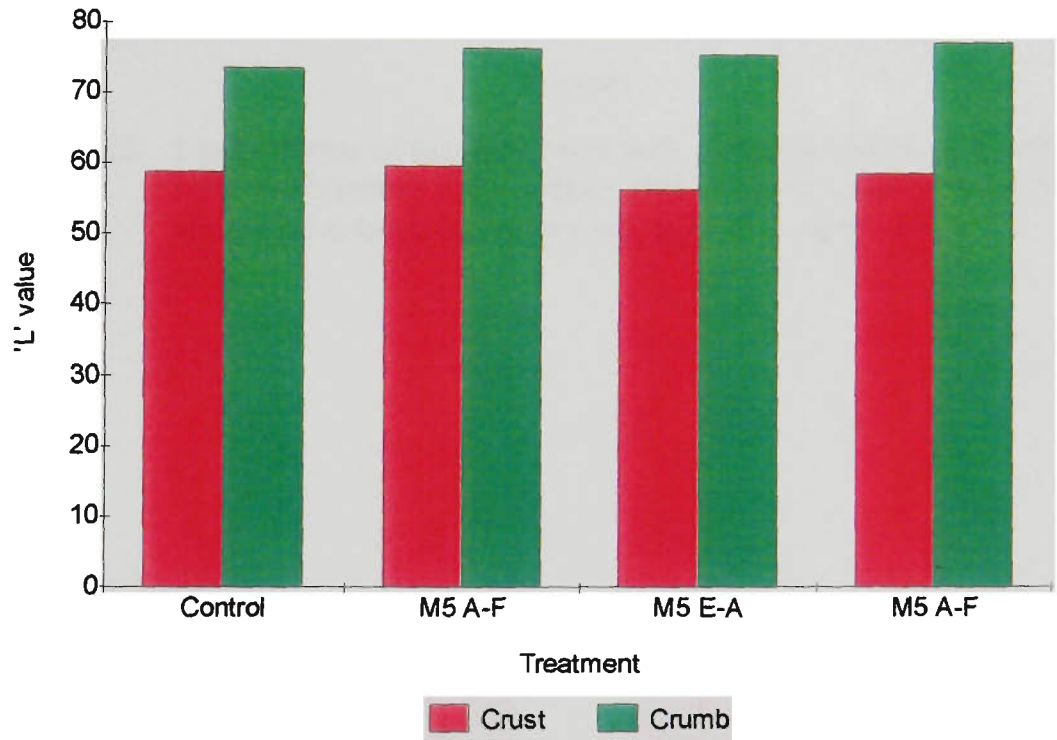


Figure 6.21 **Impact on crumb and crust colour when pentosanase composites comprising endoxylanase from *T longibrachiatum* (M5); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M5 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M5 A-F) were added to a basic bread formulation**

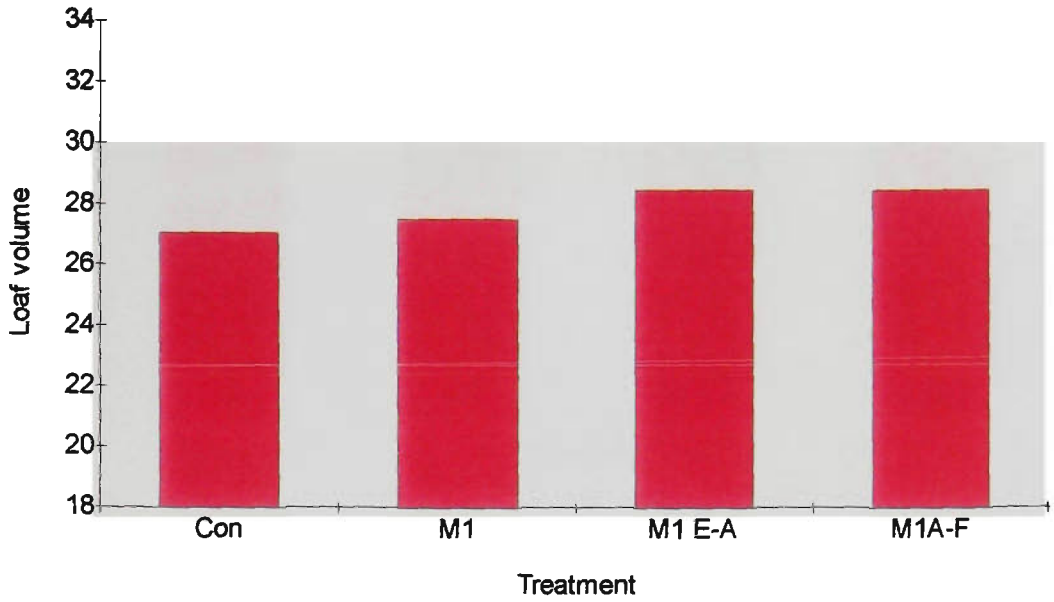


Figure 6.22 **Loaf volumes of breads treated with endoxylanase from *T viride* and *endo*-arabinanase from *A niger* (M1 E-A) and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M1 A-F)**

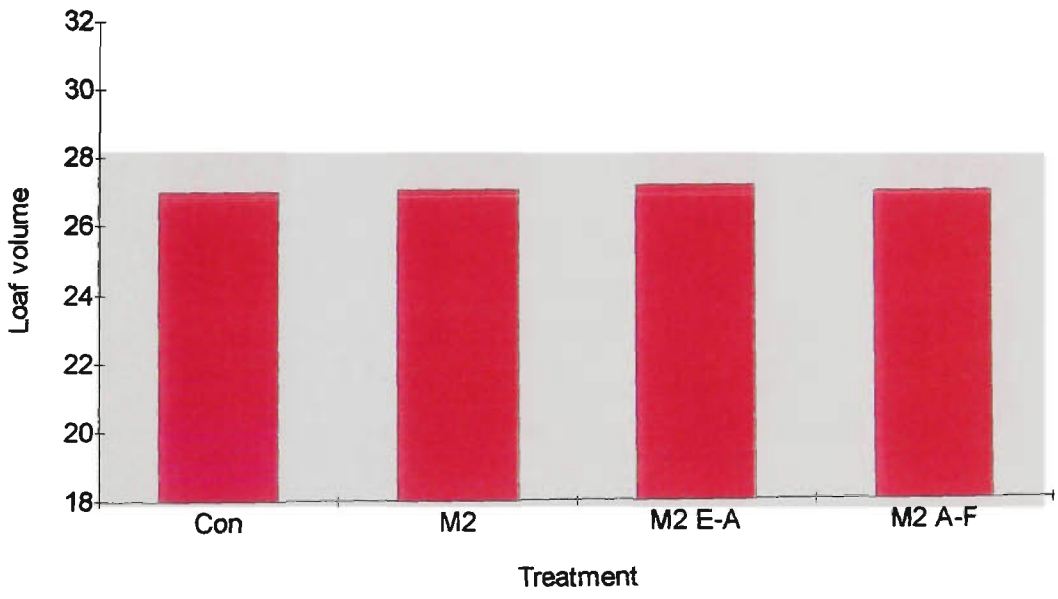


Figure 6.23 **Loaf volumes of breads treated with endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M2 E-A) and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M2 A-F).**

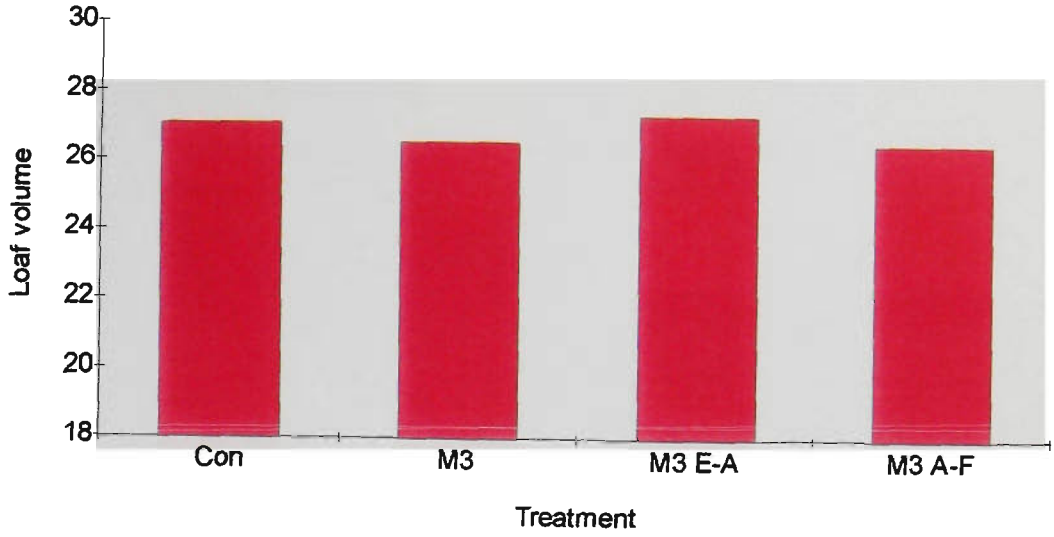


Figure 6.24 **Loaf volumes of breads treated with endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M3 E-A) and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M3 A-F)**

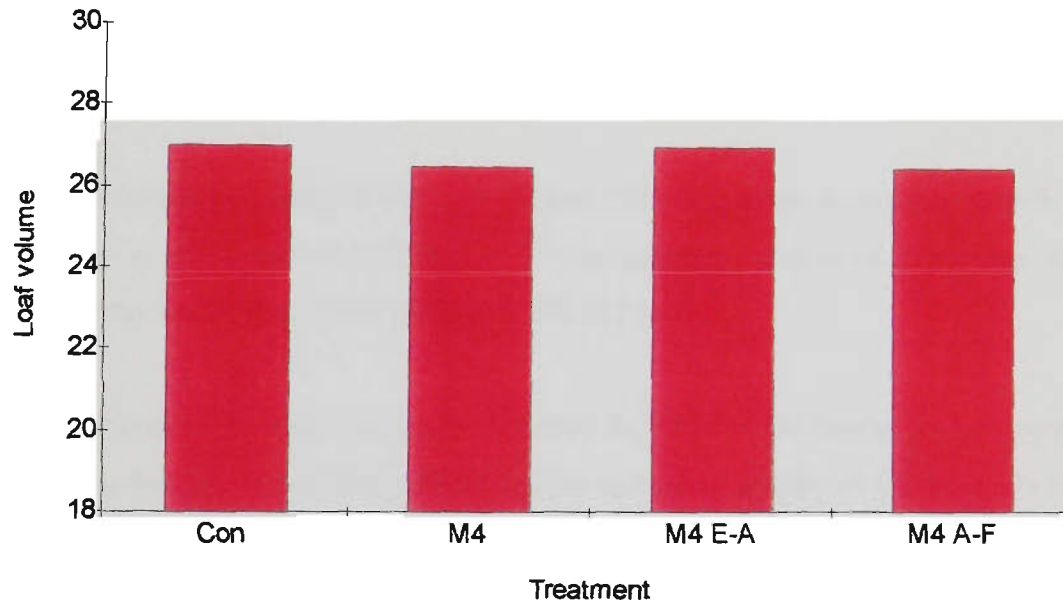


Figure 6.25 **Loaf volumes of breads treated with endoxylanase from *A niger* and *endo*-arabinanase from *A niger* (M4 E-A) and endoxylanase from *A niger* and α-L-arabinofuranosidase from *A niger* (M4 A-F)**

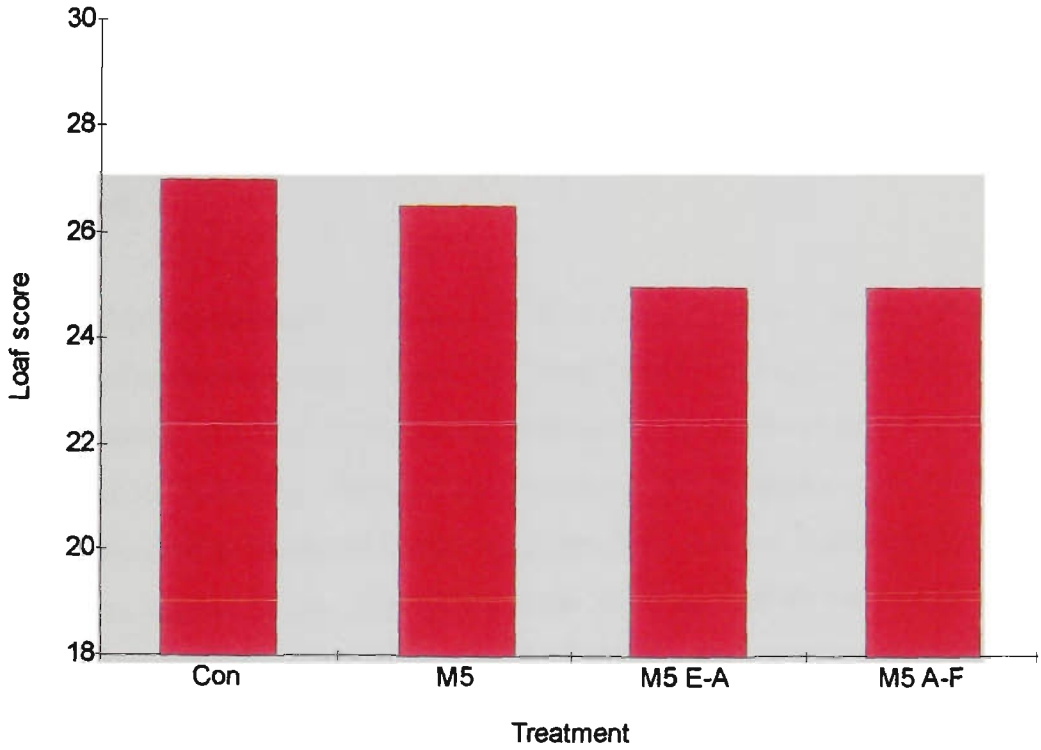


Figure 6.26 Loaf volumes of breads treated with endoxylanase from *H insolens* and *endo*-arabinanase from *A niger* (M5 E-A) and endoxylanase from *H insolens* and α-L-arabinofuranosidase from *A niger* (M5 A-F)

Of the remaining treatments both the M2 and M4 composites showed no significant difference in loaf volume while the M3 E-A preparation resulted in breads that were significantly larger than those prepared with M3 A-F.

The deterioration in crumb structure observed for some of the treated loaves suggests that the hydrolysis of arabinoxylan to smaller xylan and arabinose fractions has a destabilising effect on the gluten matrix. Dough strength is lost leading to a decrease in the ability of the gluten network to contain CO_2 produced during fermentation (van Oort et al 1995).

6.3.3 Moisture

Variation was observed in the ability of the different enzyme preparations to facilitate retention of moisture in the bread crumb. Combinations of the two different arabinanase preparations and endoxylanases from *T viride* (M1), *T longibrachiatum*

(M3) and *H insolens* (M5) did not result in any significant changes in moisture content being observed in the bread crumb (Appendix 6.19). When arabinanases and endoxylanases from *T longibrachiatum* (M2) and *A niger* (M4) were added to bread formulations significant variation in the moisture content of the resulting bread crumbs was observed.

For the freshly baked bread, it was found that the use of various combinations of xylanase and arabinanase did not produce significant differences in moisture content of the bread crumb. All of the breads both treated and untreated had crumb moistures in the range of 39.25-42.5%. To investigate the impact of pentosanase combinations on moisture retention as the bread crumb aged, moisture data was collected after allowing the breads to age for 72 hrs. The results of the moisture analysis are presented in Table 6.2. The data indicates that certain combinations of xylanase and arabinanase are more effective than others in increasing moisture retention in bread crumb. The bread crumb of breads treated with treatments M1 and M2 (*T longibrachiatum*) exhibited significant differences in moisture content between the fresh and aged loaves ($p<0.05$). The *endo*-arabinanase and α-L-arabinofuranosidase, added to the bread formulation in conjunction with M1, the moisture retention properties of the crumb showed no significant reduction in moisture loss. Moisture contents for these breads were 41.5% prior to aging and 35% after aging. A similar finding was observed for M2 and its composites. The breads showed significant loss of moisture after aging. The addition of the M3 composites, showed no significant loss of moisture as the breads aged. The M3 preparation is another enzyme sourced from *T longibrachiatum,* but it has different thermal and pH optima properties to those of the M2 preparation. Similar findings were observed for breads treated with combinations of endoxylanase from preparation M5 and *endo*-arabinanase or arabinofuranosidase. When breads were treated with the M4 composites, significantly less moisture loss was again observed in the crumb after 72hrs. Breads to which M3, M4 and M5 alone were added, did not improve the water binding capacity as the breads aged.

Table 6.2 Moisture contents of treated and control breads aged for 72hrs

Treatment	Moisture content of fresh bread (%)	Moisture content of aged bread (%)	"Δ" values
Control	42.0	34.5[a]	7.5
M1	41.5	33.0	8.5
M1 and *endo*-arabinanase	42.0	33.0	7.0
M1 and arabinofuranosidase	42.0	35.0[a]	7.0
M2	41.5	32.7[a]	9.5
M2 and *endo*-arabinanase	41.5	32.0[a]	9.5
M2 and arabinofuranosidase	40.0	32.0[a]	8.0
M3	42.0	38.5[a]	3.5
M3 and *endo*-arabinanase	41.5	40.0	1.5
M3 and arabinofuranosidase	42.5	42.0	0.5
M4	41.0	38.0[a]	3.0
M4 and *endo*-arabinanase	41.5	38.0	3.5
M4 and arabinofuranosidase	40.0	39.0	1.0
M5	42.0	38.0[a]	4.0
M5 and *endo*-arabinanase	42.0	38.0	2.0
M5 and arabinofuranosidase	39.3	39.0	2.0

[a] denotes significant moisture loss over the 72hr period ($p<0.05$ Appendix 6.19).

6.3.4 Staling studies

The role of pentosanase preparations in slowing the rate of crumb firming has been documented (van Oort et al 1995). However many of these preparations in general contain a range of enzymes that exhibit other pentosanase activity including arabinofuranosidase and galactanase activity. The presence of residual carbohydrases and small amounts of proteinase has also been found in these some of these preparations. Little has been done to document what influence if any these residual activities play in bread staling. The purpose of this section of the current study was to add combinations of purified endoxylanase, *endo*-arabinanase and α-L-arabinofuranosidase to a basic bread formulation to investigate the synergistic effects of these preparations on the crumb characteristics as bread ages. Samples of breads were analysed for crumb softness after completion of baking and cooling as well as following storage for one, two and three day periods. The results are presented in Figures 6.27 – 6.31.

The first series of loaves was prepared using treatments M1, M1 E-A and M1 A-F. Significantly softer crumbs were observed for each of the treatments in the freshly baked breads and after the first 24hrs ($p<0.05$; Appendix 6.13). However after 48hrs breads with the treatment M1 E-A had crumb that was comparable to the untreated loaves whilst breads with the other two treatments were still significantly softer than the control loaves. At the completion of the staling study all the treated loaves were again significantly softer than the control breads. Neither of the composite treatments resulted in significantly softer crumb than those breads treated with the endoxylanase preparation only. This finding indicates that action of the endoxylanase used in this phase of the study is not enhanced by the addition of either *endo*-arabinanase or α-L-arabinofuranosidase.

In the next series of staling trials endoxylanase from *T longibrachiatum* (preparation M2) was added to a basic bread formulation in conjunction with the *endo*-arabinanase and α-L-arabinofuranosidase already described. Significant differences were observed in the ability of the composite treatments to retard the rate of crumb firming as the breads aged (Figure 6.28). All the treatments used resulted in crumbs that were significantly softer than the control loaves at every stage of the study ($p<0.05$;

Appendix 6.14). In addition to this, breads treated with the M2E-A composite were significantly softer after 72hrs than those treated with M2 only ($p<0.05$).

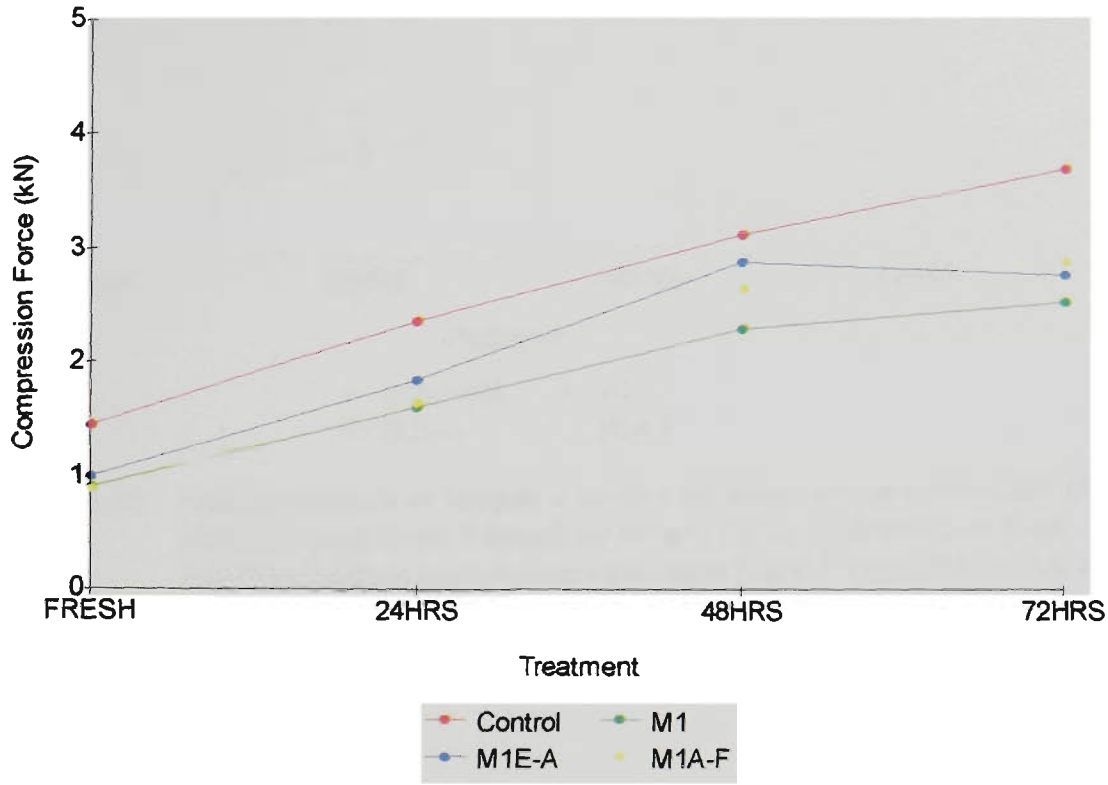


Figure 6.27 Staling analysis of breads treated with pentosanase composites of endoxylanase from *T viride* (M1); endoxylanase from *T viride* and *endo*-arabinanase from *A niger* (M1 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M1 A-F)

When bread formulations were treated with combinations of endoxylanase from preparation M3 (sourced from *T longibrachiatum*) and *endo*-arabinanase and arabinofuranosidase a different staling profile was obtained. The results are presented in Figure 6.29. The composite treatments of M3E-A and M3A-F produced significantly softer crumb than the untreated breads after 48 and 72hrs. These treatments also produced breads crumbs that were significantly softer than those treated with the xylanase preparation only for the same time frame ($p<0.05$; Appendix 6.15). In addition to this the breads treated with M3A-F had a significantly softer crumb than the control after 24hrs ($p<0.05$).

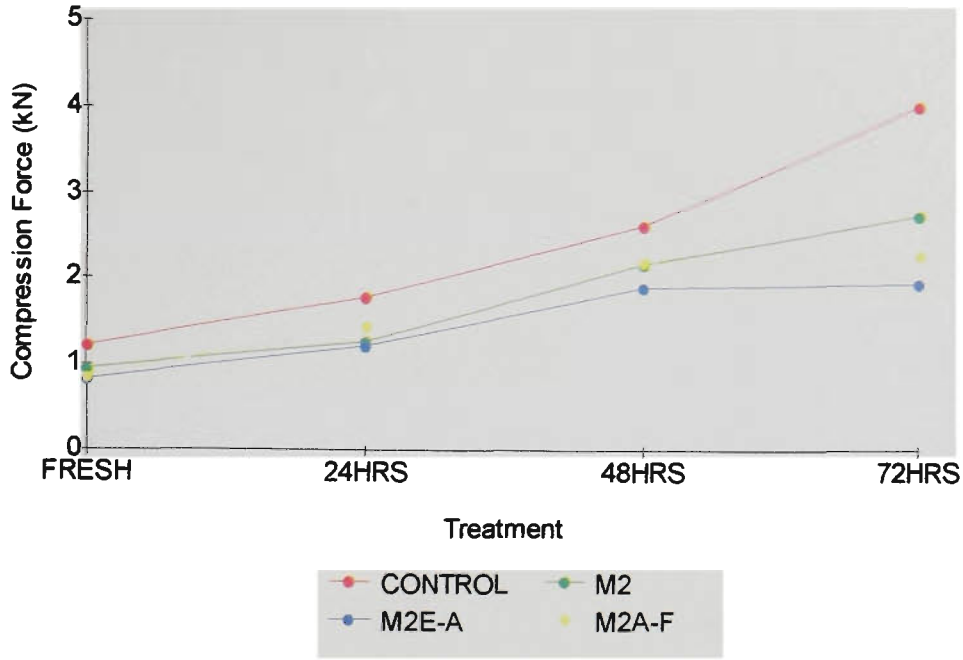


Figure 6.28 **Staling analysis of breads treated with pentosanase composites of endoxylanase from *T longibrachiatum* (M2); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from A niger (M2 E-A); and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M2 A-F)**

A similar finding was observed when breads were treated with combinations of M4 endoxylanase, from *A niger* either the *endo*-arabinanase or the arabinofuranosidase also from *A niger*. The results are presented in Figure 6.30. The addition of M4 alone did not result in any significant differences in the crumb firmness as the bread aged. However when M4E-A was added to bread dough, the resulting crumbs were significantly softer throughout the trial ($p<0.05$; Appendix 6.16). The composite M4 A-F produced crumb that was significantly softer after 48hrs and 72hrs than the control and the M4 treatment alone ($p<0.05$).

