THE ENZYMOMOLOGY OF THE MALTING, MILLING, MASHING AND FERMENTATION PROCESSES WITHIN THE SCOTCH MALT WHISKY INDUSTRY.

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ABSTRACT.

The wort from the malted barley used in the Scotch Malt Whisky process must be obtained solely from the activity of enzymes associated with the malted barley. Little or no detailed studies have been reported on the complex mixtures of enzymes found in malted barley, nor their subsequent activity and fate within the Scotch Malt Whisky process. The aims of this study were to determine assay procedures for a total of 17 enzymes, ascertain their suitability within the heterogeneous mixture of a malted barley extract and then establish the enzyme activity levels within the production process.

Method development of the assay procedures gave rise to specific assays for 13 enzymes. Especially important were those for α-amylase, using the Phadebas substrate, and the p-nitrophenyl-maltopentaose substrate for β-amylase. Both assays were found to be specific within the complex mixture of malted barley wort, with all other carbohydrase enzymes active.

Enzyme activities between malted barley varieties were studied and compared. This suggested that differences occurred between the enzyme levels of varieties, as well as within a single variety. These may be due to malting conditions or to geographical, environmental and/or climatic factors associated with barley cultivation.

The activities and fate of the enzymes were followed during the production process. Although the distillery where the study was carried out employed an infusion mashing system, the results obtained may also be valid in the semi-lauter/lauter type systems used within the industry. Malted barley enzymes were active during the initial stages of fermentation, but thereafter other enzyme systems were evident. These were most likely associated with yeast and bacteria.

CONTENTS.

LIST OF TABLES.

LIST OF FIGURES.

1 INTRODUCTION.

The enzymology of the malting, mashing and fermentation processes of the Scotch Malt Whisky industry is significant if maximum potential yields are to be attained from the raw materials. The biochemical changes that take place during the malting of barley to malt are very important in the process. This is evident from the definition of Scotch Malt Whisky, which states that Malt Whisky must be produced exclusively from a mash of 100% malted barley, fermented with yeast and batch distilled (HMSO 1990). Having completed this process the raw spirit or new make whisky, must then be allowed to mature, for at least three years, in oak casks. During mashing, the milled malted barley must be saccharified solely by the diastatic activities of the malted barley. Any treatment of the barley during the steeping or germination phases, with either growth promoters or inhibitors, whether they are natural or chemical is forbidden. From this definition clearly no additives, *i.e.*, enzymes during the mashing or fermentation stages, nor materials to aid yeast growth or activity, are permitted.

Although the production of Scotch Malt Whisky uses the same basic raw materials as the Brewing Industry, namely malted barley, yeast and water, there are several fundamental differences between the two processes. As mentioned above, there can be no additions, either chemical or enzymic to the basic raw ingredients in the Scotch Malt Whisky process. Adjuncts and substrates other than malted barley may be used in the manufacture of beer. In the Brewing process a wort boiling step is used. This does not take place in the production of Scotch Malt Whisky allowing the hydrolysis of the wort substrate by active malted barley enzymes during the fermentation stage. This is also true of the malted barley microflora, which survive the mashing stage. The protein content of the fermentation in the Scotch Malt Whisky process is higher than that of the brewery fermentation, since protein precipitation takes place during wort

boiling in the brewing process. A mixed innoculum of both brewing and distilling yeasts is often used in the fermentation of Scotch Malt Whisky, which is pitched at a high level, $2x10^7$ cells ml^{-1}. Only brewing yeast is used in the Brewing process. During fermentation in the Scotch Malt Whisky process there is no direct temperature control, thus fermentation temperatures may increase to 30-34°C, and ultimately yeast cell death and lysis occurs. Sterile conditions are not required for the Scotch Malt Whisky fermentation and so bacterial numbers can increase towards the end of fermentation (Dolan 1976). These bacteria, predominantly *Lactobacilli,* may be responsible for the production of additional flavour or off-flavour aromas, or their precursors (Priest and Pleasants 1988).

Thus, the manner in which the basic raw materials are processed are not entirely comparable between the Brewing and Distilling processes. The bulk of the scientific literature published on mashing and fermentation in the Brewing process may have only a limited value to the Scotch Malt Whisky process. Especially when enzyme studies are to be made. There is also a deficit of published scientific information concerning all stages of the Scotch Malt Whisky process.

1.1 Scotch Malt Whisky Process - An Overview.

The production of malted barley is now, in the main, carried out by specialist maltsters, and the malt supplied to the distillery will be to the specification of the distiller. The more traditional floor maltings, which were associated with distilleries have been superseded by the introduction of modern pneumatic malting plants. These modern plants are now capable of malting in excess of 250 tonne, of barley in one production cycle (Griffin 1982, Stevens 1984).

The malted barley, on delivery to the distillery, is stored in the malt bins, from where it enters the first stage of the process (Figure 1). After dressing, to remove materials, such as loose husk, partial grains and stones, the malted barley is fed to the mill. Once in the mill the malted barley can be ground to give a grist that will allow

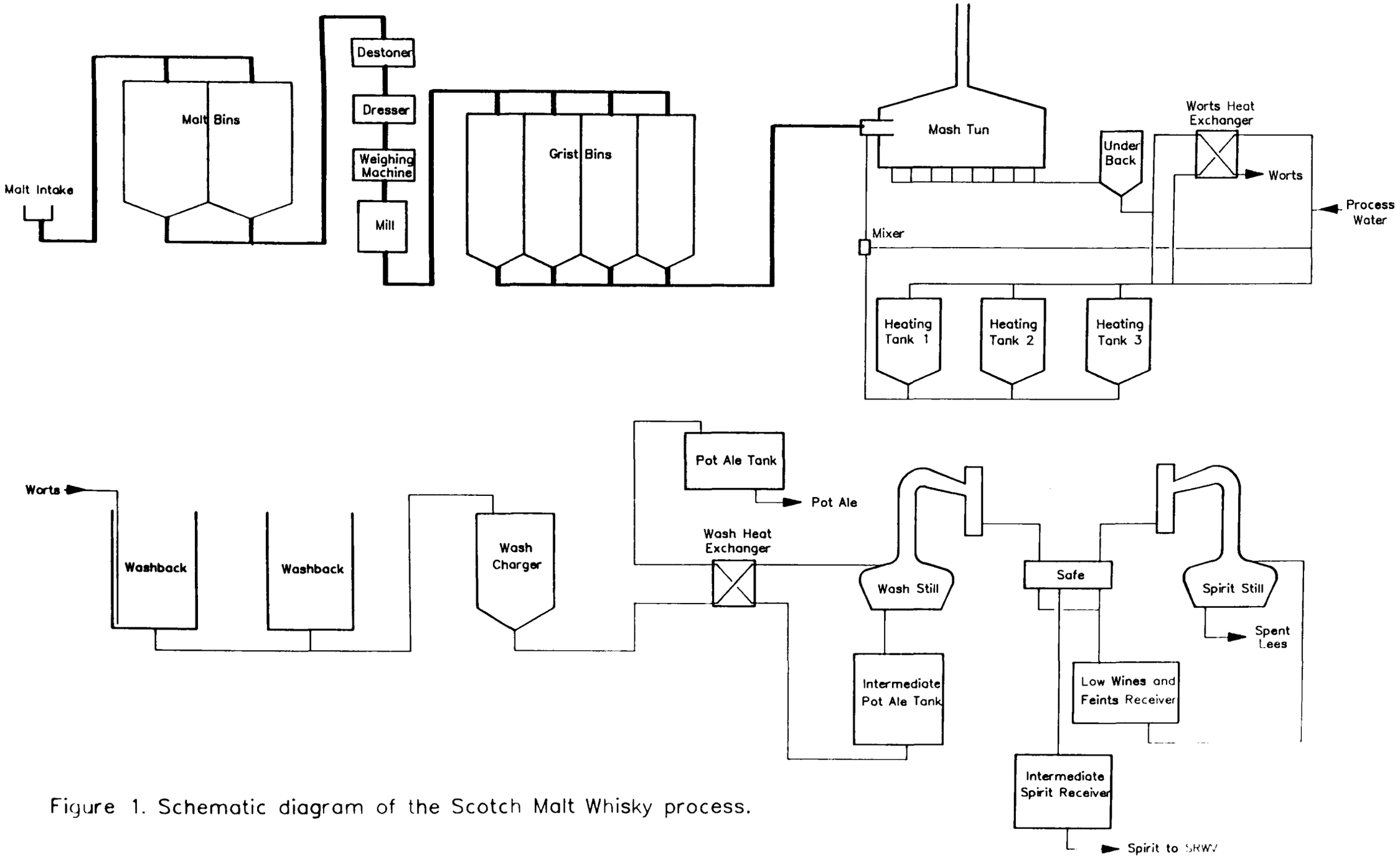


Figure 1. Schematic diagram of the Scotch Malt Whisky process.

optimal extraction of the malt polysaccharides, proteins, enzymes, minerals, *etc.* Mashing now takes place in the mash tun, this is invariably an infusion mash, although semi-lauter and lauter mashing systems are now being used. Other systems have also been evaluated, *i.e.*, mash filters and continuous mashing systems (Wilken 1983). It is during this process that the grist is mixed with the mashing liquor. Mashing liquor is obtained by sparging the previous mash. The mash is then left for a specified interval and the liquid, or wort, from the mash tun is drained through the mash bed. The wort is then cooled and pumped to the fermentation vessels known as washbacks.

Many different designs of fermentation vessels are utilized, including the traditional open-topped, wooden washback with their wooden lids. Many distilleries are now using closed stainless steel washbacks, more suited to modern requirements. Fermentation is normally carried out using a combination of both brewing and distillers' yeast strains, belonging to the genus *Saccharomyces*. The high innoculum of yeast leads to a fast fermentation that reduces the specific gravity of the wort to below 1.000 g/l within two or three days. The fully fermented wort, or as it is now known wash, is pumped, via the wash charger into the wash still for the first of the distillation steps. Normally two distillation steps are employed, but sometimes three may be found.

The first distillation produces the distillate known as Low Wines. The low wines are collected from the first runnings of the distillate until all the alcohol has been distilled off. The low wines are then mixed with feints and transferred to the second distillation step. This second distillation is more selective, with the distillate being collected in three fractions. The first fraction, containing the low boiling point organic compounds is retained in the feints storage vessel, this fraction is known as the heads. Once the heads reach a specific alcoholic strength the distillate, is then collected in the Intermediate Spirit Receiver. It is this fraction that will become the final product,

known as Scotch Malt Whisky. The cut point between the first and second fractions is selected so that undesirable compounds such as aldehydes have reached a low concentration in the distillate. Collection of the second fraction is terminated when the alcoholic strength falls to a value specified by the distiller. The third fraction, the tails, is then collected in the same vessel as the heads, both are now collectively known as feints. As previously mentioned, the feints are mixed with low wines for the next charge of the spirit still. The alcohol concentration in the feints is at a level that warrants further extraction, this type of re-circulation extracts the bulk of the alcohol from the feints.

The precursors of many flavour compounds are formed during fermentation. Distillation appears to promote reactions that contribute to final congener composition. Acids and alcohols can react to form esters, reactions of oxidation and reduction can occur, aldehydes can be reduced to acids and these acids subsequently reduced to alcohols. Many of these chemical reactions take place in the stills, lyne arms and condensers through the catalytic effect of the copper (Watson 1983). Several factors influence these reactions, but the major factor appears related to the contact time of the vapour with the copper (Watson 1985).

When distillation is complete the spirit is reduced to a suitable strength with water and filled into oak casks. These casks are normally of three sizes, and are carefully selected by the distiller. There are several different types of cask used. Examples of these include previously filled Scotch whisky casks, newly manufactured casks that can be charred or uncharred, sherry casks or sherry treated casks, casks previously used for American bourbon and sometimes casks of unknown origin.

Each of these cask types contribute differently to the maturation characteristics of the whisky and to the final quality of the whisky. The casks chosen, must allow the whisky to mature fully, and at the

correct rate (Sharp 1983). Once filled with new make whisky distillate the casks are warehoused for a minimum period of three years, or usually much longer. During the maturation process, various complex reactions take place between the wood components and the compounds in the distillate (Reazin 1983). These reactions combine to produce mature Scotch Malt Whisky, which will then be removed from the casks for blending and bottling.

1.2 Malting.

1.2.1 Barley for Malting.

Barleys used for malting may be either two or six rowed varieties. In Europe and in Australia the two row varieties are widely used for malting, however in North America the six rowed varieties are more prevalent (Schildbach 1986). Each of these groups is composed of numerous varieties. The six row barley varieties produce more grains per plant, but the larger two row grains tend to have a higher fermentable extract content. Six row varieties tend to have higher enzyme levels due to increased nitrogen content. Breeders are involved in the development of barley to ensure that varieties are high yielding, have better resistance to disease, are suitable for the climatic conditions in which they are to be grown and maintain good malting characteristics.

1.2.2 The Malting Process.

Barley in its dormant state has little or no metabolic activity. Malting commences with steeping the barley grains in water allowing them to swell by approximately one quarter of their size. The first indication of germination is the appearance of the root sheath, or coleorhiza. The barley grain is now metabolically active. If seedling growth is to proceed, the endogenous reserves of the endosperm must be degraded to provide the carbohydrates, amino acids and other metabolites. Once germination has advanced to the required degree, metabolism can be arrested by drying the green malt in the kiln.

1.2.2.1 Steeping.

Probably the most important process in the malting cycle is steeping, failure to carry out this process correctly cannot be rectified during germination and kilning. The uptake of moisture by the barley grain is critical to the quality of the malt produced (Brookes *et al.* 1976). The purpose of steeping is to activate the embryo and evenly hydrate the endosperm. The hydrolytic enzymes produced will then be able to degrade the structural components of the endosperm in a uniform manner.

Historically, barley was steeped for three days with the water being changed at regular intervals, to give a cast moisture of about 44%. Using this regime there was virtually no sign of embryo growth. Pneumatic malting plants, normally steep for about two days using a broken steeping regime. After the steep water has been drained, air is then pulled or pushed through the grain bed to remove carbon dioxide, replenish the oxygen supply and consequently stimulate the growth of the grain. For a well modified malt the cast moisture may be in the regions of 44-46% and this normally requires two or three steep cycles. The first, brings the moisture content to 32%. The second, after the first air rest, will bring the moisture content to approximately 42%. The second air rest should bring about the first emergence of the chit and the final steep will then bring the moisture content of the developing grain to the required level. Additional benefits from the steeping process include the removal of certain chemical compounds, *i.e.*, those that may have detrimental effects on the fermentation or the flavour compounds of the final product and herbicides and pesticides.

1.2.2.2 Germination.

The initial development of the grain starts during the steeping process, and the grain must then be allowed to germinate and grow. Modification is the result of the sum of the metabolic activity taking place within the barley grain. The moisture content of the grain after

steeping must be sufficient to support this modification, but not allow excessive growth. This is an important aspect, since growth is associated with a decrease in dry matter through respiration. There must be a balance between growth and modification to minimize malting losses.

During germination, the germination rate and modification levels of the grain are controlled by regulating the moisture content and the temperature of the grain. The temperature of the grain should be kept between 13-21°C, depending on the malting plant, the equipment and the type of malt to be produced. The relative humidity of the air should be kept as high as possible to ensure that the malt does not dry out, thus affecting the rate of endosperm modification (Seward 1986).

In the production of malted barley for the Scotch Malt Whisky industry, the use of additives for the control of malting losses, to allow rapid, even germination, or for any other reason is strictly prohibited.

1.2.2.3 Kilning.

The purpose of kilning is three-fold. Firstly the moisture content of the grain must be reduced to allow safe storage. Secondly, the metabolic activity of the germinating grains must be reduced and then halted when the optimal modification level has been attained. Finally, kilning allows the green malt to develop certain flavour and colour characteristics, which will depend on the requirements of the purchaser.

In the early stages of kilning the temperature of the hot air is between 50-70°C when it enters the grain bed. Depending on the malt bed depth, the temperature of the drying malt should be no more than 30°C. During this period the grain moisture decreases to 10-15%. Germination of the grain may continue to proceed while the moisture content is in the region of 40% and the grain temperature is about 30°C.

Water loss from the grain over the first stage of kilning is quite rapid and after approximately ten hours the break point is reached. At this point, water evaporation from the grains slows down, and the temperature of the bed begins to increase. Dehydration of the endosperm is now more noticeable, and over the following 8-10 hr the temperature of the grain will increase to 65-75°C. The malt moisture is now in the region of 5%. With the reduction of grain moisture level, the metabolic activity of the grain will also be reduced and finally halted. As grain temperature rises enzyme degradation can occur, with certain enzymes being more sensitive than others. The final or curing stage for certain malts occurs when the air on temperature increases to 80-85°C.

The practice of burning sulphur on all direct and some indirect fired kilns is to reduce and inhibit the formation of nitrosamines. Besides having an effect on nitrosamine levels the use of sulphur can bleach the malt resulting in less coloured malt and a decrease in wort pH may also occur. A marginal increase in extract can also occur with changes in certain enzyme activities (Sim 1983).

Malt flavour can also be influenced during kilning by the smoke from certain fuels. In malts for the Scotch Malt Whisky industry the burning of peat may have a desirable effect on the final malted barley. Peat smoke contains a variety of phenolic and nitrogenous compounds that can be absorbed by the malted barley, imparting the peaty character into certain Scotch Malt Whiskies.

1.2.3 The Biochemistry of Malting Barley.

A distiller requires a malt that can yield a free running wort containing the optimal concentrations of fermentable sugars, a balance of amino acids and polypeptides and an adequate amount of other metabolites. These are required to sustain yeast growth and metabolism. Barley itself cannot produce such a wort. Consequently the process of malting is necessary to enable the enzymes of the barley grain to be synthesized. These enzymes then utilize the natural

reserves of the barley grain. The malting of barley for the Scotch Malt Whisky industry is a natural process. The aim of malting is to utilize the food reserves of the barley grain into a substrate that can be used in the mashing and fermentation processes.

1.2.3.1 The Morphology of Barley.

The Husk.

The palae and the lemma of the barley grain are commonly known as the husk (Figure 2). This fraction comprises of up to 10% of the dry weight of the grain. The husk is a specialized leaf structure, protecting the grain from physical damage. During malting it seems that the role of the husk is in the control of water uptake into the barley grain during the initial steeping period (MacLeod 1979). It has little, or no bearing on the extract potential of the malted barley, since the husk is rich in cellulose, hemicellulose, protein and a certain amount of polyphenols (Preece 1931, MacLeod and Napier 1959, Eastmond and Gardner 1974). The husk is essential to the formation of the mash filter bed during wort separation (MacLeod 1977).

The Pericarp-Testa.

In the mature barley grain the testa (seed coat) and the pericarp (fruit wall) (Figure 2) are fused, with the contents of the cells changed in structure. Studies have shown that the testa consists of two bands of fatty and waxy materials (Freeman and Palmer 1984). The intact pericarp-testa layers can therefore act as a semi-permeable membrane, being permeable to water but not to a wide range of dissolved salts (Palmer 1974).

The Embryo.

This is the organelle of the barley grain that will develop into the viable seedling. It is consequently a very complex, metabolically active structure, with the remainder of the grain serving either to protect the embryo, or to serve as food reserves during the initial stages of germination. Representing 2-5% of the total dry weight of the barley grain it contains many soluble sugars, *e.g.*, sucrose and

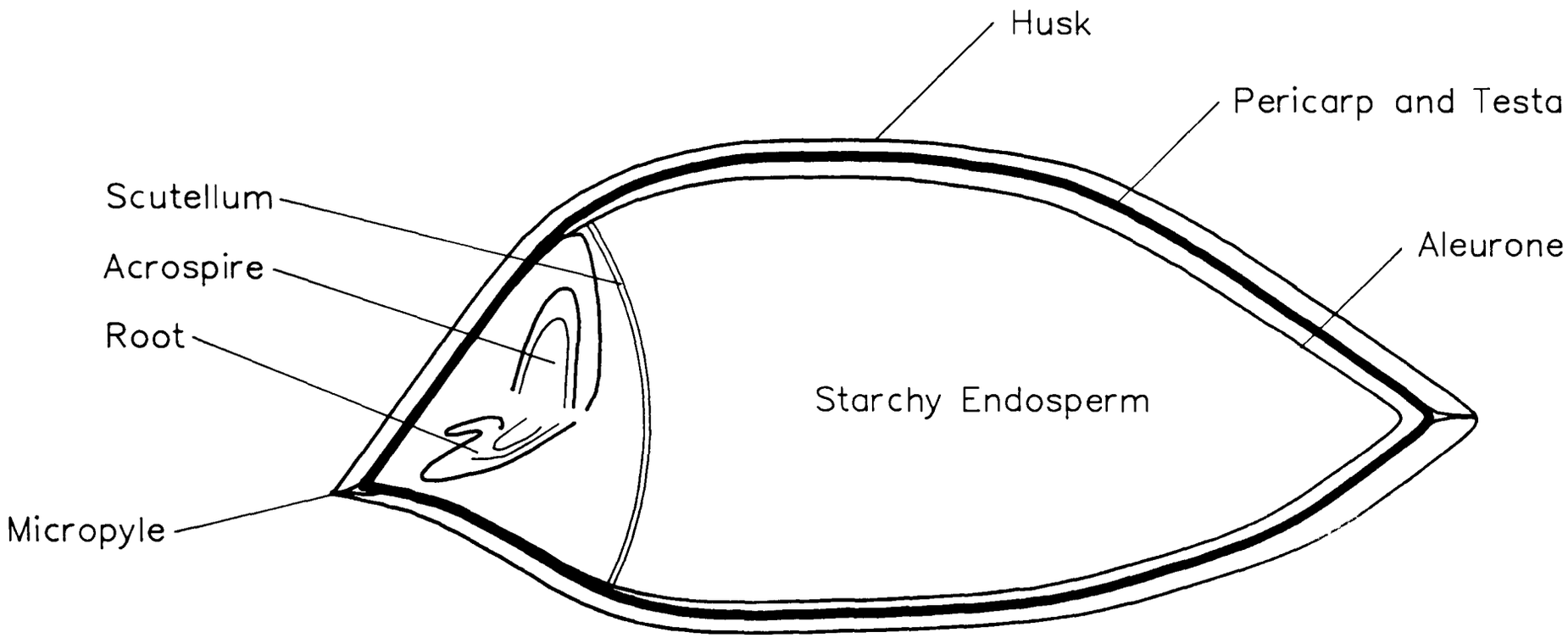


Figure 2. Longitudinal section of a barley grain.
Adapted from MacLeod 1979.

raffinose, proteins, minerals and vitamins (Palmer 1980, Kent 1983). The embryo is well stocked with reserves that are readily metabolized to sustain it while the main food reserves are being initially solubilized.

The Aleurone Layer.

In most malting barley the aleurone layer (Figure 2) is three cells deep (Pomeranz 1973). However, it is structurally and functionally very different from the endosperm. These layers of cells are specialized for the synthesis of hydrolytic enzymes (MacLeod *et al.* 1964, Varner and Ram-Chandra 1964), which are ultimately responsible for the solubilization of the food reserves in the endosperm. The synthesis and/or release of many hydrolytic enzymes is carried out after the aleurone layer has been stimulated by several plant hormones, especially the gibberellins (Cohen and Paleg 1967, Groat and Briggs 1969).

The Endosperm.

This is the main food storage organ of the cereal grain. The endosperm (Figure 2) makes up approximately 75% of the dry weight of the barley grain and contains dead cells with no respiratory action. The endosperm of the barley grain consists of approximately 65% starch, 12% protein, and 8% water soluble gums, the remainder includes hemicellulose material, lipids and other metabolites necessary for the germination of the embryo. Endosperm cells are thin walled and are large in comparison to the cells in the aleurone layer. These cells may vary in size and are packed with starch granules. The endospermic cells next to the aleurone layer are smaller and more symmetrical. In this region, which can be termed the sub-aleurone layer, the cells are comparatively rich in protein and poor in starch (Tronier and Ory 1970). The amounts of starch in the cells increase as they move towards the centre of the grain.

1.2.3.2 Plant Hormones.

The metabolism of the germinating barley grain is affected by several plant hormones, the most important of these are the gib-

berellins. Gibberellins perform a key role in the regulation of enzyme synthesis in the grain, and these have been studied in great depth (Varner and Ram-Chandra 1964, MacLeod *et al.* 1964, Jacobsen *et al.* 1970). The gibberellins were first detected in cultural filtrates of *Gibberella fujikuroi* (Kurosawa 1926). Gibberellins are formed in the barley embryo (Yomo 1958) and act as phytohormones regulating the synthesis and release of enzymes. They are a group of structurally related hormones, with gibberellin A_1 and A_3 being most effective in synthesizing and releasing the hydrolytic enzymes (Crozier *et al.* 1970). Gibberellin A_3, or gibberellic acid appears to play the major role in the control of endosperm modification.

1.2.3.3 The Development of Barley Enzymes in Response to Gibberellic Acid.

Carbohydrases.

α-Amylase, probably the most important enzyme of starch degradation, has been studied extensively in its response to gibberellic acid stimulation (Paleg 1960a, Paleg 1960b, Briggs 1963, Briggs 1964, Yomo 1960). On treatment of the aleurone layer with gibberellic acid there is a rapid increase in the production of α-amylase. It was the work of Varner and his co-workers (Varner and Ram-Chandra 1964, Jacobsen *et al.* 1970) that firmly established that the α-amylase originating from the aleurone was the product of *de novo* synthesis. During the supply of gibberellic acid to the aleurone, α-amylase will be synthesized. When sugar levels in the embryo increase an element of control is found, since gibberellic acid excretion from the embryo will cease.

Evidence has also been established for α-amylase production in the embryonic tissue. This α-amylase may account for up to 15% of the total α-amylase in the germinating barley grain (Greenwood and Thompson 1961). Gibberellic acid is therefore an absolute requirement

for the synthesis of α-amylase by *de novo* synthesis in the germinating barley grain. However, the precise roles of the various barley grain tissues have still to be clarified.