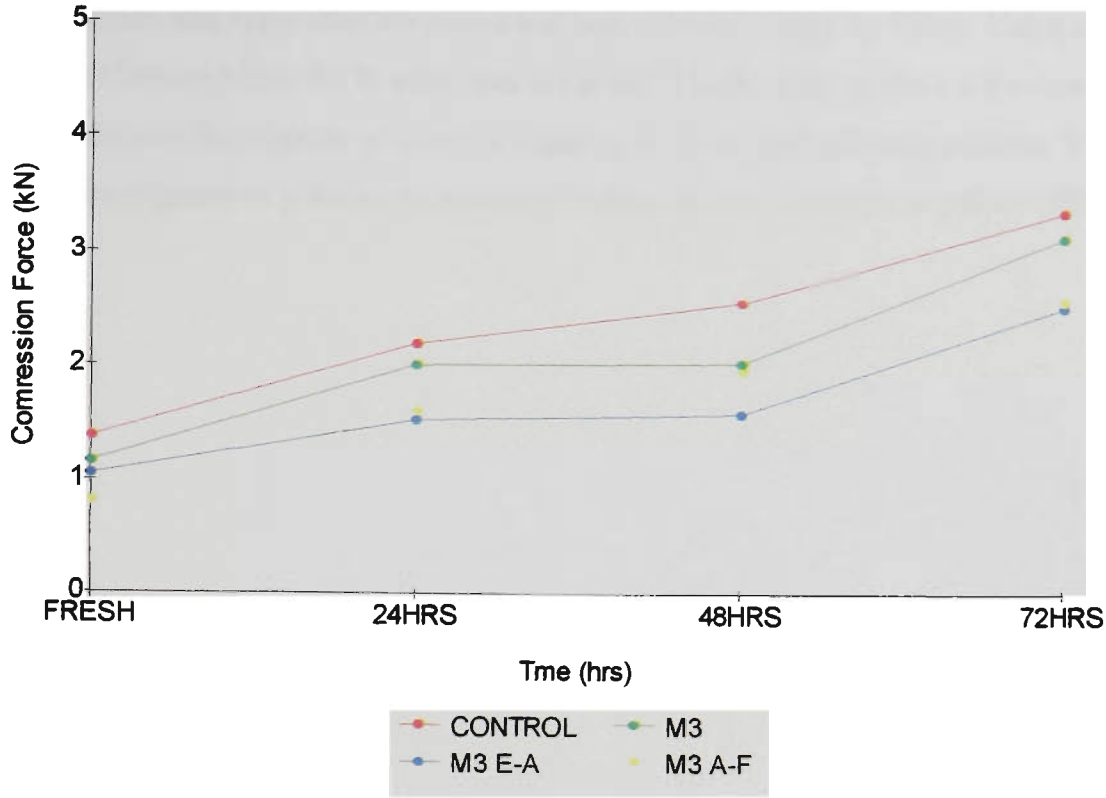


Figure 6.29 **Staling analysis of breads treated with pentosanase composites of endoxylanase from *T longibrachiatum* (M3); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M3 E-A); and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M3 A-F)**

In the final phase of the staling trials loaves were prepared using a combination of endoxylanase from *H insolens* in combination with *endo*-arabinanase and arabinofuranosidase (M5E-A and M5A-F). In this series of trials it was found that only the composite M5A-F alone was able to significantly slow the rate of crumb firming at each stage of the trial ($p<0.05$, Appendix 6.17). Breads with this treatment had crumb that was significantly softer in the fresh breads and the breads retained significantly softer crumb throughout the aging period (Figure 6.31). Again breads to which the endoxylanase preparation only was added did not exhibit softer crumb at any stage of the trial.

6.3.5 Yellow crumb

The impact of different of endoxylanase and *endo*-arabinanase preparations on the development of yellow crumb in aged bread was also investigated. As noted in earlier section of this study, the yellowing of bread crumb appears to be a natural phenomenon

in aged bread. In this part of the current study bread crumbs were assessed on freshly baked breads and again after the crumb had been allowed to age for 72hrs. Using a Minolta Chroma Meter the 'b' value was recorded. The 'b' value on the L a b colour scale indicates the intensity of green (a negative 'b' value) and yellow (a positive 'b'). The more negative or positive the reading the more intense the green or yellow colour.

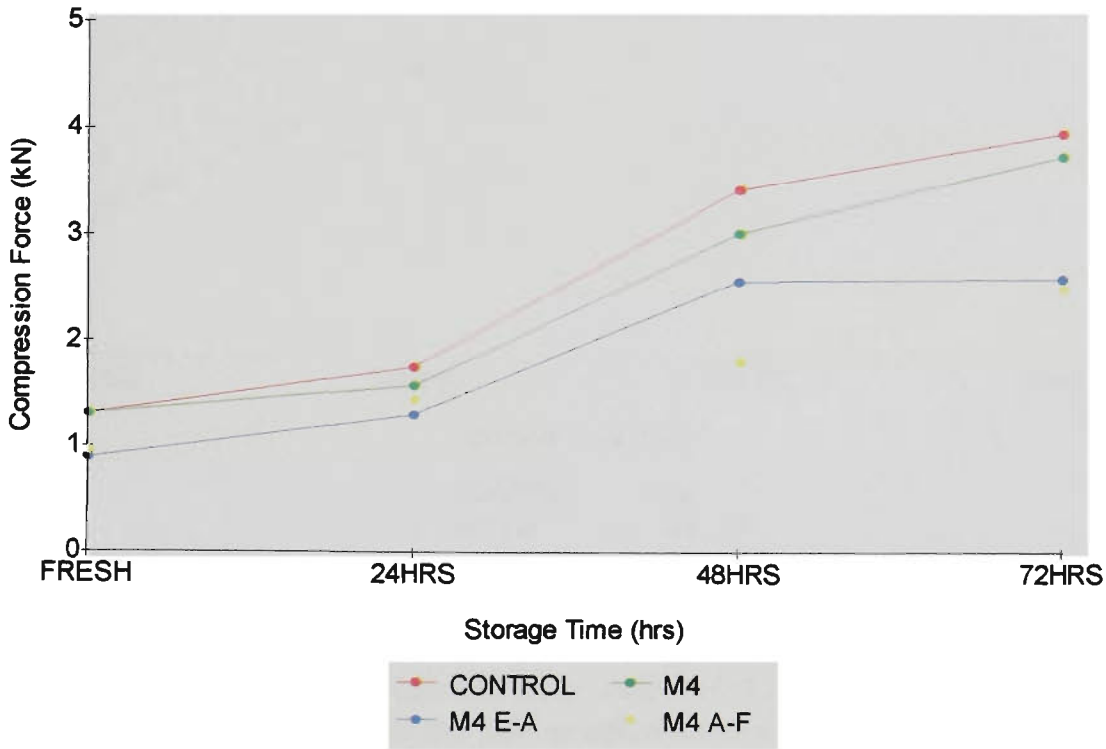


Figure 6.30 Staling analysis of breads treated with pentosanase composites of endoxylanase from *A niger* (M4); endoxylanase from *A niger* and *endo*-arabinanase from *A niger* (M4 E-A); and endoxylanase from *A niger* and α-L-arabinofuranosidase from *A niger* (M4 A-F)

Each of the experimental loaves was prepared using one of the treatments described in Table 6.1. The results are presented in Figures 6.32 – 6.36. The statistical analyses are presented in Appendix 6.18 for all of treatments utilized in Section 6.3.5. In the first part of the study breads were baked using the standard bread formulation and the M1 and M1 composite treatments. No significant difference was found between the crumb of the fresh breads and those aged for 72hrs when the various treatments were used. However the M1A-F treatment resulted in a yellower crumb in the fresh bread than the control ($p<0.05$) and the M1E-A and M1A-F aged for 72hrs also had a more yellow crumb than the fresh control ($p<0.05$).

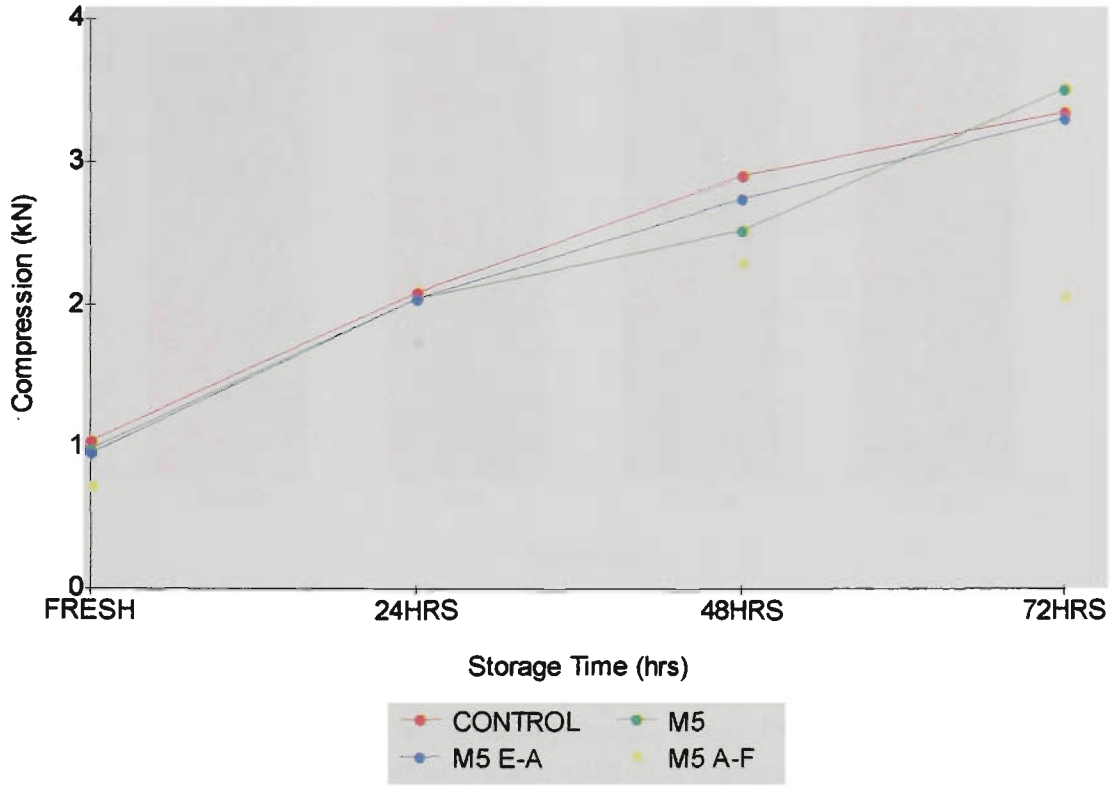


Figure 6.31 **Staling analysis of breads treated with pentosanase composites of endoxylanase from *H insolens* (M5); endoxylanase from *H insolens* and *endo*-arabinanase from *A niger* (M5 E-A); and endoxylanase from *H insolens* and α-L-arabinofuranosidase from *A niger* (M5 A-F).**

The next series of treatments involved the addition of endoxylanase from *T longibrachiatum* (M2) in conjunction with either the *endo*-arabinanase or α-L-arabinofuranosidase as described in Table 6.1 and the results are shown in Figure 6.33. Treatments M2 E-A and M2 A-F both were found to impede yellowing in the aging crumb. Crumbs of both sets of experimental breads had crumb that did not yellow significantly during aging, compared with their fresh counterparts. The control loaves by contrast yellowed significantly during aging ($p<0.05$).

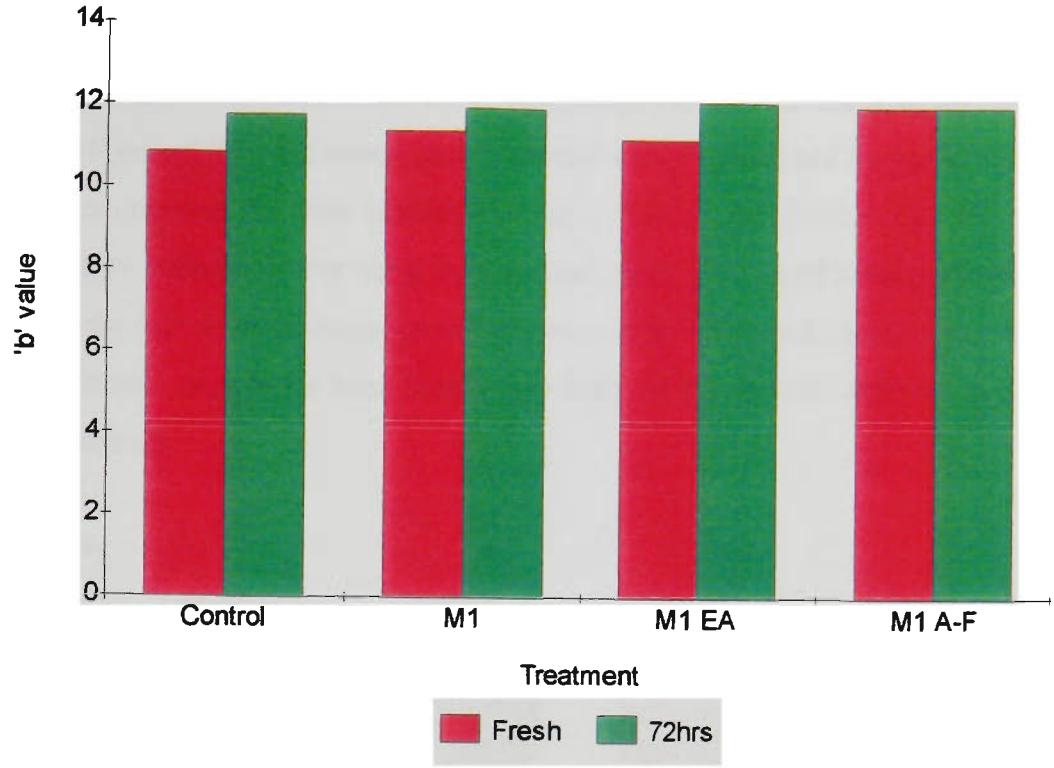


Figure 6.32 **Impact on crumb colour in aged breads treated with endoxylanase from *T viride* (M1); endoxylanase from *T viride* and *endo*-arabinanase from *A niger* (M1 E-A); and endoxylanase from *T viride* and α-L-arabinofuranosidase from *A niger* (M1 A-F)**

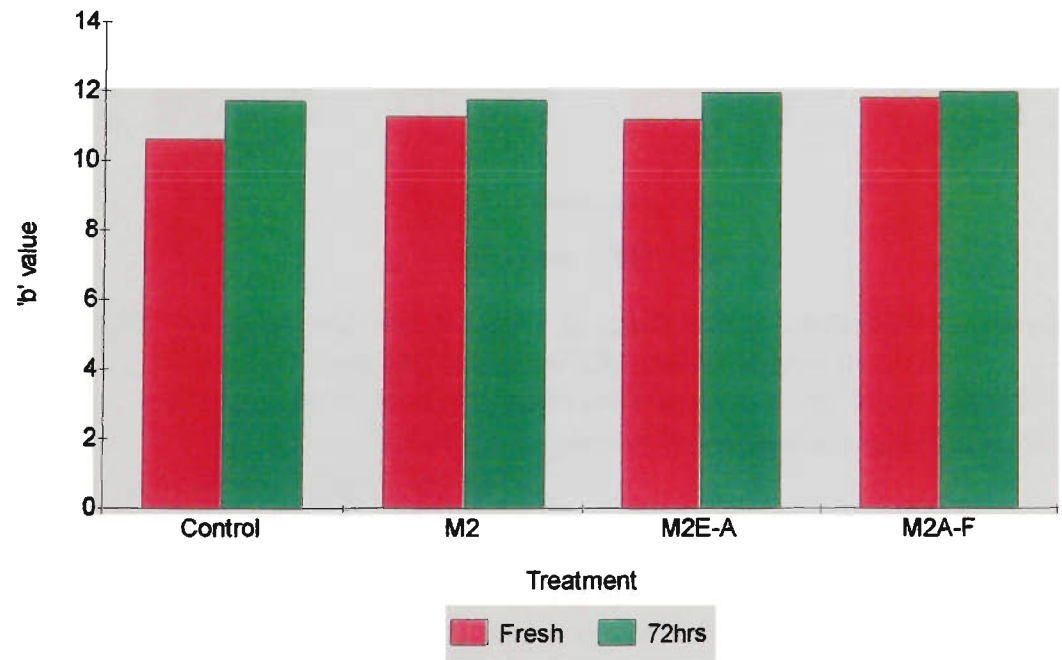


Figure 6.33 **Impact on crumb colour in aged breads treated with endoxylanase from *T longibrachiatum* (M2); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M2 E-A); and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M2 A-F)**

When breads were treated with endoxylanase treatments M3, M3 E-A and M3 A-F no significant difference was found for crumb yellowing between the fresh and the aged breads (Figure 6.34). However, breads treated with M3 E-A had a significantly more yellow crumb than the fresh untreated breads. This increased colour development was also readily visible without using the Chroma Meter. Whilst all three treatments appear to slow the rate at which bread crumb yellows, as bread ages the presence of a more yellow crumb even in the fresh breads may limit the commercial application of some of the treatments.

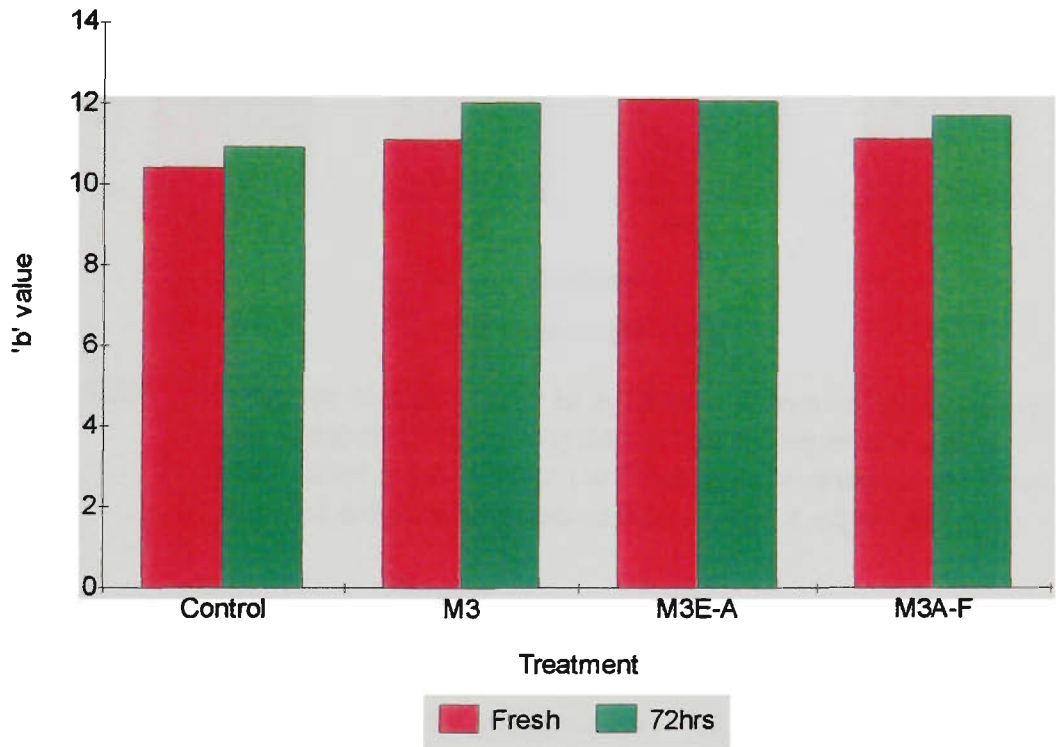


Figure 6.34 **Impact on crumb colour in aged breads treated with endoxylanase from *T longibrachiatum* (M3); endoxylanase from *T longibrachiatum* and *endo*-arabinanase from *A niger* (M3 E-A); and endoxylanase from *T longibrachiatum* and α-L-arabinofuranosidase from *A niger* (M3 A-F)**

For the next stage a series of breads were prepared using the treatments M4, M4 E-A and M4 A-F (Figure 6.35). Analysis of the data showed that only the treatment M4 was able to slow the rate of crumb yellowing in aging bread. Bread crumb from breads to which the other two treatments were added had significantly more yellow crumb than their fresh counterparts ($p<0.05$). The treatment M4 A-F also resulted in bread crumbs

that were significantly more yellow than the control breads that had been allowed to age for 72hrs.

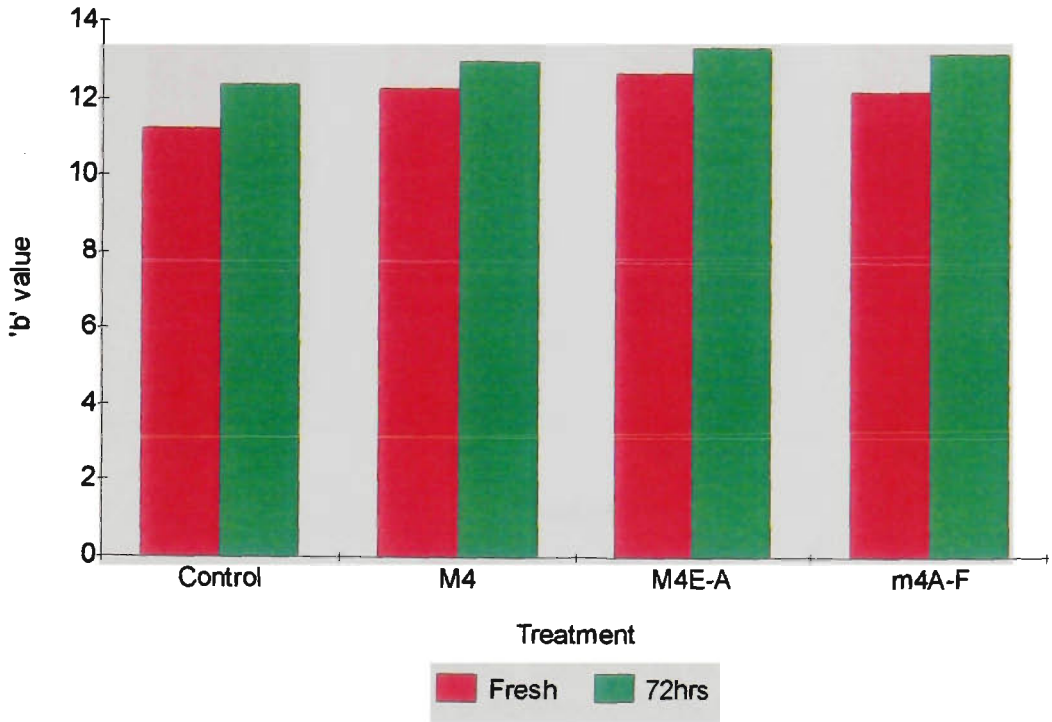


Figure 6.35 **Impact on crumb colour in aged breads treated with endoxylanase from *A niger* (M4); endoxylanase from *A niger* and *endo*-arabinanase from *A niger* (M4 E-A); and endoxylanase from *A niger* and α-L-arabinofuranosidase from *A niger* (M4 A-F)**

In the final section of the colour analysis breads were prepared with the added treatments M5, M5 E-A and M5 A-F (Figure 6.36). All the treatments appeared to impact on crumb as the bread aged. The results for these breads showed that there was no significant difference in the crumb of the fresh and aged breads for any of the treatments used. However the M5 preparation alone produced a significantly more yellow than the control after aging ($p<0.05$).

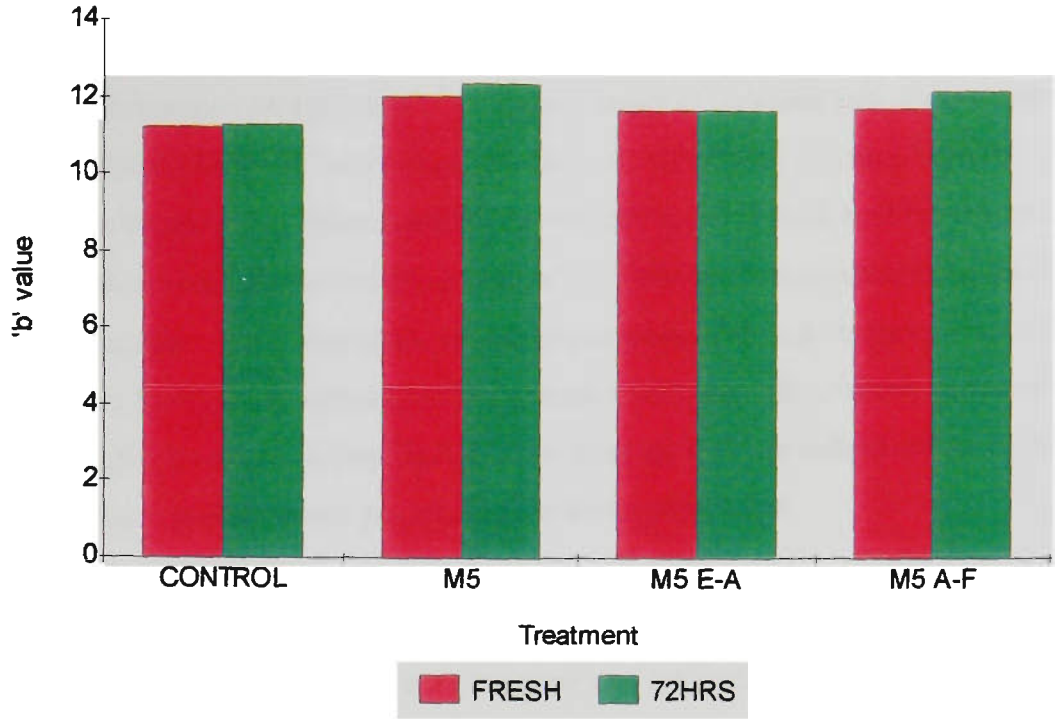


Figure 6.36 **Impact on crumb colour in aged breads treated with endoxylanase from *H insolens* (M5); endoxylanase from *T viride* and *endo*-arabinanase from *H insolens* (M5 E-A); and endoxylanase from *H insolens* and α-L-arabinofuranosidase from *A niger* (M5 A-F)**

The findings of this section of the study indicate that the selection of endoxylanases in conjunction with either *endo*-arabinanase or α-L-arabinofuranosidase for use in bread making may impact on the crumb colour both in fresh bread and in bread that is aging. More work is needed to identify the chemical processes that contribute to a yellowing crumb as bread ages. The results of such work may have commercial implications on future applications of some of the treatments investigated here. Consumer acceptance of loaf bread is affected by overall appearance, loaf symmetry as well as internal crumb structure and colour. The visibly yellower crumb observed in some of the breads crumbs examined here would impact negatively on consumer acceptance of the resulting loaves, thus rendering some of these treatments commercially unattractive.

6.4 Discussion

This study prior to this section has centred on the impact of highly purified endoxylanases on the loaf characteristics of white bread. As mentioned earlier, commercial pentosanase preparations contain a range of xylanase and arabinanase activities that are believed to act synergistically to produce the enhanced effects observed in breads when these preparations are added. This section of the current study investigated the role of *endo*-arabinanase and α-L-arabinofuranosidase enzymes in bread production. The effect of these two preparations on bread characteristics were investigated firstly alone and then in combination with the endoxylanase preparations. It was possible to establish that the use of arabinofuranosidase indeed enhances the action of some endoxylanase preparations in bread production.

Initially breads were treated with arabinofuranosidase only. The significantly darker crust colour observed in these breads suggests that the hydrolysis of arabinose residues from the xylose backbone increases the level of carbohydrate available to take part in Maillard browning and caramellisation. Both of these reactions appear to contribute to the development of crust colour during baking. The addition of arabinofuranosidase in conjunction with each of the endoxylanase preparations resulted bread crusts that were significantly darker than the control loaves. The addition of arabinofuranosidase also aided in controlling the degree of yellowing as the bread crumb aged. This finding suggests that the ability of arabinofuranosidase to enhance the activity of endoxylanase is dependant on a range of factors, including, source of enzyme and pH and temperature optima.