Limit dextrinase (E.C. 3.2.1.10), an enzyme responsible for the hydrolysis of α-1,6 linkages in dextrins derived from the α-amylolysis of starch, is also stimulated by the action of gibberellic acid on the aleurone (Manners *et al.* 1971, Hardie 1975). Levels of the enzyme α-glucosidase, which is present in ungerminated barley grains, is also found to increase during germination (Jorgensen 1965). The synthesis of this enzyme has been stimulated by treating barley grains with externally applied gibberellic acid (Clutterbuck and Briggs 1973).

Glucanases and Hemicellulases.

Several enzymes are involved in the hydrolysis of endosperm cell wall glucans and hemicelluloses. Several of these are stimulated by the effect of gibberellic acid. The enhancement of endo-β-glucanase by gibberellic acid has been established for a considerable time (MacLeod and Miller 1962). This hormone has also been shown to stimulate the production of the enzyme by the intact barley (MacLeod *et al.* 1964). Unlike the barley endo-β-glucanase enzyme, gibberellic acid is not a specific requirement for the synthesis of laminarinase (a β-1,3-glucanase). However, it is necessary for the release of this enzyme from the aleurone (Jones 1971). Gibberellic acid enhances the synthesis and secretion of a few pentosan hydrolyzing enzymes (Taiz and Honigman 1976), which include β-xylosidase, α-arabinosidase and endoxylanase.

Proteolytic Enzymes.

It has been established that gibberellic acid stimulates the general degradation of protein (Ault 1961, Macey and Stowell 1961), thus increasing the amino acid content of the germinating barley considerably. The work carried out by Jacobsen and Varner in 1967 established that gibberellic acid was required to promote protease

synthesis in the aleurone cells. The substrate used in this case was wheat gliadin protein, but the use of these enzymes' natural substrates, *e.g.*, barley hordein proteins, would enable a more realistic determination of proteolytic activity. It was further established that the time course of proteolytic activity development, during germination, was essentially similar to that of α-amylase (Jacobsen and Varner 1967). Hence, gibberellic acid controls the synthesis of proteolytic enzymes in the endosperm of the barley grain for the general degradation of proteins.

Others.

An increase in ribonuclease activity during the germination of barley after gibberellic acid application has been established (Srivastava 1964). This enzyme was shown to be synthesized in the aleurone and secreted into the endosperm (Chrispeels and Varner 1967). Acid phosphatase is secreted (Ashford and Jacobsen 1974) and phytase (Srivastava 1964, Pollard and Singh 1968) is synthesized and secreted, by the aleurone in response to gibberellic acid.

Enzymes Not Affected by Gibberellins.

There are many other enzymes that are found in germinating barley which are not related to the control of gibberellic acid. Many enzymes present in the aleurone cells may be under the control of external or internal activators. Lipase is one such example. It is synthesized in response to glutamine and indolylacetic acid (Taverner and Laidman 1971, Firn and Kende 1974). In addition, other enzymes, not affected by gibberellins, may be under the control of product inhibition (Ferrari and Varner 1969, Doig *et al.* 1975). There is a wide range of enzymes belonging to glycolysis, the operation of the Kreb's Cycle and the electron transport systems, which do not respond to gibberellic acid. These latter enzymes are all associated with either the cytosol or the mitochondria, enzymes associated with gibberellic acid stimulation are normally located with membranes (Doig *et al.* 1975). Another class of enzymes not affected by gibberellic acid are

those associated with the endosperm protein matrix (La Berge *et al.* 1967, La Berge and Meredith 1969, Allison and Swanston 1974). β-Amylase, is one such enzyme, found only in the endosperm, and released from its latent form by the action of glucanases and proteases (Hejgaard 1978). Certain proteolytic activities are also of this type (Mikola and Kolehmainen 1972).

1.2.3.4 The Enzymatic Modification of the Endosperm.

The growth of the embryo and endosperm modification allows the barley grain to control the rate of utilization of its food reserves while growing in the field. The malting process requires the complete modification of the endosperm. During malting, the barley embryo will commence germination before the hydrolytic enzymes begin to modify the endosperm. The maltster has therefore to allow the developing embryo to modify the endosperm, while keeping the amount of food reserves to the growing shoot and roots to a minimum.

Modification can be defined in a malting context as the conversion, by a complex mixture of hydrolytic enzymes that act to solubilize the hard endosperm of the barley grain into a friable malt. Many enzymes needed for this process require the stimulation of plant hormones for either their synthesis, release, or both from the aleurone layers of cells. While other enzymes are not dependent on these hormones for their subsequent development during malting.

Amylolytic Enzymes of the Germinating Barley Grain.

The starch of barley is composed of two types of glucose polymers. Amylose, the minor component, is a linear polymer formed from glucose units joined by α-1,4 glucosidic linkages. Amylopectin, the major component, contains mainly α-1,4 linked glucose units, but has branched points of glucose through α-1,6 glucosidic linkages.

Starch comprises of approximately two thirds of the dry weight of the barley grain. During germination starch is ultimately hydrolyzed

to glucose, which is used as both an energy source and to initiate the synthesis of new components necessary for the growth of the barley seedling.

Barley contains two distinct populations of starch granules that are distinguished by their physical sizes. The smaller of the granules represents approximately 90% of the total number of granules, but only 10% by weight of the total starch (Bathgate and Palmer 1972, Buttrose 1960). During malting only 5-10% of the total starch is solubilized, and this is mainly from the embryonic end of the endosperm (Greenwood and Thompson 1961). The small and large starch granules are degraded by contrasting methods. The degradation of the small granules appears to be due to amylolytic activity on the surface of the granule (Bathgate and Palmer 1973). The large granules are degraded in a more restricted fashion, with channels forming in the granule that run towards the centre (Buttrose 1960).

Microscopic observations on malted barley reveal that in the well-modified regions of the endosperm, the small starch granules are totally degraded, but the large granules are only pitted (Palmer 1972). This is consistent with α-amylolytic activity. Limit dextrinase, which is present in the endosperm, with α-glucosidase, may, like β-amylase play a secondary role in the degradation of some of the primary products of α-amylolysis of the native starch granules. During malting, starch degrading enzymes, such as, α-amylase, α-glucosidase and limit dextrinase migrate into the endosperm from the aleurone layer. Activation of the β-amylase, which originates in the starchy endosperm, as well as any endospermic located limit dextrinase, is believed to be released from the protein matrix by protease activity (Hejgaard 1978). Biochemical evidence suggests that unlike α-amylase synthesis, which is *de novo*, β-amylase is released from a bound form to a free active form as germination progresses (Bettner and Meredith 1970).

Starch must be hydrolyzed to give oligosaccharides of a more manageable size. This degradation is almost solely carried out by the enzyme α-amylase. The basic activity of barley α-amylase is to act on branched and unbranched starch molecules giving a mixture of malto-oligosaccharides and α-limit dextrins. Thus the endo-hydrolytic action of the enzyme acts randomly on the α-1,4 glucosidic linkages of the starch polymers, leaving the terminal glucose residue in the α-configuration. It must be remembered that the α-amylase from barley lacks α-1,6 activity and that a certain amount of glucose and maltose will be released by α-amylase action. α-Amylase in germinating barley is heterogeneous, consisting of several isoenzymic forms (Graber and Daussant 1964). These have been divided into three groups called α-amylase I, II and III (MacGregor and Ballance 1980, Mundy *et al.* 1983). Although group III was thought to be a distinct group, it has now been shown to be a complex of α-amylase group II and an α-amylase inhibitor (Mundy *et al.* 1983). This α-amylase inhibitor is also an inhibitor of the proteolytic enzyme subtilisin. Of the two remaining groups both show partial, but not complete cross reaction in immunochemical tests (Daussant *et al.* 1974). These two groups have recently been shown to be the products of two separate gene families (Rogers 1985). α-Amylase enzymes have not been detected, even in an inactive form, in ungerminated barley grains, but high activities appear during germination (MacGregor *et al.* 1984).

The groups of α-amylase isoenzymes, I and II, have different action patterns on the large and small starch granules (MacGregor 1980). The α-amylase I is much more efficient than the α-amylase II isoenzyme in the hydrolysis of the starch granules, since it is able to utilize both forms of granules. In contrast, the α-amylase II isoenzyme can degrade the small granules well but act much more slowly on the large granules.

The activity of the β-amylase enzyme will give the disaccharide maltose. β-Amylases are exo-enzymes and attack the starch polymers from the non-reducing end. The maltose liberated, has the free hydroxyl group at carbon number one in the β-configuration. Unlike α-amylase, β-amylase cannot by-pass the α-1,6 glucosidic linkages found in amylopectin and α-limit dextrins. Thus, activity ceases when it encounters these linkages.

The β-amylase of the barley grain consists of several isoenzymes, having different molecular weights, and differing in isoelectric points (Visuri and Nummi 1972). The separation of β-amylase by size exclusion chromatography has revealed four different components (Nummi *et al.* 1965), however all have the same antigenic properties (Daussant *et al.* 1965). From this it was deduced that the heterogeneity of the β-amylase enzymes may be due to the highly reactive nature of the monomeric form of the enzyme. It would appear that the β-amylase monomers are able to polymerize in a non-reducing environment to form a series of aggregates, due to thiol group reactions (Visuri and Nummi 1972). These aggregates may then go on to react with other molecules, including proteins (Niku-Paavola *et al.* 1973).

Barley β-amylase is synthesized in the endosperm during the development of the grain (Lauriere *et al.* 1985). It is initially synthesized in a soluble form, but as the barley grain ripens up to two-thirds of the enzyme is bound into an insoluble complex with other proteins. During germination the latent or bound form of the β-amylase decreases, with a corresponding increase in the free form of the enzyme. This increase in the free form is due to protease activity (Daussant and MacGregor 1979).

The other enzymes concerned with the degradation of the starch molecules within the endosperm, include α-glucosidase and α-1,6 hydrolyzing enzymes. Germinating barley contains at least one if not two of the so-called debranching enzymes (Manners and Rowe 1971). Limit dextrinase is probably the most active of the debranching

enzymes, enabling further α-amylase and β-amylase activity once the α-1,6 linkages in amylopectin and limit dextrins are hydrolyzed. Hydrolysis of the terminal non-reducing α-1,4 linkages of starch, with the release of α-glucose can be done by α-glucosidase (Jorgensen 1965). The same enzyme is also responsible for the hydrolysis of maltose to two glucose units.

Glucanolytic Enzymes of the Germinating Barley Grain.

The non-starch polysaccharides of the barley grain are important, since the extent of their degradation during malting can be of considerable significance in wort quality (Thompson and La Berge 1977, Masak and Basarova 1991). The hemicelluloses and gums are mainly located in the cell walls of the endosperm of the barley grain (Fincher 1975, Palmer 1975). The hemicelluloses refer to non-starch polysaccharides which are insoluble in hot water, whereas the gums are those non-starch polysaccharides which are soluble in hot water. The gums are normally β-glucans and pentosans. The endosperm cell wall is composed of approximately 70% β-glucan, 20% pentosans with the remainder other hemicelluloses, gums and proteins (Fincher and Stone 1986). These polysaccharides may also serve as a reserve polysaccharide substance, but are of much less importance than the starch granules. During germination the β-glucans and arabinoxylan polysaccharides are extensively hydrolyzed. The barley β-glucans are composed of chains of β-1,3 and β-1,4 linked glucose residues. The arabinoxylan polysaccharide, derived from the cell wall, has a more complex structure. These polysaccharides are composed of chains of D-xylose residues linked by β-1,4 linkages, carrying L-arabinose residues linked to positions 2 and/or 3 on some of the xylose residues (Vietor *et.al.* 1991).

The enzymic hydrolysis of β-glucan is extremely complex. The β-glucan of barley forms an insoluble matrix with protein (Forest and Wainwright 1977), and must be made soluble by the glucanolytic enzymes during germination. This process commences with the

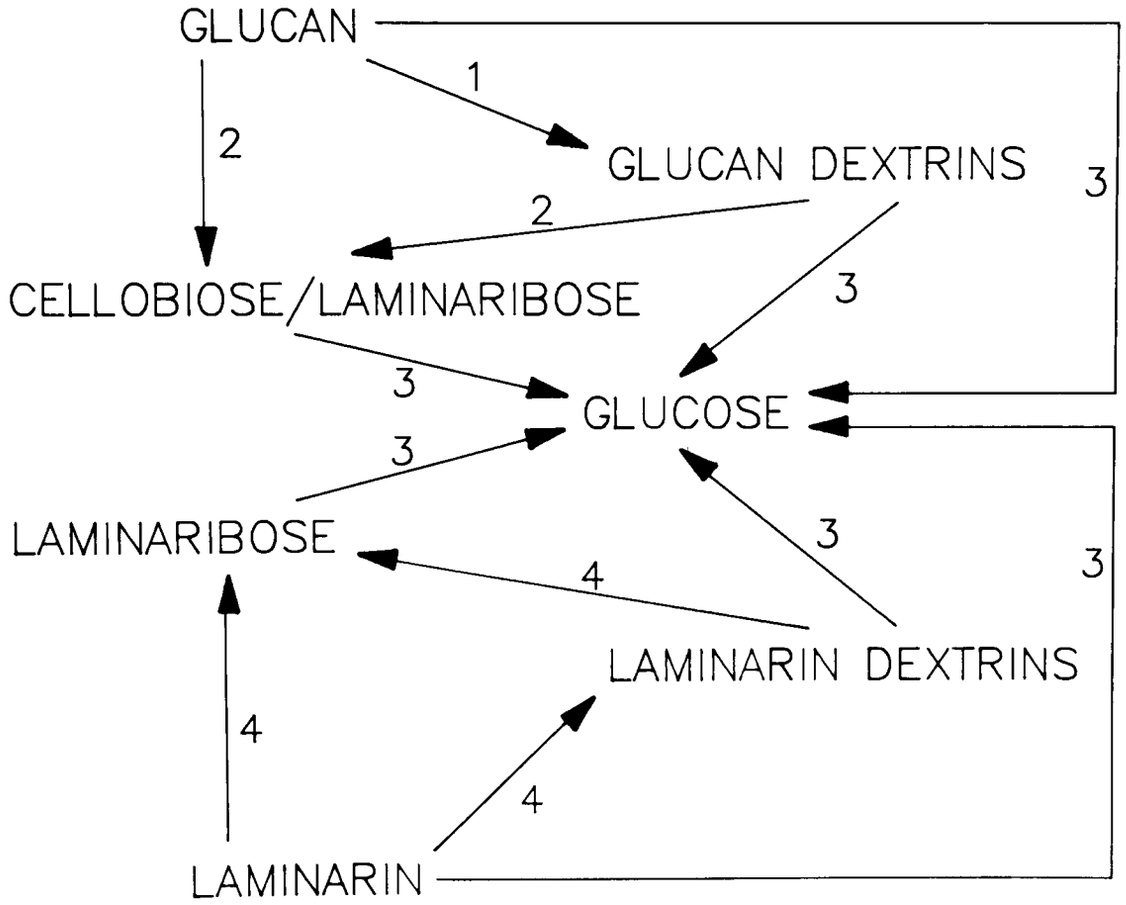


Figure 3. Routes of enzymic degradation for beta–glucan.

1 – Endo–beta–glucanase ; 2 – Exo–beta–glucanase ;
3 – beta–Glucosidase ; 4 – Laminarinase.

breakage of the β-glucan/protein ester linkage by an enzyme, β-glucansolubilase, allowing the β-glucan to become soluble (Bamforth *et al.* 1979). The soluble β-glucan can now be hydrolyzed by further enzyme activity (Thompson and La Berge 1977).

Glucans with predominantly β 1-3 linkages are hydrolyzed by the endo-β-1,3-glucanase, or laminarinase enzyme of the germinating barley (Manners and Wilson 1974). Hydrolysis products of this enzyme are mainly laminarin dextrins and laminaribiose (Figure 3). The endo-β-1,4-glucanase (Briggs 1964) will give rise to β-1,4-glucan dextrins and cellobiose. Endo-β-1,4-glucanase has, however, been linked with microbes associated with the husk, and is unlikely to be a major contributor to β-1,4-glucan hydrolysis (Hoy *et al.* 1981, Manners *et al.* 1982, Yin *et al.* 1989).

Endo-β-1,3:1,4-glucanase appears to be the main enzyme of β-glucan degradation. It is either absent or at low levels in ungerminated barley but is synthesized rapidly on hydration (Bathgate *et al.* 1974). Degradation of the β-glucan is thus carried out initially by endo-β-1,3-glucanase, to give smaller β-glucans which are then susceptible to hydrolysis by endo-β-1,3:1,4-glucanases (Bathgate and Dalgliesh 1974). The β-glucosidases are also important in this system. A number of forms of β-glucosidase have been reported (Craig and Stark 1985).

The degradation system of the arabinoxylan gums also contain a family of enzymes (Thompson and La Berge 1981), see Figure 4. β-Xylanase, β-xylosidase and an exo-xylanase are able to hydrolyse the arabinoxylan polymer into fragments (Preece and MacDougall 1958). These fragments would include arabinoxylan dextrins, xylan dextrins and xylose. Further hydrolysis of the xylan dextrins by β-xylanase and β-xylosidase would give xylose, xylobiose and possibly xylotriose. Since the arabinose residues are linked to position 2 and/or 3 on the xylose units of the main polymer (Ballance and Manners 1978),

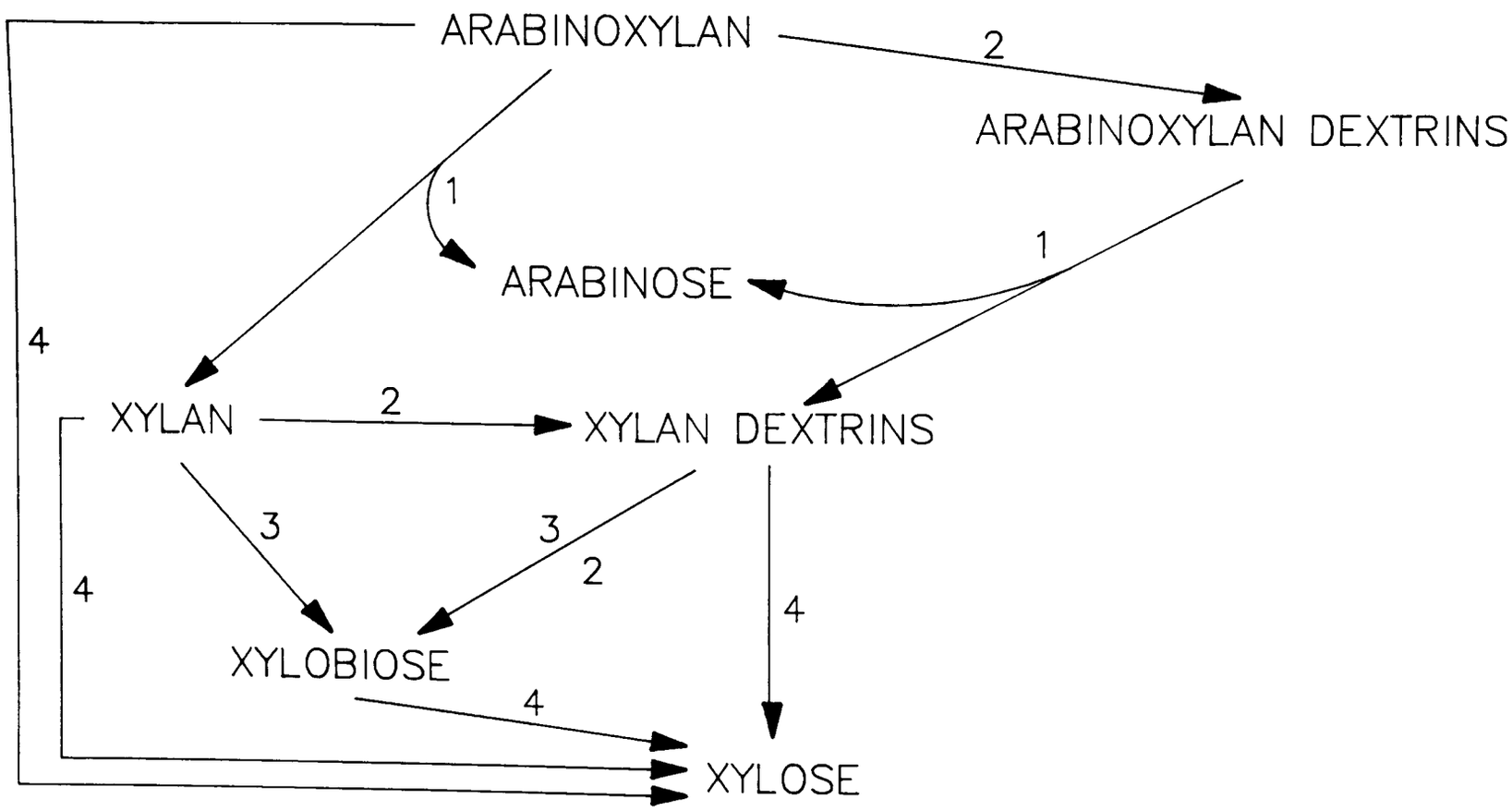


Figure 4. Routes of enzymic degradation for arabinoxylan material.
1 – Arabinosidase ; 2 – Endo–xylanase ;
3 – Exoxylanase ; 4 – beta–Xylosidase.

they can be easily removed by arabinosidases. These arabinosidases can hydrolyze the arabinose residues from both the main polymer and the arabinoxylan dextrins.

Proteolytic Enzymes of the Germinating Barley Grain.

Almost all the nitrogen found in the barley grain is incorporated in protein. The water-soluble storage proteins, hordein and glutelins, which are mainly found in the endosperm are important. During germination it is these proteins that are degraded into their constituent amino acids, which are then used to sustain growth in the seedling. Degradation of the protein matrix surrounding the starch granules allow α-amylases access into the starch granules, enabling the enzymes to commence starch granule degradation.

The endosperm shows high activities of acid-sulphydryl proteinases (endopeptidases) during germination with pH optima at 3.9 and 5.5 (Enari and Mikola 1967). Also found in the endosperm are five carboxypeptidases (Mikola 1983, Mikola and Mikola 1980), but the alkaline and neutral peptidases are not present. The insoluble reserve proteins are hydrolyzed to soluble peptides by the proteinases, and free amino acids are formed by the action of carboxypeptidases on these peptides. The scutellum has an active uptake system for amino acids and small peptides. This tissue contains high levels of the alkaline peptidases (Sopanen 1979) and moderate levels of neutral peptidase activity (Hejgaard and Boj-Hansen 1974).

There is a preponderance of protein reserves in the cells immediately below the aleurone layer and it is believed that the proteases and carboxypeptidases of the endosperm act to degrade this protein reserve (Mikola and Kolehmainen 1972). The alkaline and neutral peptidases further hydrolyze the peptides formed. The amino acids liberated in this case would be used in the synthesis of the hydrolytic enzymes of the aleurone, which will be secreted into the

endosperm. The proteolytic enzymes of the aleurone are under the control of the gibberellic acid hormonal stimulation response (Mikola and Kolehmainen 1972).

Lipolytic Enzymes of the Germinating Barley Grain.

Lipid materials are located essentially in the embryo and aleurone layer of the barley grain (MacLeod and White 1962). The major fatty acids of the barley grain are linoleic acid, oleic acid and palmitic acid. During germination, both linoleic acid and oleic acid decrease in concentration. However, palmitic acid increases (Anness and Baxter 1983). The degradation of lipids during malting by hydrolysis to low molecular weight products and conversion into starch in the scutellum (MacLeod and Palmer 1966). Lipase activity is found in the embryo with associated activity in the aleurone layer (MacLeod and White 1962). Although lipase activity fluctuates during malting, there is a nett increase after germination (Anness and Baxter 1983).

Minor Carbohydrases of the Germinating Barley Grain.

Polysaccharides contain residues of mannose, galactose, fructose and N-acetylglucosamine if hydrolyzed to a small extent, they may also provide sugars that can be used in metabolism. These enzymes, although not extensively characterized in malted barley, have been detected and include α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, and β-N-acetylglucosaminidase (Mitchell and Newman 1972, Houston *et al.* 1974).

1.3 The Milling Process.

The purpose of milling malted barley is to reduce the particle size of the malt to hasten the extraction of the soluble components, primarily the sugars and nitrogenous materials. However, the extraction of these components is not the only consideration in the preparation of the grist. The wort draining time should not be excessive. This will depend on how fine the grind is, and on what proportion of the husk remains from the coarse fraction. It is the coarser fractions that are responsible for laying down the mash

filtration bed. The milling of malt must therefore be a compromise between the need to grind as finely as possible, for the extraction of the soluble components and to maintain sufficient husk to allow efficient wort draining (Noble 1981).

It is the type of wort separation system that determines the particle size distribution of the grist. Traditional mashing systems require a coarse grind, whereas lauter and semi-lauter mashing systems can tolerate finer grinds (Wilken 1983). When the optimal grist ratio is used for a particular mashing system, high extract yields and rapid run-off rates can be accomplished. However if the grind is not optimal, low extract yields and/or slow wort run-off rates will occur, and in some circumstances the mash may set (Dixon 1977).

In the preparation of grist for the Scotch Malt Whisky process dry-milling is normally used. This is done using either four or six roller mills. The four roller mills work very well regarding extract recovery when well modified malts are used. The six roller mills, with their extra pair of rollers, allow malt with a wider range of modification to be milled successfully. Using these mills there is also greater control over the size distribution of the milled malted barley and hence wort run-off rates.

Moisture has a marked affect on the milling performance of malted barley (Klein 1982). As malt moisture decreases the percentage of the flour fraction in the grist tends to increase. However at higher moisture levels the amount of flour starts to decrease. As the moisture level within the malted barley increases, then the amount of coarse fraction produced in the grist also increases. Although the moisture content of the malt affects the milling performance of that malt, the effect is only pronounced in the range outwith that normally associated with commercial malts. Moisture also influences the recovery of extract,

as well as the wort run-off time. Malted barleys with slightly higher moisture levels can stand tighter mill settings, since the husk is more pliable and husk disintegration is less likely to occur.