The ability of composite treatments of xylanase and arabinofuranosidase to slow staling also appeared to be affected by the combination used. Freshly baked breads prepared with treatments M4A-F and M5A-F were significantly softer than the untreated breads. Significantly softer bread crumb was also observed in breads at the completion of the staling study when these treatments were added to the bread formulation ($p<0.05$). By comparison the addition of xylanase preparations M4 and M5 only, did not significantly affect crumb softness at any stage throughout the study. When breads were treated with the M3A-F composite, no significant differences were observed in crumb softness between the freshly baked treated and untreated breads. However, the compression data revealed crumbs that were significantly softer than the control breads after 24 and 48hrs

($p<0.05$). This reduction in crumb firmness was not maintained with the data revealing no significant softness in crumb at 72hrs. A possible explanation for the results presented here may be that the degree of substitution of arabinose molecules on the xylan backbone is adversely affecting the activity of endoxylanase from some fungal sources. Such enzymes may require a greater segment of unsubstituted xylose units in order to allow hydrolysis to occur. When arabinofuranosidases from *A niger* were added to a bread formulation in addition to these xylanases at the appropriate levels, a degree of depolymerisation may occur that allows the endoxylanase greater access to the xylan backbone. Further, the compression data obtained for breads with the added treatments M1A-F and M2A-F showed that whilst the addition of these composites to the bread formulations resulted in crumbs that were softer than the control throughout the trial ($p<0.05$), these crumbs were not significantly softer than bread crumbs treated with preparations M1 and M2 only. Further work is recommended to establish the degree of arabinose substitution in Australian wheat flours to determine if this is a limiting factor in the level of arabinoxylan hydrolysis for a defined endoxylanase.

Chapter 7

Results and discussion – Changes in crust and crumb characteristics when purified amylolytic enzymes are added to a basic bread formulation

7.1 Introduction

In order to establish a complete picture of the role of some of the most common carbohydrate hydrolases in bread production, the effects of α-amylases on bread properties were also investigated. The effects of α-amylases sourced from *A niger, A oryzae* and *B subtilis* have already been documented (Asp et al 1985, Kulp and Ponte 1981). However, the use of thermostable bacterial α-amylases has had little application in the baking industry to date, although the addition of a bacterial amylase of intermediate temperature stability such as Novamyl has been documented (Gerrard et al, 1997). The higher thermal stability of some of these enzymes has in the past made them unsuitable as a general-purpose amylase for the baking industry. The hydrolytic activity of bacterial amylases even after baking leads to the continued break down of amylose with the resulting sticky crumb being totally unacceptable to the consumer. However, the smaller dosage required for these enzymes may make them a serious cost effective alternative to the cereal and fungal α-amylases currently available to the food industry. In the final section of the current study the role of α-amylase in bread production from fungal and bacterial sources was examined. Whilst the influence of α-amylases on bread formulation is well documented, (Lent and Grant 2001, Leon et al. 2002, Sahltstrom and Brathen 1996, Armero and Collar 1998, Cauvain and Chamberlain 1988) the possible implementation of bacterial α-amylases as a baking aid has not been extensively explored. The purpose of this section of the current study was twofold. Firstly the study set out to establish a treatment level for a thermostable bacterial α-amylase that resulted in the production of breads with crumb and crust properties comparable to those prepared with the addition of α-amylases from more traditional sources. Having established a treatment level, the aim of the study was to investigate the breakdown of amylose due to the introduction of these preparations during baking.

The preparations used were a fungal α-amylase, sourced from *A oryzae*, and a bacterial α-amylase, sourced from *B licheniformis*. These contained 67 U/mg and 1 000U/mL, and 54 U/mg and 3 000 U/mL respectively, (see Table 4.1). Initially each preparation was measured for levels of enzyme activity as well as the presence of contaminating enzymes as described in methods and materials section 3.2.2. The results are given in Table 7.1

Table 7.1 Levels of enzyme activities of selected ingredients and commercial amylase preparations

	α-Amylase (U/mL)	Specified value (U/mL)	Endoxylanase (U/mL)	Amylo-glucosidase (U/mL)	Cellulase (U/mL)
Flour	ND	-	0.038	ND	ND
Dough improver	120	-	0.062	ND	ND
α-Amylase (fungal)	1000	1000	ND	ND	ND
α-Amylase (bacterial)	3000	3000	ND	ND	ND

One unit of activity is defined as the amount of enzyme required to release one micromole of substrate per minute under defined conditions

- no comparable literature values available

ND – not at detectable levels

Each enzyme preparation was assayed as described in Section 3.2.2. Using the appropriate substrates, each preparation was assayed for the presence of α-amylase (Table 7.1). Again the presence of contaminating enzyme activity was not detected at measurable levels. In addition to this no loss of activity of the principal enzyme was observed in either of the amylase preparations.

7.1.1 Preliminary baking studies

Breads were prepared in triplicate using both preparations used at a level of 5μL as an initial reference point. A series of untreated breads were also prepared to serve as the control. The resulting loaves appeared acceptable at the completion of the baking cycle although a visibly darker crust was observed for the breads treated with the bacterial preparation (Figure 7.1). However, when the breads treated with the bacterial α-amylase were removed from the baking pans they immediately began to collapse. Slicing of these breads showed dramatic differences in the internal crumb structure of these breads compared with those treated with the fungal preparation and the untreated breads. The crumb showed a significant degree of yellowing in conjunction with a marked degree of gumminess and loss of crumb structure. Figure 7.2 shows the degree of deterioration observed. To establish a treatment level for this preparation that resulted in breads with crumb and crust characteristics visibly comparable to those breads treated with the fungal α-amylase a series of dilutions were prepared covering the range of 1:100 to 1:2000. Bread formulations were treated with each dilution and the resulting loaves compared with breads treated with 5μL of the fungal preparation. A comparison of the compression data and visual observations was used to determine the appropriate dilution factor for the enzyme preparation. A final treatment level of 10μL of bacterial α-amylase from a dilution of 1:1000 was considered the optimum level of treatment to produce breads that had crumb and crust properties comparable to those observed in breads made the addition of α-amylase from *A oryzae*. The end products of baking studies using this treatment factor are shown in Figure 7.3.

7.1.2 Compression studies of bread crumb treated with α-amylase

The ability of the amylase preparations to produce softer crumb was determined using the Instron UTM. The results of this analysis showed that treating bread dough with 5μL of either the fungal or bacterial amylase preparations did not result in bread crumb that was softer than the control loaves in the freshly baked breads (Figure 7.4; Appendix7.1). To further investigate this finding, a series of trials were carried out in which bread formulations were supplemented with either of the amylase preparations at a level of 5, 10 and 50μL. Crumb softness was measured and the results are presented in Figures 7.5 and 7.6.

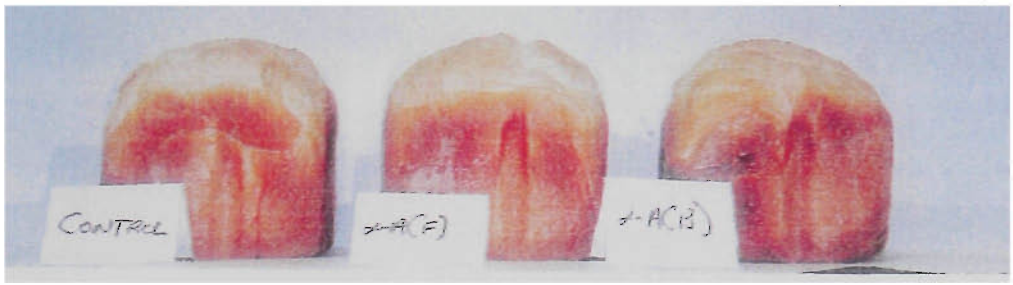


Figure 7.1 **Controls and breads treated with α-amylase from *A orzyae* and *B licheniformis* that had not been diluted showing the collapse of the loaf treated with the bacterial preparation**

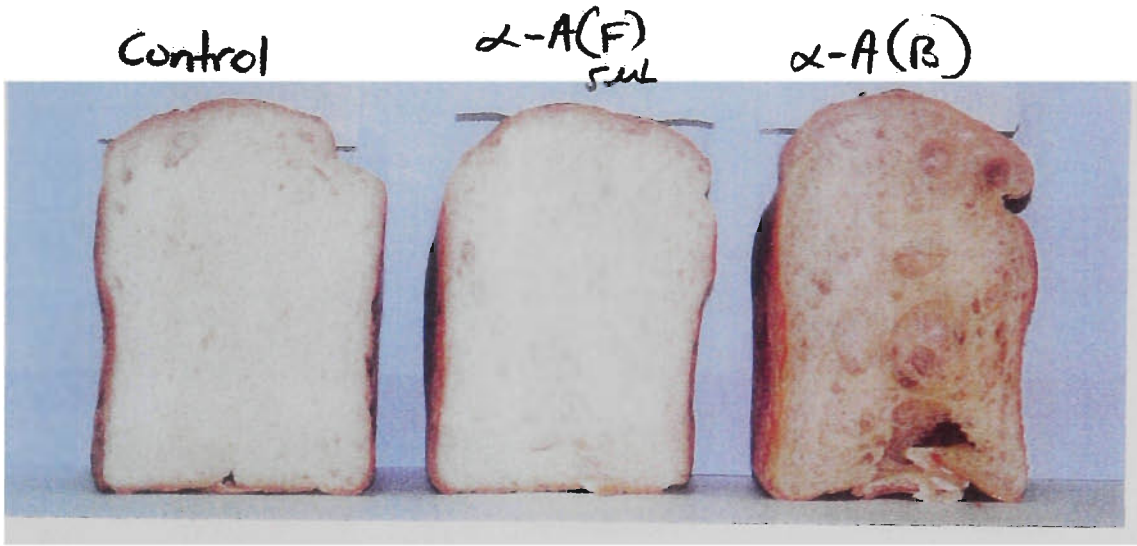


Figure 7.2 **Breads treated with α-amylase from *A oryzae* and *B licheniformis* showing the deterioration in crumb when the bacterial preparation was added in its undiluted form**

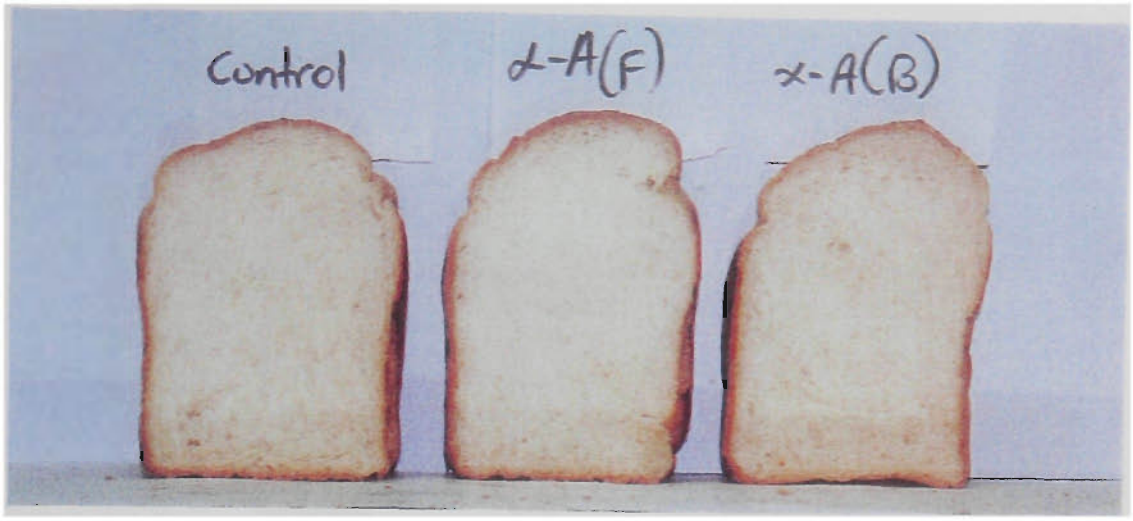


Figure 7.3 **Control bread and bread treated with 5µL of α-amylase from *A oryzae* and 5µL of α-amylase from *B licheniformis* diluted 1000 fold**

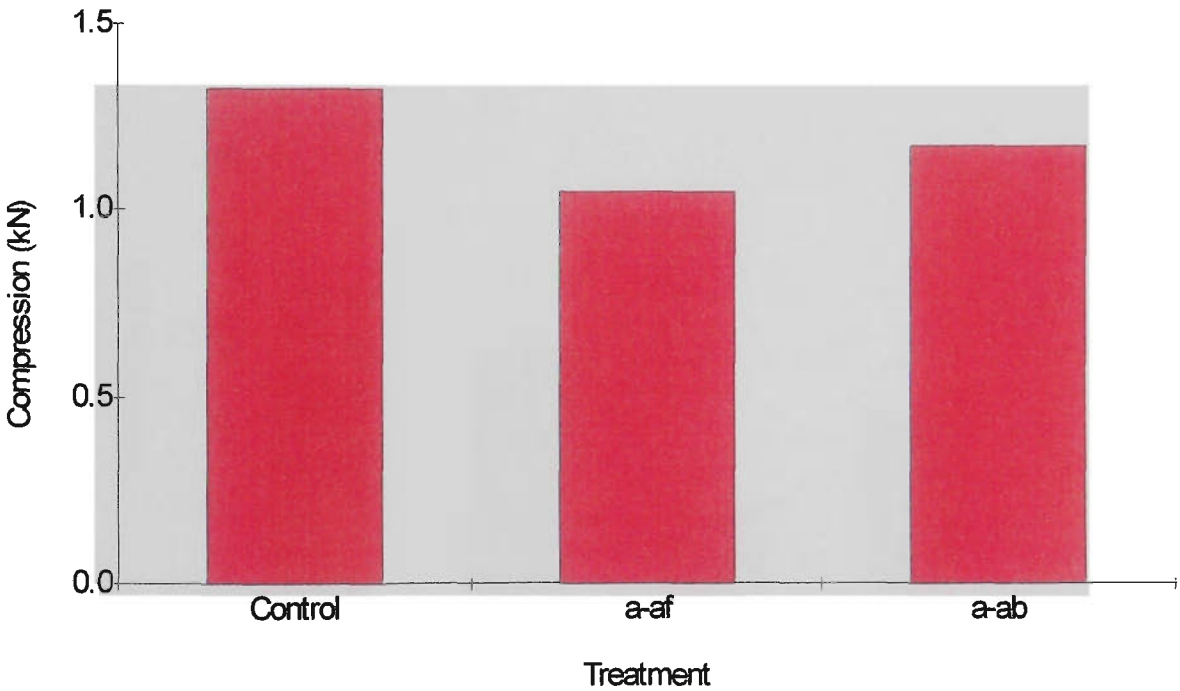


Figure 7.4 **Compression data showing no significant difference in crumb softness for breads treated with fungal α-amylase (a-af) and bacterial α-amylase at a level of 5µL compared with the control breads**

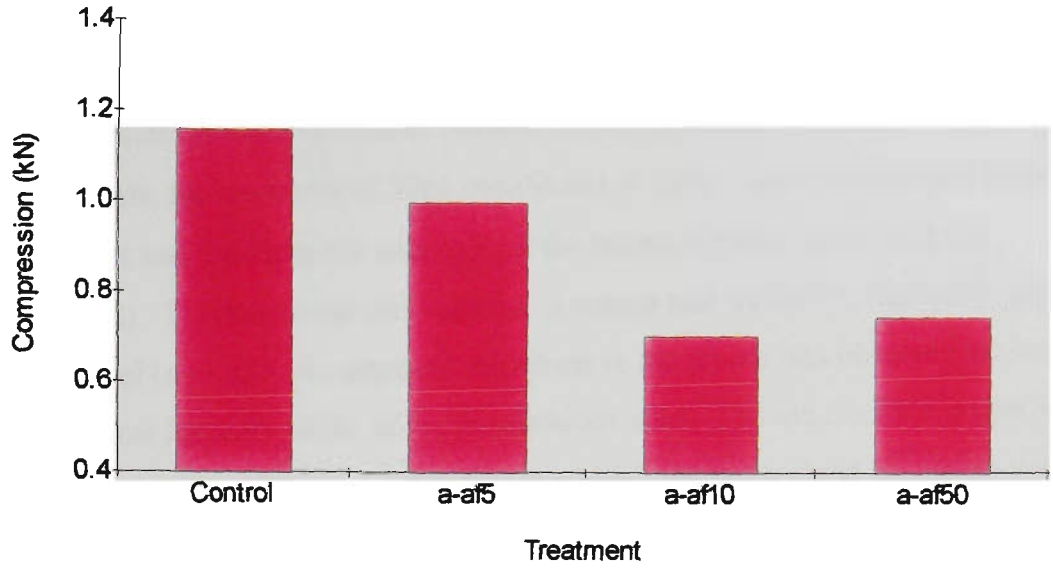


Figure 7.5 **Compression data for breads treated with fungal α-amylase at a level of 5μL (a-af5), 10μL (a-af10) and 50μL (a-af50).**

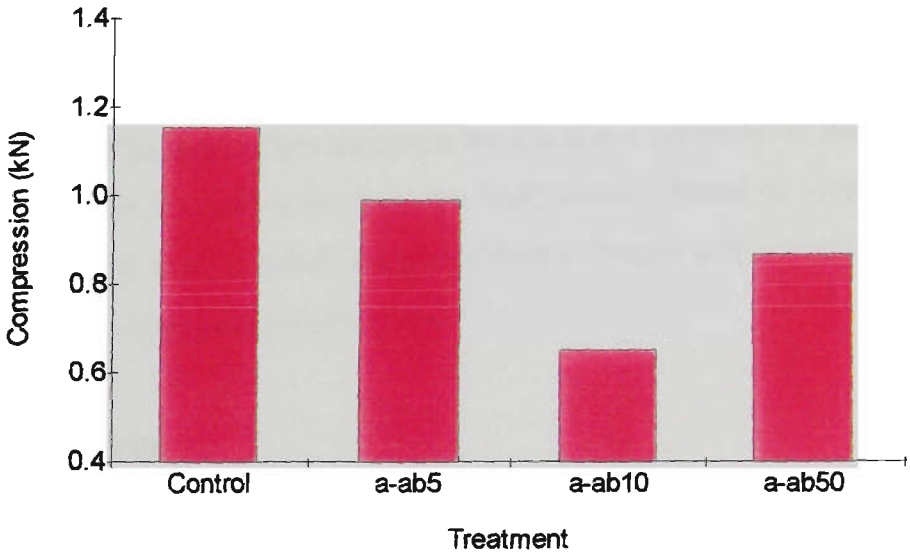


Figure 7.6 **Compression data for breads treated with bacterial α-amylase at a level of 5μL (a-ab5), 10μL (a-ab10) and 50μL (a-ab50)**

The results show softer bread crumbs were produced when bread formulations were treated with 10μL and 50μL of the fungal preparation ($p<0.05$; Appendix 7.2). No further impact was observed on crumb softness between the two treatment levels. When aliquots of the suitably diluted bacterial α-amylase were added at the same treatment levels, the treatment of 10μL was found to have a softer crumb than both the control breads and those treated with 5μL of the bacterial preparation ($p<0.05$; Appendix 7.2). This treatment also resulted in crumb that was softer than the highest treatment level ($p<0.05$). No apparent stickiness in the crumb was observed at the lower treatment level for either of the preparations used. The use of either preparation at the highest level resulted in crumbs that exhibited gummy textural properties with smaller gas vacuoles. The deterioration in crumb texture appeared to contribute to the increased crumb firmness observed in these higher treatment loaves. In addition to this, the loaf symmetry was adversely affected at the highest treatment level.

Other researchers in this area, have found that the addition of α-amylase to a bread formulation produced a significantly softer crumb in the baked bread (Akers and Hoseney 1994, Asp et al 1985, Kulp and Ponte 1981, Sahlstrom and Brathen 1996). These findings are consistent with the results of this study. However, it appears that the selection of an appropriate treatment level is also a contributing factor in the development of crumb softness in the final product. Based on these findings, further experimental work involved the preparation of breads with a treatment level of 10μL for each of the α-amylase preparations.

7.1.3 Colour

The addition of α-amylase from fungal and bacterial sources at 10μL resulted in the development of crusts that were significantly darker than the untreated breads ($p<0.05$; Appendix 7.3). The results are given in Figure 7.7. Amylases are added to bread formulations to break down starch, supplying source of energy for yeast during fermentation (Bread Research Institute of Australia 1989, Pyler 1988). The production of higher levels of maltose provides a higher concentration of reducing sugars that can then take part in Maillard browning. The darker crusts observed in the breads treated with the α-amylase preparations in this study are consistent with findings of Sahlstrom

and Brathen (1996), who found that the addition of α-amylase to a bread formulation resulted in significantly darker crust colour.

No significant differences were observed in crumb whiteness for the aged or the fresh breads to which either α-amylase preparation was added. However, when the development of a yellow crumb during aging was investigated significant differences were observed. The results of the crumb colour are presented in Figure 7.8.

No differences were observed in the fresh breads for either of the treatments used compared with the control breads. After aging for 72hrs, breads treated with the bacterial preparation had crumb that was significantly more yellow than fresh untreated breads and those treated with the fungal preparation ($p<0.05$). Again no difference was observed between the fresh and aged breads treated with the either the bacterial or the fungal preparation. The presence of the amylase preparations did not appear to impact on crumb colour as the treated breads aged. The loaves treated with the bacterial preparation were visually more yellow than the untreated loaves but this was not found to be significant (Appendix 7.4). More work is needed to identify factors that affect crumb colour as bread ages.

Loss of crumb whiteness as bread ages results in a decrease in consumer acceptance. The findings of the current study suggest that α-amylases from fungal sources do not impede the development of yellow crumb in aging bread thus consumer acceptance in not increased. Further research is needed, to investigate changes occurring in crumb colour as bread ages and whether, any of the enzymes utilized in this study have a role to play in slowing the development of a yellow crumb.

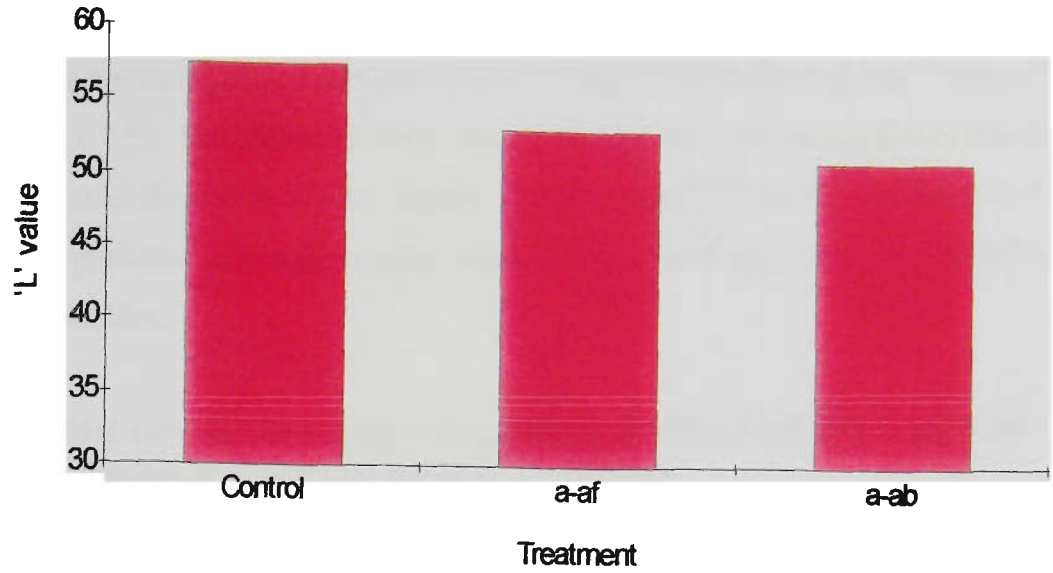


Figure 7.7 **Development of crust colour in fresh bread when α-amylases from fungal (a-af) and bacterial (a-ab) sources were incorporated into a basic bread formulation**

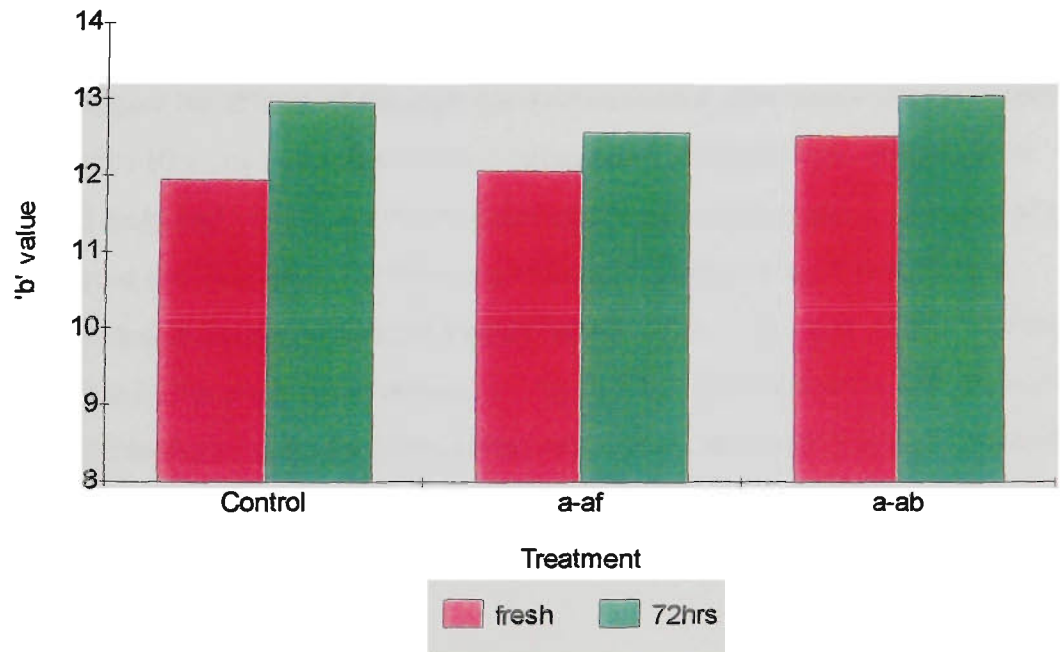


Figure 7.8 **The development of yellow crumb in aged bread when α-amylase from fungal (a-af) and bacterial (a-ab) were incorporated into a basic bread formulation**

7.1.4 The influence of fungal and bacterial α-amylases on loaf volume

No differences were observed in loaf volumes when amylases from fungal and bacterial sources were incorporated into the bread formulation at the level of 10μL (Figure 7.9; Appendix 7.5). This finding is consistent with those of other researchers in this area. For example, the results of Cauvain and Chamberlain (1988) and Valjakka et al (1994) have all indicated minimal increases in loaf volume when α-amylases are added to bread formulae.

7.1.5 Moisture and its impact on the staling of white bread baked with the addition of fungal and bacterial α-amylases

In order to investigate the effects of bacterial and fungal α-amylase during storage, breads were prepared in duplicate using the basic bread formulation. The results of the moisture analysis are given in Figure 7.10 (Appendix 7.6). The data showed that the breads treated with either of the amylase preparations used, did not exhibit significant water loss during storage ($p<0.05$). Significant moisture loss was observed for the control breads.

To investigate the effects of these preparations as bread aged experimental loaves were treated with 10μL of α-amylase from *A oryzae* or *B licheniformis*. Breads were prepared daily and stored in polyethylene bags at room temperature. A series of control loaves were also prepared. At the completion of 72hrs there were breads that corresponded to storage criteria of freshly baked, 24hrs, 48hrs and 72hrs. The results of this section of the study are presented in Figure 7.11. The data show that the use of both fungal and bacterial amylase preparations resulted in significantly higher moisture levels being retained in the bread crumb after 72hrs ($p<0.05$; Appendix 7.6).

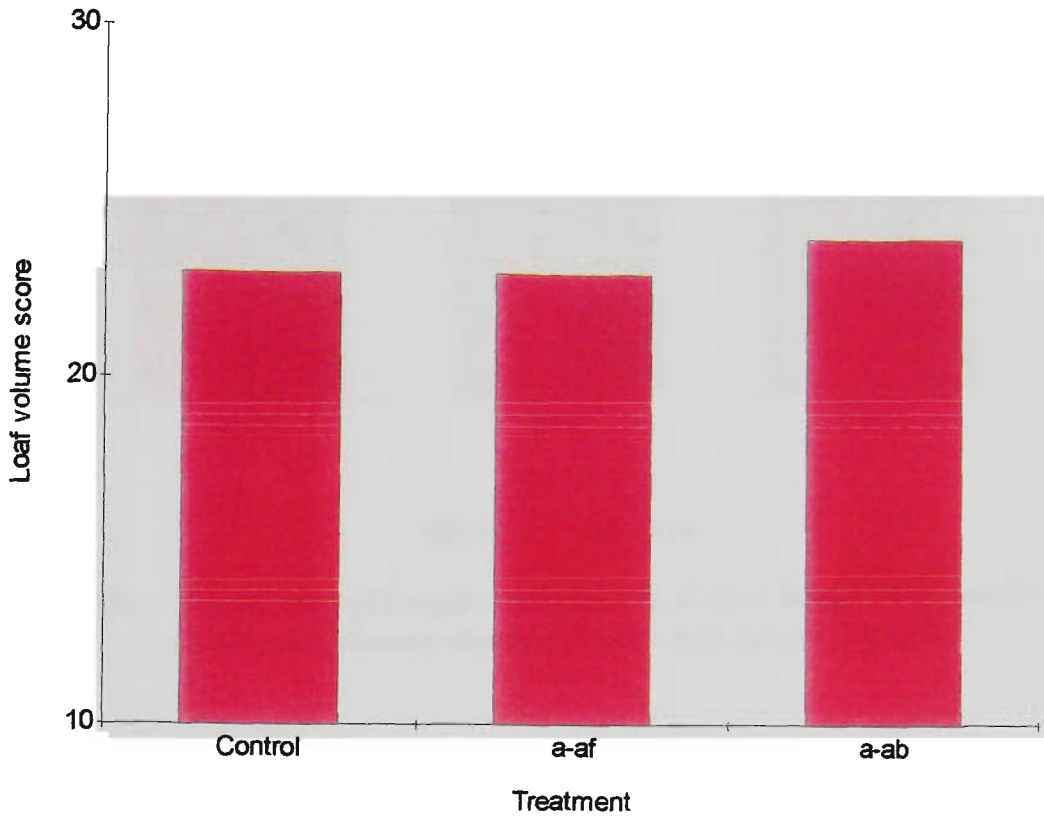


Figure 7.9 **Impact of fungal α-amylase (a-af) and bacterial α-amylase (a-ab) on loaf volume of white bread**

The addition of amylase from *B licheniformis* to the formulation resulted in bread crumb that was significantly softer than untreated breads at each stage of the trial ($p<0.05$; Appendix 7.7). This preparation also produced crumb that was softer after 72hrs than the bread treated with the fungal preparation. When amylase from *A oryzae* was added to a bread formulation the resulting breads were also softer than the control at each stage of the trial ($p<0.05$).

The use of α-amylases to delay the rate of crumb firming as bread ages has been demonstrated by a number of researchers (Defloor and Delcour 1999, Grossmann and Debarber 1997, Sahlstrom and Brathen 1996, Valjakka et al 1994). This finding was supported by the current study. In addition, the use of the bacterial amylase preparation slowed the rate of crumb firming to a greater degree than the fungal amylase. The results of the staling trials are presented in Figure 7.11.

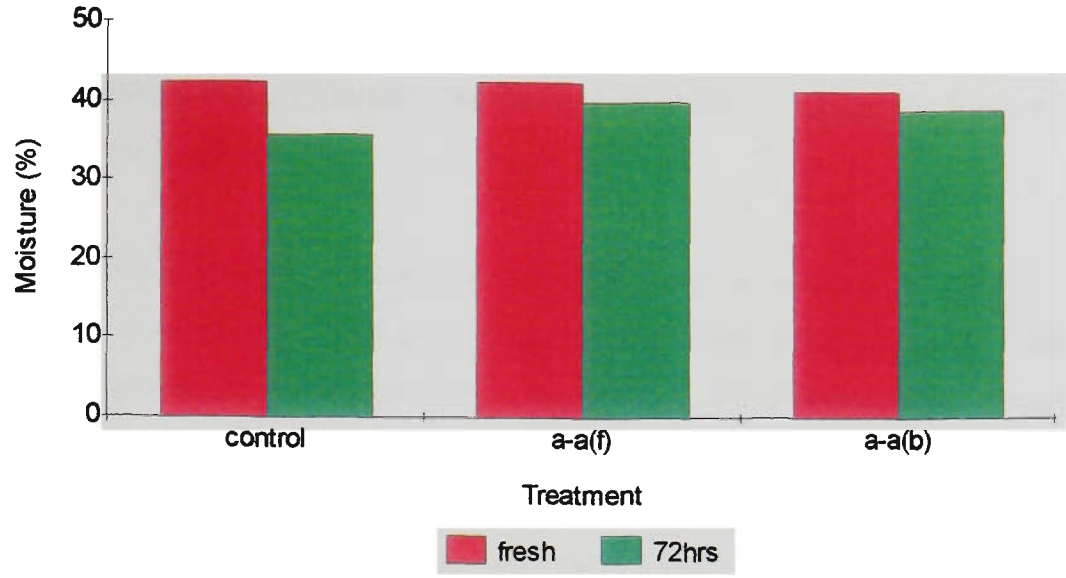


Figure 7.10 **The impact of fungal α-amylase (a-af) and bacterial α-amylase (a-ab) on moisture content of fresh and aged white bread**

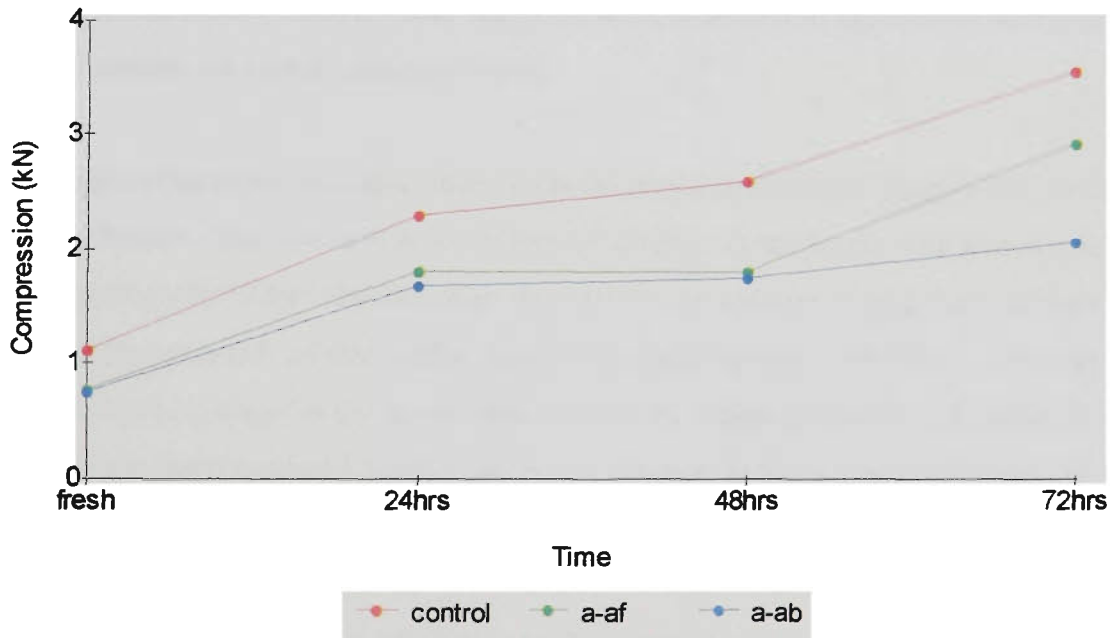


Figure 7.11 **The impact of fungal α-amylase (a-af) and bacterial α-amylase (a-ab) on staling profiles of white bread**

7.1.6 HPLC analysis of low molecular weight carbohydrates

In order to investigate the effects of added α-amylase at a molecular level, treated and untreated breads were extracted using the method of Potus et al (1994). The results are presented in Table 7.2

The addition of α-amylase from *A oryzae* and *B lichneniformis* to a basic bread formulation resulted in significantly higher levels of maltose being detected in breads that had been aged for 72hrs ($p<0.05$; Appendix 7.8). The *B licheniformis* α-amylase has a higher thermostability that the *A oryzae* α-amylase and this was reflected in the data obtained in this section. Maltose levels in the breads stored for 72hr and treated with *B licheniformis* were more than twice those found in the fresh breads to which the same preparation was added. However whether the use of this enzyme results in the production of significantly less maltose in the fresh bread, but remains active during storage to produce significantly higher levels of maltose after aging is unclear. The bacterial amylase used in this study may be inhibiting the residual cereal amylases making maltose in the control bread but more work is needed to understand how this enzyme impacts on cereal carbohydrolases.

Significant differences were also observed between the maltotriose levels of the fresh and aged breads. The use of *B licheniformis* resulted in bread crumb with a higher level of maltotriose after 72hrs than the control ($p<0.05$). In addition to this, the treatment produced higher levels of maltotriose than the fungal preparation ($p<0.05$). Fructose was found to be present in the breads treated with the fungal preparation at higher levels than both the fresh and aged control loaves. In addition to this it was also present in the fresh bread treated with the bacterial preparation but only minimal amounts could were extracted from the aged breads treated with this enzyme. The presence of fructose may have been due to the presence of sucrose in the initial formulation being broken down during the dough formation process into its individual constituents of glucose and fructose.

Table 7.2 **HPLC analysis of maltose, glucose, fructose and maltotriose content of flour, untreated breads and breads treated with fungal α-amylase (α-A(F)) and bacterial α-amylase (α-A(B)) sourced from A oryzae and B licheniformis respectively**

Treatment		Maltose g/100g	Glucose g/100g	Fructose g/100g	Maltotriose g/100g
Flour		0.23	0.29	-	0.001
Control					
	Fresh	0.55	0.10	0.07	0.02
	72 hrs	0.57	0.10	0.07	0.03
α-A(F)					
	Fresh	0.46	0.02	0.12[a]	0.01
	72 hrs	0.50[a]	0.02	0.14[a]	0.02
α-F(B)					
	Fresh	0.20	0.01	0.12[a]	0.04[b]
	72 hrs	0.56[a]	0.01	0.02	0.06[a b]

[a]Denotes significant differences between aged and fresh breads for a given treatment.
[b]Denotes significant differences between treatments.
Control - Breads made using the basic bread formulation only
α-A(F) - Breads made using the basic bread formulation to which 10 µL of α-amylase sourced from *A oryzae* has been added.
α-F(B) - Breads made using the basic bread formulation to which 10 µL of α-amylase sourced from B *licheniformis* has been added.

7.1.7 Analysis of starch retrogradation using differential scanning calorimetry

A number of methods can be used to measure the staling of bread crumb. Objective measurements such as compression tests, give clear and reproducible data that can be used to determine the rate of starch retrogradation in aged bread. These methods do not indicate what is happening at a molecular level. As bread ages there is a transition from disorder to order as the molecules in the starch granules that have gelatinized during baking realign. The formation of cross links between neighbouring molecules leads to a

gradual recrystallisation of the starch molecules. Water is excluded and the crumb becomes increasingly firmer (Pateras 1995). DSC is a technique that allows the degree of retrogradation in aged bread to be measured as a function of temperature. As the starch retrogrades over time more energy is required to break the bonds. The amount of heat required can then be related to the level of starch retrogradation that has occurred in the bread crumb. The aim of this section of the study was to determine the effects of α-amylases in relation to their ability to slow the rate of starch retrogradation and hence the rate of staling of bread crumb. A Perkin Elmer DSC-7 instrument was used with a Thermal analysis controller TAC 7/DX.

The ability of α-amylase preparations from fungal and bacterial origins to impede starch retrogradation have been widely reported in the literature (Akers and Hoseney 1994, Defloor and Delcour 1999, Dragsdorf and Varriano-Marston 1980, Gerrard et al 1997 Hebeda et al 1990, Hebeda et al 1991). The compression data reported in Figure 7.11 of the current study, demonstrated that the bacterial preparation used in this study produced softer crumb that remained softer through the aging period.

A significant slow down in crumb firming was observed in the final 24hrs of the study. The fungal preparation however showed only small differences in the slope and retained the same softness differential throughout the storage period. However whether the softer crumb is due to decreased retrogradation of the starch molecules as the bread ages or some other phenomenon is unclear. To investigate this further a series of control and treated breads was prepared to measure the retrogradation of the starch molecules in aged bread crumb.

Examples of the thermograms obtained during this study appear in Figures 7.12-7.14. The peak associated with retrograded starch in bread crumb is seen in Figures 7.12 and 7.14. For comparison purposes, a sample of ungelatinised starch from wheat was also analysed and shows the typical peak at approximately 63°C (Figure 7.13). The average measurements for retrograded starches in stored bread samples are summarised in Table 7.3. The onset of the retrogradation peak appears at approximately 42-45°C and concludes at between 61 and 63°C. Similar findings were reported by Defloor and Delcour (1999) who identified staling endotherm peaks between 41 and 72°C. Enthalpy

values indicate that significantly less starch retrogradation occurred in bread treated with bacterial α-amylase sourced from *B licheniformis* ($p<0.05$; Appendix 7.9).

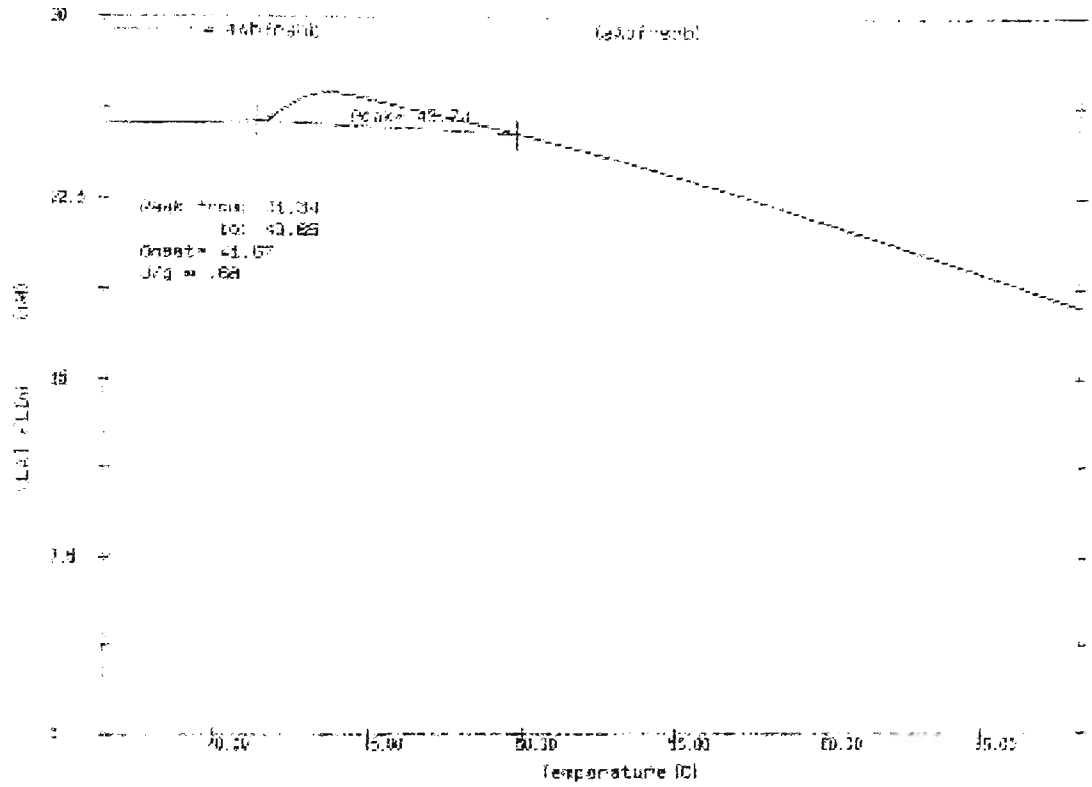


Figure 7.12 DSC pattern obtained for a sample of crumb from bread aged for 72hrs, incorporating α-amylase of bacterial origin

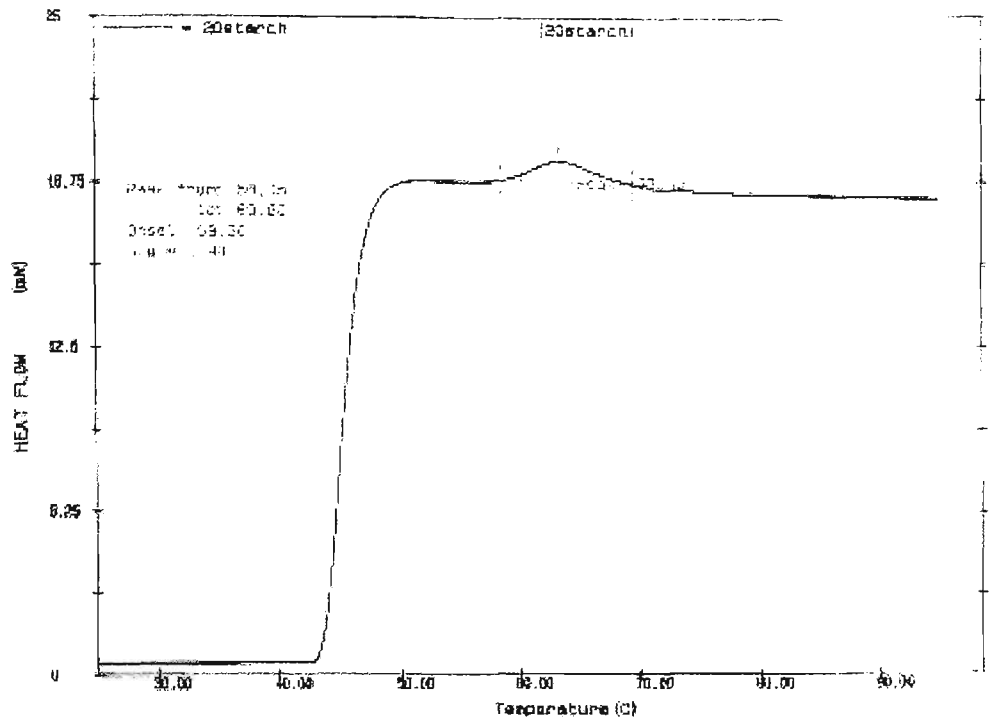


Figure 7.13 DSC pattern obtained for a sample of wheat starch showing a typical endotherm for granule gelatinization

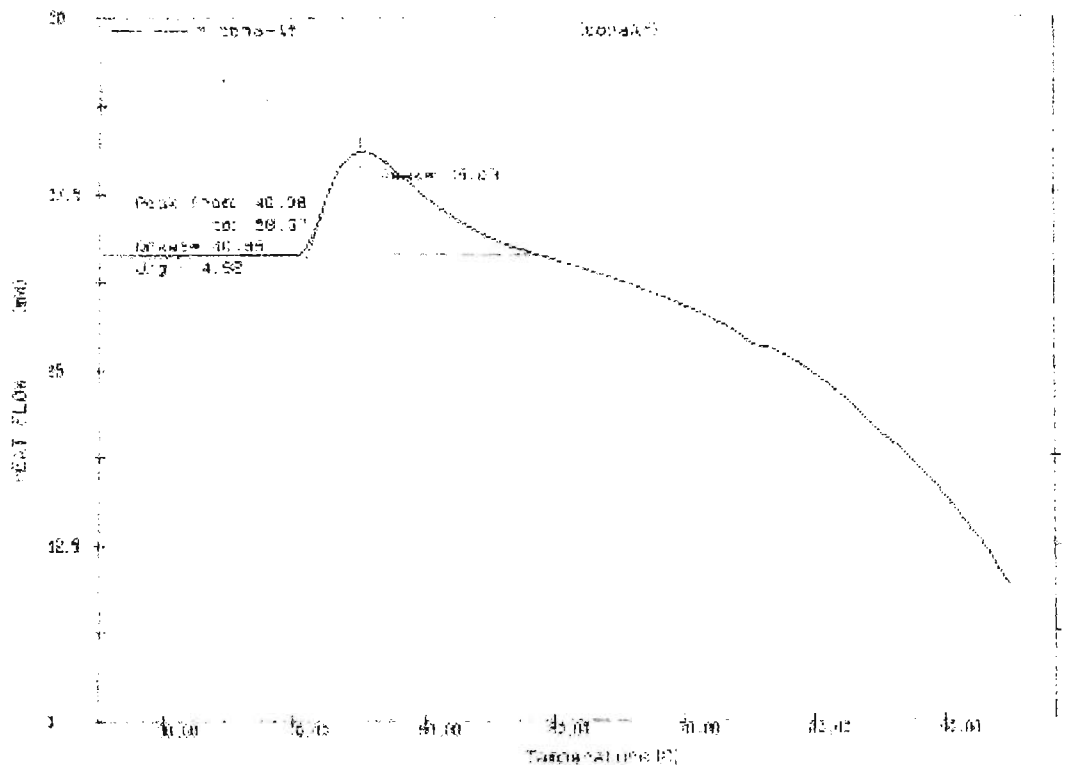


Figure 7.14 DSC pattern obtained for a sample of crumb from bread incorporating α-amylase of fungal origin and stored for 72 hrs

Table 7.3 Thermal characteristics of aged bread crumb determined by DSC analysis

Sample	Tg	Tp	Tc	ΔH (J/g)
Control 72hrs	45.9	50.3	63.0	2.61
α-A(F) 72hrs	43.5	49.8	63.2	2.56
α-A(B) 72hrs	42.2	48.0	61.2	1.83[a]

[a]Indicates enthalpy values significantly different to the enthalpy values of the controls
Tg = onset of peak (°C)
Tp = Temperature at peak (°C)
Tc = conclusion of peak (°C)
ΔH = enthalpy value

7.2 Discussion

The role of starch retrogradation in bread staling is well documented (Kulp and Ponte 1981, Maga 1975, Willhoft 1973). Further the ability of some α-amylases to slow the impact of aging on bread crumb has been well documented (Dragsdorf and Varriano-Marston 1980, Kulp and Ponte 1981, Martin et al 1991). Preparations containing α-amylase activity are sourced from fungal, cereal and bacterial origins. As such they would be expected to exhibit very different properties in terms of pH and temperature optima and substrate specificity. (Asp et al 1985).

In the final part of the current study, the ability of an α-amylase from *B licheniformis* to enhance bread crumb in freshly baked bread and to slow the rate of crumb firming during storage was investigated. Whilst the initial breads exhibited inedible crumb texture, a dilution of 1:1000, resulted in a treatment level being obtained that produced crumb and crust characteristics that were visibly comparable to the control breads. By comparing the final product with breads prepared with a fungal α-amylase, the ability of the bacterial preparation to produce comparable loaf characteristics was clearly demonstrated.

In addition the incorporation of the suitably diluted bacterial preparation was also able to slow the rate of crumb firming as the breads aged. Analysis of the HPLC data, showed significantly higher levels of maltotriose in the aged bread crumb treated with the bacterial amylase preparation, than both the control breads and those treated with the fungal preparation that had also been aged for 72hrs. These findings are consistent with those of Gerrard et al (1997) and Gil et al (1999) who demonstrated the ability of bacterial α-amylase to significantly reduce the rate of firming in bread. The authors further suggested that the dextrins present in the amylase-treated loaves were due to modifications of the starch that delays staling, but were not the direct cause of the antistaling effects observed. These findings are supported by those of Defloor and Delcour (1999) who found maltodextrins produced by antistaling enzymes reduced amylopectin retrogradation in bread stored for 7 days. Martin et al (1991) however suggest that the formation of maltodextrins with DP3-10 have an antistaling effect on bread crumb due to the formation of cross links between the gluten and starch molecules.

The findings of the current study suggest that the level of maltotriose in bread crumb does impact on the rate of crumb firming. A strong positive correlation was observed between the rate of crumb firming and the levels of maltotriose in the aged breads, suggesting that the presence of smaller maltodextrins is contributing to the delay in crumb firming observed (Appendix 7.10). No correlation was observed between the level of fructose, glucose or maltose and a delay in crumb firming. DSC analysis of starch retrogradation demonstrated the ability of bacterial α-amylase to slow the rate of starch retrogradation as bread ages. However, no correlation was found to exist between the level of retrogradation and crumb softness after 72hrs (Appendix 7.11). This finding is consistent with those of Ponte et al (1962) and Maleki et al (1980) who found that retrogradation is not the only factor contributing to crumb firming.

As mentioned earlier, one of the aims of this study was to determine if α-amylase from *B licheniformis* could be optimized to produce a loaf of bread with loaf characteristics comparable to a fungal preparation. If bread could be produced with this preparation, that had consumer appeal, then it may provide a viable cost effective alternative to the amylases currently utilized by the baking industry. The results of this study have shown, that by using a suitable dilution of the bacterial preparation, bread could be produced that had acceptable consumer appeal. Both the fungal and the bacterial preparations are readily available at a similar cost. The findings of this study therefore indicate that the bacterial α-amylase may provide a viable and effective alternative to the fungal preparations currently favoured by the industry.

Chapter 8

General discussion and conclusions

8.1 Summary of the project

The current study entailed the addition of a range of carbohydrate hydrolases to a basic bread formulation, under Australian conditions, to investigate changes in the textural properties of white bread, associated with the hydrolytic products of these enzymes. Highly purified endoxylanases, endoarabinanases, arabinofuranosidases and α-amylases were utilised in this project. The hydrolytic products of the various enzyme activities were examined using HPLC in the freshly baked breads. The findings of this section of the study were then related back to the textural analyses to determine if a relationship existed between the hydrolytic by-products and the changes in the textural characteristics observed in the breads baked with these preparations. Breads treated with endoxylanases and aged for 72hrs were also extracted and analysed to determine if any changes were occurring during storage. In addition to this, the synergistic effects of endoxylanases and endoarabinanases and arabinofuranosidases in a bread formulation were investigated. Finally, endoxylanases were added to a yeast leavened doughnut formulation, to determine if the findings of the bread studies were consistent for other yeast leavened bakery products. Bread dough formulations were also treated with α-amylases from fungal and bacterial sources. The impact of these preparations on crumb and crust characteristics were evaluated, in terms of their ability to enhance crumb softness in freshly baked breads and to delay the rate of crumb firming as bread ages.