The extent of modification can also have an effect on milling performance (Maule 1982), particularly if the malt is under-modified. Under-modified malts give grists which contain a larger quantity of the coarser fractions and lower amounts of flour. Increasing modification causes a reduction in the coarser fractions and an increase in the flour fraction. Malts which are very well- or over-modified, will give large amounts of flour and can therefore cause problems in wort separation.

The effect of the malted barley grain size is also important in milling. Obviously a mill setting for malt from one barley variety will not give the same milling performance as malt from another variety, if grain sizes are different. If ground under the milling conditions for the small grains, larger grains will give slightly less coarse and coarse/medium fractions and more fine/medium and flour fractions. Conversely, if the mill settings are for the larger grain variety then the small grain malt will give more coarse fractions and fewer fine fractions.

It is therefore necessary to ensure that there is adequate monitoring of the grist particle size distribution during milling. If this does not take place then poor extract recovery and slow wort run-off will be the consequence.

1.4 The Mashing Process.

1.4.1 The Scotch Malt Whisky Mashing Process.

The mashing process involves the conversion of the grist from the milled malted barley into a fermentable extract. The process of mashing involves the mixing of the grist with the hot mashing liquor. In the production of Scotch Malt Whisky the traditional infusion mashing system is normally used, in a mash tun of iron or steel construction. Draining of the mash is enhanced by using a false

bottom, which is composed of a number of perforated plates, covering the bottom of the mash tun. Mashing-in, when the grist and mashing liquor initially mix, is normally carried out in a modified Steel's mashing machine. Although lauter and semi-lauter mash tuns are now being installed in many Scotch Malt Whisky distilleries, the mashing procedure is still predominantly an infusion mash.

Lauter or semi-lauter systems, which are used in the production of Scotch Malt Whisky are not true lautering systems as developed for breweries. The mashing-in takes place in the tun, since there is no pre-mashing mixer. The second mashing water is normally delivered through the mashing spout rather than the sparging arm. However part, or all the sparging waters may be delivered through the spray heads or rings and the sparging arm. These lautering systems also act with much thicker mash beds than those found in breweries.

Because the mashing temperature is extremely important in the enzymatic degradation of the grist, it is essential to attain a temperature that is considered to give the best wort composition. This temperature is usually in the region of 63°C - 65.5°C in the mash tun, after the initial mashing-in. The most easily controlled variable is the mashing liquor temperature, since the temperature and moisture content of the grist can be quite variable. The striking temperature and the temperature at which mashing-in takes place, must be carefully controlled. Heat labile enzymes can be quickly denatured if the temperature is too high, or at lower temperatures starch gelatinization may not be fully achieved.

Once mashed-in, and after a resting period in the mash tun the fermentable extract, or wort, is drained through the mash bed to the fermentation vessel via the wort heat exchanger. It is the mash bed, which enables the fermentable extract to be relatively solid free. If the mash was to be drained directly from the mash tun the mashing bed would be pulled onto the mash tun plates. This would cause severe drainage problems. To avoid such problems occurring, the

fermentable extract is collected in the underback before it is pumped through the wort heat exchanger. This will ensure that the hydrostatic head on the mash is quite small. The fermentable extract is cooled to allow yeast pitching to be carried out as soon as possible after draining commences.

Since most, if not all the mashing liquor is used during mashing-in, the second mashing water is applied to the mash tun as freshly heated process water. This second mashing water is applied to give a temperature at the mash tun spout of between 71°C - 74°C. This water, like the first is then drained to the washback at a temperature of between 20°C - 23°C. The sparging waters are then applied, and these are recycled into a heating tank to be used as the mashing liquor for the next mash.

1.4.2 The Biochemistry of Mashing.

The mashing process, although a simple and straight forward procedure, houses a complicated system of chemical and biochemical reactions. Starch granules become hydrated and soluble in the mashing liquor. Other macromolecules and smaller compounds dissolve into the liquor and are then available to the catalytic activity of the malted barley enzymes. Any insoluble polymers, still associated with insoluble grist particles, may be attacked by enzymes in their micro-environment within the mash tun, due to hydration effects. The grist particles must be hydrated to enable the enzymes to hydrolyse their substrates, whether these substrates are in a soluble or an insoluble form.

The temperature of the traditional infusion mashing in brewing, is not kept constant throughout the mashing cycle. It is increased by underletting or sparging at specific times during the mash. The same is true for the Scotch Malt Whisky mash, but temperature increases are done using a different procedure. Mashing waters would normally go through the mashing spout or sparging rings, once the

previous water has been drained. The results of these variations in temperature produce changes in the mash, *e.g.*, in the solubilization of starch.

The wort produced from the mash tun is an extremely complicated fermentation substrate, to be used in the production of ethanol, by the anaerobic fermentation of yeast. It contains fermentable sugars, malto-oligosaccharides, large linear and branched dextrins, amino acids, peptides, proteins and other nitrogenous compounds necessary for yeast growth, lipid material, various vitamins and minerals, organic acids, inorganic phosphates and other inorganic salts. Since many flavour and aroma compounds of the final product are synthesized by the yeast, the precursors of these compounds must also be available, or derivable from the wort.

To obtain a consistent wort containing these metabolites from an infusion mashing system would appear to be quite difficult. Malt can be inconsistent in its level of modification, enzyme levels and enzyme activities. The length of time for which a mash rests in the mash tun and the temperatures of the mashing programme can alter the final composition of the wort. However, by using the optimal parameters for the system, a consistent wort can be produced from the infusion mashing system. Enzymes for starch degradation should always be present in excess, allowing starch conversion to proceed to a very high degree of completion.

Significant changes can be made in the wort composition due to alterations in the mashing programme. If, as an example, under modified malted barley were to be used it would be advantageous to begin the mashing programme at a lower temperature. This would enable the heat labile β-glucanases and proteases to degrade the endosperm cell walls to a certain extent. The temperature could then be increased to allow the main reaction of starch conversion to proceed at a more rapid rate.

The study of mashing enzymes, derived from the malted barley, is an extremely complicated topic. Starch hydrolysis involves α-amylase, β-amylase, α-glucosidase, limit dextrinases and other enzymes. After the initial hydration of the grist all the enzymes in the mash tun have an extremely heterogeneous and biochemically impure environment in which to carry out their hydrolysis. Infusion mashes are carried out at high temperatures, consequently some enzymes can be unaltered in their activity, some may be partially inactivated while others are completely denatured. As the first mashing water rests in the mash tun, the temperature of the mash and the pH will alter with time. The nature of the grist will also vary, since initially both enzymes and substrates are insoluble. Hydration of the grist will not be uniform, so both enzymes and substrates, will become more soluble during the first water mashing period. As substrate levels decline and while the wort is being continually drained, the protection given to the enzymes due to the heterogeneous environment decreases. This feature, with any protease type activity may quickly inactivate enzymic protein.

The composition of the mash, and hence the final wort from the mash tun is dependent on several variables, all working along with each other. Optimum conditions for enzymatic activity during mashing must involve the following variables, mashing liquor composition, the nature of the grist, mash bed thickness, mashing temperatures, pH and resting and draining times. Starch conversion has been taken as an example, and so if all enzyme/substrate interactions are considered, the whole mashing process becomes more and more complicated. This is especially so if individual enzymes are to be studied in their mash tun environment.

1.4.2.1 Starch Degradation.

The single most important biochemical event taking place during mashing is the hydrolysis of starch to produce fermentable sugars (MacWilliam 1968). Many of the large starch granules are partially

degraded during malting (Bathgate and Palmer 1973), these more modified areas of the malted barley grains will be converted in the mash tun more rapidly. The major part of the starch will be only made soluble after the gelatinization temperature has been achieved (Schur *et al.* 1973). During the mashing-in and resting periods the major products are glucose, maltose and maltotriose, with large and small dextrins. These dextrins can be linear (unbranched α-1,4 linked glucosidic oligosaccharides) or branched (maltodextrinyl α-1,6 linked maltodextrins) (Manners 1974).

The larger dextrins are liberated by the action of α-amylase and then become more susceptible to β-amylase hydrolysis. β-Amylases are incapable of attacking native starch granules (Manners 1974), and have therefore a secondary role in starch degradation. The product of β-amylase hydrolysis, maltose, is the main fermentable sugar of the wort. β-Amylase is more heat sensitive than the calcium ion stabilized α-amylase (Piendl 1973). Although, in the mash tun environment it may be more stable to elevated temperatures than in the purified form. Mashing temperatures are therefore important in regulating the amounts of fermentable sugars, especially maltose, in the final wort.

At the temperatures found in the mash tun during the first mashing water, there is limited secondary hydrolysis of the products from α-amylolysis. The action of limit dextrinase (Manners 1974) and α-glucosidase (Jorgensen 1964) are not substantial at these temperatures. Although there may be slight limit dextrinase activity at the lower temperature of 63°C. At these lower temperatures, limit dextrinase may hydrolyse in the region of 20% of the total α-1,6 linkages in the starch molecules (Enevoldsen and Bathgate 1969). Trace amounts of active limit dextrinase may be found in the wort in the washbacks, allowing dextrins to be hydrolyzed to some extent in the fermenting wort.

During the second mashing water, the critical starch hydrolysis enzyme will be α-amylase. At this stage, many remaining starch granules will gelatinize into the wort, to be hydrolyzed into larger dextrins. Further sparging at higher temperatures enable more of the large, undegraded starch granules to gelatinize and these may be hydrolyzed during the next mash.

1.4.2.2 Gums and Hemicelluloses.

The non-starch polysaccharides of β-glucan and arabinoxylan are usually known as the malt gums and/or hemicelluloses. During mashing, some of these gums are immediately extracted from the endosperm cell walls and solubilized into the wort. If the malted barley is deficient in endo-β-glucanase, and with mashing temperatures of 63°C - 65.5°C, then these gums may remain intact in the wort and contribute to wort viscosity (Bourne and Pierce 1972). When this is the case, difficulties may arise during wort draining from the mash tun. Gums can become more susceptible to degradation by endo-β-glucanases if a lightly kilned malted barley is used, *i.e.*, drying the malted barley over a longer period of time using lower temperatures (Erdal and Gjertsen 1971). This is also true if the mashing conditions employ a lower first water mashing temperature. In both cases the enzymes of gum hydrolysis will remain active for longer periods.

As mashing water temperatures are increased then more of the gums will be released from the grist particles into the wort. Removal of the starch granules and the protein matrix, due to enzymic solubilization, make these gums more readily extracted by the sparging waters. Prolonged sparging at higher temperatures also results in greater yields of the viscous gums and hemicelluloses (Scott 1972). During sparging no enzymatic activity towards the gums and hemicelluloses would be found. The highly viscous β-glucans and arabinoxylans may therefore be present in the mashing liquor of the

next mash. From here they will be able to pass through into the fermentation, since their enzymatic hydrolysis in the first water of mashing will be limited.

The degradation of the arabinoxylan from the endosperm cell walls during mashing is not well understood. However, a limited degree of hydrolysis of the pentosans may take place at 65°C in infusion mashing systems by endopentosanases.

1.4.2.3 Nitrogenous Material of Wort.

Wort must contain the correct balance of proteins and amino acids to maintain yeast nutrition and growth. The vast bulk of the soluble free α-amino nitrogen in the wort comes directly from the malted barley (Barrett and Kirsop 1971), but some additional amino acids are formed by hydrolysis in the mash tun (Mikola *et al.* 1972). The principle reactions that take place during mashing are the breakdown and dissolution of protein to form polypeptides of low to medium molecular weights. The second stage of hydrolysis involves the production of amino acids by peptidase activity, mainly carboxy-peptidases. Almost 80% of the α-amino nitrogen in the wort is directly attributable to carboxypeptidase activity (Enari 1972). Proteolytic activity in the mash tun is necessary for the release of starch granules from the starch/protein matrix within the endosperm cells.

In wort, the distribution of nitrogenous material derived from protein was approximated by Clapperton (1971) as 60% amino acids, 20% polypeptides and 20% high molecular weight protein. These figures are not entirely precise, due to variations that may occur in the amounts of temporary and permanent soluble nitrogen content in the wort.

Nucleic acids are also found in malted barley and these molecules are solubilized and degraded during mashing (Prentice 1976). The monomeric components of nucleic acids, the nucleotides, are not found in quantity in the wort. These are degraded to give the purine and

pyrimidine nucleosides by the action of the very active phosphatases found during mashing (Bradee 1970). The nucleosides can then be further degraded to give their free bases and sugars.

1.4.2.4 Lipids and Fatty Acids of Wort.

In mashing the most important factors in determining the release of lipids into the wort are the mashing temperature, the pH and the sparging conditions (Holmberg and Sellman-Persson 1967). If the mashing-in temperature is high, then the lipid concentration will also be high. Similarly, high sparging temperatures and the squeezing of the mash during mash filtration will cause the lipid levels of the wort to increase. If excessive lipids are extracted into the wort then this can produce subsequent effects on the final product. High concentrations of unsaturated fatty acids in cloudy wort can suppress the formation of esters (Ayrapaa and Lindstrom 1973). During anaerobic fermentation an adequate supply of unsaturated fatty acids and sterols are necessary for successful yeast growth (Nordheim 1965).

1.4.2.5 Vitamins and Minerals in Wort.

The quantity of vitamins that occur in wort varies considerably, but are present in adequate amounts not to limit yeast performance. Some are present in bound forms that may require enzyme hydrolysis for their release during mashing, *e.g.*, *myo*-inositol (MacWilliam 1968). The minerals present in the mashing liquor and in the malted barley can have a major effect on the final composition of the wort. They can act as buffering agents or influence the pH of the mash. They affect the stability and activity of enzymes, with resulting effects on the fermentable extract. Certain ions may act as enzyme inhibitors *e.g.*, copper or iron. The mineral content of the wort is also important in yeast metabolism and growth during the fermentation process (Mandl 1974).

Phytic acid contains approximately half of the malted barley's combined phosphate and occurs in the aleurone (Pyliotis *et al* 1979). During malting phosphatases, which are able to hydrolyze phytic

acid, increase in amount and inorganic phosphate is released from the aleurone. During this process 10-30 % of the phytic acid in the grain is hydrolyzed. Phytase activity is greatly influenced by temperature, especially during malt kilning (Lee 1990).

Phosphate residues are sequentially hydrolyzed from phytic acid, producing inorganic phosphate and eventually give myo-inositol, a B-vitamin, essential for yeast growth.

Phytic acid has a much higher affinity for calcium ions than inorganic phosphate and can therefore act as a chelating agent in the mash tun. The pH control of the wort includes chemical reactions between phosphates from the malt and calcium ions. During mashing calcium/phosphate and calcium/protein complexes form precipitates. This leads to an increase in the hydrogen ion concentration, with a resulting drop in pH.

1.5 The Fermentation Process.

In the production of Scotch Malt Whisky the fermentation is carried out by yeast, *Saccharomyces cerevisiae* on the malted barley wort, produced during the mashing process. Unlike the fermentation from breweries, the wort is neither boiled nor hopped. An advantage of the former is that the malt enzymes, surviving mashing, are viable in the fermentation. The associated malt microflora will also survive, to a certain extent, into the fermentation and this may result in competition for the malt wort substrates.

Malt wort is the source of carbon, nitrogen and other elements required for yeast growth. Wort is therefore the supplier of new cell material, the energy requirements for the metabolizing cells, the main products of primary metabolism, namely ethanol and carbon dioxide and other organic compounds that are produced in smaller quantities. These compounds, which include higher alcohols, fatty acids and esters, can be responsible for flavour and aroma compounds in the final distillate.

1.5.1 Factors Influencing Fermentation.

Yeast is innoculated into the washbacks during the first runnings from the mash tun, and brewery and/or distilling yeast strains can be used. A short lag phase allows sugar utilization to proceed very quickly and the fermentation normally commences before the washback is completely full. The growth phase of the yeast is normally completed after 12-16 hours (Watson 1983). Yeast is pitched at approximately 2 x 10^7 cells per ml in Scotch Malt Whisky production and after the growth phase there are approximately 2 x 10^8 cells per ml (Pyke 1965, Watson 1983). The major limiting factor of yeast growth, in the anaerobic Scotch Malt Whisky fermentation is the levels of free amino nitrogen that can be accumulated from the wort (Berry and Ramsey 1983).

During the growth phase of fermentation, heat is generated by the yeast cells. The setting temperature at the beginning of fermentation is extremely important in attaining the final temperature of the wash. Although the setting temperature varies with the ambient temperature, the washback is normally set between 20°C and 23°C. The temperature is found to remain stable over the initial stages of the fermentation, but rises to 32°C to 34°C before slightly decreasing towards the end of the fermentation (Watson 1981). The effects of pH can influence many biochemical reactions, especially on the activity of malted barley amylolytic enzymes present in the wort. As the wort pH falls, the activity of these enzymes may also decline.

The specific gravity of the wort in the washback at the beginning of fermentation varies through-out the industry, but would normally be in the range 1.050 to 1.057. As the fermentation proceeds, this value will decrease until at the end of alcohol production it has a specific gravity of approximately 0.998 to 1.000. At this stage many yeast cells die and autolyse, this autolysis is, however, dependent on the yeast strain used.

At the commencement of fermentation, initial levels of bacteria are low depending on the malted barley, yeast quality and plant hygiene. It is towards the end of the stationary phase of yeast growth when bacterial numbers, mainly Lactobacilli, increase (Dolan 1976). Although wash associated bacteria have little effect at their normal levels, if the wash is retained for too long a period before distillation, then a substantial bacterial contamination may take place (Dolan 1976). This may lead to a loss of yield and utilization of some fatty acids and esters that are important flavour and aroma compounds. The production of bacterial fermentation compounds may cause undesirable flavour and aroma compounds in the final distilled spirit (Dolan 1979).

1.5.2 Yeast Properties.

The yeast strains used in the production of Scotch Malt Whisky include both primary and secondary strains. The primary strains are used as the main fermenting or ethanol producing yeasts, while the secondary strains maintain the inoculation levels and may also add to the congener profile of the distilled spirit (Sharp and Watson 1979). The main secondary strains, which are the brewery yeasts, are now collectively known as *S. cerevisiae*, but include *S. uvarum*, *S. carlsbergensis* and *S. diastaticus* (Barnett *et al.* 1983).

The yeasts used with a Scotch Malt Whisky fermentation must possess several desirable characteristics. These include the ability to be fast fermenters and to attain high alcohol yields. These yeasts must therefore have an efficient substrate utilization capability, with a high alcohol tolerance. The necessity for a suitable organoleptic profile in the final distillate is also required, since different strains may produce different congeners or concentrations of these congeners.

1.5.3 The Biochemistry of Fermentation.

1.5.3.1 Carbohydrate Metabolism.

The carbohydrates of the wort are the major source of carbon compounds for the structural and functional synthesis of the yeast

cells during growth. In addition to the fermentable sugars of glucose, maltose and maltotriose, the malt wort contains larger malto-oligosaccharides that may be degraded by yeast (Erratt and Stewart 1978) or malted barley enzymes. During fermentation the fermentable sugars are removed by the yeast from the wort in a particular order. The first sugars to be metabolized are sucrose, glucose and fructose, followed by maltose and then maltotriose (Pyke 1965).

Sugars do not permeate freely through lipid membranes, and some form of transportation must be present before they cross the cell membrane (Cooper 1982). These permeases, which are responsible for transportation are normally quite specific and can be suppressed in the presence of other sugars (Masschelain 1967). An example of this would be the suppression of maltose transportation in the presence of glucose (Cheng and Michels 1991). Sucrose is hydrolyzed by a periplasmic associated invertase, the products of this hydrolysis, glucose and fructose, can then be transported into the cell (Lampen 1968). The transportation of maltotriose is inhibited by maltose (Stewart 1978). It is only after the bulk of the maltose in the wort is metabolized that the maltotriose permease becomes operational (Harris and Thompson 1960). Yeast α-glucosidase is responsible for the cytosolic hydrolysis of both maltose and maltotriose.

The major energy yielding process of yeast is carried out by the Embden-Meyerhoff-Parnas (EMP) pathway (Figure 5). Fermentable sugars from the wort are converted via pyruvate to the final products of ethanol and carbon dioxide. Briefly, hexose sugars are phosphorylated, cleaved to give triose phosphates and then to the three carbon compound, pyruvate, which may then be decarboxylated to give ethanol and carbon dioxide. During the conversion of glucose to pyruvate via the EMP pathway energy is absorbed during the phosphorylation of glucose to glucose-6-phosphate and fructose-6-phosphate to fructose-1,6-diphosphate, as the energy rich bond of adenosine triphosphate (ATP). On subsequent hydrolysis of the

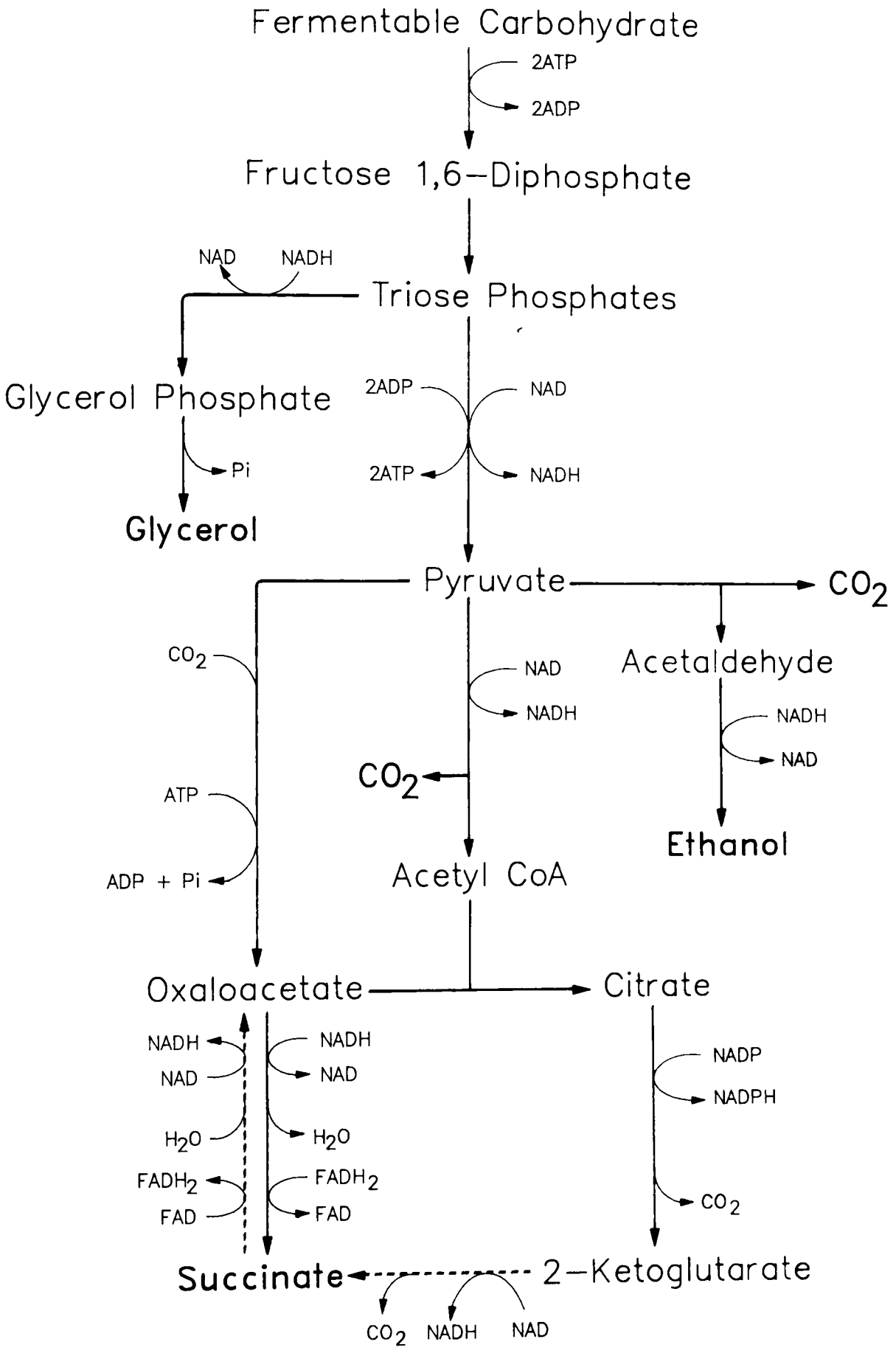


Figure 5. Summary of yeast carbohydrate metabolism under fermentation conditions.

phosphorylated trioses a total of four ATP molecules are formed. Thus a nett gain in energy of two ATP molecules for every glucose molecule oxidized to pyruvate is found.

The reduced nicotinamide adenine dinucleotide (NADH), formed on phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, is re-oxidized to NAD^+ by the reduction of acetaldehyde to ethanol. The ethanol, with the carbon dioxide formed are the major products of fermentation. In this way, the continued operation of the EMP pathway can proceed in the absence of molecular oxygen, with the continued formation of ATP for use in biosynthetic reactions.

During the initial stages of fermentation, yeast growth is significant. Since pyruvate is at this stage removed for biosynthesis a build up in NADH could be envisaged, resulting in a halt to catabolism. To compensate for this, the yeast cell reduces dihydroxyacetone phosphate to glycerol phosphate with the subsequent oxidation of NADH to NAD^+. The resulting glycerol from the glycerol phosphate is then excreted into the wash via passive diffusion (Oura 1977).