8.2 Discussion

The hypothesis upon which the current study was based, is that one or a limited number of specific enzymes are responsible for the enhanced bread quality observed, when commercial enzyme preparations with pentosanase activity are used in bread making. To investigate this, five purified fungal endoxylanases, an endoarabinanase and an arabinofuranosidase preparation were added to a basic bread formulation in increasing amounts. The data presented in Chapter 4 showed that when the treatment levels for endoxylanase were increased above an optimum level adverse changes occurred in the textural properties of the crumb and crust for each of the preparations used. Crust

bloom was lost and the crumb structure became irregular, with the development of large air cells particularly at the crust crumb margin. The crumb of these breads also exhibited noticeable stickiness. The treatment levels selected for this study were 5, 10 and 50μL of each preparation. Although the greatest loss of crumb structure was observed at the highest treatment levels, all breads continued to exhibit less exaggerated deterioration when treated with 10μL of each preparation. In addition to this, significantly firmer crumbs were observed at the higher treatment levels for most of the preparations used. However when the treatment level was lowered to 5μL in the next section of the study, only the addition of preparations M1 and M2 resulted in significantly softer crumbs in the final product. Table 4.1 shows that preparations M1 and M2 had activity levels of 730U/mL and 360U/mL respectively. Preparations M3, M4 and M5 had 1900U/mL, 1000U/mL and 220U/mL respectively. Since there appears to be no correlation between the level of enzyme activity present and textural characteristics present in the baked breads it appeared that something else was accounting for the enhanced crumb softness produced by preparations M1 and M2. Each endoxylanase preparation had its own temperature and pH optima. A study of the dough pH at various stages of preparation (see Table 5.1) showed that both preparations had pH optima close to that of the bread dough suggesting that dough pH was limiting the ability of preparations M3, M4 and M5 to hydrolyse arabinoxylan. HPLC analysis of the pentosan profiles examined the levels of xylose and arabinose in terms of their solubility properties. The data showed significant differences in the pentosan makeup of the treated and control breads in addition to significant differences between the treatments used. Of particular interest was a comparison of the ratios of xylose solubilities. Breads found to have approximately equal proportions of the arabinoxylan fragments had significantly softer crumbs than those with a much greater proportion of the larger or smaller arabinoxylan residues. This finding suggests, that the end product of pentosanase hydrolysis, may be an important factor when selecting the most appropriate xylanase preparation for the baking industry.

The addition of endo-arabinanase and arabinofuranosidase only to a bread formulation did not result in changes in textural characteristics of the resulting loaves. Even when added at levels that were excessive for endoxylanase there appeared to be no apparent impact on crumb structure or texture. However, the addition of higher levels of

arabinofuranosidase produced loaf breads that were significantly larger than the untreated breads but did not exhibit the crumb deterioration observed when higher treatments of the endoxylanases were added. Whilst these preparations did not impact on crumb softness, or the keeping properties of the bread, the commercial viability of producing significantly larger loaves without a corresponding increase in raw materials and energy costs requires further investigation.

The next stage of the study involved investigating the synergistic effects of these preparations in conjunction with endoxylanases. The object was to determine if the presence of these ancillary enzymes played a role in the production of breads with increased loaf volumes observed when commercial pentosanases preparations are used. The effects of endoxylanase and endo-arabinanase were also studied since the use of endo-arabinanase alone appeared to impact positively on moisture content of aged breads. The synergistic effects of endoxylanases and arabinofuranosidases on loaf volume were mixed. The combination of M1 and arabinofuranosidase and M1 endo-arabinanase both produced loaves that were significantly larger than when M1 alone was used. When the endoxylanase M1 was replaced with M2, M3 and M4 endoxylanases the resulting breads had volumes that were comparable, to the control breads and those produced when only the endoxylanases were used. In addition to this the breads treated with the M1 composite exhibited deterioration in crumb structure. Since M1 had demonstrated a strong affinity for its substrate, these findings suggest that the addition of arabinose hydrolysing enzymes and the subsequent release of arabinose residues allowed excessive breakdown of the xylan backbone to occur. This in turn may have contributed to the deterioration in crumb structure observed in the treated loaves. Breads treated with the M5 composites were significantly smaller than the control breads and had a denser crumb structure with a more uniform arrangement of gas cells. No significant difference was observed for breads treated with M2, M3 and M4 composites. These findings suggest that endo-arabinanase and arabinofuranosidase from *A oryzae* are acting synergistically with only one of the endoxylanase preparations that being *T viride*. Breads treated with the composite preparations also exhibited deterioration in crumb structure and hence, an overall loss of loaf quality. The increased loaf volume without a loss of crumb structure observed when commercial preparations are used suggests that other residual enzymes present in these preparations are influencing the final loaf quality.

The current study has demonstrated the ability of various enzymes, with pentosanase activity, to affect the physical properties of loaf bread. However, further research is required to determine how the various residual enzyme acitivities interact, to produce breads with enhanced loaf volumes without the inherent loss of quality observed here.

Analysis of the moisture data also demonstrated the ability of some pentosan enzymes to enhance water retention as bread ages. When enzymes with arabinanase and arabinofuranosidase activity, were added as part of composite preparations with the endoxylanases, combinations M3, M4 and M5 were found to significantly slow the rate of moisture migration and loss as the breads aged. The hypothesis that slowing the rate of moisture loss as bread ages will also slow the rate of crumb firming is not supported by the current study. Statistical analysis of these findings did not show a correlation between increased moisture and crumb softness in the aged breads. In fact, the current study demonstrated that while endoxylanases with lower substrate specificity are enhanced by the presence of anxillary pentosanases enzymes, a high substrate specificity appears to be of primary importance in determining the impact on the final crumb softness.

In the final section of the current study the ability of α-amylase from *B licheniformis* and *A oryzae*, to impact on crumb and crust characteristics in fresh and aged breads was investigated. Control breads were prepared using the basic bread formulation only. Both preparations were found to slow the development of a yellow crumb as the bread aged, although the bacterial preparation also produced a significantly more yellow crumb than the control initially. As crumb colour can impact on the overall consumer acceptance of loaf bread the ability of the enzyme preparations to retain crumb whiteness during storage was an important finding. Darker crusts were also noted in the treated loaves. Higher levels of reducing sugars produced by enzymic hydrolysis of starch components enhanced Maillard reactions occurring during baking thus causing a darker crust in the resultant loaves.

HPLC analysis of the carbohydrate profile of fresh and aged breads showed significantly higher levels of maltotriose in breads treated with α-amylase from *B licheniformis*. The compression data clearly demonstrated the ability of both preparations to produce breads with significantly softer crumb however only the

bacterial preparation was found to significantly reduce the level of firming as the bread aged. DSC analysis showed significantly less starch retrogradation in the aged bread treated with the bacterial preparation compared with that treated with the fungal α-amylase. However, no correlation was observed between the level of retrogradation and the degree of crumb firming observed in the aged breads. As a bacterial preparation, α-amylase from *B licheniformis* was expected to have a higher thermal tolerance than the fungal α-amylase. The staling trials clearly demonstrated this as evidenced by the increase in maltotriose levels during aging suggesting that the enzymes are retaining some level of activity during and after the baking process.

In addition to this the findings of the initial baking trials demonstrated a high substrate specificity of the bacterial preparation necessitating a 1:1000 dilution of the original preparation to produce loaf breads that were acceptable to the consumer. As both preparations are similar in terms of cost and keeping properties careful use of the bacterial preparation the dilution factor required to use the bacterial preparation could be a viable cost effective alternative to fungal and cereal preparations currently available.

8.3 Conclusions

1. Endoxylanases from a range of fungal sources with differing pH and temperature optima can produce significant changes in the textural properties of white bread prepared under Australian bread making conditions.

2. Not all endoxylanases were effective in producing softer crumb and enhanced keeping properties. Factors including pH optima and substrate specificity resulted in significant variation in the ability of the individual preparations to bring about textural changes in white bread.

3. Substrate specificity appears to be a determining factor in the ability of the various endoxylanases utilised in this study to impact on the textural properties of white bread.

4. The endoxylanases used in this study did not slow the rate of starch retrogradation under the test conditions utilized. Thus the positive impact observed on staling, when breads were supplemented with preparations M1 and M2, were due to factors other than a slowing of amylopectin crystallisation.

5. The use of endoarabinanase and arabinofuranosidase alone do impact on crumb softness or the shelf life of white bread. However, when used as part of a composite pentosanase preparation, enzymes with the anxillary activity of arabinofurnanosidase can enhance the action of endoxylanases that, when used alone, do not produce significant changes in crumb softness. These findings warrant further investigation. Scaling up production may help determine if these preparations could be a natural, 'consummer acceptable,' alternative for use in dough improvers currently used in the Australian bread industry. Whilst the use of some of the pentosanase preparations slowed moisture loss significantly, no correlation was observed between the moisture content of the aged breads and crumb firmness.

6. α-Amylases from *A oryzae* and *B licheniformis* produce softer crumb in baked breads, however, only the *B licheniformis* preparation was successful in enhancing the keeping properties of white bread.

7. The thermal stability of the bacterial preparation may account for the continuing hydrolytic activity as the bread ages, resulting in an increase in the levels of maltotriose in the aged bread crumb.

8. The ability of α-amylases from *B licheniformis* to retard, staling at the levels used in this study, may make it a cost effective alternative for use in the bread industry in Australia. Further pilot scale studies of this preparation are warranted.

References

Note In this thesis referencing has followed the recommendations to authors for the journal Cereal Chemistry (see AACC 2003 below).

AACC 2003 Instructions to authors. Downloaded from www.aaccnet.org, April 2003.

Ablett, S., Attenburrow, G. E., and Lillford, P. J., 1988, The significance of water in the baking process, in Chemistry and physics of baking, Blanshard, J. M.V., Frazier, P. J. and Galliard, T. Eds. Royal society of chemistry, London. Pg 30-41.

Adams, M., 1953. Amylases: their kinds and properties and factors which influence their activity. Food Technology, 7:35-38.

Akers, A.A. and Hoseney, R. C. 1994. Water-soluble dextrins from alpha-amylase-treated bread and their relationship to bread. Cereal Chem. 71:223-226.

Armero, E.; Collar, C. 1998. Crumb firming kinetics of wheat breads with anti-staling additives. J-cer-sci., 28(2) 165-174

Asp, E.H., Midness, L.T., Bakshi, A. S., and Smith, L. B. 1985. Changes in activity of cereal, fungal or bacterial alpha-amylase in yeast dough during fermentation and baking. Food Tech. in Australia, 37:198-201.

Asp, E.H., Midness, L.T., and Spelhaug, S. 1988. Alpha-amylase activities in yeasted doughs with added surfactants. Food Tech. in Aust. 40(8) 330-331.

Axford, D. W. E., Colwell, K. H., Cornford, S. J. and Elton, G. A. H. 1968. Effect of loaf specific volume on the rate and extent of staling in bread. J. Sci. Food Agric. 19:95.

Baker, J. C., Parker, H. K. and Fortmann, K. L. 1953. Flavour of bread. Cereal Chem., 30:22-30.

Betchel, D.B., Pomeranz, Y., and De Francisco, A. 1978. Bread making studied by light and transmission electron microscopy. Cereal Chem. 55:392.

Biebaut, D. 1991. Flour improvers and raising agents. Pages 242-256 in Food Additive Users Handbook. J. Smith Ed, Blackie, London.

Bird, R. and Hopkins, R.H. 1954. The action of some α-amylases on amylose. Biochemisty. 56:86-99.

Blanco Gormas, D., 1992. HPLC analysis for organic acids in Food analysis by HPLC, L. M. L. Ed. Marcel Dekker Inc. NY. Pages 371-385.

Blanshard, J. M. V., Frazier, P. J. and Galliard, T. 1988. Chemistry and physics of baking. Royal Society of Chemistry. Burlington House. London.

Bonnin, E., Legoff, A., Saulnier, L., Chaurand, M. and Thibault, J. K. 1998. Preliminary characterization of endogenous wheat arabinoxylan-degrading enzymic extracts. Journal of Cereal Science. 28:53-62.

Brady, P. L., and Mayer, S. M. 1985. Correlation of sensory and instrumental measures of bread texture. Cereal Chem. 62:70-71.

Bread Research Institute of Australia. 1989. Australian breadmaking handbook Kensington, N.S.W. TAFE Educational Books,

Bushuk W., Rasper V.F. 1994. Wheat production, properties and quality. London: Blackie Academic and Professional. Page 239

Cauvain S.P. and Young L.S. 1998. Technology of Breadmaking ed. Stanley P. and Linda S. London: Blackie Academic & Professional.

Cauvain, S. P., Chamberlain, N. 1988. The bread improving effect of fungal amylase. J Cereal Sci.. 8:239-244.

Charamlambous, G. 1993. Shelf life studies of foods and beveridges. Elsevier Science Publishers. London. Page 1144.

Cleemput, G., Booij, C., Hessing, M., Gruppen, H., Declour. J. A. 1997. Solubilisation and changes in molecular weight distribution of arabinoxylans and protein in wheat flours during bread-making, and the effects of endogenous arabinxylan hydroysing enzymes. J. Cer. Sci 26:55-66

Cleemput, G., Roels, P., Van Oort, M., Grobet, P.J., Delcour, J. A. 1993. Heterogeneity in the structure of water soluble arabinxylans in wheat flours of variable bread-making quality. Cer. Chem. 70:324-329.

Cleemput, G., van Oort, M., Hessing, M., Bergmans, M.E.F., Gruppen, H., Grobet, P.J., Delcour, J.A. 1995. Variation in the degree of D-xylose substitution in arabinoxylans extracted from a European wheat flour. J. Cereal Sci. 22:73-84.

Coffman, J.R. 1965. Bread flavour. Symp Foods: Chem. Physiol. Flavours. P185-202.

Collar, C., Martinez, J.C., Andreu, P., Armero, E. 2000. Effects of enzyme associations on bread dough performance. A response surface analysis. Food Sci. & Tech. Int. 6(3):217-226.

Corder, A. M. and Henry, R.J. 1989. Carbohydrate degrading enzymes in germinating wheat. Cereal Chem., 66:435-439.

Coultate T. P. 1996 Food: the chemistry of its components Cambridge : Royal Society of Chemistry 3rd edn Page 360

Courtin, C. M., Roelants, A., and Delcour, J.A., 1999. Fractionation-reconstitution experiments provide insight into the role of endoxylanases in bread making. J. Agric. Food Chem., 47(5):180-187.

D'Appolonia, B. L. 1971. Role of pentosans in bread and dough. Bakers Digest. 45:20-25.

D'Appolonia, B. L. 1980. Structure and importance of nonstrachy polysaccharides of wheat flour. Food and Nutrition Press Inc. p201-210

D'Appolonia, B.L., and Morad, M.M. 1981. Bread staling. Cereal Chem. 58:186-190.

Debsyer, W., Peumans, W. J., Van Damme, E. J. M., and Delcour, J. A. 1999. *Triticum aestivum* Xylanase inhibitor (TAXI) a new class of enzyme inhibitor affecting breadmaking.

Defloor, I., Delcour, J. A. 1999. Impact of maltodextrins and antistaling enzymes on the differential scanning calorimetry staling endotherm of baked bread doughs. J. Agric. Food Chem. 47:737-741.

Dragsdorf, R. D., Varriano-Marston, E. 1980. Bread staling: X-ray diffraction studies on bread supplemented with α-amylases from different sources. Cer. Chem. 57:310-314

Eliasson., A.C., and Larsson, K. 1993. Cereals in breadmaking. New York, NY 10016, USA; Marcel Dekker Page 376

Fance, W. J. 1971. Bread science and technology.. AVI Publishing CO. Inc. Conneticut Page 145

Fance W.J., (1991), Bread making and flour confectionary. Wilfred James Fance London. Page 74-83

Faridi and Faubion. 1990. Dough rheology and baked product texture. Van Nostrand Reinhold, USA. Page145-146

Fearn, T. and Russell, P.L. 1981. A kinetic study of bread staling by differential scanning calorimetry. The effect of loaf specific volume. J. Sci. Food Agric. 33:537-548.

Fessas, D., Schiraldi, A. 1998. Texture and staling of wheat bread crumb: effects of water extractable proteins and pentosans. Thermochimica Acta 323:17-26.

Folkes, D. J. and Gramshaw, J. W. 1977. Volatile constituents of white bread crust. Journal of Food Technology. 12:1-8.

Gerrard, J. A., Every, D., Sutton, K. H., Gilpin, M. J. 1997. The role of maltdextrins in the staling of bread. J. Cereal Sci. 26:201-209.

Gan, Z., Ellis, P. R., and Schofield, J. D. 1995 Gas cell stabilization and gas retention in wheat bread dough. J. of Cereal Sci, 21(3)215-230.

Gil, M.J., Callejo, M.J., Rodriguez, g. and Ruiz, M.V. 1999. Keeping qualities of white pan bread upon storage: effect of selected enzymes on bread firmness and elasticity. 208:394-399

Godfrey, T. and West, S. (Ed). 1996. Industrial enzymology. 2nd edn. Stockton Press. New York.

Grossmann, M.V. E., Debarber, C. B. 1997. Bread staling-Simultaneous effect of bacterial alpha-amylase and emulsifer on firmness and pasting properties of bread crumb. Archivos Latinomericanos de Nutricion. 47:229-233.

Harado, O., Lysenko, E.D., Preston, K.R. 2000. Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics. Cer. Chem. 77_1):70-76

Hebeda, R. E., Bowles, L.K., Teague, W. M. 1990. Developments in enzymes for retarding staling of baked goods. Cereal Foods World 35:453-457.

Hebeda, R. E., Bowles, L.K., Teague, W. M. 1991. Use of intermediate temperature stability enzymes for retarding staling in baked goods. Cereal Foods World. 36:619-624.

Hebeda, R. E., Zobel, H. E. 1996. Baked goods freshness: Technology, Evaluation and inhibition of staling. Marcel and Dekker Publisher.

Herz, K.O. 1965. Staling of bread – a review. Food Technology. 9:90-103.

Higgins, J.M., and Small, D.M. 1996 A study of enzyme preparations and their impact on bread quality. Honours project.

Hilhorst, R., Dunnewind, B. Orsel, R. Stegman, P. van Vilet, T., Gruppen, H. Schols, H. A. 1999. Baking ferformance, rheology, and chemical composition of wheat dough and gluten affected by xylanase and oxidative enzymes. Journal of Food Science. 64:808-813.

Holas, J. and Tippler, K. H. 1978. Factors affecting farinograph and baking absorption. 1. Quality characteristics of flour streams. Cereal Chem. 55:637-652.

Hood, L., F., and Liboff, M. 1983. Starch Ultrastructure in New Frontiers in Food Microstructure. Bechtel, D.B. ed AACC. Minnesota. Page 341-370.

Hoseney, R. C. and Faubion, J. M. 1981. A mechanism for oxidative gelation of wheat flour water soluble pentosans. Cereal Chemistry. 63:421-424.

Hoseney, R. C.1994. Principles of Cereal Science and Technology, Second ed. AACC. St Paul, Minnesota.

Hoseney, R.C. 1984. Functional properties of pentosans in baked foods. Food Tech. 38:114.

Howard, J. 2001. www.touregyptnet/...

Hutchings J. B. 1999. Food colour and appearance. Great Britain: Blackie Academic and Professional. Page 610

Inglebrecht, J. A., Verwimp, T., and Delcour, J. A. 2000. Endoxylanases in durum wheat semolina processing: solubilisation of arabinoxylans, action of endogenous inhibitors, and effects on rheological properties. J. Agric. Food Chem. 48:2017-2022.

Jorgensen, O.B., Sia, J. Q. and Jakobsen, T.S. 1999. Xylanolytic enzyme as bread improver. hppt://www.sciencedirect.com/scie...

Jelaca, S.L. and Hlynka, I. 1978. Water binding capacity of wheat flour crude pentosans and their relation to mixing characteristics of dough. Cereal Chemistry. 48:211-222.

Jiminez, T. and Martinez-Anaya. M. A. 2000. Characteristisation of water soluble pentosans of enzyme supplemented dough and breads. Food Science and Technology International. 6:197-205.

Karmel and Stauffer. 1993. Advances in baking technology. Copyright Chapman & Hall. London. Page 237-239

Kent, N. L., and Evers, A.D. 1993. Technology of Cereals. CRC Press

Kim, S. K., and D' Appolonia, B. L. 1977c. Effects of pentosans on dough, bread and bread staling. Cereal Chem. 54:225-231.

Krishnarau, L., Hoseney R. C. 1994. Enzymes increase loaf volume of bread supplemented with starch tailings and insoluble pentosans. Journal of Food Science. 59:1251-1254.

Kroh, L. W. 1994. Caramelisation in food and beverages. Food Chemistry. 51:373-379.

Kulp, K. 1967. Enzymolysis of flour pentosans. Cereal Chem. 45:339-350.

Kulp, K., and Bechtel, W. G. 1968. Effects of water insoluble pentosan of wheat endosperm on the quality of white bread. Cereal Chemistry 40:493-504.

Kulp, K., Ponte, J. G, Jr. 1981. Staling of white pan bread: Fundamental causes. CRC. Crit. Rev. Food Sci. Nutr. 15:1-48.

Kuracina, K. A., Lorenz, K., Kulp, K. 1987. Starch functionality as affected by amylases from different sources. Cereal Chem. 64:182-186.

Laurikainen, T., Harkonen, H., Autio, K. and Poutanen, K. 1998. Effects of enzymes in fibre-enriched baking. Journal of the Science of Food and Agriculture. 76:239-249.

Lent P. J., Grant L.A. 2001 Effects of additives and storage temperature on staling properties of bagels. Cereal chemistry 78(5):619-624

Leon, A. E,; Duran, E., Benedito-deBarber, C. 2000 Utilisation of enzyme mixtures to retard bread crumb firming. J-agric-food-chem. 50(6):1416-1419

Maga, J. A. 1974. Bread flavour. Critical Review. Food Technology. 5:55-142.

Maga, J. A. 1975. Bread staling. Critical Review. Food Technology. 5:443-452.

Mainder, M., Joergensoen, O. B. 1983. Interactions of starch and fungal alpha-amylase in bread making. Starch. 35:419.

Maleki, M., Hoseney, R. C. and Mattern, P. J. 1980. Effects of loaf volume, moisture content and protein quality on the softness and staling rate of bread. Cereal Chem. 57:138-140.

Mani, K., Tragadh, C., Eliasson, A.C. and Lindahl, L. 1992. Water content, water soluble fraction and mixing affect fundamental rheological properties of wheat flour doughs. Journal of Food Science. 57:1198-1201.

Martin, M. L., Zeleznak K. J., and Hoseney, R. C. 1991a. A mechanism of bread firming II. Role of starch hydrolyzing enzymes. Cereal Chem. 68:503-507.

Martin, M. L., Zeleznak K. J., and Hoseney, R. C. 1991b. A mechanism of bread firming. I. Role of starch swelling. Cereal Chem 68:498-503.

Martinez- Anaya, M. A. 1996. Enzymes and bread flavour. Journal of Agriculture and Food Chemistry. 44:469-481.

Martinezanaya, M. A. and Jiminez, T. 1998. Physical properties of enzyme-supplemented doughs and relationship with bread quality parameters. A- Food Research and Technology. 206:134-142.

Matz SA. 1992. Bakery technology and engineering. 3rd edn New York: Van Nostrand Reinhold

McCleary B. V., Gibson, T.S., Allen, J., and Gams, T. C. 1986. Enzyme hydrolysis and industrial importance of barley beta-glucans and wheat flour pentosans, Starch. 35:433-437.

McKean C. 1999. Australian food - The complete reference to the Australian food industry. East Melbourne: Agri Food Media in association with Australian Food and Grocery Council. Page 408

Megazyme International 2002a. webpage Agricultural production statistical database. [Online. Internet.] Available: http://www.apps.fao.org. Accessed 5 Nov 2002.

Megazyme International 2002b. webpage

Megazyme International 2002c. webpage

Michniewicz, C, Biliaderis, G. and Bushuk, W. 1990. Water-insoluble pentosans of wheat composition and some physical properties. Cereal Chemistry. 67:434-439.

Minolta Camera Co Ltd, (publication year unknown). Operating manual: Minolta Chroma Meter CR 300. Minolta Camera Co Ltd, Osaka, Japan.

Moskowitz H. R., 1983. Product testing and sensory evaluation of foods. Food and Nutrition Press Inc. Conneticut USA. Page 208-218.

Mueser, F. and Sukow, P. 1986. Non-starch polysaccharides in Chemistry and physics of baking, Blanshard, J. M.V., Frazier, P. J. and Galliard, T. Eds. Royal society of chemistry, London. Page 42-61.

Muesser f., and Suckow, P. 1988., in Chemistry and physics of baking, Blanshard, J. M.V., Frazier, P. J. and Galliard, T. Eds. Royal society of chemistry, London. Page 42-61.

O'Brien, J., and Morrisey, P. A. 1989. Nutritional and toxicological aspects of the Maillard browning reaction in foods. Critical Reviews in Food Science and Nutrition. 28:211-248.

Pateras, I. M. C., 1995. Bread spoilage and staling in Technology of bread making, Cauvain, S.P and Young, L.S Eds, Blackie Academic and Professional, NY. Page 240-259.

Pence, J. W., Elder, A. H. and Mecham D. K., 1951. Some effects of soluble components on baking behaviour. Cereal Chem. 28:94-104.

Pomeranz, Y. 1987 Modern cereal science and technology New York, N.Y : VCH,

Pomeranz, Y., and Finney, K. F. 1975. Sugars in breadmaking. Bakers Digest. 49:20-27.

Pomeranz, Y., and Shellenberger, J.A. 1971, Bread science and technology. AVI Publishing Co. Inc. Conneticut, USA.

Ponte, J. G. Jr., Titcomb, S. T, and Cotton, R. H. 1962. Flour as a factor in bread firming. Cereal Chem, 36:437-441.

Potus, J. Poiffait, A. and Drapron, R. 1994. Influence of dough-making conditions on the concentration of individual sugars and their utilisation during fermentation. Cereal Chem. 71:505-508.

Poutanen, K. 1997. Enzymes-an important tool in the improvement of quality of cereal foods. Trends in Food Science and Technology. 8:300-306.

Pyler, E. J. 1988b. Baking Science and Technology. 3rd edn, Vol 2. SoslandPublishing Company. USA. Pp 34-38, 827.

RACI Royal Australian Chemical Institute, Cereal Chemistry Division (RACI) Official testing methods. North Melbourne

Rogers, D. E., Zeleznak, K. J., Lai, C. S., and Hoseney, R. C. 1988. Effect of native lipids, shortening, and bread moisture on bread firming. Cereal Chem. 65:398-401.

Rouau, X. 1993. Investigations into the effects of an enzyme preparation for baking on wheat flour dough pentosans. J Cereal Sci. 18:145-157.

Rouau, X., Surget, A. 1998. Evidence for the presence of a pentosanases inhibitor in wheat flours. J Cer. Sci. 28:63-70.