Besides glycerol, another minor by-product of ethanol fermentation is succinic acid. Together they may lower the yield of ethanol from the fermentable sugar by approximately 3% to 5% (Oura 1977). Under aerobic conditions succinic acid is formed by the oxidation of α-ketogluterate in the Kreb's Cycle, and can then be used in other biosynthetic reactions and cell growth. Under the anaerobic conditions of fermentation, the oxidative function of the Kreb's Cycle will be disrupted due to low levels of the enzyme α-ketogluterate dehydrogenase. If this is the case, the cell would have to synthesize additional enzymes leading to the formation of succinic acid via a reductive pathway (Sols *et al.* 1971). A second mechanism envisages that the levels of α-ketogluterate dehydrogenase are high enough to account for the concentration of succinic acid excreted by the yeast. The Kreb's Cycle would therefore function in its oxidative mode, but

at a much reduced rate (Oura 1977). The transport of succinic acid through the cell membrane is believed to be driven by a membrane potential (Duro and Serrano 1981).

The synthesis of pentose sugars are essential to the biosynthesis of nucleic acid precursors and many enzyme co-factors. Due to the inability of *Saccharomyces* to accumulate pentose sugars (Barnett 1976) from the wort it is essential that these sugars are synthesized. This is done using the Hexose Monophosphate (HMP) pathway.

1.5.3.2 Nitrogen Metabolism.

Yeasts require a source of nitrogen for the synthesis of amino acids, proteins, nucleic acids and other nitrogenous cell components. In wort the main source of nitrogen is the free amino acids, formed by the hydrolysis of the barley and malt proteins. Selective di- or tri-peptides may also be taken up by the yeast cells (Marder *et al.* 1977, Parker *et al.* 1980). Amino acids, like sugars, do not enter the cell by simple diffusion. There is a regulated uptake by several transport systems (Eddy 1982).

During the initial stages of a brewery fermentation, it has been observed that only eight amino acids (arginine, asparagine, aspartic acid, glutamic acid, glutamine, lysine, serine and threonine) are absorbed rapidly (Jones and Pierce 1969). The remaining amino acids are transported slowly or not at all until later in the fermentation (Rose and Keenan 1981). Only when the levels of the preferential amino acids have fallen, are the permeases capable of transporting the remaining amino acids. Protein synthesis requires all twenty of the essential amino acids, and so those not transported into the cell, have to be synthesized by the yeast.

The synthesis of amino acids within the yeast cell requires the production of the corresponding α-keto acid. When an amino acid enters the cell a transaminase system removes the amino group, thus allowing the remainder of the molecule to be metabolized. The main transamination system present employs α-ketoglutarate as the amino

acceptor (Ayrapaa and Palmqvist 1970). The glutamic acid formed may then be used as an amino donor for the synthesis of other amino acids from carbon skeletons, which have been synthesized through anaerobic metabolism. Transaminase reactions are readily reversible and with anabolic pathways are responsible for the generation of the α-keto acid pool.

Only the necessary amounts of α-keto acids are produced for synthesis of the required amino acids. The production of α-keto acids being controlled by feedback inhibition (Holzer 1968). During the initial stages of fermentation, the active synthesis of slowly transported amino acids has no serious side effects. However, as nitrogen deficiency develops later in fermentation, the feedback control breaks down. In effect, larger amounts of the various α-keto acids are produced in an attempt to ensure synthesis of the missing amino acids. In the absence of the necessary nitrogen, such synthesis is impossible. The accumulation of these α-keto acids cannot be tolerated under anaerobic conditions and are rapidly reduced to their corresponding alcohols (Ayrapaa 1965). The α-keto acid is firstly decarboxylated with the release of carbon dioxide to form an aldehyde. The reduction of the carboxyl group by an alcohol dehydrogenase gives the corresponding alcohol from the aldehyde, with the release of NAD^+. These reductions are carried out by specific decarboxylases, but the alcohol dehydrogenase enzyme may be sufficiently non-specific to reduce a number of aldehydes to their corresponding alcohols.

1.5.3.3 Lipid Metabolism.

Although fermentation is intrinsically an anaerobic process, the levels of dissolved oxygen in the wort during the initial stages of fermentation are important (Kirsop 1974). If molecular oxygen is not present then reduced yeast growth and viability may occur, since the cells cannot produce sufficient unsaturated lipids for membrane synthesis (David and Kirsop 1973). Yeast lipid can be divided into three categories, triglycerols, phospholipids and sterols. All are

necessary components of the cell membrane, since it is the cell membrane that controls the entry and excretion of compounds into or from the cell.

Yeast lipid is primarily composed of fatty acids of chain length of 16 or 18 carbon atoms. These fatty acids can be either saturated or unsaturated. Palmitic and stearic acids are the major saturated fatty acids and palmitoleic, oleic and linoleic the major unsaturated fatty acids (Henry 1982). Many major phospholipids have been identified in yeast (Pilkington and Rose 1991), and the major sterols of yeast are ergosterol and zymosterol (Rattery *et al.* 1975).

The fatty acids synthetase complex is responsible for the biosynthesis of saturated fatty acids from acetyl CoA, which is derived from the fermentable carbohydrate. Unsaturated fatty acids can then be synthesized from their corresponding saturated fatty acids by a $NADP^+$ dependent oxidase. This reaction, is however, dependent on molecular oxygen. The same types of reaction are responsible for sterol synthesis. Sterols are also unsaturated molecules but the key reaction is the production of mevalonic acid from acetyl CoA. The requirement for molecular oxygen for sterol synthesis is greater than that for unsaturated fatty acid synthesis in the yeast cell (David 1974).

1.6 Aims Of This Study.

The aims of this study were to determine assay procedures for a total of seventeen malted barley enzymes (α-amylase, β-amylase, α-glucosidase β-glucosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, N-acetyl-β-glucosaminidase, arabinosidase, β-xylosidase, acid phosphatase, phytase, a general proteinase assay, serine protease, leucine aminopeptidase and carboxypeptidase A), using specific, synthetic substrates. Using the developed or modified assays, their suitability within the heterogeneous mixture of a malted barley extract could then be determined.

Of particular interest was the development of specific assay procedures for the amylolytic enzymes and other carbohydrases.

Having ascertained the suitability of the assays, their application to production samples from the malting, mashing wort draining and fermentation processes within the Scotch Malt Whisky industry were studied.

2 MATERIALS AND METHODS.

2.1 Materials.

2.1.1 Chemicals and Biochemicals.

α-Amylase enzymes from *Bacillus licheniformis*, malted barley, porcine pancreas and human saliva were all obtained from the Sigma Chemical Co., Poole, Dorset with *B. subtilis* α-amylase purchased from BCL, Lewes, Sussex. Malted barley purified β-amylases were from BDH (Poole, Dorset), Cloder (Manchester), and Uniscience Ltd, (London). The sweet potato β-amylases were obtained from BCL and BDH. Yeast α-glucosidase was purchased from Uniscience Ltd. All the p-nitrophenyl carbohydrate substrates for the minor carbohydrase studies were obtained from Sigma, as was p-nitrophenyl maltose. All other p-nitrophenyl substrates for the α- and β-amylase studies were from BCL. Glucose and maltose were from Sigma while maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were purchased from BCL. Phadebas amylose test tablets (Batch Number LH78920) were from Pharmacia Wallac (UK), Milton Keynes. Substrates for proteinase and protease assays were purchased from Sigma, as was phytic acid and p-nitrophenyl phosphate, the substrates used for the phytase and acid phosphatase asseys respectively.

The Sigma Phosphate Kit (Kit number 670) was used for the determination of phosphate and the Sigma Protein Kit (Kit number P 5656) for the determination of protein in solution. The assay of succinic acid, lactic acid, acetic acid, glycerol and ethanol was carried out using their respective food analysis kits from BCL.

Bond Elut C_{18} sample preparation columns were manufactured by Analytichem International and supplied by Crawford Scientific, Strathaven.

All other chemicals and biochemicals were of analytical grade and purchased from either BDH or Sigma.

2.1.2 Malted Barley Samples.

Micro malted barley samples, from the varieties Golden Promise, Natasha and Triumph, were kindly supplied by Dr. G.N. Bathgate, (then of) Moray Firth Maltings, Inverness. The production malted barley samples were obtained from the Production Control Laboratory, Chivas Brothers Ltd., Keith, which had been obtained from the distilleries. Samples were from the following maltsters Baird's, DCCL, Moray Firth, Paul's, Portgordon and Simpson's.

2.1.3 Distillery Samples.

Samples of distillery mash, wort and wash were all obtained from Glen Keith Distillery and were obtained as stated in the sampling procedures (**Section 2.2.9**).

2.2 Methods.

2.2.1 Malted Barley Extraction Procedures.

Enzyme extraction of malted barley was carried out using a similar method to that in the Institute of Brewing, Recommended Methods of Analysis (IoB 1982) for the Alpha Amylase International Method. However, a series of extraction volumes were compared, using 5.0 g of the ground malted barley to determine if the stated method was the most efficient.

The malted barley was finely ground, setting 2 on a Buhler-Miag Universal Laboratory Disc Mill (type DLFU). Samples of the resulting flour (5.0 g) were accurately weighed into the extraction flasks. These were then extracted with 25 ml, 50 ml, 75 ml and 100 ml of 0.5% (w/v) sodium chloride. The flasks were covered with parafilm and allowed to stand in a 20°C water bath for 2.5 hr, with gentle swirling every 20 min. The suspensions were then filtered through Whatman No.1 filter paper, with the first 10 ml of the filtrate being returned to the funnel. Once filtration was complete, the bright, clear filtrate was stored at 4°C in a stoppered universal container for a maximum period of 3 hr. Extracts were carried out in triplicate and were assayed for protein concentration (**Section 2.2.10.6**).

The results given in Table 1 indicate that there is little difference between the 50, 75 and 100 ml extract volumes. The 50 ml extraction, *i.e.*, 1.0 g malted barley flour per 10 ml 0.5 (w/v) sodium chloride, was used in all further extractions of malted barley.

Table 1. Protein content (mg/ml) from malted barley extractions.

	Protein Content (mg/ml)	To the 100 ml Extract
25 ml	8.25	2.06
50 ml	4.69	2.35
75 ml	3.16	2.37
100 ml	2.33	2.33

2.2.2 Standard Assays.

2.2.2.1 Determination of α-Amylase Activity.

To determine α-amylase activity, in the presence of an enzyme extract from malted barley, the substrate used should be specific only for α-amylase activity, or the activity should be clearly quantified for α-amylase activity. A method for the former was chosen to be applied to a malted barley enzyme extract. The Phadebas α-amylase method was one which is routinely used for clinical chemistry applications (Ceska *et al.*, 1969) and was developed for use with cereal α-amylase (Barnes and Blakeney 1974). Subsequently, it was found to be a substitute method for α-amylase activity as detailed by the EBC Analytical (1975). Although similar, the method described here and that of the EBC were not identical.

The liberation of a water soluble blue dye from the water insoluble cross linked starch polymer under the standard conditions of the assay is a measurement of the enzyme activity of the α-amylase enzyme. The α-amylase in the malted barley extract, degraded the starch to release the dye into solution. The absorption at 620 nm of the blue solution was a function of the α-amylase activity. The

Phadebas test tablets also contain Bovine Serum Albumin to prevent any interference from compounds of the extract with the test procedure.

Before commencing the assay, by the addition of one Phadebas tablet per test, the tubes containing 5.0 ml of the assay buffer (0.1 M acetate buffer / 1 mM calcium chloride, pH 5.5) were incubated at 65°C for 5 min. An aliquot, 0.05 ml of suitably diluted malt enzyme extract solution, mashing liquor, wort or fermented worts or wash was added and the assay initiated. The solution was mixed, and the tablet dispersed by vortex mixing. The resulting solution was incubated for 5 min at exactly 65°C. Termination of the reaction was carried out by the addition of 1.0 ml 0.5 M sodium hydroxide, followed by 3.95 ml distiled water, mix by inversion. The terminated assay was then filtered through Whatman No.3 filter paper and the absorption of the filtrate determined at 620 nm. All tests were carried out in duplicate and were measured against an extract solution blank.

The enzyme activity can now be calculated, using the absorbance of the filtrate. The activity units (A) of the enzyme were obtained from the standard curve which accompanies the tablets. The levels of α-amylase enzyme activity (U) can be calculated as follows:

$$U = \frac{(A \times DF)}{Time\ of\ Incubation\ (\text{min})}$$

Where DF was the dilution factor.

Because of the nature of the Phadebas tablets a number of precautions must be strictly adhered to. Contamination of the tablets, test-tubes and glassware with perspiration, saliva or fingers should be prevented. Extracts should be assayed immediately or stored at 4°C and assayed the same day, preferably within 3 hr. The malt extract should be diluted prior to beginning the assay.

2.2.2.2 Determination of β-Amylase Activity.

Like the determination of α-amylase, the assay for β-amylase activity in a heterogeneous malted barley extract required a very

specific substrate, or the activity should be clearly distinguished as that of β-amylase activity. A number of researchers (Warchalewski and Tkachuk 1978, Mathewson and Seabourn 1983) have intimated the use of specific substrates for the quantification of β-amylase, but these have been found not to be totally specific for β-amylase. It was therefore necessary to use a similar type of substrate (p-nitrophenyl malto-oligosaccharides), but to ensure that it was specific for β-amylase, with no interference from other carbohydrases present in the malted barley extract.

The method developed in this study utilized the substrate p-nitrophenyl maltopentaose in combination with purified yeast α-glucosidase. After cleavage of the p-nitrophenyl maltopentaose, the α-glucosidase began to hydrolyze the remaining p-nitrophenyl saccharides to glucose and p-nitrophenol. The amount of p-nitrophenol liberated per time unit, under the standard assay conditions was a measure of the enzymatic activity of the β-amylase. Once the reaction mixture had been terminated by the addition of an alkaline solution, the increase in absorption at 410 nm was determined. The amount of p-nitrophenol liberated was stoichiometric with the amount of substrate hydrolyse, and can be measured from a standard curve.

The reaction mixture contained a suitably diluted aliquot (0.25 ml) of malted barley extract, mashing liquor, wort or fermented wort or wash (to give an increase in absorption at 410 nm of between 0.5 and 1.0 absorbance units), and 0.75 ml of the working assay buffer. The working assay buffer, which was prepared daily, consisted of a stock 0.1 M phosphate buffer with added 0.5 M ethylenediaminetetraacetic acid (EDTA), giving a final pH of 7.1. Yeast α-glucosidase (30 U/ml) was added to the stock buffer solution to give the working assay buffer. (The stock phosphate/EDTA buffer was prepared on a weekly basis.) The assay commenced with the addition of 0.25 ml of 5 mM p-nitrophenyl maltopentaose and was incubated at 45°C for exactly 10 min.

Each test was carried out in duplicate and measured against a substrate blank. The reaction was terminated by the addition of 1.0 ml of 0.4 M sodium carbonate and the liberated p-nitrophenol measured spectrophotometrically at 410 nm. The liberated p-nitrophenol was determined from a standard curve of OD 410 nm against μg p-nitrophenol.

One unit of enzyme activity (EU) was determined as the amount that hydrolysis 1 mmole of p-nitrophenyl maltopentaose per minute under the standard assay conditions. This was determined using the following equation:

$$EU = \frac{\mu g \ p\text{-}Nitrophenol \ released \ x \ 7.189 \ x \ DF}{Time \ of \ Incubation \ (\text{min}) \ x \ 1000}$$

Where DF was the dilution factor and 7.189 converts μg p-nitrophenol released into nmol p-nitrophenol released.

The standard curve illustrated in Figure 6 was an example of those carried out. Standard curves were prepared on a daily basis by dilution from a stock p-nitrophenol solution. Dilutions of the stock p-nitrophenol solution (20 μg/ml) were made up to 1.25 ml with distiled water and then 1.0 ml 0.4 M sodium carbonate added. The standard curves indicated a high level of correlation (0.9998) over the range of p-nitrophenol concentrations used.

2.2.2.3 Determination of Minor Carbohydrase Activities.

A number of the activities of minor carbohydrases from malted barley can be determined using p-nitrophenyl derivatives of their common substrates (Mitchell and Newman 1972). These enzymes, along with the substrates used are listed in Table 2. The minor carbohydrases can be grouped into enzymes which hydrolyze selected types of substrates. The glucosidases may hydrolyze α- and β-glucans, and molecules derived from these polymers. Arabinosidases and xylanases, which may be exo or endo acting, on arabinoxylans or products from the degradation of these polymers. The others include α- and

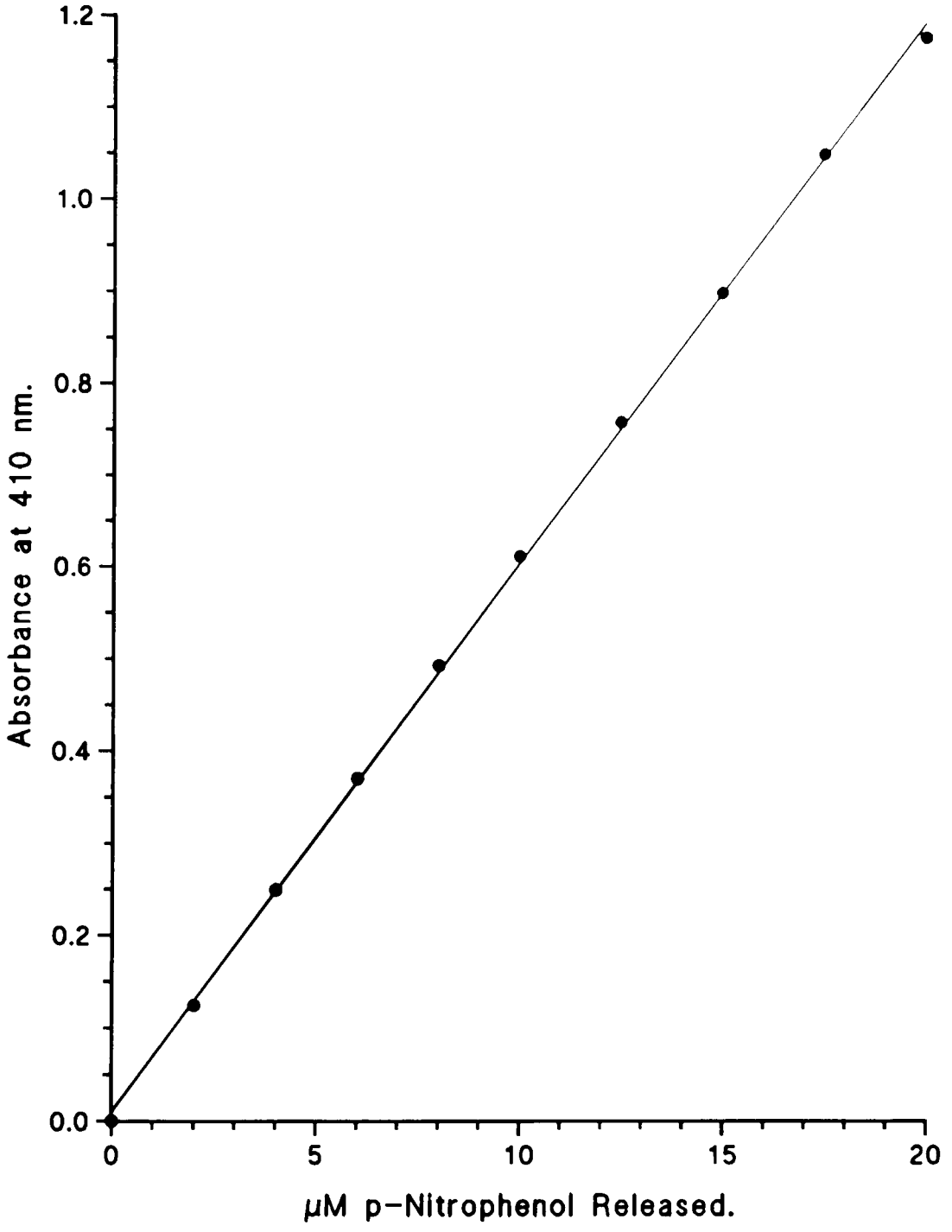


Figure 6. Standard curve for determining the amount of p-nitrophenol released due to enzyme activity on p-nitrophenyl substrates at an absorption of 410 nm.

β-galactosidases, α- and β-mannosidases and N-acetylglucosaminidase, all of which aid in the degradation of carbohydrate moieties in polysaccharides, glycoproteins and glycolipid.

Table 2. Standard assay conditions and substrate requirements for the various minor carbohydrases studied from malted barley. (NP - nitrophenyl, Glu - Glucose, Gal - Galactose, Man - Mannose, NAG - N-acetylglucosamine, Arab - Arabinose, Xyl - Xylose)

Enzyme	Substrate	Citrate Buffer	Incub Temp °C	Incub Time min
α-Glucosidase	p-NP α Glu	0.1 M, pH 6.1	50	20
β-Glucosidase	p-NP β Glu	0.1 M, pH 5.1	35	20
α-Galactosidase	p-NP α Gal	0.1 M, pH 5.1	55	20
β-Galactosidase	p-NP β Gal	0.1 M, pH 4.0	35	20
α-Mannosidase	p-NP α Man	0.1 M, pH 4.7	55	20
β-Mannosidase	p-NP β Man	0.1 M, pH 4.9	55	20
N-Acetylglucosaminidase	p-NP NAG	0.1 M, pH 4.9	55	20
Arabinosidase	p-NP Arab	0.1 M, pH 4.6	55	20
β-Xylosidase	p-NP β Xyl	0.1 M, pH 4.4	55	20

The principle of all the assays was very similar. The p-nitrophenol liberated per time unit under the standard conditions of the assay was an indication of the relative activity of the enzyme. If the reaction mixture was made alkaline after a definite time, the solution becomes yellow and the reaction terminated. The increase in absorbance at 410 nm, due to the p-nitrophenol liberated, was correlated with the amount of substrate hydrolyse by means of a calibration curve.

The use of the calibration curve for determining the liberated p-nitrophenol was due to the temperature variability of the extinction coefficient when using p-nitrophenol.

Other carbohydrase assays from other plant materials were suitable for adaptation, although the assays developed or modified in this study were done so specifically for the malted barley enzymes.

α-Glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, N-acetylglucosaminidase, arabinosidase and xylosidase activities were determined by measuring the amounts of p-nitrophenol released from their respective p-nitrophenyl glycosides (see Table 2). The reaction mixture contained a suitably diluted aliquot (0.25 ml) of the malted barley extract, mash liquor, worts or fermented wort or wash, and 0.75 ml of the optimal buffer. The assay was initiated by the addition of 0.25 ml of the substrate (5 mM) and incubated at the optimal temperature for 20 min. (See Table 2 for the optimal requirements for the individual enzymes). Each test was carried out in duplicate and measured against a substrate blank. Enzyme activity was terminated by the addition of 1.0 ml 0.4 M sodium carbonate, and the liberated p-nitrophenol measured at 410 nm. The liberated p-nitrophenol was determined from a standard curve of OD 410 nm against μg p-nitrophenol.

One unit of enzyme activity (EU) was defined as the amount that hydrolyses 1 nmol of respective p-nitrophenyl derivative per minute under the conditions of the standard assay for that enzyme. This was determined using the following formula:

$$EU = \frac{\mu g \ \underline{p}\text{-}Nitrophenol \ released \ x \ 7.189 \ x \ DF}{Time \ of \ Incubation \ (\text{min})}$$

Where DF was the dilution factor and 7.189 converts μg p-nitrophenol released into nmol p-nitrophenol released.

The standard curve illustrated in Figure 6 was an example of those carried out. Standard curves were prepared on a daily basis from dilution of a stock p-nitrophenol solution. Dilutions of the stock p-nitrophenol solution (20 μg/ml) were made up to 1.25 ml with distiled water and then 1.0 ml 0.4 M sodium carbonate added.

The standard curves indicated a high level of correlation (0.9998) over the range of p-nitrophenol concentrations used. It was decided to restrict the p-nitrophenol released in the assays to below 1.0 absorption units by using suitably dilute extract solutions, mashing liquor, wort or fermented wort or wash samples.

2.2.2.4 Determination of Acid Phosphatase Activity.

Acid phosphate isoenzymes are widely distributed throughout the plant kingdom. A number have been associated with the aleurone layer of cereals (Jacobsen *et al.* 1971, Yamagata *et al.* 1979, Akiyama *et al.* 1981). This enzyme may be assayed from a malted barley extract using the substrate p-nitrophenyl phosphate. The hydrolysis of the p-nitrophenyl phosphate by the acid phosphatases liberated p-nitrophenol, the increase of which can be followed spectrophotometrically at OD 410 nm.

The reaction mixture contains a suitably diluted aliquot (0.25 ml) of malted barley extract, mashing liquor, wort or fermented wort or wash, and 0.75 ml of 0.1 M citrate buffer, pH 4.9. The assay commenced with the addition of 0.25 ml 5 mM p-nitrophenyl phosphate and was incubated at 35°C for 20 min. Termination of the reaction was carried out by the addition of 1.0 ml 0.4 M sodium carbonate and the liberated p-nitrophenol measured at OD 410 nm. The liberated p-nitrophenol was determined from a standard curve and the calculation of acid phosphatase activity (EU) was carried out using the following formula:

$$EU = \frac{\mu g \ p\text{-}Nitrophenol \ released \ x \ 7.189 \ x \ DF}{Time \ of \ Incubation \ (\text{min})}$$

Where DF was the dilution factor and 7.189 converts μg p-nitrophenol released into nmol p-nitrophenol released.

Each test was performed in duplicate and both a substrate and extract solution blank was carried out. Both the substrate and extract blanks were subtracted from the test. A substrate blank was necessary

due to slight substrate degradation during the assay procedure. In all assays performed the substrate blank was less than 0.003 absorption units.

2.2.2.5 Determination of Phytase Activity.

The inorganic phosphate, liberated by the catalytic action of phytase on the phytic acid, was determined by the Fiske and SubbaRow (1925) method, according to the Sigma Diagnostic Kit procedure (**Section 2.2.10.7**)

Extraction of the malted barley flour was carried out as detailed in the Malted Barley Extraction (**Section 2.2.1**). Dilution of the malt extract, mashing liquor, wort or fermented wort or wash was not normally required for the assay of this enzyme. Care must be taken at every stage of the assay to minimize all pipetting errors, since the phytic acid substrate also occurs within the malt grains.

To 1.0 ml of 0.1 M acetate buffer, pH 4.8 add 0.5 ml of the extract solution. The assay commenced with the addition of 0.5 ml 2 mM phytic acid. The resulting solution was mixed and incubated at 50°C for exactly 20 min. Termination of the assay was achieved by the addition of 4.5 ml 10% (w/v) trichloroacetic acid. After mixing, the solution was allowed to stand for 10 min. An aliquot was then centrifuged for 10 min in an MSE microfuge at high speed (13400 x g). The assay for inorganic phosphate was carried out using the supernatent as detailed in **Section 2.2.10.7.**

The enzyme activity (EU) of malted barley phytase (nM phosphate released.min^{-1}) was calculated using the formula:

$$EU = \frac{\mu g \ Phosphate \ released \ x \ 325}{Time \ of \ Incubation \ (\text{min})}$$

A substrate blank was carried out for each test and must be subtracted from the test result to obtained the true phytase activity on the phytic acid substrate. All assays, both tests and blanks, were carried out in duplicate.

2.2.2.6 Determination of General Proteolytic Activity.

Proteinases are most easily detected by using proteins as their substrates. Many assays are known and some well documented (Ansun 1938, Moore 1969). This class of enzymes catalyse, the hydrolysis of peptide bonds within proteins and peptides, forming peptides or in some cases amino acids. Their mode of action can therefore be classed as endo. Enzyme specificity can vary over a wide range, from recognizing a specific protein and hydrolyzing only one or a few peptide bonds in it, to very non-specific, hydrolyzing many peptide bonds in most proteins.

The use of proteins for the detection of proteinases has the advantage that many different types of amino acids are found within polypeptides. Hence, most proteinases, including the relatively specific ones can be measured when overall proteolytic activity is required. The more common methods for measuring the general proteolytic activity, fall into two main categories. The first measures the release of amino groups (Reimerdes and Klostmeyer 1976). This method initially seemed attractive since every proteolytic cleavage should give a response. An assay for this method was developed using a buffered gelatine solution, with subsequent detection of the release of free amino nitrogen. This method, and those similar to it, have very high blank values, making the assays less sensitive, and also less reproducible.

The second category allows the measurement of the sum of the product peptides. The dye-stained protein used in the method adopted was azo-albumin which is a soluble, trichloroacetic acid precipitable protein (Lambert *et al.* 1978).

Extracts of the malted barley flour were carried out as detailed for Malted Barley Extraction (**Section 2.2.1**). Dilution of the malted barley extract, mashing liquor, wort or fermented wort or wash was not normally required for the assay of this enzyme. Each test sample was carried out in duplicate and measured against a substrate blank.

The substrate solution was prepared by obtaining a 1 % (w/v) azo-albumin solution in 0.1 M phosphate buffer, pH 6.5. Since the azo-albumin was soluble in aqueous buffers, any unreacted protein/dye complex must be precipitated out of solution. The assay was initiated by the addition of 0.3 ml of the extract solution to 0.75 ml of the buffered azo-albumin solution. Incubation took place at 55°C for exactly 20 min. At the end of incubation, the assay was terminated and the protein/dye complex precipitated using 0.75 ml of 5 % (w/v) trichloroacetic acid. The precipitate was removed by centrifugation in an MSE microfuge at high speed (13400 x g) for 5 min. To 0.75 ml of the supernatent, add 0.75 ml 0.5 M sodium hydroxide and the absorption at 440 nm was determined against a substrate blank.

The enzyme activity (EU) of the extract was defined as the amount of enzyme which will catalyse the release of azo dye causing an increase in absorption (ΔA) per minute equal to 0.001 absorption units. The enzyme activity was given by the following formula:

$$EU = \frac{1000 \; x \; \Delta A}{Time \; of \; incubation \; (\text{min})}$$

2.2.2.7 Determination of Serine Protease Type Activity.

Several peptidases have been found in malt which are inactivated by the reduction of sulphydryl groups, others are slightly activated by reducing agents. Jones and Pierce (1967b) examined the metabolism of amino acids during barley germination and found that peptidase activity in the endosperm provided the major part of the amino acid activity. Mikola and Kolehmainen (1972) examined the distribution of peptidases in germinated barley. Malted barley also contains peptidases which exhibit trypsin-like activity (Jones and Pierce 1967a). These enzymes are only slightly affected by activators and inhibitors.

Trypsin-like serine protease activity can be measured by the degradation of denatured standard preparations of haemoglobin and/or casein, or by the hydrolysis of synthetic amino acids or substrates.

These synthetic substrates are not specific for trypsin, as they can also be hydrolyzed, more or less rapidly, by many other trypsin-like serine proteases (Muller-Estert and Fritz 1980).

The substrate used in this assay was N-benzoyl-L-arginine-4-nitroanilide. The 4-nitroaniline released was determined directly by measurement of the absorbance at 405 nm. Thus, the enzyme activity was directly proportional to the quantity of 4-nitroaniline liberated per time unit.

Extracts of the malted barley flour were carried out as detailed for Malted Barley Extraction (**Section 2.2.1**). Dilution of the malted barley extract, mashing liquor, wort or fermented wort or wash was not normally required for the assay of this enzyme. Each test sample was carried out in duplicate and measured against a substrate blank. The method used is that of Samorin *et al.* (1979), and the N-benzoyl-L-arginine-4-nitroanilide should be of a highly pure grade.

To 0.75 ml 0.2 M triethanolamine / 20 mM calcium chloride buffer, pH 7.8, add 0.05 ml extract solution. The assay was initiated by the addition of 0.20 ml 4 mM N-benzoyl-L-arginine-4-nitroanilide hydrochloride. Mix the solution by inversion and incubate at 25°C for exactly 10 min. At the end of the incubation period immediately read the absorbance at 405 nm against the substrate blank.

The calculation of the enzyme activity was given as follows:

$$\Delta A = OD_{405nm}test - OD_{405nm}blank$$

$$EU/l = \frac{1961 \times \Delta A}{Time\ of\ Incubation}$$

2.2.2.8 Determination of Leucine Aminopeptidase Type Activity.

Leucine aminopeptidase is an exopeptidase which liberates amino acids, especially leucine, from the N-terminal end of proteins and polypeptides. Many aliphatic amides and thio-esters can also be hydrolyzed. The enzyme is so called due to its rapid activity on leucine residues. Leucine aminopeptidase is a zinc metallo-enzyme which is widespread in the animal and plant kingdoms.

The substrate used in this assay is L-leucine-4-nitroanilide. This substrate was not solely specific for leucine aminopeptidase since amino acid acylaminase may also liberate the 4-nitroaniline. However, the activity of the microsomally associated amino acid acylaminase was much lower when using this substrate. This is because the preferred substrate of this enzyme would be L-alanine-4-nitroanilide. The 4-nitroaniline released was determined directly in the assay cuvette by measurement of the increase in absorbance at 405 nm. The enzyme activity was therefore directly proportional to the quantity of 4-nitroanaline liberated per time unit.

Extracts of the malted barley flour were carried out as detailed for Malted Barley Extraction (**Section 2.2.1**). Dilution of the malted barley extract, mashing liquor, wort or fermented wort or wash was not normally required for the assay of the above enzyme. Each test sample was carried out in duplicate and measured against a substrate blank. The method used was adapted from Hafkenscheid (1984).

The assay was initiated by the addition of 0.05 ml 65 mM L-leucine-4-nitroanilide hydrochloride to 1.50 ml 0.2 M Tris.HCl buffer, pH 7.8 and 0.05 ml of the extract solution. The assay solution was mixed by inversion and incubated for exactly 10 min at 25°C. At the end of the incubation period the absorbance of the test sample was immediately determined at 405 nm against the substrate blank.

The calculation of the enzyme activity (EU) was determined as follows:

$$\Delta A = OD_{405nm}\,test - OD_{405nm}\,blank$$

$$EU/l = \frac{3333 \times \Delta A}{Time\ of\ Incubation\ (\text{min})}$$

2.2.2.9 Determination of Carboxypeptidase A Type Activity.

The carboxypeptidases hydrolyze the C-terminal amide bond of peptides. Dipeptides are poor substrates since the free amino group in the penultimate position prevents rapid hydrolysis. The best substrates are suitably N-blocked dipeptides or longer homologues.

Carboxypeptidases are normally zinc metalloenzymes, however the enzymes from various plants and yeast are known to be non-zinc requiring (Zuber 1964).

N-(3-[2-furyl]acryloyl)-L-phenylalanine-L-phenylalanine (FAPP) was used as the substrate for this assay. The substrate was specific for carboxypeptidase A (Peterson *et al.* 1982). Carboxypeptidase A preferentially cleaves C-terminal amino acids which have aromatic or branched side chains from the peptide chain. When the phenylalanine-phenylalanine bond is hydrolyzed a decrease in absorption occurs. This is probably due to a spectral shift to a lower wavelength of the furyloacryloyl absorption band. It was this change which was used to monitor the hydrolysis spectrophotometrically.

Extracts of the malted barley flour were carried out as detailed for Malted Barley Extraction (**Section 2.2.1**). Dilution of the malted barley extract, mashing liquor, wort or fermented wort or wash was not normally required for the assay of this enzyme. Each test sample was carried out in duplicate and measured against an air blank.

The substrate solution was prepared by dissolving the FAPP to give a concentration of 0.25 mM in a 50 mM Tris.HCL / 70 mM sodium chloride buffer, pH 7.5. To 0.8 ml of the buffered substrate solution add 0.2 ml of the extract solution, mix by inversion and immediately read the absorbance at 330 nm. The test solution was then incubated at 35°C for exactly 30 min and the absorbance was again determined at the end of the incubation period.

The enzyme activity (EU/l) was calculated from the following formulae:

$$\Delta A = OD_{330nm}\,before - OD_{330nm}\,after$$

$$EU/l = \frac{2500 \times \Delta A}{Time\ of\ Incubation\ (\text{min})}$$

2.2.3 Method Development of Amylase Assays.

2.2.3.1 α-Amylase.

In order to determine the specificity of the Phadebas assay for malted barley α-amylase it was necessary to study the assay using different buffer conditions. The stock buffer consisted of 0.1 M acetate buffer, pH 5.5. The standard assay buffer system, contained the stock buffer with 1 mM calcium chloride added. To establish the chelating effects of EDTA on the assay, the stock buffer was prepared and made to 10 mM with respect to EDTA.

In order to test the specificity of the crude α-amylase preparation on the p-nitrophenyl-oligosaccharide substrates it was necessary to use the standard β-amylase assay. To remove β-amylase activity from the crude α-amylase preparation the solution was heat treated (**Section 2.2.7**). The heat treated crude α-amylase preparation (10 mg/ml) was then used in the assay, with the substrates p-nitrophenyl maltotetraose, p-nitrophenyl maltopentaose, p-nitrophenyl maltohexaose and p-nitrophenyl maltoheptaose.

2.2.3.2 β-Amylase.

Since the β-amylase assay was a coupled reaction with yeast α-glucosidase, it was necessary to study the affects of the buffer systems and substrates on the yeast α-glucosidase.

Substrate utilization of the yeast α-glucosidase was carried out in the working assay buffer for the standard β-amylase assay without the addition of the yeast α-glucosidase. Yeast α-glucosidase was prepared in a 1 mg/ml solution with distilled water and used as the enzyme source in the assay. The assay commenced with the addition of the substrate solution, p-nitrophenyl maltose, p-nitrophenyl maltotriose, p-nitrophenyl maltotetraose, p-nitrophenyl maltopentaose, p-nitrophenyl maltohexaose or p-nitrophenyl maltoheptaose. All other conditions were similar for that of the standard β-amylase assay.

Activation and inhibition studies of the yeast α-glucosidase were carried out using the buffer compositions detailed in Table 3. A 1 mg/ml solution of the enzyme was used and the substrate employed was p-nitrophenyl maltose. The assay conditions were those of the standard β-amylase assay.

Table 3. Key to buffer compositions used in activation and inhibition studies of yeast α-glucosidase and malted barley amylases. For β-amylase studies addition of yeast α-glucosidase to the buffers was necessary. (EDTA - Ethylenediaminetetraacetic acid).

Buffer Code	Buffer Composition.
1	0.1 M Phosphate, pH 7.1
2	0.1 M Phosphate, pH 7.1 + 1 mM Ca^{2+}
3	0.1 M Phosphate, pH 7.1 + 10 mM EDTA
4	0.1 M Phosphate, pH 7.1 + 10 mM Ascorbate
5	0.1 M Phosphate, pH 7.1 + 10 mM Ascorbate + 1 mM Ca^{2+}
6	0.1 M Phosphate, pH 7.1 + 10 mM Ascorbate + 10 mM EDTA
7	0.1 M Phosphate, pH 7.1 + 1 mM Cu^{2+}
8	0.1 M Phosphate, pH 7.1 + 1 mM Cu^{2+} + 1 mM Ca^{2+}
9	0.1 M Phosphate, pH 7.1 + 1 mM Hg^{2+}
10	0.1 M Phosphate, pH 7.1 + 1 mM Hg^{2+} + 1 mM Ca^{2+}

The assay for purified β-amylase, (Cloder, 1 mg/ml), was carried out under the conditions of the standard malted barley β-amylase assay. The assay commenced with the addition of the substrate, either p-nitrophenyl maltotetraose, p-nitrophenyl maltopentaose, p-nitrophenyl maltohexaose or p-nitrophenyl maltoheptaose. In order to determine the β-amylase activity on the Phadebas tablets, the conditions of the α-amylase standard assay was used. However, in this case the β-amylase working buffer without α-glucosidase was used in place of the α-amylase buffer. All other conditions were as the α-amylase assay.

The activation and inhibition studies of the purified malted barley β-amylase enzyme (Cloder, 1 mg/ml) were carried out using the buffer compositions detailed in Table 3. All buffers contained yeast α-glucosidase (30 U/ml). All other conditions were as the standard β-amylase assay. The crude α-amylase preparation, was also used in a similar experiment, the enzyme preparation was prepared as standard, *ie* 10 mg/ml.

Table 4. Buffer composition key for additions to the standard buffer for α-amylase during source comparison studies. (EDTA - Ethylenediaminetetraacetic acid).

Buffer Code	Buffer Composition.
1	0.1 M Acetate, pH 5.5
2	0.1 M Acetate, pH 5.5 + 1 mM Ca^{2+}
3	0.1 M Acetate, pH 5.5 + 10 mM EDTA
4	0.1 M Acetate, pH 5.5 + 10 mM Ascorbate + 1 mM Ca^{2+}
5	0.1 M Acetate, pH 5.5 + 10 mM Ascorbate + 10 mM EDTA
6	0.1 M Acetate, pH 5.5 + 1 mM Cu^{2+}

2.2.4 Comparisons of α-Amylases From Different Sources.

The standard assay for malted barley α-amylase was employed in the comparison of the α-amylases from different sources. All enzymes were prepared at a concentration of 1 mg/ml except for the crude malted barley enzyme which was at 10 mg/ml. The buffer solutions used in this comparison are detailed in Table 4. The comparison of the p-nitrophenyl maltopentaose and p-nitrophenyl maltoheptaose substrates was determined using the standard p-nitrophenyl substrate assay using a 0.1 M acetate / 1 mM calcium chloride buffer, pH 5.5 with 30 U/ml yeast α-glucosidase in the buffer solution. These assays were incubated for 20 min at 65°C.

2.2.5 Comparisons of β-Amylases From Different Sources.

The standard assay for the malted barley β-amylase enzyme was employed in the comparison of β-amylases from different sources. All enzymes were prepared at a concentration of 1 mg/ml. The buffer solutions used in this study are detailed in Table 5. Substrate comparison for p-nitrophenyl maltoheptaose was carried out using the 0.1 M phosphate / 10 mM EDTA buffer, pH 7.1 using the standard β-amylase assay. The α-amylase assay using 0.1 M phosphate / 1 mM Ca^{2+} buffer, pH 7.1 was used for the Phadebas substrate comparison. In this case the assays were incubated for 10 min at 45°C.

Table 5. Buffer composition key for additions to the standard buffer for β-amylase during source comparison studies. (EDTA - Ethylene-diaminetetraacetic acid).

Buffer Code	Buffer Composition.
1	0.1 M Phosphate, pH 7.1
2	0.1 M Phosphate, pH 7.1 + 1 mM Ca^{2+}
3	0.1 M Phosphate, pH 7.1 + 10 mM EDTA
4	0.1 M Phosphate, pH 7.1 + 10 mM Ascorbate
5	0.1 M Phosphate, pH 7.1 + 10 mM Ascorbate + 10 mM EDTA
6	0.1 M Phosphate, pH 7.1 + 1 mM Cu^{2+}

2.2.6 Method Development of Enzyme Assays.

Method development of the enzyme assays was performed for the following enzymes α-amylase, β-amylase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, N-acetylglucosaminidase, arabinosidase and xylosidase. All assays relate to the enzyme present in the malted barley extract, from the variety Triumph.

2.2.6.1 Kinetic Studies.

The k_m values were determined from double reciprocal plots, using data from at least seven or nine concentrations of the p-nitrophenyl substrate. Malted barley extracts were incubated under standard conditions for 20 min. Reactions rates were linear for this incubation time.

2.2.6.2 Effect of pH.

The pH dependency of the specific enzymes was determined by incubation of the malted barley extract with 5 mM p-nitrophenyl substrate for 20 min. The buffers used were either 0.1 M citrate between the range pH 4.0-6.0 or 0.1 M phosphate between the range pH 6.2-7.8.

2.2.6.3 Effect of Temperature.

The temperature dependency of the specific enzyme from the malted barley extract was determined over the range 4°C to 100°C. The extracts were incubated under the standard assay conditions for the particular enzyme for 20 min. The pH optimum being determined as above.

2.2.6.4 Inhibition Effects.

All assays were performed under the standard conditions for the enzyme, with 5 mM substrate. Inhibitors (Cu^{2+} and Hg^{2+}) were incorporated at a concentration of 1 mM into the assay buffer. The extract and buffer were pre-incubated for 5 min before substrate addition.

2.2.6.5 Effect of Incubation Time.

The effect of the length of incubation time was determined over the period 0 to 60 min after the assay was initiated. The malted barley extracts were incubated under the standard conditions of pH and temperature as determined above.

2.2.6.6 Effects of Extract Volume.

The effect of extract volume on the rate of the enzymic reaction was determined by varying both the extract volume and the volume

of buffer used in the assay. Total assay volume remained at 1.25 ml. The extracts were incubated under standard conditions of pH and temperature for 20 min.

2.2.7 Heat Treatment of Crude α-Amylase Preparation.

Previous studies on α-amylase by Bathgate and Palmer (1973) indicated that a heat treatment step of a crude α-amylase extract was sufficient to destroy interfering enzyme activity. Although the method also reduced α-amylase activity it was used to indicate buffer and substrate specificity of the crude Sigma α-amylase preparation.

In order to determine the effectiveness of the heat treatment, on the crude α-amylase preparation, the effects of the crude and heat treated preparations on maltotetraose, maltopentaose, maltohexaose and maltoheptulaose were studied.

To 0.75 ml of 0.1 M acetate / 1 mM calcium chloride buffer, pH 5.5, add 0.25 ml of the substrate solution (approximately 1.0 mg/ml). The assay was initiated by the addition of 0.25 ml of a 10 mg/ml solution of the crude α-amylase preparation, or a heat treated preparation of the crude α-amylase solution. All assays were incubated at 65°C for 30 min, and terminated in a boiling water bath for 5 min.

The analysis of the hydrolysis products was carried out using high pressure liquid chromatography (HPLC). Sample preparation and the HPLC analysis was carried out as in **Section 2.2.8.** All assays were performed in triplicate and the results reported were an average of these.

2.2.8 HPLC Methods.

The isocratic high pressure liquid chromatography (HPLC) system was composed as follows. A Gilson 302 HPLC pump with its associated manometric module, this module is necessary to ensure pulse-free eluent flow rates. Detection of the carbohydrate peaks eluting from the HPLC column was carried out using a Gilson 131 refractive index detector, which was linked to a chart recorder and data acquisition system. Samples were injected automatically using a Gilson 231/401

auto-injector supplied with a Rheodyne 7010, electrically activated, HPLC valve. The auto-injector programme was written to ensure sufficient rinsing and washing of the injection valve, mixing and loading of the sample and to ensure that injections were correctly timed with activation of the data acquisition system (see **Appendix I**).

The HPLC column used for these separations was an Aminex HPX-42A (30 cm x 0.5 cm) from BioRad Laboratories Ltd.. A guard column (Carbo-C from BioRad Laboratories Ltd.) was used to increase the working life of the analytical column. The analytical column was maintained at a temperature of 70°C using a Jones Chromatography column heater (Model Number 7930). The eleunt used was degassed distiled water at a flow rate of 0.6 ml/min. Total separation time was 30 min.

Sample preparation consisted of removing the pariculate material from the sample by centrifugation, and then passing a 0.5 ml aliquot of the supernatent through a conditioned C_{18} Bond Elut cartridge (Analyitichem International, supplied by Crawford Scientific, Strathaven). The cartridge was conditioned by passing one column volume of methanol through the cartridge followed by two column volumes of distilled water. Once the supernatent has passed through the column, it was then washed with 0.5 ml of distilled water, which was collected and pooled with the treated supernatent. The sample was now ready for injection onto the HPLC system or stored frozen until analysis takes place. During sample preparation it was imperative that the Bond Elut cartridge remains "wet" at all times.

Standards were prepared by using weighed amounts of glucose, maltose, maltotriose, maltotetraose, maltopentaose maltohexaose and maltoheptaose, dissolved in a 5 mg/ml soluble starch solution to achieve a 5 mg/ml standard solution for each carbohydrate. Appropriate dilutions can then be carried out for the calibration of the

data acquisition software. From this the quantification of the starch, glucose, maltose, maltotriose, maltotetraose, maltopentaose maltohexaose and maltoheptaose fraction in the unknown can be determined.

2.2.9 Sampling Procedures Employed During The Distillery Process.

2.2.9.1 Mashing.

Sampling during mashing was found to be quite difficult due to the heterogenous composition of the mash. However, it was decided to extract a 500 ml aliquot from the mash tun at specific times through-out the mashing programme. These samples were immediately centrifuges in a MSE Mistral 2000 at 3200 x g for 5 min, refrigerated at 4°C. The bright supernatent was then distributed into 20 ml aliquots and immediately frozen. These aliquots were used, at a later date, for the analysis of enzyme activity and other compounds of interest.

Mashing was carried out using the normal mashing procedures at the distillery. The initial process, was that of mashing-in, where the grist and mashing liquor are mixed in the mashing machine. The first sample drawn, was taken from this mixture as it came from the spout of the mashing machine. Mashing-in took approximately 45 min. The second sample at 22 min, was drawn from the mash tun, directly across the circumference of the mash tun from the mashing machine. Once mashing-in was complete, the whole mash was mixed by raking. After raking, sampling was carried out at 20 min intervals from the mash tun bed, directly opposite the mashing machine.

As wort draining from the mash tun progressed, the amount of wort in the mash tun decreased. Hence, the amount of extracted grist in the samples increased.

Further samples were drawn during the second water and the sparging waters. These were extracted from the mash tun at the same point as those from the first water. Again they were collected at intervals after raking at the beginning of the water.

2.2.9.2 Wort Draining.

Mashing was carried out using the normal mashing procedures at the distillery. Malt of the same variety, from the same maltings was used for the wort draining study, similar to that used during the mashing study. The wort, drained from the mash tun, was cooled by passing it through the wort heat exchanger and then pumped to the washback via the wort line. The washback was filled from the wort line using a down pipe. Samples were drawn from the down pipe. This was achieved by incorporating a 12 mm valve on the underside of the horizontal portion of the down pipe (see Figure 7).

Samples of approximately 1000 ml were drawn and the gravity and temperature of the wort were immediately determined. A 500 ml aliquot of the sample was then removed for immediate centrifugation in a MSE Mistral 2000 at 3200 x g for 5 min, refrigerated at 4°C. The residual wort was returned to the washback. The bright supernatent was then distributed into 20 ml aliquots and immediately frozen. These aliquots were used for the analysis of enzyme activity and other compounds of interest at a later date. Samples were drawn at 10 min intervals throughout the entire period of wort draining, and also from the washback at the end of first and second water draining.

2.2.9.3 Fermentation.

Mashing and wort draining was carried out as previously described, with the malt variety and maltster being consistent for all three studies. Since a comprehensive study was to take place over the period of fermentation, it was decided to monitor four successive washbacks. Each, had been declared at eight hour intervals. All procedures and raw materials were similar for each washback.