Rubenthaler, G., Finney, K. F. and Pomeranz, Y. 1965. Effects on loaf volume and bread characteristics of alpha-amylases from cereal, fungi and bacterial sources. Food Technology. 19:239-243.

Russel, P. L. 1983. A kinetic study of bread staling by differential scanning calorimetry and compressibility measurements. The effect of different grists. J. Cereal Sci. 1:285

Saha., B.C. 2000. α-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnology Advances, 18(5):403-423.

Sahlstrom, S., Brathen, E. 1996. Effects of enzyme preparations for baking, mixing time and resting time on bread quality and bread staling. Food Chemistry. 58:75-80.

Schiraldi, S., Piazza, L. Brenna, O. and Vittadini, E. 1996. Structure and properties of bread dough and crumb calorimetric, rheological and mechanical investigations on the effect produced by hydrocolloids, pentosans and soluble proteins. J. Ther. Anal. 47(5):1339-1360.

Schoch, T. J. and French, D. 1947. Studies on bread staling. I. The role of starch. Cereal Chem. 24:231-242.

Stacey, M. V. 1964. The role of wheat flour pentosans in baking. 1: Enzymic destruction of pentosans in situ. J. Sci. Food Agric. 15:607

Stear, C. A. 1990. Handbook of breadmaking technology. Elseviers Science Publishers Ltd. New York.

Tolstoguzov, V. 1997. Thermodynamic aspects of dough formation and functionality. Food Hydrocolloids 11:181-193.

Valjakka, T.T., Ponte, J.G. Kulp, K. 1994. Studies on a raw-starch digesting enzyme. I. Comparison to fungal and bacterial enzymes and an emulsifier in white pan bread. Cereal Chem. 71:139-144.

van Eijk, J.H., and Hille, Jd, R. 1996.Non amylolytic enzymes. In: Hebeda, R.E., Zobel, H., editors. Baked goods freshness. New York, Marcel Dekker. Page 131-150

van Oort, M., von Stratten, F., and Laanne, C. 1995. Pentosans and pentosanases in bread making. International Food Ingredients (IFI) NR.2 Page 23.

Vinkx, C. J.A., Reynaert, H. R., Grobet, P. J., and Delcour, J. A. 1993. Physicochemical and functional properties of nonstarch polysaccharides. V. Variability in the structure of water-soluble arabinoxylans. J. Cereal Sci. 30:39-43.

Westerlund, E., Andersson, R., Aman, P., and Theander, O. 1990. Effects of baking on water soluble non-starch polysaccharides in white bread fractions. J. Cereal Sci 12:33-41.

Whistler RL and Daniel JR 1985. Carbohydrates. Chapter 3 in Fennema, O. R. 1986. Food Chemistry pp69-137.

Willhoft, E. M. A. 1973. Recent developments in the bread staling problem. Bakers Digest. 47:14-25.

Wootton, M., Acone, L. and Wills, R.B.H. 1995. Pentosans in Australian wheats, Aust. J. Agric. Research 46:389-392.

Wong, D. W. S., 1995. Food enzymes: structure and mechanism. Chapman and Hall, New York. Page 37-72.

Yeh, Y.F., Hoseney, R.C. and Lineback, R.R. 1980. Changes in wheat flour pentosans as a result of dough mixing and oxidation. Cereal Chem. 57:144-148.

Zeleznak., R.J. and Hoseney, R.C. 1986. The role of water in the retrogradation of wheat starch gels and bread crumb. Cereal Chem. 62:407-411.

Zoebel, H. E. 1973. A review of bread staling. Bakers Digest. 47:52-55.

Appendices

Appendix 1 Curves used in calculating the endoxylanase activity of ingredients and enzyme preparations

(these graphs were sourced from Megazyme International 2002b)

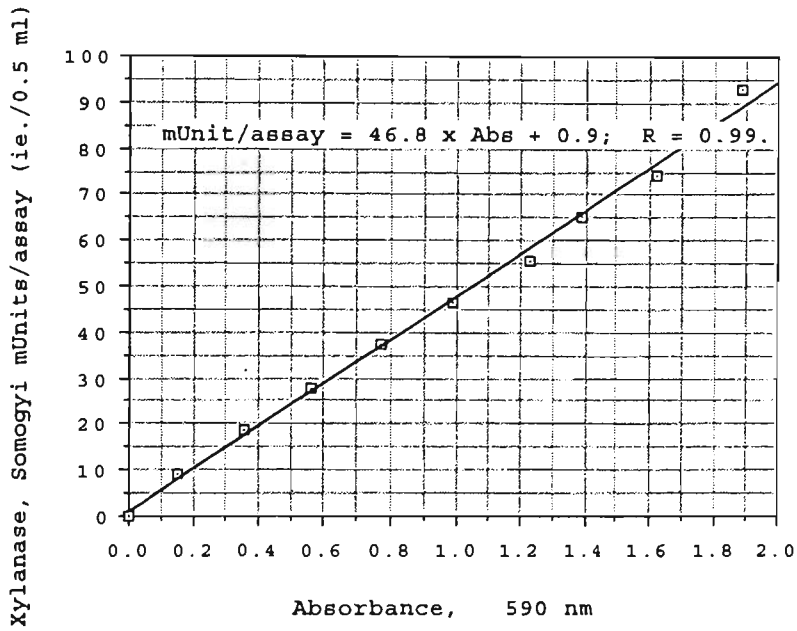


Figure 1. Pure *Aspergillus niger* xylanase standard curve on Xylazyme AX (Lot 40602) at pH 4.7. Reaction stopped with Trizma base.

Appendix 1 continued

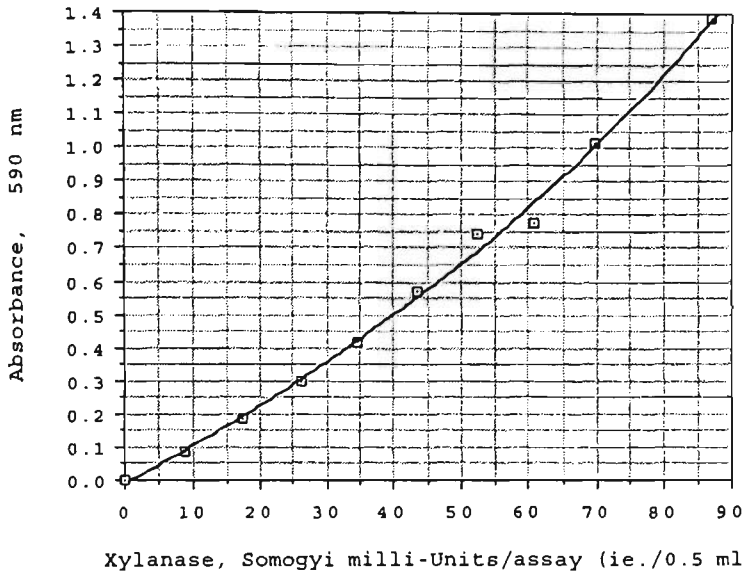


Figure 2. *Trichoderma longibrachiatum* xylanase (pI 9.0 form) standard curve on Xylazyme AX (Lot 40602) at pH 6.0. Reaction stopped with Trizma base.

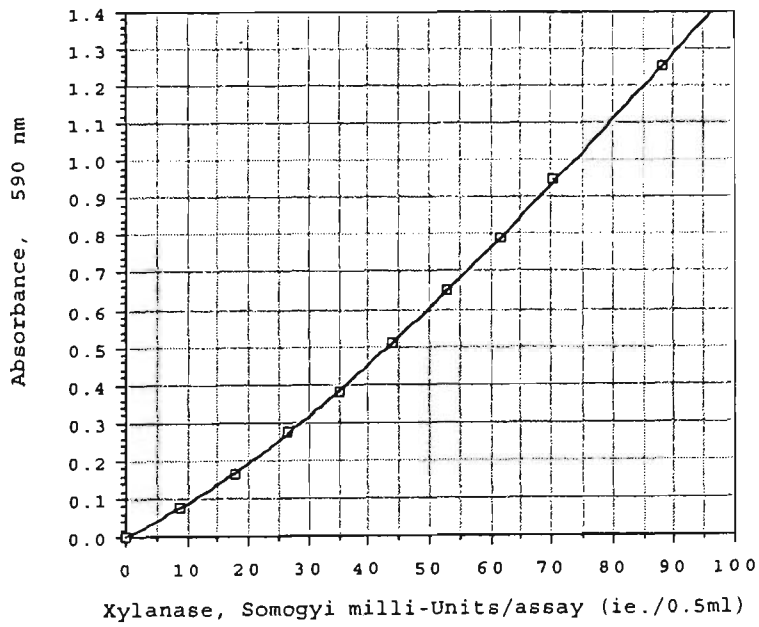


Figure 3. Pure *Humicola insolens* xylanase standard curve on Xylazyme AX (Lot 40602) at pH 6.0. Reaction stopped with Trizma base.

Appendix 2 Curves used in calculating the α amylase activity of ingredients and enzyme preparations

(these graphs were sourced from Megazyme International 2002c)

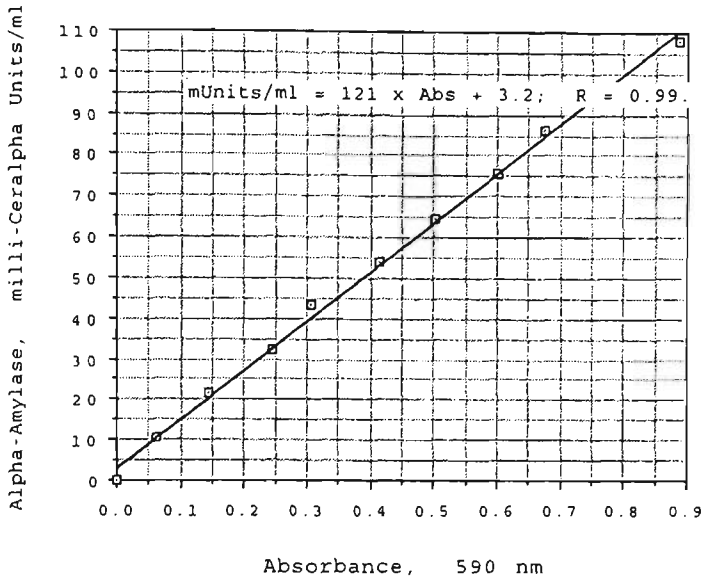


Figure 1. **Malted barley α-amylase standard curve on Amylazyme (Lot 50504).**

Amylazyme assay was performed at pH 6.0 and Ceralpha assay at pH 5.2 under standard conditions as described in this booklet and in Megazyme Booklet CER 6/93 (Ceralpha), using purified malt α-amylase.

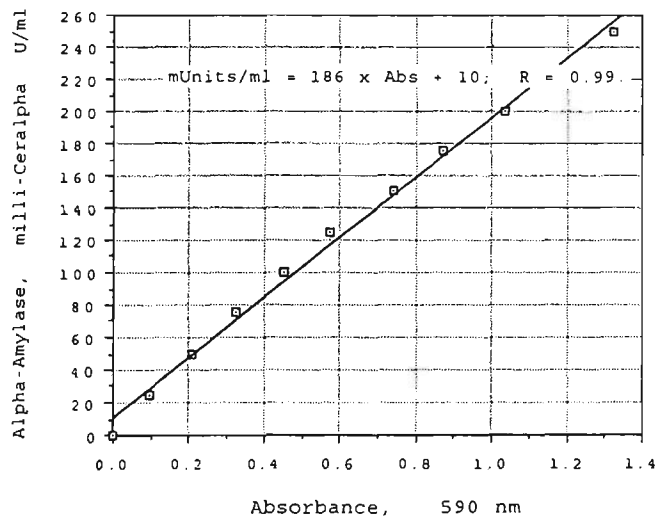


Figure 2. Aspergillus oryzae **α-amylase standard curve on Amylazyme (Lot 50504).**

The Amylazyme assay was performed at pH 4.4 and the Ceralpha assay at pH 5.0 under standard conditions as described in this booklet and in Megazyme Booklet CER 6/93 (Ceralpha), using pure *A. oryzae* α-amylase (Megazyme cat. no. E-ANAAM).

Appendix 2 continued

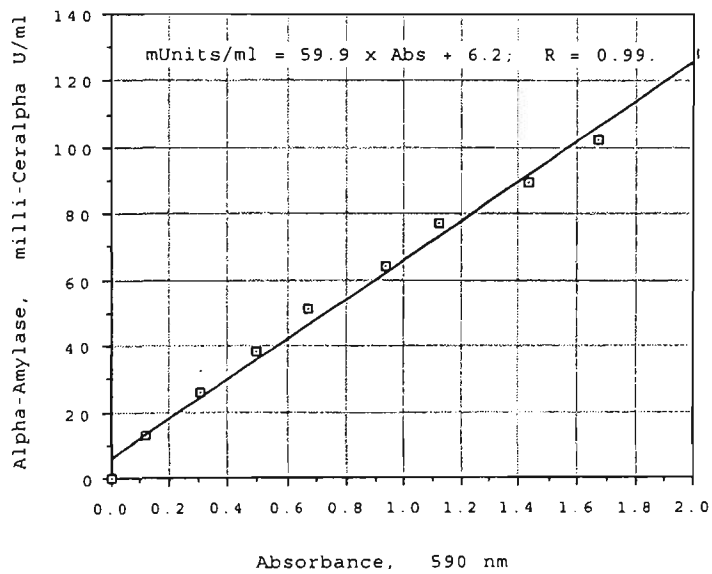
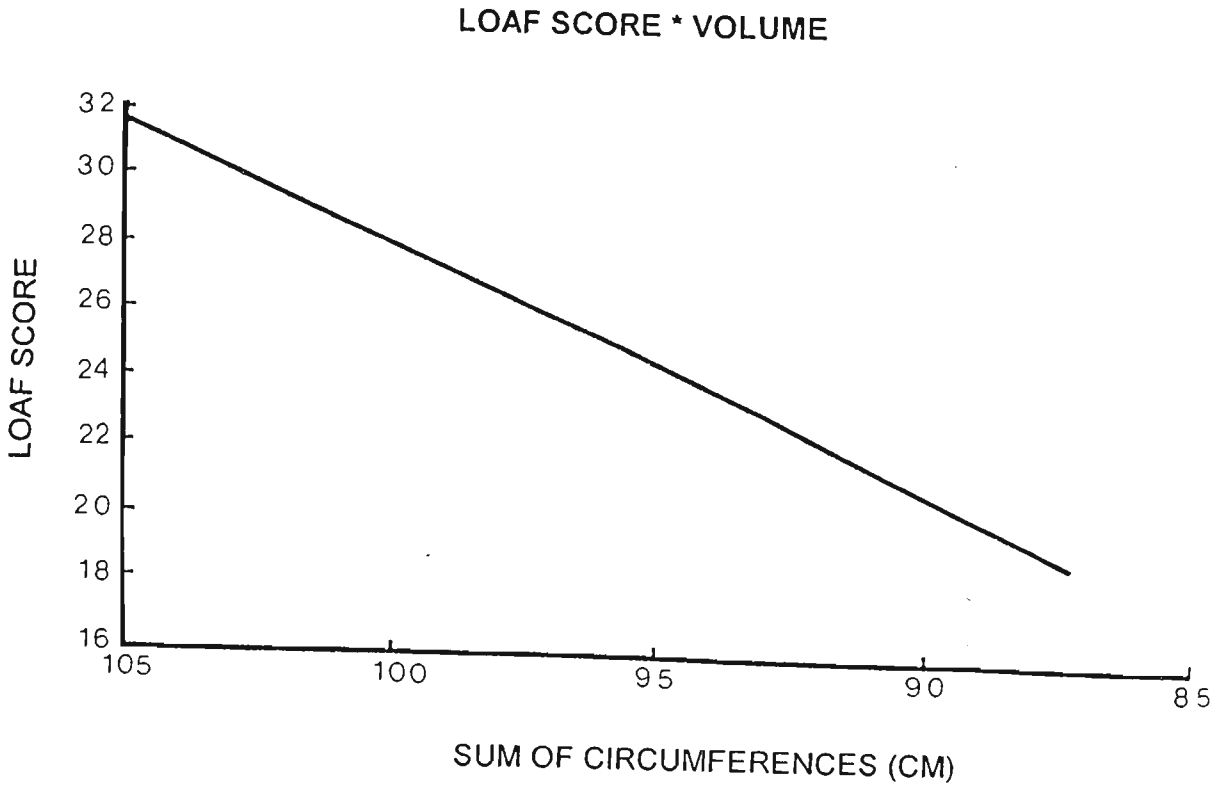


***Figure 3. Bacillus licheniformis* α–amylase standard curve on Amylazyme (Lot 50504).**
The Amylazyme assay was performed at pH 7.0 and Ceralpha assay at pH 6.0 under standard conditions as described in this booklet and in Megazyme Booklet CER 6/93 (Ceralpha). The enzyme employed was purified *Bacillus licheniformis* α-amylase (Megazyme cat. no. E-BLAAM).

Appendix 3 **Graph used in calculating the loaf volume scores of baked loaves**
(this graph was sourced from RACI 1995 method 07-01)



Appendix 4.1

Crust colour when endoxylanases are used at 5microlitres

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	64.551	4.932	1.273
M1	15	0	51.421	4.218	1.089
M2	15	0	51.523	4.085	1.055
M3	15	0	56.671	3.465	0.895
M4	15	0	60.249	4.692	1.211
M5	15	0	54.363	6.549	1.691

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M1	13.131	6	10.693	<0.001	Yes
con vs. M2	13.029	6	10.61	<0.001	Yes
con vs. M5	10.188	6	8.297	<0.001	Yes
con vs. M3	7.881	6	6.418	<0.001	Yes
M4 vs. M1	8.828	6	7.189	<0.001	Yes
M4 vs. M2	8.726	6	7.106	<0.001	Yes
M4 vs. M5	5.885	6	4.793	0.013	Yes
M3 vs. M1	5.25	6	4.275	0.038	Yes
M3 vs. M2	5.148	6	4.192	0.044	Yes

Crust colour when endoxylanases are added at 10microlitres

Group Name	N	Missing	Mean	Std Dev	SEM
con	15	0	64.551	4.932	1.273
M1	15	0	55.315	3.551	0.917
M2	15	0	51.465	4	1.033
M3	15	0	54.622	3.787	0.978
M4	15	0	53.113	6.011	1.552
M5	15	0	48.942	5.73	1.479

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M5	15.609	6	12.687	<0.001	Yes
con vs. M2	13.087	6	10.636	<0.001	Yes
con vs. M4	11.439	6	9.297	<0.001	Yes
con vs. M3	9.929	6	8.07	<0.001	Yes
con vs. M1	9.237	6	7.507	<0.001	Yes
M1 vs. M5	6.373	6	5.179	0.006	Yes
M3 vs. M5	5.68	6	4.616	0.019	Yes

Crust colour when endoxylanases were added at 50microlitres

Group Name	N	Missing	Mean	Std Dev	SEM
con	15	0	64.551	4.932	1.273
M1	15	0	49.779	5.563	1.436
M2	15	0	54.49	4.788	1.236
M3	15	0	67.26	3.3	0.852
M4	15	0	63.575	2.794	0.721
M5	15	0	61.386	3.778	0.976

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 13

Comparison	Diff of Means	p	q	P	P<0.050
M3 vs. M1	17.481	6	15.729	<0.001	Yes
M3 vs. M2	12.77	6	11.49	<0.001	Yes
M3 vs. M5	5.874	6	5.285	0.005	Yes
con vs. M1	14.773	6	13.292	<0.001	Yes
con vs. M2	10.061	6	9.053	<0.001	Yes
M4 vs. M1	13.796	6	12.413	<0.001	Yes
M4 vs. M2	9.085	6	8.174	<0.001	Yes
M5 vs. M1	11.607	6	10.444	<0.001	Yes
M5 vs. M2	6.896	6	6.205	<0.001	Yes
M2 vs. M1	4.711	6	4.239	0.04	Yes

Appendix 4.2

Crumb whiteness when endoxylanses were added at 5microlitres

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	75.797	2.528	0.653
M1	15	0	76.705	1.191	0.308
M2	15	5	76.196	1.703	0.539
M3	15	0	76.86	1.518	0.392
M4	15	0	77.501	1.725	0.445
M5	15	0	77.833	1.67	0.431

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.027).

Comparisons for factor: Col 4

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. con	2.036	6	4.447	0.027	Yes
M5 vs. M2	1.637	6	3.197	0.223	No

Crumb whiteness when endoxylanases were added at 10microlitres

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	75.797	2.528	0.653
M1	15	0	76.24	1.63	0.421
M2	15	0	76.055	1.408	0.363
M3	15	0	75.535	1.945	0.502
M4	15	0	78.236	1.855	0.479
M5	15	0	78.465	1.246	0.322

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 10

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M3	2.93	6	6.245	<0.001	Yes
M5 vs. con	2.668	6	5.688	0.002	Yes
M5 vs. M2	2.41	6	5.138	0.006	Yes
M5 vs. M1	2.225	6	4.743	0.015	Yes
M4 vs. M3	2.701	6	5.757	0.002	Yes
M4 vs. con	2.439	6	5.2	0.006	Yes
M4 vs. M2	2.181	6	4.65	0.018	Yes
M4 vs. M1	1.996	6	4.255	0.039	Yes

Crumb whiteness when endoxylanases were added at 50microlitres

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	75.797	2.528	0.653
M1	15	0	73.957	1.439	0.372
M2	15	1	76.207	1.496	0.4
M3	15	0	73.587	1.593	0.411
M4	15	0	74.877	2.515	0.649
M5	15	0	74.957	1.816	0.469

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003).

Comparisons for factor: Col 16

Comparison	Diff of Means	p	q	P	P<0.050
M2 vs. M3	2.62	6	5.095	0.007	Yes
M2 vs. M1	2.25	6	4.377	0.031	Yes
con vs. M3	2.209	6	4.373	0.031	Yes

Appendix 4.3

Loaf volumes for breads treated with 5UL of xylanase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	26.1	0.2	0.115
M1	3	0	26.933	0.252	0.145
M2	3	0	26.4	0.3	0.173
M3	3	0	27.167	0.0577	0.0333
M4	3	0	27.067	0.0577	0.0333
M5	3	0	25.967	0.153	0.0882

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
M3 vs. M5	1.2	6	10.773	<0.001	Yes
M3 vs. Con	1.067	6	9.576	<0.001	Yes
M3 vs. M2	0.767	6	6.883	0.004	Yes
M4 vs. M5	1.1	6	9.875	<0.001	Yes
M4 vs. Con	0.967	6	8.678	<0.001	Yes
M4 vs. M2	0.667	6	5.985	0.012	Yes
M1 vs. M5	0.967	6	8.678	<0.001	Yes
M1 vs. Con	0.833	6	7.481	0.002	Yes
M1 vs. M2	0.533	6	4.788	0.048	Yes

Loaf volumes of breads treated with 10UL of xylanase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	26.1	0.2	0.115
M1	3	0	27.467	0.351	0.203
M2	3	0	27.867	0.252	0.145
M3	3	0	28.1	0.3	0.173
M4	3	0	28.1	0.1	0.0577
M5	3	0	26.933	0.404	0.233

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
M3 vs. Con	2	6	12.122	<0.001	Yes
M3 vs. M5	1.167	6	7.071	0.003	Yes
M4 vs. Con	2	6	12.122	<0.001	Yes
M4 vs. M5	1.167	6	7.071	0.003	Yes
M2 vs. Con	1.767	6	10.708	<0.001	Yes
M2 vs. M5	0.933	6	5.657	0.017	Yes
M1 vs. Con	1.367	6	8.283	<0.001	Yes
M5 vs. Con	0.833	6	5.051	0.035	Yes

Loaf volumes of breads treated with 50UL of xylanase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	26.1	0.2	0.115
M1	3	0	31.933	0.404	0.233
M2	3	0	32	1	0.577
M3	3	0	32.2	0.265	0.153
M4	3	0	31.4	1.389	0.802
M5	3	0	32.067	0.115	0.0667

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
M3 vs. Con	6.1	6	14.43	<0.001	Yes
M5 vs. Con	5.967	6	14.114	<0.001	Yes
M2 vs. Con	5.9	6	13.957	<0.001	Yes
M1 vs. Con	5.833	6	13.799	<0.001	Yes
M4 vs. Con	5.3	6	12.537	<0.001	Yes

Appendix 4.4

Instron data for breads treated with 5UL of xylanase

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	0.938	0.137	0.0432
M2	10	0	1.148	0.212	0.067
M3	10	0	1.108	0.309	0.0977
M4	10	0	1.421	0.41	0.13
M5	10	0	1.638	0.305	0.0965

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: treatment

Comparison	Diff of	p	q	P	P<0.050

	Means				
M5 vs. M1	0.7	6	8.085	<0.001	Yes
M5 vs. M3	0.53	6	6.122	<0.001	Yes
M5 vs. M2	0.49	6	5.657	0.003	Yes
Con vs. M1	0.648	6	7.488	<0.001	Yes
Con vs. M3	0.478	6	5.525	0.004	Yes
Con vs. M2	0.438	6	5.06	0.009	Yes
M4 vs. M1	0.483	6	5.573	0.003	Yes

Instron data for breads treated with 10UL xylanase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	1.086	0.0311	0.00985
M2	10	0	1.166	0.0237	0.00748
M3	10	0	1.498	0.0382	0.0121
M4	10	0	1.079	0.00244	0.000772
M5	10	0	1.118	0.0166	0.00526

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. M4	0.507	6	21.582	<0.001	Yes
Con vs. M1	0.501	6	21.293	<0.001	Yes
Con vs. M5	0.469	6	19.944	<0.001	Yes
Con vs. M2	0.421	6	17.889	<0.001	Yes
M3 vs. M4	0.419	6	17.817	<0.001	Yes
M3 vs. M1	0.412	6	17.528	<0.001	Yes
M3 vs. M5	0.38	6	16.179	<0.001	Yes
M3 vs. M2	0.332	6	14.124	<0.001	Yes

Instron data for breads treated with 50UL of xylanase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	0.857	0.0579	0.0183
M2	10	0	0.737	0.321	0.101
M3	10	0	1.192	0.282	0.0891
M4	10	0	1.26	0.213	0.0674
M5	10	0	1.029	0.00652	0.00206

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. M2	0.849	6	12.875	<0.001	Yes

Con vs. M1	0.73	6	11.064	<0.001	Yes
Con vs. M5	0.558	6	8.456	<0.001	Yes
Con vs. M3	0.394	6	5.979	0.001	Yes
Con vs. M4	0.326	6	4.944	0.012	Yes
M4 vs. M2	0.523	6	7.932	<0.001	Yes
M4 vs. M1	0.404	6	6.12	0.001	Yes
M3 vs. M2	0.455	6	6.896	<0.001	Yes
M3 vs. M1	0.335	6	5.085	0.009	Yes
M5 vs. M2	0.291	6	4.419	0.032	Yes

Appendix 4.5

Crust colour of E-A breads treated with 5, 10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
E-A5	15	0	50.313	4.771	1.232
E-A10	15	0	54.874	5.15	1.33
E-A50	15	0	60.932	3.896	1.006
CON	15	0	55.985	5.073	1.31

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
E-A50 vs. E-A5	10.619	4	8.661	<0.001	Yes
E-A50 vs. E-A10	6.058	4	4.941	0.005	Yes
E-A50 vs. CON	4.947	4	4.035	0.03	Yes
CON vs. E-A5	5.672	4	4.626	0.01	Yes

Appendix 4.6

Crumb whiteness of E-A breads treated with 5, 10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
E-A5	15	0	78.269	1.991	0.514
E-A10	15	0	78.823	1.113	0.287
E-A50	15	0	77.796	1.468	0.379
CON	15	0	78.359	1.216	0.314

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.317).