Sampling consisted of simply filling the sampling jar, to a similar depth within the bulk of the wash, and then removing the sampling jar. The temperature and gravity were immediately recorded, and a 500 ml sample was removed for centrifugation in a MSE Mistral 2000 at 3200 x g for 5 min, refrigerated at 4°C. Any remaining wash was

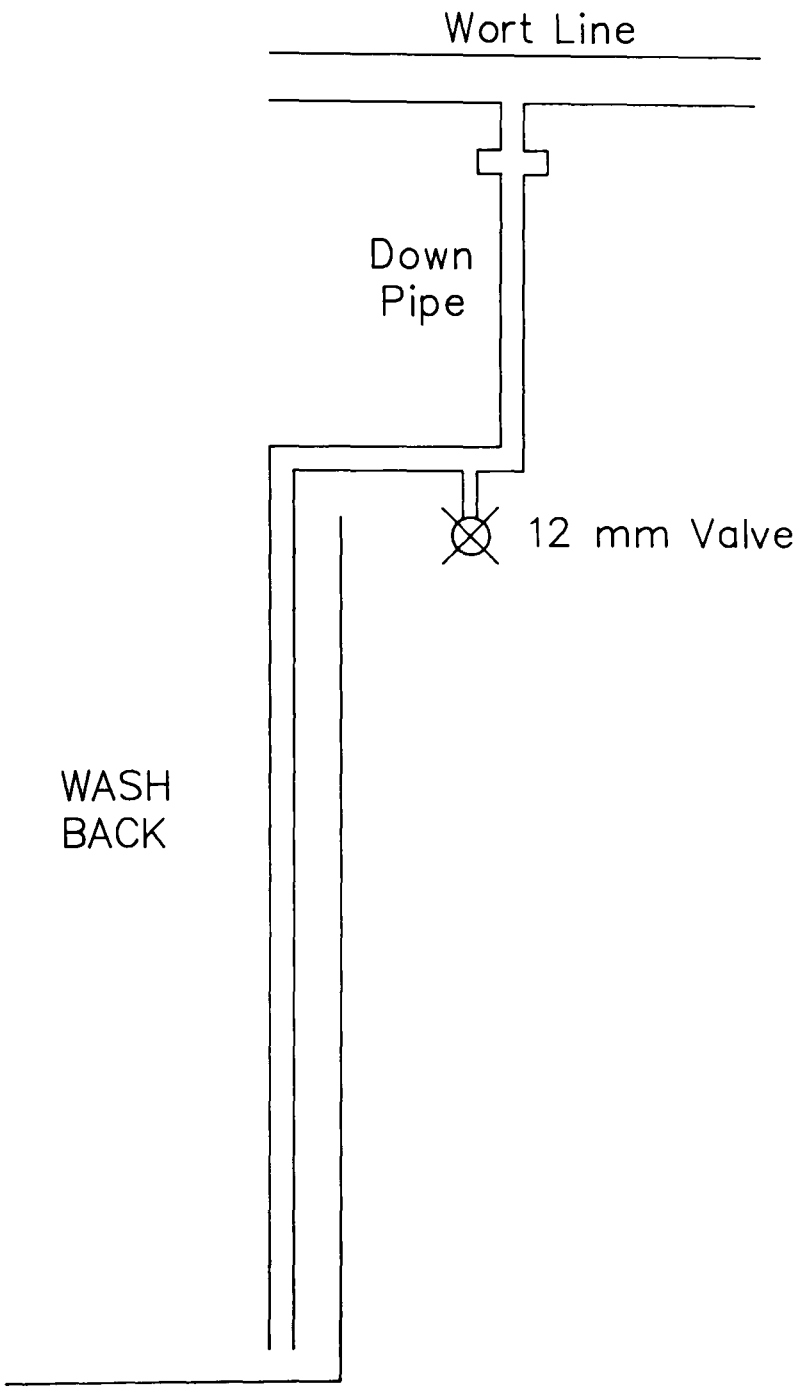


Figure 7. Diagram of the sampling point used for drawing wort draining samples.

returned to the washback. The bright supernatent was then distributed in 20 ml aliquots and stored frozen until further analysis took place. Sampling commenced on an hourly basis from 0 to 12 hr, every 2 hr from 14 to 26 hr and every 4 hr from 30 to 46 hr.

2.2.10 Non-enzymatic Analysis Carried Out On Distillery Samples.

2.2.10.1 Temperature and pH.

The temperature and pH of all samples were recorded as soon as possible after the sample was drawn, except for the mashing study. In this case the temperature was determined in the mash tun, at the point were the sample was to be drawn, using a mash tun spear thermometer. Temperatures of the wort from the wort draining study and the fermenting wort or wash from the fermentation study were carried out immediately after the sample was drawn in the tun room of the distillery.

The pH was determined on all samples prior to centrifugation using a Corning 240 pH meter, previously calibrated to pH 4.00 and pH 7.00.

Both the temperature and pH readings were carried out on the uncentrifuged sample because enzyme studies were to be performed using these conditions in addition to the optimum enzyme conditions.

2.2.10.2 Specific Gravity.

The specific gravity of the wort and fermenting wort or wash was determined using the distillery saccharometers. The saccharometer and thermometer were immersed in the first sample and allowed to attemperate. Another sample was drawn, and again the saccharometer and thermometer immersed, the temperature was recorded and the thermometer withdrawn. The saccharometer was allowed to float free from the sides of the testing jar and the specific gravity was read at the miniscus. Any temperature corrections can now be applied to ensure that the correct specific gravity is recorded.

2.2.10.3 Free α-Amino Nitrogen.

The method employed was the ninhydrin method of Lie (1973) as detailed in the Recommended Procedures of the Institute of Brewing (IoB 1982). Ninhydrin is used as an oxidizing reagent, causing the oxidative decarboxylation of α-amino acids to yield carbon dioxide, ammonia and an aldehyde with one less carbon atom than that of the original amino acid. The reduced ninhydrin will then react with the unreduced ninhydrin and liberated ammonia to form a coloured complex. Fructose is incorporated into the Colour Reagent to act as a reducing substance. The addition of potassium iodate in the Dilution Reagent ensures that unreduced ninhydrin remains oxidized and prevents further colour reaction.

The diluted samples were heated with the buffered Colour Reagent and the colour complex produced is measured spectrophotometrically at 570 nm. Samples were diluted to a concentration of 1-3 mg free α-amino nitrogen per litre and a 2.0 ml aliquot pipetted into a test-tube which was immediately stoppered. To this 1.0 ml of the Colour Reagent (25.0 g di-sodium hydrogen phosphate, dodecahydrate, 15.0 g potassium di-hydrogen phosphate, 1.25 g ninhydrin and 0.75 g fructose all dissolved in 250 ml distiled water) was added. On mixing, the tubes were placed in a boiling water bath for exactly 16 min and were then cooled in a water bath at 20°C for 20 min. The reaction was terminated by the addition of 5.0 ml of Dilution Reagent (2.0 g potassium iodate dissolved in 600 ml distiled water, with 400 ml 96.6% (v/v) ethanol). The solution was then thoroughly mixed, and the absorption recorded at 570 nm against a reagent blank.

With each set of analysis, four replicate standards were included. The stock standard solution contained 107.2 mg glycine, dissolved in 100 ml distiled water. This solution was stable for long periods if stored refrigerated at 4°C. The working standard is prepared daily by diluting the stock solution 1 in 100 with distilled water to give a standard solution of 2 mg free α-amino nitrogen per litre.

Results are given to one decimal place and are expressed as follows:

$$mg/litre\ FAN = \frac{OD\ of\ Test}{Mean\ OD\ of\ Std} \times 2 \times Dilution$$

2.2.10.4 Yeast Viability.

The methylene blue staining method of Pierce (1970) was used. In this method, viable cells are able to reduce the methylene blue to a colourless compound. The non-viable cells do not have the active enzyme required to reduce the methylene blue, and so retain the blue colour. The percentage of unstained cells is a measure of the viability.

Methylene blue, 0.01 g, was dissolved in 10 ml distilled water and then 2.0 g of sodium citrate, dihydrate dissolved in the solution by stirring. The resulting solution was then filtered and the filtrate was made up to 100 ml with distilled water.

An equal volume of the dye solution was mixed with the fermenting wort or wash and examined microscopically. The fermenting wort or wash may require diluting to enable approximately 60 cells to be viewed in a single microscope field. The viability was reported as the number of unstained as a percentage of the whole. Yeast cell buds are only counted individually if they are greater than half the size of the parent cell.

2.2.10.5 Wash Solids.

This was used as a very basic guide to yeast growth during fermentation. In addition to the yeast, wash solids and bacteria will also be present, the former throughout the fermentation, and the latter towards the end of fermentation. Samples of well mixed, uncentrifuged wort or wash (4 x 75 ml) were centrifuged for 10 min in a Baird and Tatlock Auto Bench Centrifuge (Mark IV) at 2775 x g, in tared 100 ml centrifuge tubes. After centrifugation, the supernatent was decanted off and the surplus supernatent drained off, by inverting the tubes for 10 min. The weights of the pellets were determined and the results reported in g/l.

2.2.10.6 Protein Determination.

The method used for protein determination was that of Lowry *et al.* (1951) based on Peterson's modification (1979), which is available in kit form from Sigma. The Lowry method combines the use of the Biuret reaction of proteins with copper ions in alkali, with the reduction of the Folin-Ciocalteu phenol reagent (phosphomolybdic-phosphotungstic acid) by tyrosine and tryptophan residues in the protein molecules. This latter reaction is intensified by the copper-protein complex.

The absorbance can be read at a suitable wavelength between 500 nm and 800 nm and the protein concentration determined from a calibration curve. The calibration curve was prepared daily.

For many proteins, the Lowry reaction can be run directly on the protein solution. However, interference can be caused by commonly used chemicals, *e.g.*, Tris, ammonium sulphate, ethylenediaminetetraacetic acid (EDTA), sucrose, citric acid, amino acid and peptide buffers and also phenols. The procedure used, included protein precipitation with trichloroacetic acid. Trichloroacetic acid alone, does not precipitate proteins reliably and quantitatively at low levels (1 - 25 μg) of protein. This difficulty may be overcome by the combined use of trichloroacetic acid and deoxycholate (Bensadoun and Weinstein 1976).

The method used was that detailed in the Sigma Procedure Sheet (P 5656). The absorbance of the solution after the reaction was complete was determined at 600 nm, within 30 min of the end of the reaction.

The protein concentration was determined from the standard curve, (Figure 8) and the resulting protein concentration multiplied by the appropriate dilution factor. The calibration curve illustrated was an example of those carried out. The calibration curve was prepared on a daily basis.

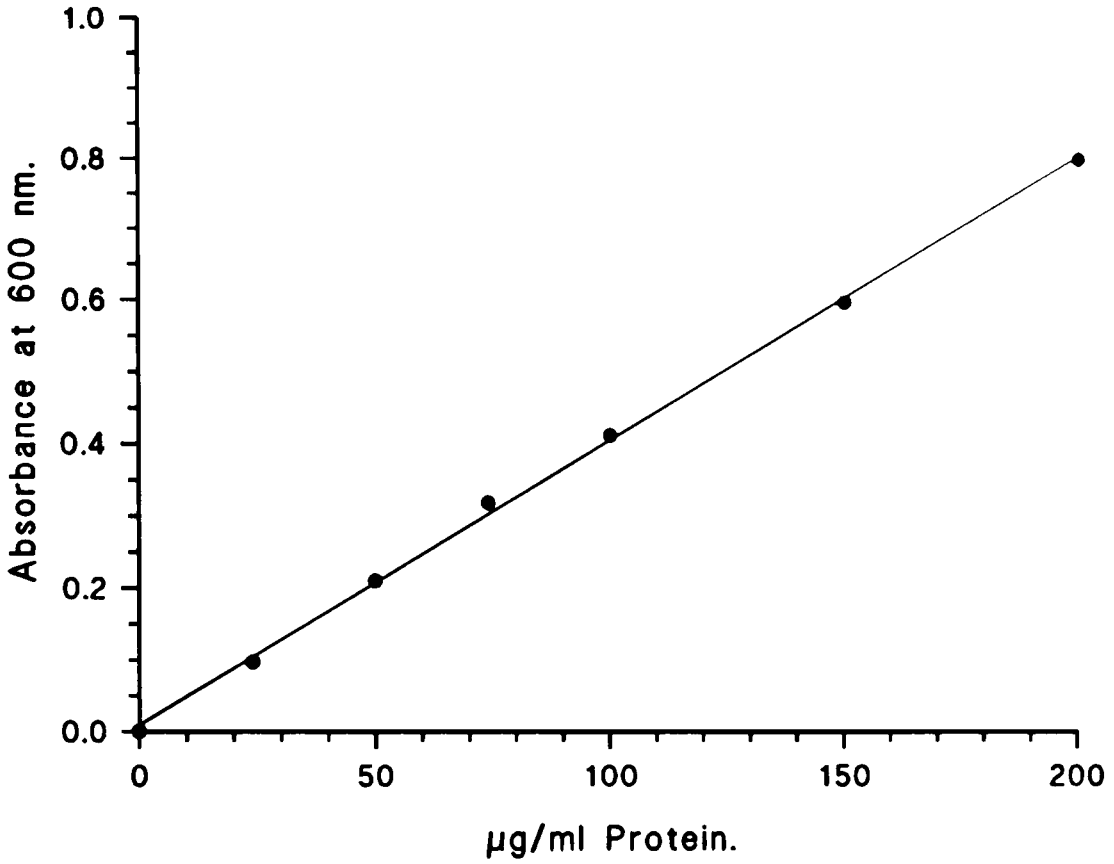


Figure 8. Standard curve for protein concentration using the modified Lowry method at 600 nm.

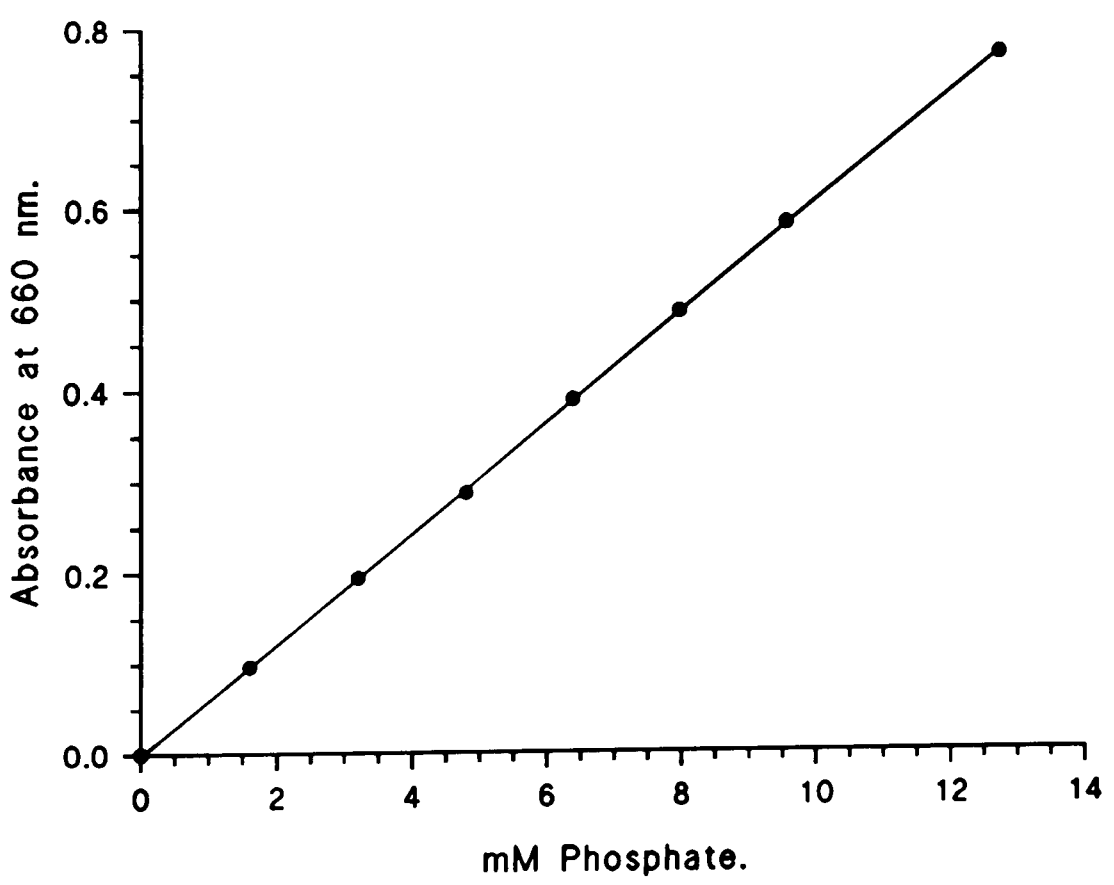


Figure 9. Standard curve for phosphate concentration using the Fiske and SubbaRow method at 660 nm.

2.2.10.7 Phosphate Determination.

The method used for phosphate determination during the mashing, wort draining and fermentation studies was a modified Fiske and SubbaRow procedure. This procedure made use of the inorganic phosphorus test kit from Sigma Diagnostics. Numerous modifications of the molybdate acid and reducer concentration have been made to overcome various technical problems. However, the test kit supplied by Sigma allows the use of a single molybdate reagent and a purified reducer ready for use.

Protein and lipid phosphates were removed by treatment of the sample with trichloroacetic acid, with the resulting supernatent being reacted with ammonium molybdate in an acid solution to form phosphomolybdate. The Fiske and SubbaRow reducer, comprising of sodium bisulphite, sodium sulphite and 1-amino-2-naphthol-4-sulphonic acid reduced the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour was proportional to the phosphate concentration and is measured at 660 nm.

A 0.5 ml aliquot of the sample, along with 4.5 ml of a 10% (w/v) trichloroacetic acid solution was mixed in a test-tube and allowed to stand for 5 min. At the end of this period the mixture was centrifuged for 10 min in a MSE Microfuge at high speed. 1.0 ml of the supernatent was pipetted into a test-tube with 1.5 ml distilled water and 0.5 ml acid molybdate solution. The resulting solution was mixed by inversion and 0.125 ml of the Fiske and SubbaRow reducer solution added. After mixing by inversion, the resulting solution was allowed to stand for 10 min in order that colour development may take place. On transfer to a cuvette the solutions are read at an absorbance of 660 nm against a reagent blank. For the reagent blank, the supernatent was replaced with 1.0 ml 10% (w/v) trichloroacetic acid. The absorbance readings must be completed within 10 min of colour development.

The concentration of inorganic phosphorus can now be determined by referring to the calibration curve. The calibration curve illustrated in Figure 9 was an example of those carried out, the calibration curve was prepared on a daily basis.

2.2.10.8 Determination of Acetic Acid.

Acetic acid was determined according to the method of Beutler (1984b) as stated in the instructions of the BCL Test Kit for acetic acid. Samples were centrifuged to remove particulate matter and stored frozen until asseyed, as previously described, **Section 2.2.9.**

The acetic acid present in the sample was converted to acetyl CoA by the enzyme acetyl CoA synthetase in the presence of ATP and CoA. Acetyl CoA then reacted with oxaloacetic acid to form citric acid using the enzyme citrate synthetase. The oxaloacetic acid required for this reaction was formed from malic acid and NAD in the presence of malate dehydrogenase. The NAD was reduced to NADH. The determination of acetic acid was therefore based on the formation of NADH measured by the increase in absorbance at 340 nm. This type of assay employs the use of a preceding indicator reaction. There is no linear proportionality between the absorbance difference and the acetic acid concentration. In this case the formula given below should be used (Bergmeyer 1984).

The assay procedure was given in the BCL test kit sheet. Since a spectrophotometric method was used, the calculation was based on the Beer-Lambert Law. The absorption coefficient (ϵ) of NADH at 340 nm was equal to 6.3 l x $mmol^{-1}$ x cm^{-1}. It follows that the acetic acid concentration (g/l) in the sample can be calculated from the following formula:

$$\Delta A_{Acetic\ Acid} = \left((A_2 - A_0)_{sample} - \frac{(A_1 - A_0)^2_{sample}}{(A_2 - A_0)_{sample}}\right) - \left((A_2 - A_0)_{blank} - \frac{(A_1 - A_0)^2_{blank}}{(A_2 - A_0)_{blank}}\right)$$

Where $\Delta A_{Acetic\ Acid}$ was the absorbance difference between the sample and the blank at 340 nm, A_0 was the absorption, at 340 nm, of the

initial assay mixture with no added enzymes, A_1 was the absorption, at 340 nm, after the addition of malate dehydrogenase and A_2 was the absorption, at 340 nm, after the addition of acetyl CoA synthetase.

$$C_{Acetic\ Acid}\ (g/l) = \frac{1.940}{\epsilon} \times \Delta A_{Acetic\ Acid} \times F$$

If the sample was diluted during preparation, the result must be multiplied by the dilution factor, F.

2.2.10.9 Determination of Ethanol.

The concentration of ethanol in the samples was determined using the UV method of Beutler (1984a). This method was adapted from the BCL ethanol test kit for use with distillery samples. Samples were centrifuged to remove particulate matter and stored frozen until asseyed, as previously described, **Section 2.2.9.**

Ethanol was oxidized by alcohol dehydrogenase in the presence of NAD to acetaldehyde. However, since the equilibrium of this reaction lies towards ethanol, it must be displaced to the acetaldehyde side by employing alkaline conditions. The acetaldehyde was then oxidized in the presence of acetaldehyde dehydrogenase to acetic acid. It was the increase in NADH present at the end of the reaction, which was measured. This increase in the NADH concentration was stoichiometric to half the amount of the ethanol initially present in the sample.

The assay was carried out as indicated in the BCL test procedure, great care must be taken to minimize ethanol losses during the dilution, assay procedures and measurement. The calculation was based on the Beer-Lambert Law. The absorption coefficient (ϵ) of NADH at 340 nm is equal to 6.3 l x $mmol^{-1}$ x cm^{-1}. It follows that the ethanol concentration (g/l) in the sample can be calculated from the following formula:

$$C_{Ethanol}\ (g/l) = \frac{0.7256}{\epsilon} \times \Delta A_{Ethanol} \times F$$

Where $\Delta A_{Ethanol}$ was the difference between the absorptions of the sample and the blank at 340 nm. If the sample was diluted during preparation, the result must be multiplied by the dilution factor, F.

2.2.10.10 Determination of Glycerol.

The amount of glycerol in the distillery samples can be determined quite accurately using the BCL Glycerol Test Kit. This method was adapted from Wieland (1984), but requires the formation of pyruvic acid as an intermediate. Samples were centrifuged to remove particulate matter and stored frozen until asseyed, as previously described, **Section 2.2.9.**

Initially glycerol was phosphorylated using ATP by the enzyme glycerokinase to glycerol-3-phosphate and ADP. The ADP was then re-phosphorylated to ATP using phosphoenolpyruvate with the formation of pyruvic acid. The enzyme pyruvate kinase was responsible for this reaction. The pyruvate can now be reduced to lactic acid with the corresponding oxidation of NADH to NAD. The amount of NADH oxidized was stoichiometric with the initial concentration of glycerol.

The assay procedure was given in the BCL test procedure. Because, 1.5 ml cuvettes were used, all volumes were divided by two. The calculation was based on the Beer-Lambert Law, since a spectrophotometric method was used. The absorption coefficient (ϵ) of NADH at 340 nm was equal to 6.3 l x $mmol^{-1}$ x cm^{-1}. It follows that the glycerol concentration (g/l) in the sample can be calculated from the following formula:

$$C_{Glycerol}\ (g/l) = \frac{2.781}{\epsilon} \times \Delta A_{Glycerol} \times F$$

Where $\Delta A_{Glycerol}$ was the difference between the absorptions of the sample and the blank at 340 nm. If the sample was diluted during preparation, the result must be multiplied by the dilution factor, F.

2.2.10.11 Determination of Lactic Acid.

The concentration of lactic acid in the distillery samples was determined using the UV method of Noll (1984). This method was adapted for use with the BCL Lactic Acid Test Kit. Samples were centrifuged to remove particulate matter and stored frozen until asseyed, as previously described, **Section 2.2.9.**

Lactic acid was oxidized by NAD in the presence of lactate dehydrogenase to pyruvic acid. Since the equilibrium of this reaction was completely on the lactic acid side, it was necessary to remove the pyruvic acid. This was achieved using the enzyme glutamate-pyruvate transaminase, in the presence of glutamate. The pyruvic acid was thus catalyzed to alanine. The amount of NADH formed in the reaction was stoichiometric with the amount of lactic acid, and the increase in NADH was determined spectrophotometrically at 340 nm.

Using the assay procedures set out in the BCL test sheets the Beer-Lambert Law can be used to calculate the concentration of lactic acid. The absorption coefficient (ϵ) of NADH at 340 nm was equal to 6.3 l x $mmol^{-1}$ x cm^{-1}. It follows that the lactic acid concentration (g/l) in the sample can be calculated from the following formula:

$$C_{Lactic\ Acid}\ (g/l) = \frac{2.018}{\epsilon}\ x\ \Delta A_{Lactic\ Acid}\ x\ F$$

Where $\Delta A_{Lactic\ Acid}$ was the difference between the absorptions of the sample and the blank at 340 nm. If the sample was diluted during preparation, the result must be multiplied by the dilution factor, F.

2.2.10.12 Determination of Succinic Acid.

The levels of succinic acid in the distillery samples can be determined using the BCL Succinic Acid Test Kit. This method was adapted from Beutler (1984c). Samples were centrifuged to remove particulate matter and stored frozen until asseyed, as previously described, **Section 2.2.9.**

Succinic acid was converted to succinyl CoA by the enzyme succinyl CoA synthetase, in the presence of CoA and inosine 5'-triphosphate. The inosine 5'-triphosphate formed can then react with phosphoenolpyruvate, the phosphoenolpyruvate in turn was de-phosphorylated to pyruvic acid in the presence of pyruvate kinase. pyruvic acid was then reduced to lactic acid by the enzyme lactate dehydrogenase in the presence of NADH, with the formation of NAD.

The formation of NAD, or the consumption of NADH, was proportional to the succinic acid concentration. The consumption of NADH was measured by the change in absorption at 340 nm.

Using the assay procedures set out in the BCL test sheets the Beer-Lambert Law can be used to calculate the concentration of succinic acid. The absorption coefficient (ϵ) of NADH at 340 nm was equal to 6.3 l x $mmol^{-1}$ x cm^{-1}. It follows that the succinic acid concentration (g/l) in the sample can be calculated from the following formula:

$$C_{Succinic\ Acid}\ (g/l) = \frac{3.625}{\epsilon} \times \Delta A_{Succinic\ Acid} \times F$$

Where $\Delta A_{Succinic\ Acid}$ was the difference between the absorptions of the sample and the blank at 340 nm. If the sample was diluted during preparation, the result must be multiplied by the dilution factor, F.

3 RESULTS.

3.1 Assay Method Development of Malted Barley Carbohydrases.

3.1.1 α-Amylase.

Initial experiments using malt extracts (Section 2.2.2.1) suggested that the Phadebas amylase substrate was indeed hydrolyzed by malted barley amylases. However further development of the assay procedures were needed since the assay protocol was that for serum and urine α-amylase.

Under the standard assay conditions, the optimum parameters for the activity of α-amylase from malted barley were determined (Section 2.2.2.1). It was ascertained that the optimum temperature for enzyme activity was between 57°C and 65°C. Activity was found to declines rapidly after 65°C. The optimal pH for enzyme activity was pH 5.5, although activity between pH 5.4 and 5.8 was still high.

Enzyme activity was linear (r = 0.9866) with respect to extract volume up to 0.5 ml. The reaction was also linear (r = 0.9939) with regard to incubation time up to 20 min. Thereafter a less rapid release of the dye-labelled starch fragments were detected.

Having established the assay parameters of α-amylase from malted barley extract, further work was carried out using a malted barley α-amylase from a crude commercial preparation. This preparation contained both α-amylase and β-amylase. The use of this crude preparation eliminated the need for the extraction procedure, required to obtain the malted barley enzyme extract. Since β-amylase was present in the preparation, the use of the dye-labelled Phadebas assay was still required for the specific determination of α-amylase. The results obtained using a 10 mg/ml solution of the crude α-amylase preparation showed that the Phadebas assay system was suitable for the specific determination of malted barley α-amylase.

Table 6 indicates that the Phadebas substrate was specific for α-amylase, see Section 2.2.3.1. Malted barley α-amylase has a

requirement for added Ca^{2+} ions in the buffer, although 16.3% of the activity remained when the buffer was used with no added Ca^{2+}. The activity of the enzyme was least in the presence of EDTA.

Table 6. The effect of added Ca^{2+} and EDTA to the Phadebas assay buffer system for α-amylase determination. Results are given as Enzyme Units.

Enzyme Used.	Buffer	Buffer plus Ca^{2+}	Buffer plus EDTA
α-Amylase (Crude)	14.652	89.783	0.006
β-Amylase (Purified)	0.000	0.587	0.000

Several assays, as indicated previously, have used p-nitrophenyl malto-oligosaccharides as substrates for α-amylase. All these assays were developed for clinical applications where α-amylase would be the sole amylolytic enzyme. A comparison of substrates that could be used by malt α-amylase using certain p-nitrophenyl malto-oligosaccharides and the Phadebas substrate was made. Before such a comparison could be made it was necessary to denature any β-amylase activity present.

The activity of α-amylase was determined on four p-nitrophenyl malto-oligosaccharides, besides the Phadebas substrate. The results are given in Figure 10. Heat inactivation of the crude malted barley α-amylase preparation, reduced the α-amylase activity, as determined by the Phadebas method, by 86%. When using p-nitrophenyl maltohexaose or p-nitrophenyl maltoheptaose as substrate, 45% and 60% respectively of the activity in the non heat treated preparation was detected. The activity from the heat treated preparation is least when p-nitrophenyl maltotetraose or p-nitrophenyl maltopentaose were used

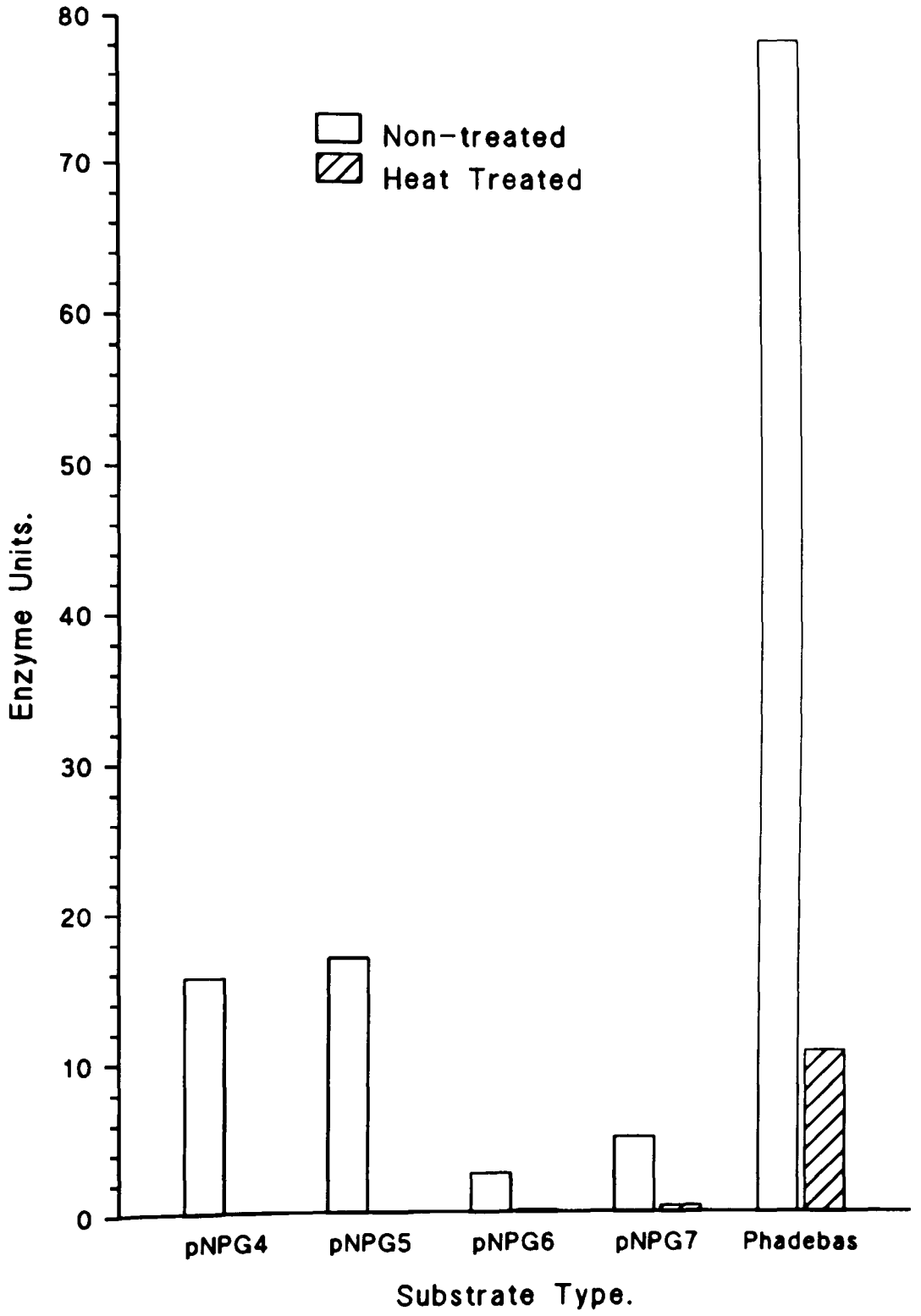


Figure 10. Substrate utilization by the crude α-amylase preparation before and after heat treatment. (pNPG4 - p-nitrophenyl maltotetraose, pNPG5 - p-nitrophenyl maltopentaose, pNPG6 - p-nitrophenly maltohexaose, pNPG7 - p-nitrophenyl maltoheptaose)

as the substrate. Both heat treated activities were 0.4% of the original non heat treated α-amylase preparation. Figure 10 indicates that α-amylase activity was not the single enzyme activity present. However, heat treatment (Section 2.2.7) denatured any additional enzyme activities.

3.1.2 β-Amylase.

It was Mathewson and Seabourn (1983) who initially reported an alternative assay for cereal β-amylase. This method made use of 'Pantrak' reagent, a commercial product for the determination of α-amylase in human serum and urine. The substrates used in this diagnostic test were p-nitrophenyl maltopentaose and p-nitrophenyl maltohexaose.

The products released from a p-nitrophenyl malto-oligosaccharide when assaying for β-amylase, invariably depend on the substrate. They are normally maltose, besides p-nitrophenyl maltose, p-nitrophenyl maltotriose or p-nitrophenyl maltotetraose. It is necessary to introduce an α-glucosidase to hydrolyze the p-nitrophenyl malto-saccharides to release glucose and p-nitrophenol. The rate of release of the p-nitrophenol will be dependent on the initial hydrolysis of the p-nitrophenyl malto-oligosaccharide by the β-amylase. The β-amylase substrate must, preferably, be hydrolyzed only once by β-amylase. The degradation products give maltose and a p-nitrophenyl malto-saccharide, the latter will be rapidly hydrolyzed by the α-glucosidase enzyme.

The activity of yeast α-glucosidase on p-nitrophenyl malto-saccharides is demonstrated in Figure 11. Yeast α-glucosidase readily hydrolyzed the p-nitrophenyl maltose and p-nitrophenyl maltotriose, with p-nitrophenyl maltotetraose hydrolyzed to a lesser extent. p-Nitrophenyl malto-saccharides with molecular weights greater than p-nitrophenyl maltotetraose are not hydrolyzed. From Figure 10 it was demonstrated that α-amylase could not hydrolyze p-nitrophenyl malto-oligosaccharides below the molecular weight of

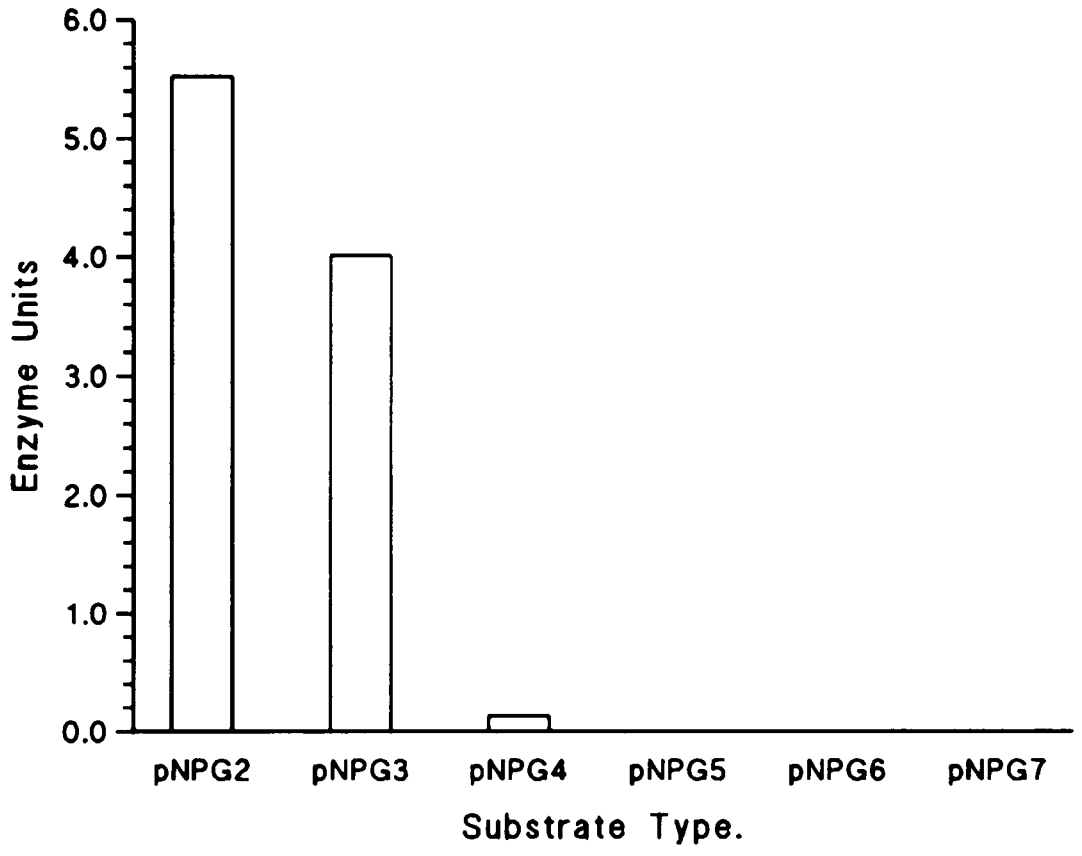


Figure 11. Substrate utilization of the p-nitrophenyl malto-oligosaccharides by the α-glucosidase of yeast.

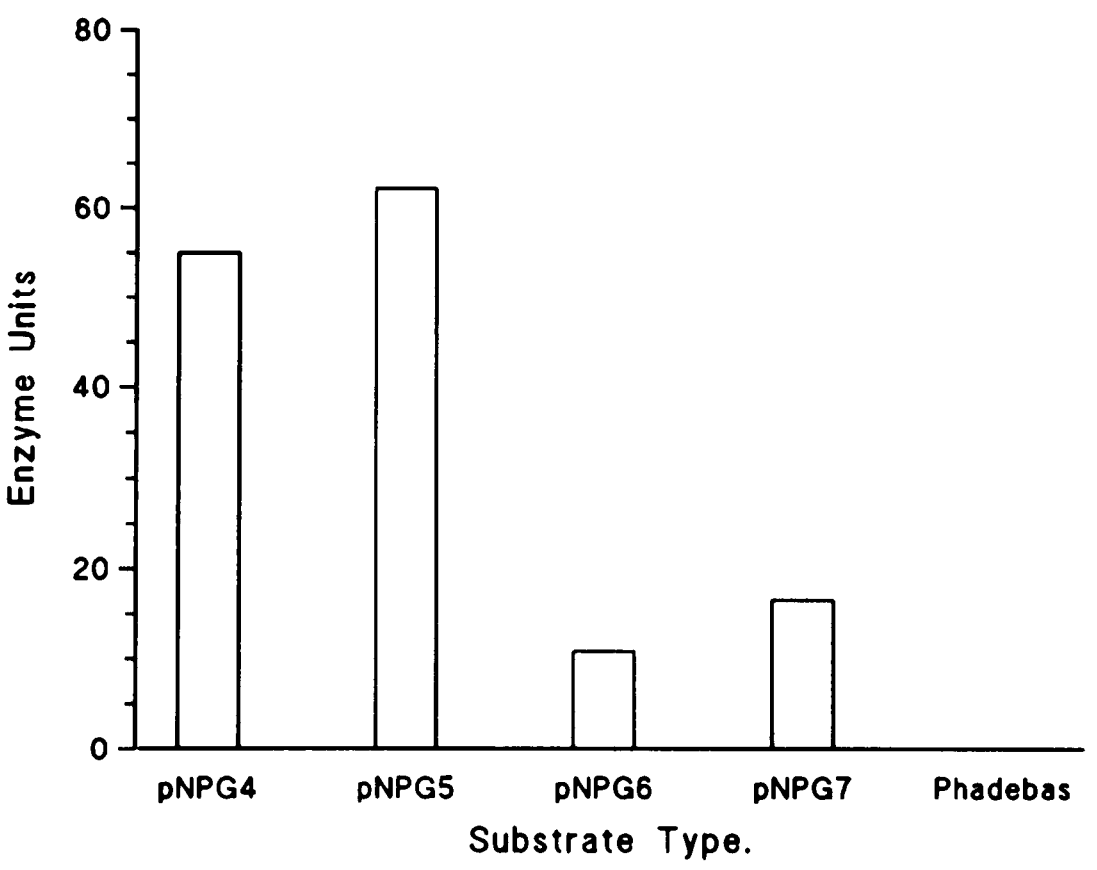


Figure 12. Substrate utilization of purified malted barley β-amylase.

p-nitrophenyl maltohexaose. The hydrolysis of p-nitrophenyl maltotetraose may be due to β-amylase and/or α-glucosidase activity. However activity on the p-nitrophenyl maltopentaose must be due to β-amylase activity alone. Purified Cloder malted barley β-amylase (judged to be standardized at DP 2000 Lintner and free of α-amylase by the supplier) was demonstrated (Figure 12) to hydrolyze p-nitrophenyl maltotetraose, p-nitrophenyl maltopentaose, p-nitrophenyl maltohexaose and p-nitrophenyl maltoheptaose. This β-amylase, did not hydrolyze the α-amylase Phadebas substrate. Activity was greatest with p-nitrophenyl maltopentaose and p-nitrophenyl maltotetraose. Some of this activity on the p-nitrophenyl maltotetraose may be attributed to the α-glucosidase coupling enzyme. The rate of hydrolysis on the p-nitrophenyl maltohexaose and p-nitrophenyl maltoheptaose substrates was much lower. This may have been due to an increase in β-amylase activity on the substrate molecules.

Further investigations into the substrate specificities of β-amylase were carried out using the malto-oligosaccharide substrates maltotetraose, maltopentaose, maltohexaose and maltoheptaose. The crude α-amylase preparation (see Methods and Materials) was used as the enzyme source. The results are given in Table 7.

The non-heat treated enzyme preparation gave hydrolysis of all substrates with the final hydrolysis products suggesting β-amylase activity on maltotetraose, maltopentaose and maltohexaose. The hydrolysis pattern from the maltoheptaose substrate showed a slight α-amylase activity. As observed by the low concentrations of glucose in the final hydrolysis products. Maltohexaose was not present when maltoheptaose was the substrate. This would be consistent with β-amylase activity. No hydrolysis products were detected when maltotetraose, maltopentaose and maltohexaose were used as substrates for the heat treated enzyme preparation. Only a limited activity was observed when maltoheptaose was the substrate.

Table 7. Hydrolysis products of malto-oligosaccharide substrates from the crude α-amylase preparation, indicating the inability of α-amylase to hydrolyze malto-oligosaccharides less than seven glucose units, using non-treated and heat treated extracts. Hydrolysis of substrates from the non treated extract indicates β-amylase activity. (G1 - Glucose, G2 - Maltose, G3 - Maltotriose, G4 - Maltotetraose, G5 - Maltopentaose, G6 - Maltohexaose, G7 - Maltoheptaose)

Treatment & Substrate.	G 7 mg/ml	G 6 mg/ml	G 5 mg/ml	G 4 mg/ml	G 3 mg/ml	G 2 mg/ml	G 1 mg/ml
None G 4				0.909		0.286	
None G 5			0.256		0.298	0.304	
None G 6		0.138		0.479		0.549	
None G 7	0.257			0.064	0.159	0.428	0.028
Heat G 4				1.111			
Heat G 5			0.880				
Heat G 6		1.103					
Heat G 7	1.170	0.027					0.034

These results are consistent with α-amylase unable to hydrolyze malto-oligosaccharides smaller than p-nitrophenyl maltoheptaose. The results from the heat treated enzyme preparation of Table 7 were consistent with those found by MacGregor and MacGregor (1985).

So as to carry out more meaningful determinations of β-amylase activity in the presence of other carbohydrases, a study into the buffer composition of the assay was undertaken. If a buffer system that inhibited α-amylase could be found, suitable for α-glucosidase and β-amylase activity, then it could be classed as a specific assay for malted barley β-amylase. The substrate to be employed would be p-nitrophenyl maltopentaose. The ten buffer systems used are given in Table 3.

The basic buffer system consisted of a 0.1 M phosphate buffer at pH 7.1. Additions to this buffer system included calcium ions to determine supplementary α-amylase activity, EDTA was used to chelate any metal ions present. Ascorbic acid is a known inhibitor of β-amylase (Baker and Smiley 1985) as is Cu^{2+} and Hg^{2+}.

Since the assay for β-amylase involves a coupling reaction, then the activity of α-glucosidase would have to be studied with respect to the different buffer systems. The results of α-glucosidase activity in these buffers are given in Figure 13. As detailed in the Materials and Methods the α-glucosidase solution used in this assay was 0.5 mg/ml (approximately 30 U/ml), with p-nitrophenyl glucose as substrate.

The results suggest that buffer systems 1, 2 and 3 have no effect on α-glucosidase activity. Ascorbic acid addition to the buffer gave complete inhibition of the enzyme. When the assay with ascorbic acid addition was repeated, with the α-glucosidase concentration similar to that in the final β-amylase assay, activity was extremely high. α-Glucosidase activity was therefore deemed not to be rate-limiting when used in this buffer system at 3 mg/ml (approximately 180 U/ml). Activity of α-glucosidase under buffer system 5 was relatively similar to that of the control, buffer system 1. In the presence of added EDTA as well as ascorbic acid, α-glucosidase activity was activated. Activity in the presence of Cu^{2+} ions was decreased by 61.3% when compared to the control, and Hg^{2+} ions completely inhibited activity.

When the standard β-amylase assay was used, with the buffers in Table 3, the results in Figure 14 were obtained. These results report the activities obtained from β-amylase, which was purified from malted barley and from the crude α-amylase preparation, which contained substantial β-amylase activity.

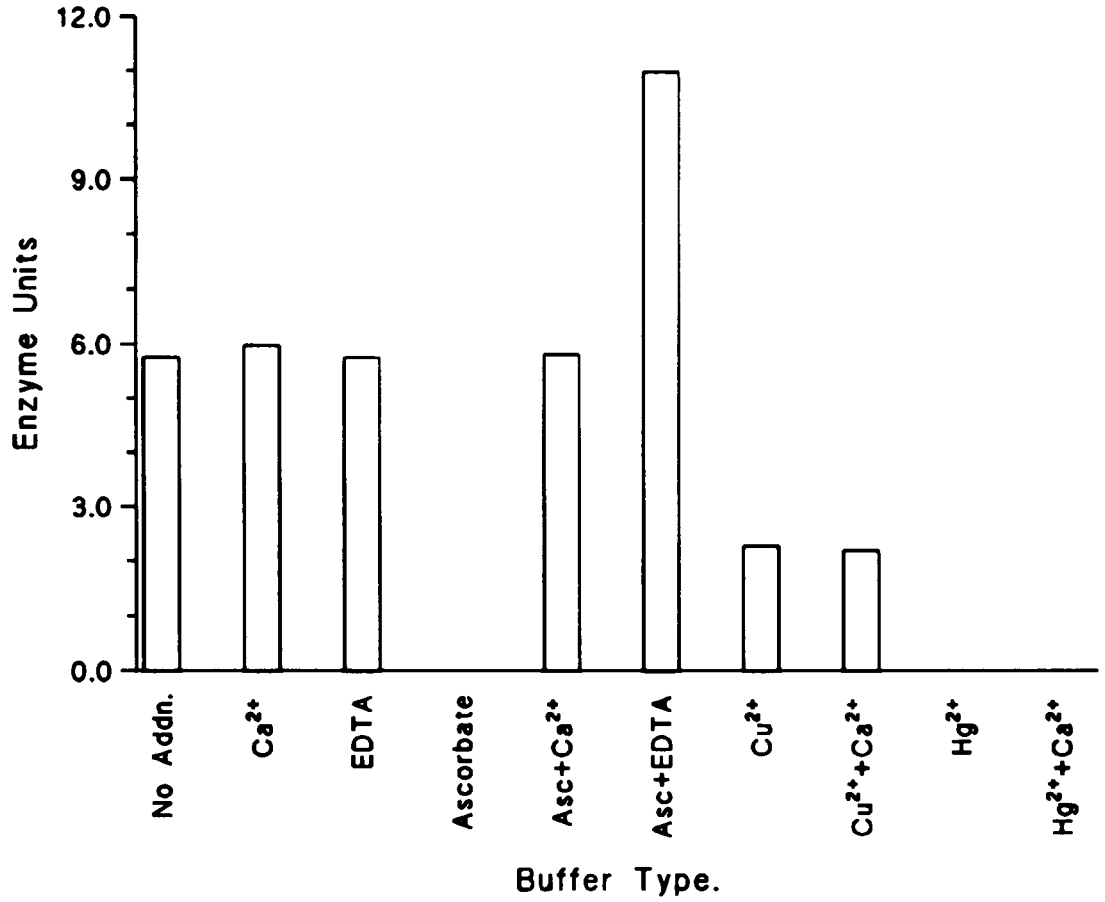


Figure 13. Activation and inhibition of the yeast α-glucosidase using the 0.1 Phosphate buffer, pH7.1 with the additions shown.

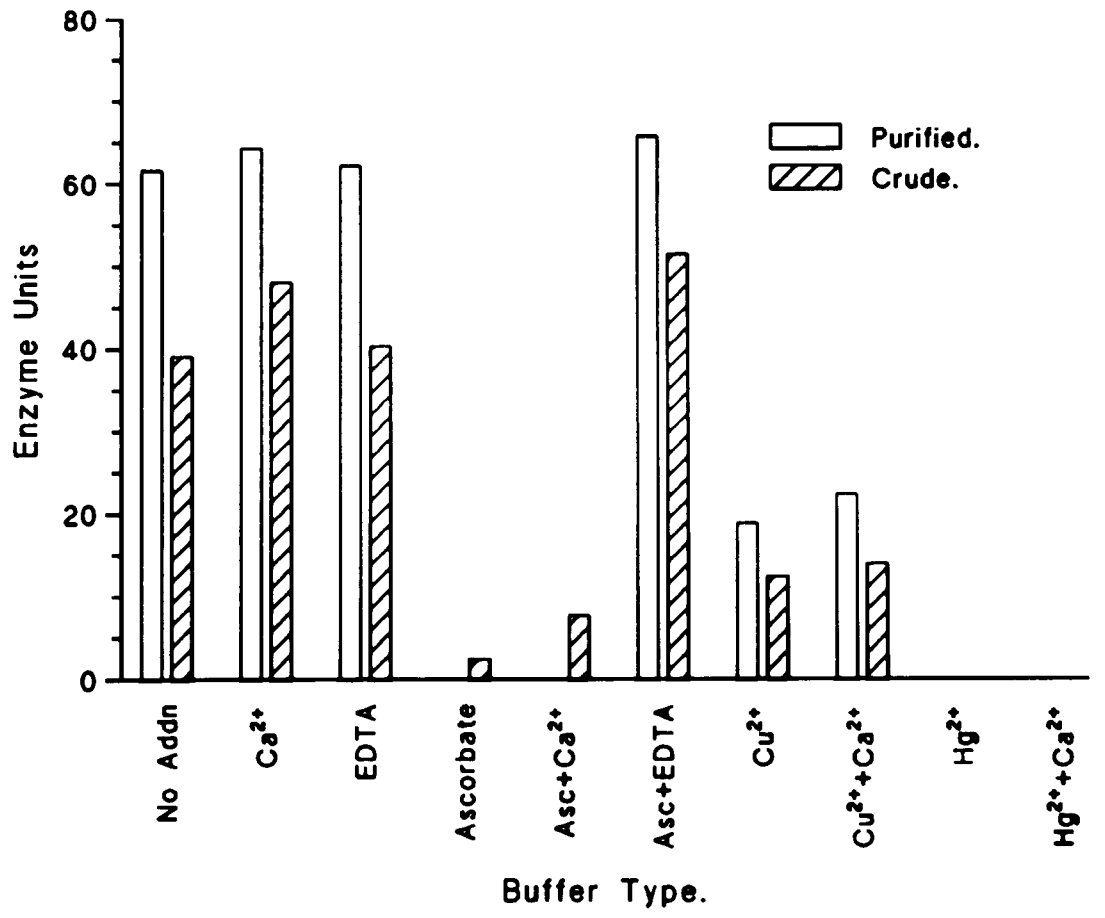


Figure 14. Activation and inhibition of malted barley β-amylase using the 0.1M Phosphate buffer, pH7.1, with the additions shown.