Appendix 4.7

Instron data of E-A breads treated with 5, 10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
E-A5	10	0	4.163	0.127	0.0402
E-A10	10	0	4.282	0.273	0.0863
E-A50	10	0	4.184	0.209	0.0662
CON	10	0	3.842	0.111	0.035

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
E-A10 vs. CON	0.441	4	7.28	<0.001	Yes
E-A50 vs. CON	0.343	4	5.657	0.002	Yes
E-A5 vs. CON	0.321	4	5.303	0.003	Yes

Appendix 4.8

Loaf volumes of E-A breads treated with 5, 10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	28.3	0.2	0.115
E-A5	3	0	28.167	0.153	0.0882
E-A10	3	0	27.533	0.351	0.203
E-A50	3	0	29.267	0.208	0.12

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 9

Comparison	Diff of Means	p	q	P	P<0.050
E-A50 vs. E-A10	1.733	4	12.52	<0.001	Yes
E-A50 vs. E-A5	1.1	4	7.945	0.002	Yes
E-A50 vs. Con	0.967	4	6.982	0.005	Yes
Con vs. E-A10	0.767	4	5.538	0.019	Yes
E-A5 vs. E-A10	0.633	4	4.575	0.048	Yes

Appendix 4.9

Crust colour of A-F bread treated with 5, 10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
CON	15	0	58.829	4.522	1.168
A-F	15	0	51.411	7.493	1.935
AF10	15	0	52.773	5.688	1.469
AF50	15	0	57.511	4.73	1.221

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).

Comparisons for factor: Col 18

Comparison	Diff of Means	p	q	P	P<0.050
CON vs. A-F	7.419	4	5.014	0.004	Yes
CON vs. AF10	6.057	4	4.094	0.027	Yes
AF50 vs. A-F	6.101	4	4.124	0.026	Yes

Appendix 4.10

Crumb colour of A-F bread treated with 5,10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
CON	15	0	76.501	2.097	0.541
A-F	15	0	76.551	1.772	0.458
AF10	15	0	77.754	1.716	0.443
AF50	15	0	77.685	2.059	0.532

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.134).

Appendix 4.11

Instron data for A-F breads treated at 5,10 and 50µL

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.732	0.205	0.0648
a-af5	10	0	2.81	0.0876	0.0277
a-af10	10	0	2.831	0.0665	0.021
a-af50	10	0	2.74	0.209	0.066

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.405).

Appendix 4.12

Loaf volume of A-F breads treated with 5, 10 and 50µL

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Con	3	0	28.9	28.825	28.975
a-f5	3	0	28.4	26.45	28.7
a-f10	3	0	28.75	28.675	28.788
a-f50	3	0	31.5	31.275	31.725

H = 9.024 with 3 degrees of freedom. (P = 0.029)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.029) To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
a-f50 vs a-f5	24	3.843	Yes

Appendix 5.1

Moisture data for fresh treated and untreated breads and after aging for 72hrs

Group	N	Missing	Median	25%	75%
con	4	0	43.5	43.2	43.6
m1	4	0	45.55	45.3	45.65
m2	4	0	45	44.9	45.15
m3	4	0	44.05	43.9	44.2
m4	4	0	44.45	44.35	44.7
m5	4	0	44.6	44.3	44.75
con1	4	0	35.5	34.5	36
m1a	4	0	36	34.5	36
m2a	4	0	35.5	35	36
m3a	4	0	35	34.5	36
m4a	4	0	37.5	34.5	39.5
m5a	4	0	39	38	40

H = 43.161 with 11 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	q	P<0.05
m1 vs m3a	149.5	5.339	Yes
m1 vs con1	147.5	5.268	Yes
m1 vs m2a	144	5.143	Yes
m1 vs m1a	143	5.107	Yes
m2 vs m3a	134	4.786	Yes
m2 vs con1	132	4.714	Yes

Appendix 5.2

Loaf volumes for xylanase treatments M1-M5

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	5	0	26.08	0.148	0.0663
M1	5	0	26.56	0.541	0.242
M2	5	0	26.46	0.219	0.098
M3	5	0	26.9	0.524	0.235
M4	5	0	27.04	0.195	0.0872
M5	5	0	26.14	0.114	
Total	17	4.469			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of	p	q	P	P<0.050

	Means				
M3 vs. M5	1.2	6	10.773	<0.001	Yes
M3 vs. Con	1.067	6	9.576	<0.001	Yes
M3 vs. M2	0.767	6	6.883	0.004	Yes
M4 vs. M5	1.1	6	9.875	<0.001	Yes
M4 vs. Con	0.967	6	8.678	<0.001	Yes
M4 vs. M2	0.667	6	5.985	0.012	Yes
M1 vs. M5	0.967	6	8.678	<0.001	Yes
M1 vs. Con	0.833	6	7.481	0.002	Yes
M1 vs. M2	0.533	6	4.788	0.048	Yes

Appendix 5.3

Crumb whiteness in fresh treated and untreated breads

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	76.785	1.728	0.546
m1	10	0	77.377	1.497	0.473
m2	10	0	77.774	1.562	0.494
m3	10	0	78.389	1.013	0.32
m4	10	0	77.097	1.425	0.451
m5	10	0	76.959	2.242	0.709

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.254).

Crust colour in fresh treated and untreated breads

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	65.602	5.241	1.657
m1	10	0	48.176	3.628	1.147
m2	10	0	49.374	4.933	1.56
m3	10	0	50.201	4.115	1.301
m4	10	0	57.592	2.574	0.814
m5	10	0	63.362	3.269	1.034

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. m1	17.426	6	13.552	<0.001	Yes
con vs. m2	16.228	6	12.62	<0.001	Yes
con vs. m3	15.401	6	11.977	<0.001	Yes
con vs. m4	8.01	6	6.229	<0.001	Yes
m5 vs. m1	15.186	6	11.81	<0.001	Yes
m5 vs. m2	13.988	6	10.878	<0.001	Yes

m5 vs. m3	13.161	6	10.235	<0.001	Yes
m5 vs. m4	5.77	6	4.487	0.029	Yes
m4 vs. m1	9.416	6	7.322	<0.001	Yes
m4 vs. m2	8.218	6	6.391	<0.001	Yes
m4 vs. m3	7.391	6	5.748	0.002	Yes

Appendix 5.4

Crumb compression of fresh bread

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	0.938	0.137	0.0432
M2	10	0	1.148	0.212	0.067
M3	10	0	1.108	0.309	0.0977
M4	10	0	1.421	0.41	0.13
M5	10	0	1.638	0.305	0.0965

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: treatment

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M1	0.7	6	8.085	<0.001	Yes
M5 vs. M3	0.53	6	6.122	<0.001	Yes
M5 vs. M2	0.49	6	5.657	0.003	Yes
Con vs. M1	0.648	6	7.488	<0.001	Yes
Con vs. M3	0.478	6	5.525	0.004	Yes
Con vs. M2	0.438	6	5.06	0.009	Yes
M4 vs. M1	0.483	6	5.573	0.003	Yes

Appendix 5.5

Ratio of xylose sugars produced by xylanases M1 to M5

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
con	6	0	0.32	0.31	0.32
M1	6	0	0.81	0.8	0.815
M2	6	0	0.845	0.83	0.86
M3	6	0	0.4	0.39	0.41
M4	6	0	1.91	1.89	1.93
M5	6	0	1.31	1.3	1.34

H = 33.561 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	q	P<0.05
M4 vs con	180	6.975	Yes
M4 vs M3	144	5.58	Yes
M5 vs con	144	5.58	Yes
M5 vs M3	108	4.185	Yes
M2 vs con	102	3.952	No

Appendix 5.6

Crumb colour of treated and untreated breads after aging for 72hrs

One Way Analysis of Variance

Group	N	Missing	Median	25%	75%
con	10	0	78.915	77.5	80.32
M1	10	0	79.82	79.51	80.25
M2	10	0	79.47	79.21	79.58
M3	10	0	78.81	78.07	79.38
M4	10	0	78.23	75.73	78.54
M5	10	0	78.27	77.16	78.9

H = 19.698 with 5 degrees of freedom. (P = 0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001)

Comparison	Diff of Ranks	q	P<0.05
M1 vs m4	279.5	5.061	Yes
M1 vs m5	274	4.961	Yes

Appendix 5.7

Measurement of crumb yellowing after aging for 72hrs

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
con1	10	0	15.075	14.75	15.99
M1a	10	0	13.015	12.55	13.24
M2a	10	0	13.24	12.74	14.13
M3a	10	0	13.02	12.82	13.32
M4a	10	0	14.045	13.33	14.25
M5a	10	0	13.895	12.85	14.55

H = 27.498 with 5 degrees of freedom. (P = <0.001)

Comparison	Diff of Ranks	q	P<0.05
con1 vs M1a	357.5	6.473	Yes
con1 vs M3a	338.5	6.129	Yes
con1 vs M2a	271	4.907	Yes

Measurement of crumb yellowing in fresh bread

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
CON	10	0	13.309	0.769	0.243
M1	10	0	12.396	0.788	0.249
M2	10	0	13.268	0.442	0.14
M3	10	0	12.102	0.66	0.209
M4	10	0	12.667	0.717	0.227
M5	10	0	12.984	0.86	0.272

Comparisons for factor: SAMPLE

Comparison	Diff of Means	p	q	P	P<0.050
CON vs. M3	1.207	6	5.312	0.005	Yes
M2 vs. M3	1.166	6	5.132	0.008	Yes

Appendix 5.8

Staling data of treated and control breads tested fresh

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	0.938	0.137	0.0432
M2	10	0	1.148	0.212	0.067
M3	10	0	1.108	0.309	0.0977
M4	10	0	1.421	0.41	0.13
M5	10	0	1.638	0.305	0.0965

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: treatment

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M1	0.7	6	8.085	<0.001	Yes
M5 vs. M3	0.53	6	6.122	<0.001	Yes
M5 vs. M2	0.49	6	5.657	0.003	Yes
Con vs. M1	0.648	6	7.488	<0.001	Yes
Con vs. M3	0.478	6	5.525	0.004	Yes
Con vs. M2	0.438	6	5.06	0.009	Yes
M4 vs. M1	0.483	6	5.573	0.003	Yes

Staling data of treated and control breads aged for 24hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.396	0.439	0.139
M1	10	0	1.608	0.158	0.0501
M2	10	0	1.789	0.373	0.118
M3	10	0	2.105	0.288	0.0909
M4	10	0	2.844	0.211	0.0666
M5	10	0	3.038	0.355	0.112

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M1	1.43	6	14.183	<0.001	Yes
M5 vs. M2	1.249	6	12.386	<0.001	Yes
M5 vs. M3	0.932	6	9.246	<0.001	Yes
M5 vs. Con	0.642	6	6.366	<0.001	Yes
M4 vs. M1	1.236	6	12.257	<0.001	Yes
M4 vs. M2	1.055	6	10.46	<0.001	Yes
M4 vs. M3	0.738	6	7.32	<0.001	Yes
M4 vs. Con	0.448	6	4.44	0.031	Yes
Con vs. M1	0.788	6	7.817	<0.001	Yes
Con vs. M2	0.607	6	6.02	0.001	Yes
M3 vs. M1	0.498	6	4.937	0.012	Yes

Staling data of treated and control breads aged for 48hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.731	0.805	0.254
M1	10	0	1.856	0.26	0.0821
M2	10	0	2.269	0.409	0.129
M3	10	0	2.279	0.468	0.148
M4	10	0	3.661	0.619	0.196
M5	10	0	3.772	0.461	0.146

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M1	1.916	6	11.392	<0.001	Yes
M5 vs. M2	1.504	6	8.942	<0.001	Yes
M5 vs. M3	1.493	6	8.877	<0.001	Yes
M5 vs. Con	1.041	6	6.189	<0.001	Yes
M4 vs. M1	1.804	6	10.727	<0.001	Yes
M4 vs. M2	1.392	6	8.277	<0.001	Yes
M4 vs. M3	1.381	6	8.212	<0.001	Yes

M4 vs. Con	0.929	6	5.525	0.004	Yes
Con vs. M1	0.875	6	5.202	0.007	Yes

Staling data of treated and control breads aged for 72hrs

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	4.449	0.798	0.252
M1	10	0	2.867	0.394	0.125
M2	10	0	2.899	0.434	0.137
M3	10	0	3.575	0.64	0.202
M4	10	0	4.135	0.544	0.172
M5	10	0	5.033	0.925	0.292

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
M5 vs. M1	2.166	6	10.525	<0.001	Yes
M5 vs. M2	2.134	6	10.371	<0.001	Yes
M5 vs. M3	1.458	6	7.085	<0.001	Yes
M5 vs. M4	0.899	6	4.367	0.036	Yes
Con vs. M1	1.582	6	7.686	<0.001	Yes
Con vs. M2	1.55	6	7.532	<0.001	Yes
Con vs. M3	0.874	6	4.246	0.044	Yes
M4 vs. M1	1.267	6	6.158	<0.001	Yes
M4 vs. M2	1.236	6	6.004	0.001	Yes

Appendix 5.9

Statistical analysis of doughnuts treated with endoxylanase preparations

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
confresh	6	0	10.921	1.778	0.726
M1fresh	8	0	10.099	0.94	0.332
M2fresh	8	0	14.31	2.03	0.718
M3fresh	8	0	8.829	2.223	0.786
M4fresh	8	0	8.811	1.373	0.485
M5fresh	8	0	8.03	0.625	0.221

Comparisons for factor: Column A

Comparison	Diff of Means	p	q	P	P<0.050
M2fresh vs. M5fresh	6.28	6	11.157	<0.001	Yes
M2fresh vs. M4fresh	5.499	6	9.769	<0.001	Yes
M2fresh vs. M3fresh	5.481	6	9.738	<0.001	Yes
M2fresh vs. M1fresh	4.211	6	7.481	<0.001	Yes
M2fresh vs. confresh	3.389	6	5.574	0.004	Yes
confresh vs. M5fresh	2.891	6	4.755	0.02	Yes

Appendix 5.10

Pentosans extracted - fresh and 72hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	6	0	56.117	0.747	0.305
M1	6	0	50.783	0.826	0.337
M2	6	0	54.033	0.413	0.169
M3	6	0	61.017	0.382	0.156
M4	6	0	61	0.245	0.1
M5	6	0	58.067	0.301	0.123
con72	6	0	63.867	0.441	0.18
m172	6	0	68.05	0.468	0.191
m272	6	0	87.9	0.374	0.153
m372	6	0	80.983	0.387	0.158
m472	6	0	87.033	0.388	0.158
m572	6	0	87.733	0.745	0.304

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
m272 vs. M1	37.117	12	178.446	<0.001	Yes
m272 vs. M2	33.867	12	162.821	<0.001	Yes
m272 vs. con	31.783	12	152.805	<0.001	Yes
m272 vs. M5	29.833	12	143.43	<0.001	Yes
m272 vs. M4	26.9	12	129.327	<0.001	Yes
m272 vs. M3	26.883	12	129.247	<0.001	Yes
m272 vs. con72	24.033	12	115.545	<0.001	Yes
m272 vs. m172	19.85	12	95.433	<0.001	Yes
m272 vs. m372	6.917	12	33.253	<0.001	Yes
m272 vs. m472	0.867	12	4.167	0.151	No
m572 vs. M1	36.95	12	177.644	<0.001	Yes
m572 vs. M2	33.7	12	162.019	<0.001	Yes
m572 vs. con	31.617	12	152.003	<0.001	Yes
m572 vs. M5	29.667	12	142.628	<0.001	Yes
m572 vs. M4	26.733	12	128.526	<0.001	Yes
m572 vs. M3	26.717	12	128.446	<0.001	Yes
m572 vs. con72	23.867	12	114.744	<0.001	Yes
m572 vs. m172	19.683	12	94.632	<0.001	Yes
m572 vs. m372	6.75	12	32.452	<0.001	Yes

Appendix 6.1

Loaf volume of breads treated with E-A and A-F

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	28.933	0.252	0.145
e-a	3	0	28.467	0.0577	0.0333
a-f	3	0	28.5	0.2	0.115

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.041).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. e-a	0.467	3	4.287	0.052	No
con vs. a-f	0.433	3	3.98	0.069	Do Not Test
a-f vs. e-a	0.0333	3	0.306	0.975	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would no

Appendix 6.2

Crust colour of controls and breads treated with preparations E-A and A-F

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
CON	15	0	55.985	5.073	1.31
E-A	15	0	50.313	4.771	1.232
A-F	15	0	51.847	3.046	0.786

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003).

Comparisons for factor: Col 12

Comparison	Diff of Means	p	q	P	P<0.050
CON vs. E-A	5.672	3	5.006	0.003	Yes
CON vs. A-F	4.138	3	3.652	0.035	Yes

Appendix 6.3

Crumb colour of control breads and loaves treated with preparations E-A and A-F

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
CON	15	0	78.24	77.815	79.435
E-A	15	0	78.11	77.052	80.093
A-F	15	0	77.63	74.597	78.382

H = 5.126 with 2 degrees of freedom. (P = 0.077)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability: there is not a statistically significant difference (P = 0.077)

Appendix 6.4

Yellow crumb data for E-A and A-F breads - fresh and 72hrs

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Con	15	0	12.49	12.395	12.551
e-a	15	0	12.67	12.633	12.703
a-f	15	0	11.547	11.532	11.562
con72	15	0	12.803	12.799	12.817
e-a72	15	0	12.553	12.542	12.595
a-f72	15	0	11.874	11.853	11.896

H = 81.184 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance: there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	q	P<0.05
con72 vs a-f	1121.5	11.084	Yes
con72 vs a-f72	896.5	8.86	Yes
con72 vs con	583	5.762	Yes
con72 vs e-a72	479	4.734	Yes
e-a vs a-f	847.5	8.376	Yes
e-a vs a-f72	622.5	6.152	Yes
e-a72 vs a-f	642.5	6.35	Yes
e-a72 vs a-f72	417.5	4.126	Yes
con vs a-f	538.5	5.322	Yes

Appendix 6.5

Moisture analysis of aged breads treated with E-A and A-F

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	4	0	35	0	0
e-a	4	0	37.5	0.577	0.289
a-f	4	0	39	1.414	0.707

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
a-f vs. con	4	3	9.071	<0.001	Yes
e-a vs. con	2.5	3	5.669	0.008	Yes

Appendix 6.6

Staling of E-A and A-F breads - fresh

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.549	0.324	0.102
E-A	10	0	1.347	0.356	0.112
A-F	10	0	1.438	0.289	0.0914

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.389).

Staling data of E-A and A-F breads - 24hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	3.308	0.507	0.16
E-A	10	0	3.105	0.842	0.266
A-F	10	0	3.124	0.354	0.112

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.712).

Staling data of E-A and A-F breads - 48hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	3.52	0.289	0.0913
E-A	10	0	3.239	0.387	0.122
A-F	10	0	3.126	0.759	0.24

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.825	0.413	1.53	0.235
Residual	27	7.282	0.27		
Total	29	8.107			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.235).

Staling data for E-A and A-F breads - 72hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	3.563	0.789	0.249
E-A	10	0	3.285	0.598	0.189
A-F	10	0	3.095	0.536	0.169

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.108	0.554	1.313	0.286
Residual	27	11.397	0.422		
Total	29	12.505			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.286).

Appendix 6.7

Crumb and crust colour for controls and breads treated with M1and its composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
CON	15	0	58.694	3.971	1.025
M1	15	0	53.447	3.286	0.848
M1E-A	15	0	50.479	5.858	1.513
M1A-F	15	0	52.834	5.92	1.529

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
CON vs. M1E-A	8.215	4	6.497	<0.001	Yes
CON vs. M1A-F	5.86	4	4.634	0.01	Yes
CON vs. M1	5.247	4	4.149	0.024	Yes

Appendix 6.8

Crumb and crust analysis of controls and breads treated with M2 composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	58.589	4.627	1.195
M2	14	0	50.596	6.411	1.713
M2 E-A	15	0	53.657	5.266	1.36
M2 A-F	15	0	52.245	7.465	1.928

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M2	7.993	4	5.041	0.004	Yes
con vs. M2 A-F	6.344	4	4.072	0.028	Yes
M2 A-F vs. M2	1.649	4	1.04	0.883	Do Not Test

Analysis of crumb for controls and breads treated with M2 composite

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	75.226	1.828	0.472
M2	14	0	76.173	1.375	0.368
M2 E-A	15	0	76.267	2.182	0.563
M2 A-F	15	0	76.681	1.215	0.314

Source of Variation	DF	SS	MS	F	P
Between Groups	3	16.994	5.665	1.963	0.13
Residual	55	158.681	2.885		
Total	58	175.674			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.130).

Appendix 6.9

Analysis of crust colour for controls and breads treated with M3 composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	58.315	3.617	0.934
M3	15	0	51.989	5.644	1.457
M3E-A	15	0	53.28	7.148	1.846
M3A-F	15	2	58.8	4.458	1.236

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
M3A-F vs. M3	6.811	4	4.695	0.009	Yes
M3A-F vs. M3E-A	5.52	4	3.805	0.046	Yes
con vs. M3	6.327	4	4.526	0.012	Yes

Analysis of crumb colour of controls and breads treated with M3 composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	76.292	0.965	0.249
M3	15	0	76.586	0.892	0.23
M3E-A	15	0	76.615	1.186	0.306
M3A-F	15	0	76.965	1.551	0.4

Source of Variation	DF	SS	MS	F	P
Between Groups	3	3.412	1.137	0.821	0.488
Residual	56	77.524	1.384		
Total	59	80.936			

Appendix 6.10

Crust colour data of M4 and composites

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Con	10	0	57.39	56.66	60.34
M4	10	0	60.44	53.08	64.22
M4E-A	10	1	54.37	50.07	59.027
M4A-F	10	0	55.23	52.78	56.66

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.160)

Crumb colour data of M4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	76.948	1.44	0.455
M4	10	0	76.228	2.331	0.737
M4E-A	10	1	75.34	2.771	0.924
M4A-F	10	0	76.139	2.316	0.732

Source of Variation	DF	SS	MS	F	P
Between Groups	3	12.288	4.096	0.809	0.498
Residual	35	177.283	5.065		
Total	38	189.571			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.498).

Appendix 6.11

Crust colour produced by M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	59.799	2.825	0.893
M5	10	0	59.818	4.73	1.496
M5E-A	10	0	56.563	3.634	1.149
M5A-F	10	0	58.92	3.398	1.075

Source of Variation	DF	SS	MS	F	P
Between Groups	3	70.504	23.501	1.706	0.183
Residual	36	495.979	13.777		
Total	39	566.483			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.183).

Appendix 6.12

Volume analysis of breads treated with M1 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	27	0.173	0.1
M1	3	0	27.067	0.0577	0.0333
M1E-A	3	0	28.4	0.265	0.153
M1A-F	3	0	28.467	0.208	0.12

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
M1A-F vs. con	1.467	4	13.266	<0.001	Yes
M1A-F vs. M1	1.4	4	12.663	<0.001	Yes
M1E-A vs. con	1.4	4	12.663	<0.001	Yes
M1E-A vs. M1	1.333	4	12.06	<0.001	Yes

Volume analysis of breads treated with M2 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	27	0.2	0.115
M2	3	0	27.033	0.306	0.176
M2E-A	3	0	27.2	0.1	0.0577
M2A-F	3	0	26.983	0.126	0.0726

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.555).

Volume analysis of breads treated with M3 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	27	0.173	0.1
M3	3	0	26.933	0.153	0.0882
M3E-A	3	0	27.333	0.321	0.186
M3A-F	3	0	26.7	0.2	0.115

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.047).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
M3E-A vs. M3A-F	0.633	4	4.947	0.033	Yes

Volume analysis of breads treated with m4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	27	0.173	0.1
M4	3	0	26.667	0.306	0.176
M4E-A	3	0	27	0.173	0.1
M4A-F	3	0	26.733	0.153	0.0882

Source of Variation	DF	SS	MS	F	P
Between Groups	3	0.277	0.0922	2.088	0.18
Residual	8	0.353	0.0442		
Total	11	0.63			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.180).

Volume analysis of breads treated with M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	3	0	27	0.173	0.1
M5	3	0	26.633	0.153	0.0882
M5E-A	3	0	25.033	0.153	0.0882
M5A-F	3	0	24.967	0.252	0.145

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 9

Comparison	Diff of Means	p	q	P	P<0.050

con vs. M5A-F	2.033	4	18.825	<0.001	Yes
con vs. M5E-A	1.967	4	18.208	<0.001	Yes
M5 vs. M5A-F	1.667	4	15.43	<0.001	Yes
M5 vs. M5E-A	1.6	4	14.813	<0.001	Yes

Appendix 6.13

Compression data for fresh untreated breads and breads treated with M1 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.587	0.173	0.0547
M1	10	0	0.906	0.115	0.0364
m1e-a	10	0	0.986	0.0543	0.0172
M1A-F	10	0	0.883	0.0899	0.0284

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. M1A-F	0.703	4	19.116	<0.001	Yes
Con vs. M1	0.681	4	18.503	<0.001	Yes
Con vs. m1e-a	0.6	4	16.311	<0.001	Yes

Compression data for breads aged for 24hrs treated with M1 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.37	0.231	0.0729
M1	10	0	1.615	0.15	0.0474
m1e-a	10	0	1.865	0.0825	0.0261
M1A-F	10	0	1.656	0.259	0.0818

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M1	0.755	4	12.352	<0.001	Yes
con vs. M1A-F	0.714	4	11.687	<0.001	Yes
con vs. m1e-a	0.505	4	8.261	<0.001	Yes
m1e-a vs. M1	0.25	4	4.091	0.031	Yes

Compression data for breads aged for 48 hrs treated with M1 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	3.147	0.356	0.112

M1	10	0	2.313	0.375	0.119
m1e-a	10	0	2.901	0.586	0.185
M1A-F	10	0	2.659	0.59	0.187

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.004).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M1	0.834	4	5.383	0.003	Yes

Compression data of breads aged for 72hrs treated withM1 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	3.725	0.196	0.0619
M1	10	0	2.551	0.386	0.122
m1e-a	10	0	2.799	0.399	0.126
M1A-F	10	0	2.908	0.403	0.128

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M1	1.174	4	10.406	<0.001	Yes
con vs. m1e-a	0.926	4	8.211	<0.001	Yes
con vs. M1A-F	0.817	4	7.238	<0.001	Yes

Appendix 6.14

Compression data of fresh breads treated with M2 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	1.229	0.158	0.05
M2	10	0	0.967	0.218	0.0689
M2E-A	10	0	0.833	0.153	0.0483
M2A-F	10	0	0.869	0.0982	0.031

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M2E-A	0.395	4	7.703	<0.001	Yes
con vs. M2A-F	0.36	4	7.01	<0.001	Yes
con vs. M2	0.262	4	5.109	0.005	Yes

Compression data for breads aged for 24hrs treated with M2 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.788	0.179	0.0567
M2	10	0	1.279	0.169	0.0533
M2E-A	10	0	1.237	0.143	0.0452
M2A-F	10	0	1.437	0.195	0.0616

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M2E-A	0.551	4	10.105	<0.001	Yes
con vs. M2	0.509	4	9.335	<0.001	Yes
con vs. M2A-F	0.352	4	6.446	<0.001	Yes

Compression data of breads aged for 48hrs and treated with M2 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	2.621	0.262	0.0829
M2	10	0	2.188	0.31	0.098
M2E-A	10	0	1.896	0.192	0.0608
M2A-F	10	0	2.195	0.321	0.101

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M2E-A	0.726	4	8.315	<0.001	Yes
con vs. M2	0.433	4	4.967	0.006	Yes
con vs. M2A-F	0.426	4	4.887	0.008	Yes

Compression data of breads aged for 72hrs treated with M2 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	4.109	0.549	0.174
M2	10	0	2.737	0.507	0.16
M2E-A	10	0	1.962	0.456	0.144
M2A-F	10	0	2.288	0.44	0.139

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of	p	q	P	P<0.050

	Means				
con vs. M2E-A	2.147	4	13.853	<0.001	Yes
con vs. M2A-F	1.821	4	11.751	<0.001	Yes
con vs. M2	1.371	4	8.85	<0.001	Yes
M2 vs. M2E-A	0.775	4	5.004	0.006	Yes

Appendix 6.15

Compression data of fresh breads treated with M3 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	1.369	0.174	0.0549
M3	10	0	1.151	0.336	0.106
M3E-A	10	0	1.045	0.181	0.0574
M3A-F	10	0	0.81	0.185	0.0586

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M3A-F	0.559	4	7.71	<0.001	Yes
con vs. M3E-A	0.323	4	4.462	0.016	Yes
M3 vs. M3A-F	0.341	4	4.708	0.011	Yes

Compression data of breads aged for 24hrs treated with M3 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	2.187	0.343	0.108
M3	10	0	1.925	0.354	0.112
M3E-A	10	0	1.542	0.29	0.0918
M3A-F	10	0	1.622	0.138	0.0437

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M3E-A	0.645	4	6.927	<0.001	Yes
con vs. M3A-F	0.565	4	6.073	<0.001	Yes
M3 vs. M3E-A	0.382	4	4.105	0.031	Yes

Compression data of breads aged for 48hrs and treated with M3 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM

con	10	0	2.565	0.368	0.116
M3	10	0	2.043	0.289	0.0915
M3E-A	10	0	1.6	0.292	0.0923
M3A-F	10	0	1.972	0.138	0.0437

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M3E-A	0.965	4	10.744	<0.001	Yes
con vs. M3A-F	0.594	4	6.608	<0.001	Yes
con vs. M3	0.522	4	5.813	0.001	Yes
M3 vs. M3E-A	0.443	4	4.932	0.007	Yes
M3A-F vs. M3E-A	0.372	4	4.136	0.029	Yes

Compression data for breads aged for 72hrs treated with M3 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	3.364	0.737	0.233
M3	10	0	3.149	0.379	0.12
M3E-A	10	0	2.539	0.275	0.0869
M3A-F	10	0	2.596	0.207	0.0654

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M3E-A	0.825	4	5.816	0.001	Yes
con vs. M3A-F	0.768	4	5.413	0.003	Yes
M3 vs. M3E-A	0.61	4	4.299	0.022	Yes
M3 vs. M3A-F	0.552	4	3.896	0.043	Yes

Appendix 6.16

Compression data for bread aged for 24hrs treated with M4 and composites

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	1.76	0.377	0.119
M4	10	0	1.586	0.366	0.116
M4E-A	10	0	1.308	0.233	0.0736
M4A-F	10	0	1.455	0.258	0.0817

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.020).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M4E-A	0.452	4	4.533	0.014	Yes

Compression data of bread aged for 48hrs and treated with M4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	3.443	0.754	0.239
M4	10	0	3.032	0.381	0.12
M4E-A	10	0	2.595	0.771	0.244
M4A-F	10	0	1.82	0.277	0.0876

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M4A-F	1.623	4	8.721	<0.001	Yes
con vs. M4E-A	0.849	4	4.56	0.014	Yes
M4 vs. M4A-F	1.212	4	6.51	<0.001	Yes
M4E-A vs. M4A-F	0.774	4	4.161	0.028	Yes

Compression data of bread aged for 72hrs and treated with M4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	10	0	3.975	0.68	0.215
M4	10	0	3.773	0.521	0.165
M4E-A	10	0	2.602	0.262	0.0827
M4A-F	10	0	2.514	0.486	0.154

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M4A-F	1.461	4	9.072	<0.001	Yes
con vs. M4E-A	1.373	4	8.525	<0.001	Yes
M4 vs. M4A-F	1.259	4	7.816	<0.001	Yes
M4 vs. M4E-A	1.171	4	7.269	<0.001	Yes

Compression data for fresh breads treated with M4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.308	0.125	0.0394
M4	10	0	1.306	0.0799	0.0253
M4E-A	10	0	0.896	0.0989	0.0313
M1A-F	10	0	0.964	0.0881	0.0279

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. M4E-A	0.412	4	13.127	<0.001	Yes
Con vs. M1A-F	0.344	4	10.961	<0.001	Yes
M4 vs. M4E-A	0.41	4	13.057	<0.001	Yes
M4 vs. M1A-F	0.342	4	10.891	<0.001	Yes

Appendix 6.17

Compression data for fresh bread treated with M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.039	0.0351	0.0111
M5	10	0	0.984	0.0327	0.0103
M5E-A	10	0	0.959	0.0213	0.00674
M5A-F	10	0	0.716	0.0945	0.0299

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M5A-F	0.323	4	18.89	<0.001	Yes
con vs. M5E-A	0.08	4	4.679	0.011	Yes
M5 vs. M5A-F	0.268	4	15.673	<0.001	Yes
M5E-A vs. M5A-F	0.243	4	14.211	<0.001	Yes

Compression data for breads aged for 24hrs and treated with M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.104	0.0406	0.0128
M5	10	0	2.06	0.0271	0.00856
M5E-A	10	0	2.054	0.0392	0.0124
M5A-F	10	0	1.752	0.0702	0.0222

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M5A-F	0.352	4	23.666	<0.001	Yes
M5 vs. M5A-F	0.308	4	20.708	<0.001	Yes
M5E-A vs. M5A-F	0.302	4	20.305	<0.001	Yes

Compression data for breads aged for 48hrs and treated with M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.952	0.0607	0.0192
M5	10	0	2.898	0.0618	0.0195
M5E-A	10	0	2.907	0.0724	0.0229
M5A-F	10	0	2.335	0.147	0.0465

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of Means	p	q	P	P<0.050
con vs. M5A-F	0.617	4	21.042	<0.001	Yes
M5E-A vs. M5A-F	0.572	4	19.511	<0.001	Yes
M5 vs. M5A-F	0.563	4	19.204	<0.001	Yes

Compression data for breads aged for 72hrs and treated with M5 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	3.362	0.0955	0.0302
M5	10	0	3.525	0.0363	0.0115
M5E-A	10	0	3.325	0.053	0.0167
M5A-F	10	0	2.072	0.0416	0.0131

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of	p	q	P	P<0.050

	Means				
M5 vs. M5A-F	1.453	4	75.113	<0.001	Yes
M5 vs. M5E-A	0.2	4	10.339	<0.001	Yes
M5 vs. con	0.163	4	8.4	<0.001	Yes
con vs. M5A-F	1.29	4	66.713	<0.001	Yes
M5E-A vs. M5A-F	1.253	4	64.774	<0.001	Yes

Appendix 6.18

Yellow crumb in M1 and composite treated breads

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	10.839	0.7	0.181
M1	15	0	11.402	0.975	0.252
m1e-a	15	0	11.245	1	0.258
M1A-F	15	0	12.033	1.023	0.264
CONA	15	0	11.74	0.939	0.242
M1A	15	0	11.961	1.339	0.346
M1E-AA	15	0	12.152	1.166	0.301
M1A-FA	15	0	12.057	0.53	0.137

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003).
Comparisons for factor: Col 16

Comparison	Diff of Means	p	q	P	P<0.050
M1E-AA vs. con	1.313	8	5.147	0.01	Yes
M1A-FA vs. con	1.218	8	4.776	0.022	Yes
M1A-F vs. con	1.194	8	4.682	0.027	Yes
M1A vs. con	1.122	8	4.4	0.047	Yes

Yellow crumb for M2 and composite treated breads

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	10.745	0.585	0.151
M2	15	0	11.261	0.805	0.208
M2E-A	15	0	11.245	1	0.258
M2A-F	15	0	11.837	0.871	0.225
CONA	15	0	11.74	0.939	0.242
M2A	15	0	11.761	1.114	0.288
M2E-AA	15	0	12.03	0.996	0.257
M2A-FA	15	0	12.039	0.516	0.133

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 16

Comparison	Diff of	p	q	P	P<0.050

	Means				
M2A-FA vs. con	1.295	8	5.728	0.002	Yes
M2E-AA vs. con	1.285	8	5.687	0.003	Yes
M2A-F vs. con	1.092	8	4.832	0.019	Yes
M2A vs. con	1.017	8	4.498	0.039	Yes
CONA vs. con	0.995	8	4.404	0.047	Yes

Yellow crumb for aged breads treated with M3 and composites.

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	10.409	0.963	0.249
M3	15	0	11.135	1.295	0.334
M3E-A	15	1	12.134	0.829	0.222
M3A-F	15	1	11.271	1.367	0.365
CONA	15	0	10.898	0.916	0.236
M3A	15	1	12.068	0.514	0.137
M3E-AA	15	0	12.105	0.829	0.214
M3A-FA	15	1	11.745	0.668	0.178

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 14

Comparison	Diff of Means	p	q	P	P<0.050
M3E-A vs. con	1.726	8	6.815	<0.001	Yes
M3E-A vs. CONA	1.236	8	4.882	0.018	Yes
M3E-AA vs. con	1.697	8	6.819	<0.001	Yes
M3E-AA vs. CONA	1.207	8	4.852	0.019	Yes
M3A vs. con	1.659	8	6.552	<0.001	Yes
M3A vs. CONA	1.17	8	4.62	0.031	Yes
M3A-FA vs. con	1.336	8	5.277	0.007	Yes

Yellow crumb of breads treated with M4 and composites

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	11.236	0.981	0.253
M4	15	0	12.318	0.971	0.251
M4E-A	15	0	12.723	1.025	0.265
M4A-F	15	0	12.265	0.822	0.212
CONA	15	0	12.371	0.503	0.13
M4A	15	0	13.029	0.916	0.236
M4E-A72	15	0	13.396	1.265	0.327
M4A-F72	15	0	13.243	0.541	0.14

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 18

Comparison	Diff of Means	p	q	P	P<0.050
M4E-A72 vs. con	2.16	8	9.2	<0.001	Yes
M4E-A72 vs. M4A-F	1.131	8	4.818	0.02	Yes
M4E-A72 vs. M4	1.078	8	4.591	0.032	Yes
M4A-F72 vs. con	2.007	8	8.547	<0.001	Yes
M4A vs. con	1.794	8	7.638	<0.001	Yes
M4E-A vs. con	1.487	8	6.332	<0.001	Yes
CONA vs. con	1.136	8	4.836	0.019	Yes

Yellow crumb of breads treated with M5 and composites.

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	15	0	11.215	0.838	0.216
M5	15	1	12.024	1.083	0.289
M5E-A	15	0	11.697	0.963	0.249
M5A-F	15	0	11.781	0.714	0.184
CONA	15	0	11.269	0.945	0.244
M5A	15	0	12.361	0.864	0.223
M5E-AA	15	0	11.727	0.974	0.252
M5A-FA	15	0	12.197	0.843	0.218

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 14

Comparison	Diff of Means	p	q	P	P<0.050
M5A vs. con	1.146	8	4.891	0.017	Yes
M5A vs. CONA	1.092	8	4.661	0.028	Yes

Appendix 6.19

Mositure data of endoxylanases and composites.

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
con	4	0	42.5	42	43
m1	4	0	42	40.5	42.5
e-a	4	0	42	42	42
a-f	4	0	42	42	42
con72	4	0	34.65	34.42	34.85
m172	4	0	32.95	32.85	33.2
e-a72	4	0	35.05	34.9	35.2
af-72	1	0	34.9	34.9	34.9
a-f72	3	0	35.1	35.025	35.175

H = 28.407 with 8 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	Q	P<0.05
con vs m172	24.75	3.731	Yes
m1 vs m172	21.25	3.204	Yes

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
con	4	0	42	41.5	42
m2	4	0	41.5	40.5	42.5
e-a	4	0	41.5	41	42
a-f	4	0	40	39.5	40
con72	4	0	34.65	34.42	34.85
m272	4	0	32.05	31.85	32.15
e-a72	4	0	32.1	32	32.2
a-f72	4	0	32	31.9	32.05

H = 27.967 with 7 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	Q	P<0.05
con vs a-f72	22.125	3.335	Yes
con vs m272	20.875	3.147	Yes
e-a vs a-f72	20.875	3.147	Yes
m2 vs a-f72	20.75	3.128	Yes

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Con	4	0	43	42.5	43.5
m3	4	0	43	40	43.5
e-a	4	0	40	37	43
a-f	4	0	42.5	41.5	43
con72	4	0	34.65	34.42	34.85
m372	4	0	39	37.35	40.25
e-a72	4	0	39.95	39.65	40.55
a-f72	4	0	41.95	41.85	42.1

H = 19.385 with 7 degrees of freedom. (P = 0.007)

The differences in the median values among the treatment groups are greater than would be expected by chance: there is a statistically significant difference (P = 0.007)

Comparison	Diff of Ranks	Q	P<0.05
con vs con72	23.625	3.562	Yes

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	4	0	41.25	0.957	0.479
m4	4	0	41.25	0.5	0.25
e-a	4	0	41.75	0.5	0.25
a-f	4	0	39.25	0.957	0.479
con72	4	0	34.635	0.26	0.13
m472	4	0	38.025	0.359	0.18
e-a72	4	0	38.1	0.374	0.187
a-f72	4	0	39.05	0.311	0.155

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor:
Col 7

Comparison	Diff of Means	p	q	P	P<0.050
e-a vs. con72	7.115	8	24.197	<0.001	Yes
e-a vs. m472	3.725	8	12.668	<0.001	Yes
e-a vs. e-a72	3.65	8	12.413	<0.001	Yes
e-a vs. a-f72	2.7	8	9.182	<0.001	Yes
e-a vs. a-f	2.5	8	8.502	<0.001	Yes
con vs. con72	6.615	8	22.497	<0.001	Yes
con vs. m472	3.225	8	10.968	<0.001	Yes
con vs. e-a72	3.15	8	10.713	<0.001	Yes
con vs. a-f72	2.2	8	7.482	<0.001	Yes
con vs. a-f	2	8	6.802	0.002	Yes
m4 vs. con72	6.615	8	22.497	<0.001	Yes
m4 vs. m472	3.225	8	10.968	<0.001	Yes
m4 vs. e-a72	3.15	8	10.713	<0.001	Yes
m4 vs. a-f72	2.2	8	7.482	<0.001	Yes
m4 vs. a-f	2	8	6.802	0.002	Yes
a-f vs. con72	4.615	8	15.695	<0.001	Yes
a-f vs. m472	1.225	8	4.166	0.107	No

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	4	0	42.75	1.5	0.75
m5	4	0	42	1.414	0.707

e-a	4	0	42	0.816	0.408
a-f	4	0	42.25	1.5	0.75
con72	4	0	34.635	0.26	0.13
m572	4	0	38.025	0.737	0.368
e-a72	4	0	39.75	0.705	0.352
a-f72	4	0	40.4	0.216	0.108

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparison	Diff of Means	p	q	P	P<0.050
con vs. con72	8.115	8	15.915	<0.001	Yes
con vs. m572	4.725	8	9.266	<0.001	Yes
con vs. e-a72	3	8	5.883	0.007	Yes
a-f vs. con72	7.615	8	14.934	<0.001	Yes
a-f vs. m572	4.225	8	8.286	<0.001	Yes
a-f vs. e-a72	2.5	8	4.903	0.036	Yes
e-a vs. con72	7.365	8	14.444	<0.001	Yes
e-a vs. m572	3.975	8	7.796	<0.001	Yes
m5 vs. con72	7.365	8	14.444	<0.001	Yes
m5 vs. m572	3.975	8	7.796	<0.001	Yes
a-f72 vs. con72	5.765	8	11.306	<0.001	Yes

Appendix 7.1

Compression data of amylase preparations

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.255	0.0756	0.0239
a-af	10	0	1.119	0.114	0.036
a-ab	10	0	1.13	0.186	0.0589

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.115	0.0576	3.24	0.055
Residual	27	0.48	0.0178		
Total	29	0.596			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.055).

Appendix 7.2

Compression data for fungal amylase at different treatment levels

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
5a	10	0	0.996	0.19	0.0602
10a	10	0	0.703	0.217	0.0685
50a	10	0	0.743	0.104	0.033
Con	10	0	1.152	0.178	0.0563

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. 10a	0.449	4	8.013	<0.001	Yes
con vs. 50a	0.409	4	7.292	<0.001	Yes
5a vs. 10a	0.294	4	5.239	0.004	Yes
5a vs. 50a	0.253	4	4.518	0.015	Yes

Compression data for bacterial amylase at different treatment levels

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
5b	10	0	0.99	0.0391	0.0124
10b	10	0	0.654	0.205	0.065
50b	10	0	0.872	0.111	0.035

Con	10	0	1.152	0.178	0.0563

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 4

Comparison	Diff of Means	p	q	P	P<0.050
con vs. 10b	0.498	4	10.629	<0.001	Yes
con vs. 50b	0.28	4	5.983	<0.001	Yes
5b vs. 10b	0.336	4	7.177	<0.001	Yes
50b vs. 10b	0.218	4	4.646	0.012	Yes

Appendix 7.3

Development of crust colour when fungal and bacterial amylases are added.

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	57.363	4.903	1.266
a-AF	15	0	52.912	3.841	0.992
a-AB	15	0	50.911	3.996	1.032

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. a-AB	6.453	3	5.85	<0.001	Yes
Con vs. a-AF	4.451	3	4.036	0.018	Yes

Appendix 7.4

Crumb colour in fresh and aged bread treated with baterial or fungal amylase

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	15	0	11.959	1.107	0.286
a-AF	15	1	12.071	0.941	0.252
a-AB	15	0	12.565	0.619	0.16
CONA	15	1	12.977	1.071	0.286
A-AFA	15	0	12.595	0.552	0.142
A-ABA	15	0	13.119	0.879	0.227

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Comparisons for factor: Col 7

Comparison	Diff of	p	q	P	P<0.050

	Means				
A-ABA vs. con	1.16	6	5.084	0.007	Yes
A-ABA vs. a-AF	1.049	6	4.516	0.024	Yes
CONA vs. con	1.018	6	4.383	0.031	Yes

Appendix 7.5

Loaf volume of breads treated with fungal and baceterial amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	22.75	1.146	0.661
a-af	3	0	23	0	0
a-ab	3	0	24.167	0.52	0.3

Source of Variation	DF	SS	MS	F	P
Between Groups	2	3.431	1.715	3.25	0.111
Residual	6	3.167	0.528		
Total	8	6.597			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.111).

Appendix 7.6

Moisture data of fresh and aged breads treated with amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	3	0	42.167	1.258	0.726
a-af	3	0	42.167	0.764	0.441
a-ab	3	0	41.167	0.764	0.441
Cona	3	0	35.6	2.6	1.501
a-faa	3	0	39.9	0.458	0.265
a-ba	3	0	38.933	0.306	0.176

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 11

Comparison	Diff of Means	p	q	P	P<0.050
con vs. cona	6.567	6	8.894	<0.001	Yes
a-af vs. cona	6.567	6	8.894	<0.001	Yes
a-faa vs. cona	4.3	6	5.824	0.014	Yes

Appendix 7.7

Compression data of bread treated with amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	1.102	0.181	0.0571
a-af	10	0	0.775	0.177	0.0561
a-ab	10	0	0.755	0.198	0.0628

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
con vs. a-ab	0.347	3	5.9	<0.001	Yes
con vs. a-af	0.327	3	5.566	0.002	Yes

Compression data for breads aged for 24hrs and treated with amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.351	0.42	0.133
a-af	10	0	1.812	0.285	0.0902
a-ab	10	0	1.695	0.213	0.0673

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. a-ab	0.656	3	6.522	<0.001	Yes
con vs. a-af	0.538	3	5.354	0.002	Yes

Compression data for breads aged for 48hrs and treated with amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	2.615	0.587	0.186
a-af	10	0	1.825	0.256	0.081
a-ab	10	0	1.761	0.225	0.0713

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 5

Comparison	Diff of	p	q	P	P<0.050

	Means				
con vs. a-ab	0.853	3	6.887	<0.001	Yes
con vs. a-af	0.789	3	6.368	<0.001	Yes

Compression data for breads aged for 72hrs and treated with amylases

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	10	0	3.563	0.634	0.201
a-af	10	0	2.941	0.268	0.0848
a-ab	10	0	2.069	0.266	0.0841

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
con vs. a-ab	1.493	3	11.079	<0.001	Yes
con vs. a-af	0.622	3	4.612	0.008	Yes
a-af vs. a-ab	0.872	3	6.467	<0.001	Yes

Appendix 7.8

HPLC data of fresh and aged breads - maltose

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
con	6	0	0.553	0.0137	0.00558
aafa	6	0	0.458	0.0147	0.00601
aaba	6	0	0.202	0.00753	0.00307
conb	6	0	0.567	0.0103	0.00422
aafb	6	0	0.503	0.0103	0.00422
aabb	6	0	0.56	0.0141	0.00577

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 1

Comparison	Diff of Means	p	q	P	P<0.050
conb vs. aaba	0.365	6	74.106	<0.001	Yes
conb vs. aafa	0.108	6	21.995	<0.001	Yes
conb vs. aafb	0.0633	6	12.859	<0.001	Yes
aabb vs. aaba	0.358	6	72.753	<0.001	Yes
aabb vs. aafa	0.102	6	20.641	<0.001	Yes
aabb vs. aafb	0.0567	6	11.505	<0.001	Yes
con vs. aaba	0.352	6	71.399	<0.001	Yes

con vs. aafa	0.095	6	19.288	<0.001	Yes
con vs. aafb	0.05	6	10.152	<0.001	Yes
aafb vs. aaba	0.302	6	61.248	<0.001	Yes
aafb vs. aafa	0.045	6	9.136	<0.001	Yes
aafa vs. aaba	0.257	6	52.111	<0.001	Yes

HPLC data for fresh and aged breads - glucose

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	6	0	0.1	0.00894	0.00365
aafa	6	0	0.0182	0.00492	0.00201
aaba	6	0	0.01	0.000894	0.000365
conb	6	0	0.042	0.0415	0.0169
aafb	6	0	0.0175	0.00423	0.00173
aabb	6	0	0.01	0.000894	0.000365

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
Con vs. aabb	0.09	6	12.567	<0.001	Yes
Con vs. aaba	0.09	6	12.567	<0.001	Yes
Con vs. aafb	0.0825	6	11.52	<0.001	Yes
Con vs. aafa	0.0818	6	11.426	<0.001	Yes
Con vs. conb	0.058	6	8.099	<0.001	Yes
conb vs. aabb	0.032	6	4.468	0.038	Yes
conb vs. aaba	0.032	6	4.468	0.038	Yes

HPLC data for fresh and aged breads - fructose

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
con	6	0	0.071	0.069	0.073
aafa	6	0	0.121	0.119	0.123
aaba	6	0	0.123	0.12	0.125
conb	6	0	0.071	0.069	0.073
aafb	6	0	0.142	0.141	0.144
aabb	6	0	0.02	0.019	0.021

H = 32.229 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be

expected by chance; there is a statistically significant difference (P = <0.001)

Comparison	Diff of Ranks	q	P<0.05
Aafb vs aabb	180	6.975	Yes
Aafb vs conb	126	4.882	Yes
Aafb vs con	126	4.882	Yes
aaba vs aabb	131	5.076	Yes
Aafa vs aabb	121	4.689	Yes

HPLC data for fresh and aged breads - maltotriose

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	6	0	0.0215	0.00308	0.00126
Aafa	6	0	0.0125	0.00105	0.000428
aaba	6	0	0.0403	0.00216	0.000882
conb	6	0	0.0305	0.00164	0.000671
Aafb	6	0	0.0207	0.00207	0.000843
aabb	6	0	0.0612	0.00279	0.00114

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 7

Comparison	Diff of Means	p	q	P	P<0.050
aabb vs. aafa	0.0487	6	53.312	<0.001	Yes
aabb vs. aafb	0.0405	6	44.366	<0.001	Yes
aabb vs. con	0.0397	6	43.453	<0.001	Yes
aabb vs. conb	0.0307	6	33.594	<0.001	Yes
aabb vs. aaba	0.0208	6	22.822	<0.001	Yes
aaba vs. aafa	0.0278	6	30.49	<0.001	Yes
aaba vs. aafb	0.0197	6	21.544	<0.001	Yes
aaba vs. con	0.0188	6	20.631	<0.001	Yes
aaba vs. conb	0.00983	6	10.772	<0.001	Yes
conb vs. aafa	0.018	6	19.718	<0.001	Yes
conb vs. aafb	0.00983	6	10.772	<0.001	Yes
conb vs. con	0.009	6	9.859	<0.001	Yes
con vs. aafa	0.009	6	9.859	<0.001	Yes
aafb vs. aafa	0.00817	6	8.946	<0.001	Yes

Appendix 7.9

Thermal effects of a-amylase preparations after 72hrs

One Way Analysis of Variance

Group Name	N	Missing	Mean	Std Dev	SEM
Con	6	0	2.607	0.0258	0.0105
Aafa	6	0	2.565	0.0378	0.0154
Aaba	6	0	1.832	0.036	0.0147

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Comparisons for factor: Col 3

Comparison	Diff of Means	p	q	P	P<0.050
con vs. aaba	0.775	3	56.445	<0.001	Yes
aafa vs. aaba	0.733	3	53.41	<0.001	Yes

Appendix 7.10

Correlation of maltotriose and compression data after 72hrs

Pearson Product Moment Correlation

Cell Contents:
Correlation Coefficient
P Value
Number of Samples

	aged 72hs
Maltotriose	-0.666
	0.000058
	4
	30

aged 72hs

The pair(s) of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases.

Appendix 7.11

Correlation between retrogradation and crumb firming

Spearman Rank Order Correlation

Cell Contents:
Correlation Coefficient
P Value
Number of Samples

	72hrs
retrograd	0.417
	0.243
	9

72hrs

There are no significant relationships between any pair of variables in the correlation table (P > 0.050).