The activity patterns for both the crude and purified β-amylase preparations follow similar patterns. The activity in buffer systems 1, 2 and 3 were as expected since Ca^{2+} and EDTA should have no effects on the β-amylase enzyme. The addition of ascorbic acid and ascorbic acid plus Ca^{2+} gave predictable results when purified β-amylase was used. However, activity was present, with these buffers, when the crude α-amylase preparation was used as the enzyme source. The addition of ascorbic acid plus EDTA was found to have an activating effect on both enzyme preparations. In the presence of Cu^{2+} ions, β-amylase activity was decreased by 70%, however activity was only decreased by 65% in buffer system 7 when compared to the control, buffer system 1. No activity was detected with added Hg^{2+} ions, either with or without Ca^{2+} ions.

The low levels of β-amylase activity in buffer systems 4 and 5, when using the crude enzyme preparation was probably due to the stabilizing effect of increased protein concentration on β-amylase activity in the enzyme solution Spradlin & Thoma (1970). Activity was not due to the α-amylase activity since p-nitrophenyl maltopentaose is not hydrolyzed by α-amylase from malted barley (see Figure 10).

The slight activation by Ca^{2+} ions is consistent with the results of Baker and Smiley (1985). A possible explanation for this may be that the Ca^{2+} ions can promote subunit association.

The optimum parameters for the β-amylase assay from malted barley were determined under the standard assay conditions (see Materials and Methods). It must be remembered that this is a coupled reaction, with α-glucosidase present in the assay buffer. The assay conditions are not necessarily optimal for purified malted barley β-amylase activity but are judged to be optimal for the assay system using a malted barley extract. To ensure that activity, or any part of the activity, was not due to α-amylase hydrolysis on the p-nitrophenyl maltopentaose, an aliquote of the extract was heat treated before the analysis was carried out. No activity was found in the

heat treated extract in any of the experiments carried out. Table 8 contains the results from the determination of the assay parameters from malted barley β-amylase.

Table 8. Summary of the assay conditions for β-amylase from malted barley.

Temperature (°C)	pH	Extract Volume (ml)	Incubation Time (min)
45.0	7.1	0.3	30

The optimal temperature for the assay system was between 35°C and 45°C with a rapid decrease in activity between 55°C and 65°C. The optimal pH for the assay system was pH 7.1, However activity between pH 5.5 and pH 7.5 was in excess of 65% of the optimal activity found at pH 7.1, which was 14.088 E.U.

Enzyme activity was linear with respect to extract volume up to 0.3 ml. The β-amylase activity was also linear with regard to incubation time, up to 30 min.

Table 9. Summary of the kinetic parameters for β-amylase from malted barley using p-nitrophenyl maltopentaose as the substrate.

Lineweaver Burk Plot.		Eadie-Hofstee Plot.		Hanes-Woolf Plot.		Direct Linear Plot.	
k_m	V_{max}	k_m	V_{max}	k_m	V_{max}	k_m	V_{max}
1.13	54.9	1.10	54.1	0.99	51.9	1.12	55.4

The effect of substrate concentration on rate of reaction is shown in Figure 15. The k_m value was found to be 1.13 mM by the Lineweaver-Burk plot using p-nitrophenyl maltopentaose as the

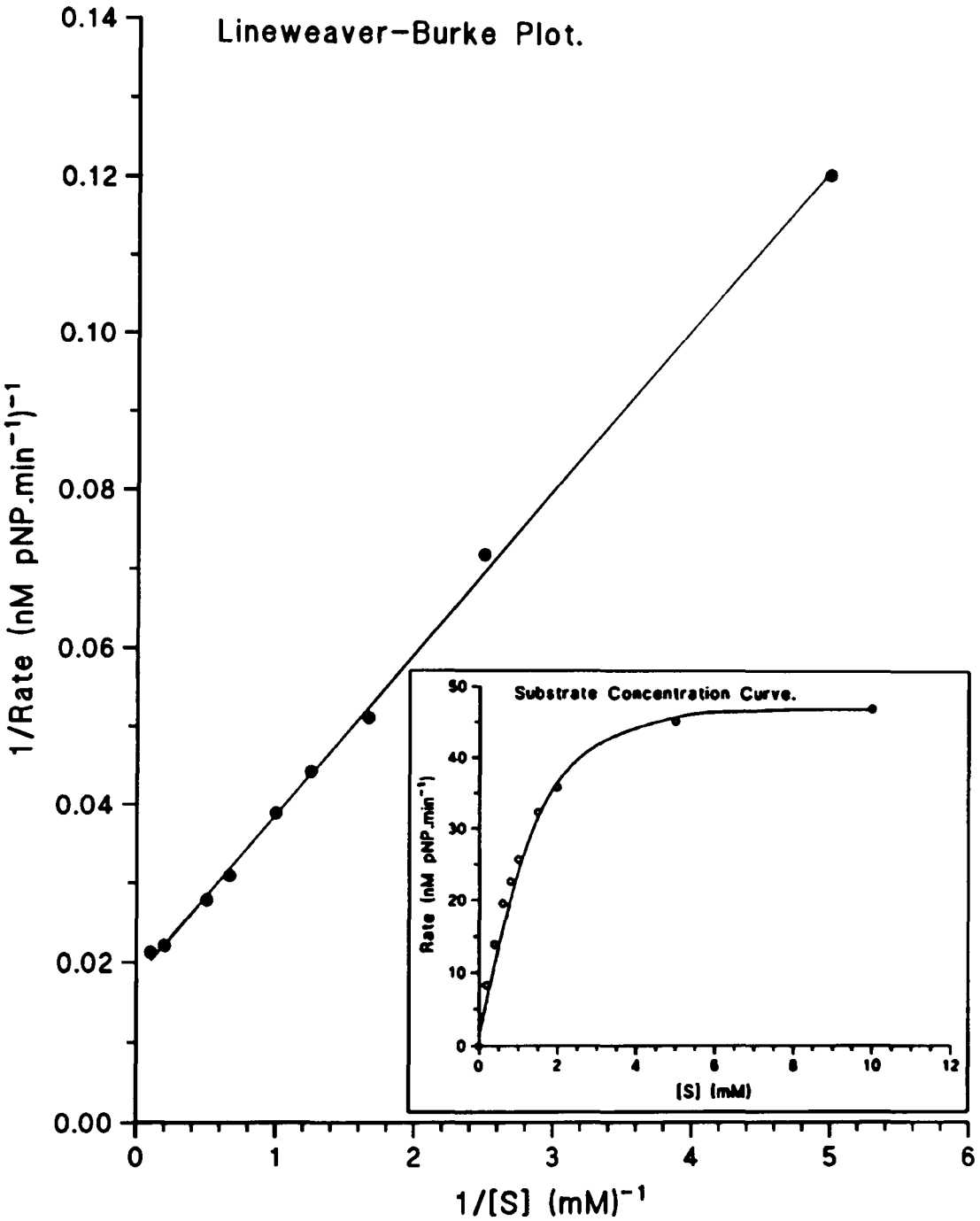


Figure 15. Lineweaver-Burk plot for β-amylase using p-nitrophenyl maltopentaose as the substrate. The insert indicates the substrate concentration curve.

substrate in the standard assay system. Calculation of both the k_m and V_{max} values was carried out using four different methods and these values are given in Table 9.

3.1.3 Minor Carbohydrases.

Although α-amylase and β-amylase are the major starch hydrolyzing enzymes of malted barley, other enzymes are present in the malt that will contribute to the degradation of starch and other carbohydrates. The use of p-nitrophenol labelled substrates would enable many carbohydrases to be assayed in the complex mixture found in a malted barley extract. Many of these enzymes have already been characterized from other fields of research, but few have been studied from distilling malt, nor through the processes involved in the production of Scotch Malt Whisky. To ensure optimum activity for each enzyme under study, the enzyme parameters had to be established. The following enzymes were examined: α- and β-glucosidase, α- and β-galactosidase, α- and β-mannosidase, β-N-acetyl-glucosaminidase, arabinosidase and β-xylosidase.

3.1.3.1 α-Glucosidase.

The enzyme parameters of α-glucosidase from malted barley are given in Table 10. It was found that the optimal temperature for this enzyme was at 50°C, although the temperature range was determined between 40°C and 65°C. The optimal pH for enzyme activity was at pH 6.0 but the enzyme was stable between pH 5.7 and pH 6.8. Under the standard assay conditions the enzyme activity was linear with respect to extract volume up to 0.4 ml, and with regard to incubation time up to 40 min.

The effect of substrate concentration on the enzyme is shown in Figure 16. The k_m value was found to be 37.7 mM by the Lineweaver-Burk plot using p-nitrophenyl-α-D-glucose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} was carried out using four different methods, these are given in Table 11.

Table 10. Summary of the assay conditions for the enzymes studied from malted barley.

Enzyme.	Temp. (°C)	pH	Extract Volume (ml)	Incubation Time (min)
α-Glucosidase	50.0	6.0	0.4	40
β-Glucosidase	35.0	5.1	0.3	60
α-Galactosidase	55.0	5.1	0.3	30
β-Galactosidase	35.0	4.0	0.5	60
α-Mannosidase	55.0	4.7	0.3	60
β-Mannosidase	55.0	4.9	0.5	60
N-Acetylglucosaminidase	55.0	4.9	0.5	60
Arabinosidase	55.0	4.6	0.5	45
β-Xylosidase	55.0	4.4	0.3	30

Table 11. Summary of the kinetic parameters for the enzymes studied from malted barley.

Enzyme	Lineweaver Burk Plot		Eadie-Hofstee Plot		Hanes-Woolf Plot		Direct Linear Plot	
	k_m	V_{max}	k_m	V_{max}	k_m	V_{max}	k_m	V_{max}
α-Glucosidase	37.70	1.7	36.00	1.7	31.80	1.6	42.80	2.0
β-Glucosidase	4.15	35.2	4.51	36.2	6.11	39.0	3.91	37.0
α-Galactosidase	2.89	208.0	3.06	211.0	4.15	225.0	3.36	223.0
β-Galactosidase	3.76	81.7	4.41	86.5	5.94	94.3	4.24	92.4
α-Mannosidase	5.04	11.8	4.96	11.7	4.57	11.5	4.09	11.8
β-Mannosidase	3.34	59.5	3.16	58.5	2.55	56.4	2.29	57.6
N-Acetylglucosaminidase	1.54	32.4	1.53	32.4	1.36	32.0	0.97	31.9
Arabinosidase	48.50	24.8	24.80	15.5	21.00	14.3	25.55	29.9
β-Xylosidase	4.80	26.9	5.06	27.5	5.66	28.3	4.85	28.8

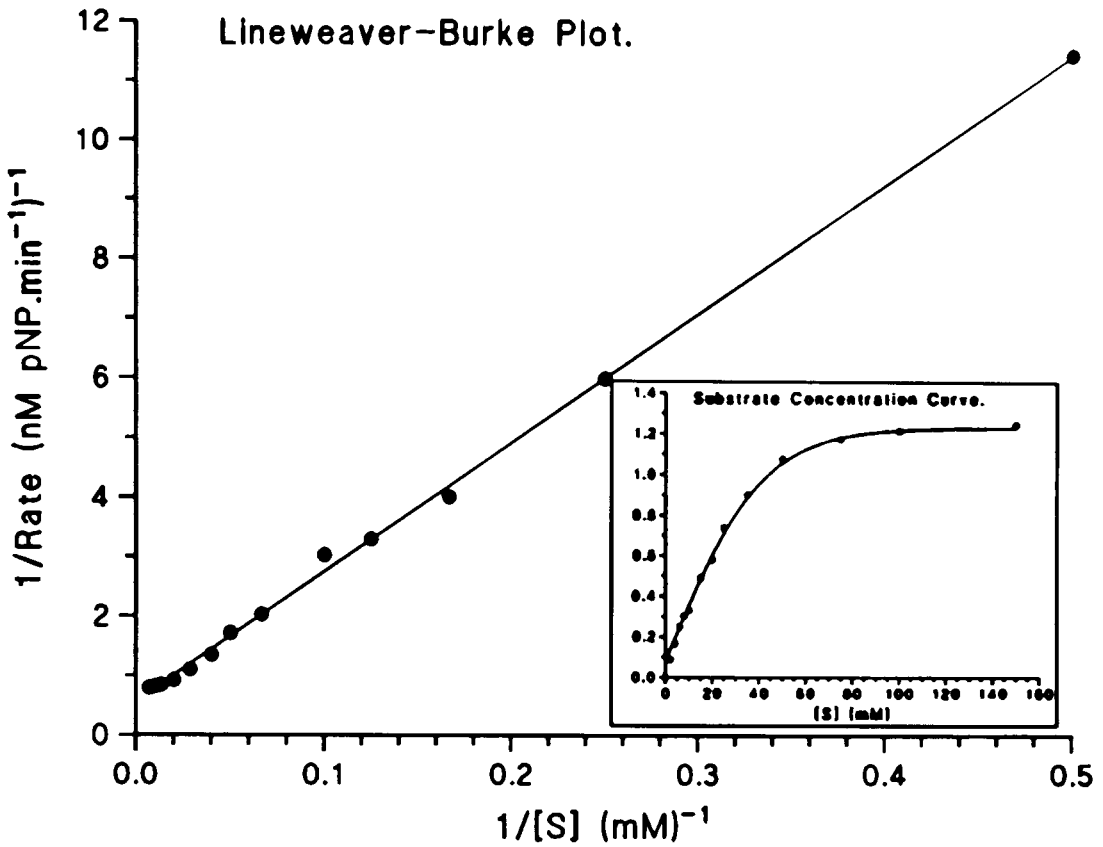


Figure 16. Lineweaver-Burk plot for α-glucosidase using p-nitrophenyl α-D-glucose as the substrate. The insert indicates the substrate concentration curve.

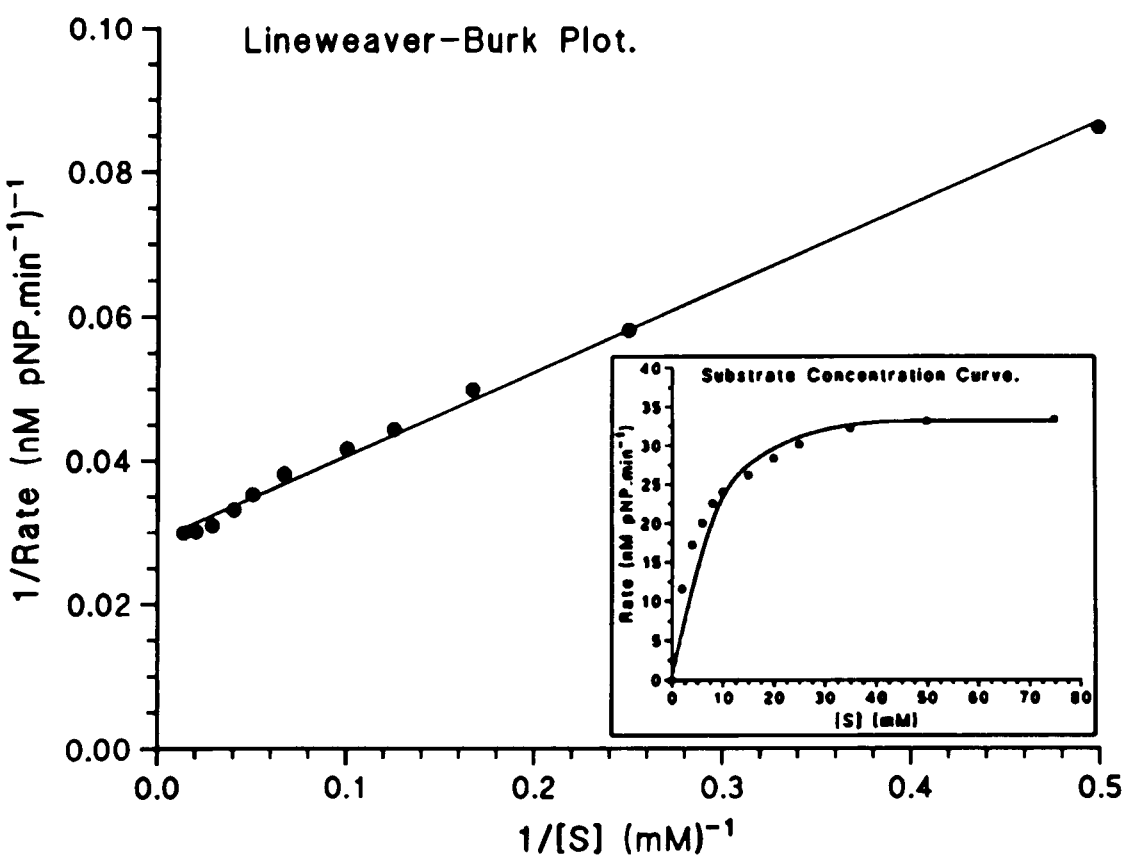


Figure 17. Lineweaver-Burke plot for β-glucosidase using p-nitrophenyl β-D-glucose as the substrate. The insert indicates the substrate concentration curve.

3.1.3.2 β-Glucosidase.

The enzyme parameters of β-glucosidase from malted barley are given in Table 10. The optimal temperature for this enzyme was 35°C and the temperature range was between 32°C and 47°C. Optimum pH of enzyme activity in this assay system was found to be pH 5.1. Under the standard assay conditions the enzyme activity was linear with respect to incubation time up to 60 min and with regard to extract volume up to 0.3 ml.

The effect of substrate concentration on activity is shown in Figure 17. The k_m value was determined as 4.15 mM by the Lineweaver-Burk plot, using p-nitrophenyl-β-D-glucose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} was carried out using four different methods and these are given in Table 11.

3.1.3.3 α-Galactosidase.

The enzyme parameters of α-galactosidase from malted barley are given in Table 10. It was found that the optimum temperature for enzyme activity was 55°C and activity decreased rapidly after this temperature. The optimal pH for enzyme activity was at pH 5.1, although 90% of the activity lay between pH 4.8 and pH 5.9. Under the standard assay conditions the enzyme activity was linear with extract volumes up to 0.3 ml and with regard to incubation time of up to 30 min.

The effect of substrate concentration on the rate of reaction is shown in Figure 18. The k_m value was 2.89 mM by the Lineweaver-Burk plot using p-nitrophenyl-α-D-galactose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} was carried out using four different methods, and these are given in Table 11.

3.1.3.4 β-Galactosidase.

The enzyme parameters for β-galactosidase from malted barley are given in Table 10. The optimal temperature for this enzyme was

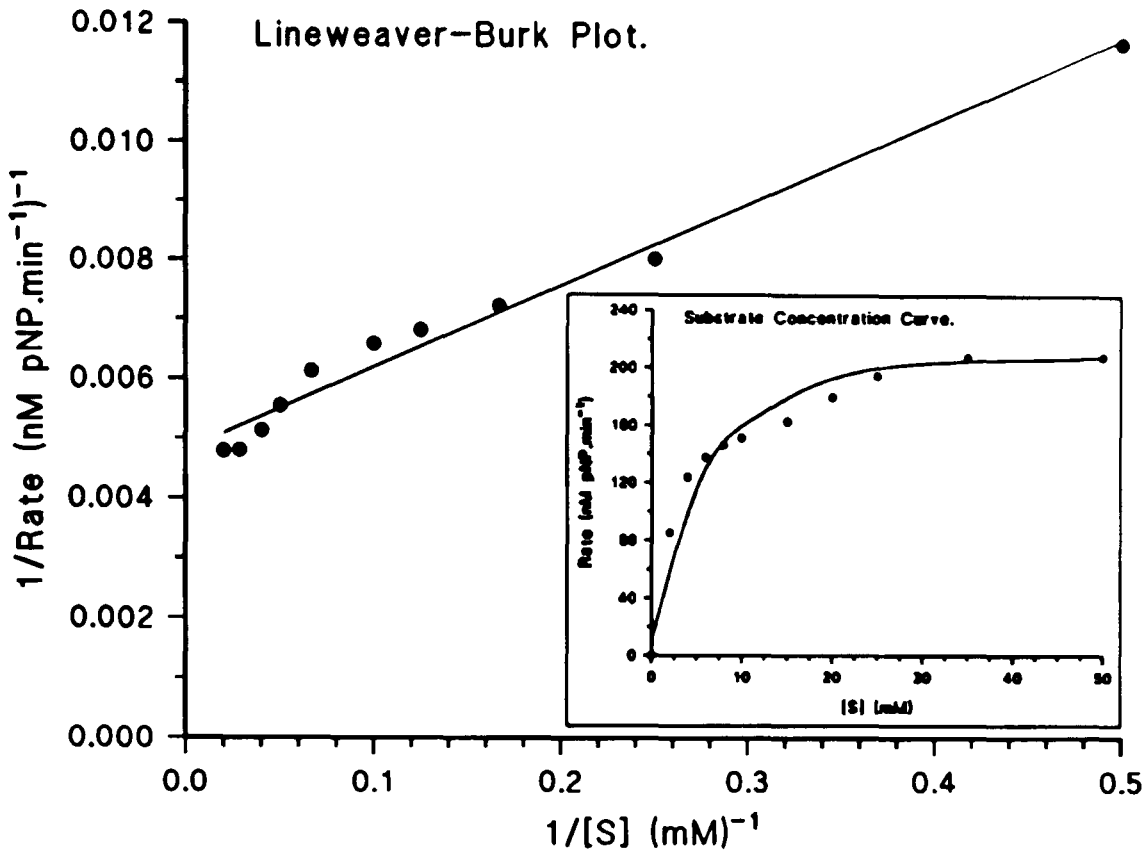


Figure 18. Lineweaver-Burk plot for α-galactosidase using p-nitrophenyl α-D-galactose as the substrate. The insert indicates the substrate concentration curve.

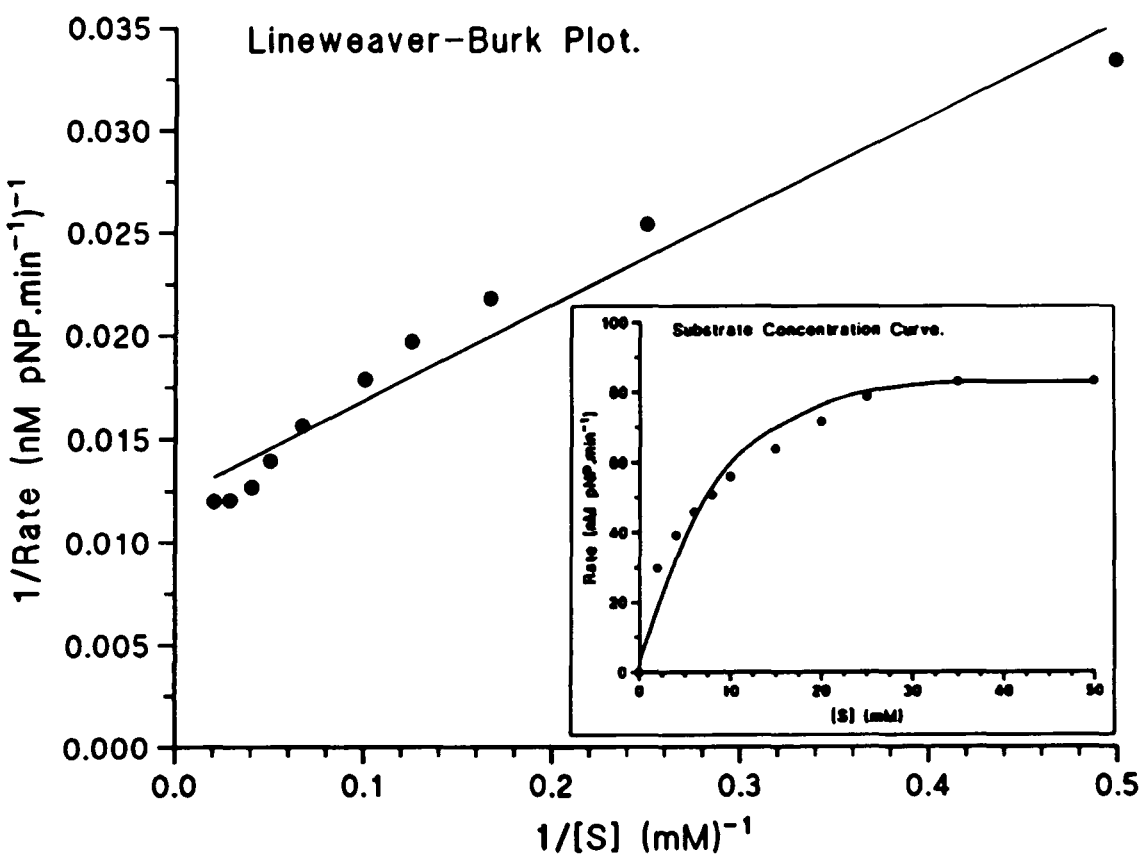


Figure 19. Lineweaver-Burk plot for β-galactosidase using p-nitrophenyl β-D-galactose as the substrate. The insert indicates the substrate concentration curve.

35°C, activity above this temperature decreased rapidly. The optimum pH for enzyme activity was determined as pH 4.0, although 90% of the activity was found between pH 3.8 and pH 4.3. Under the standard assay conditions, enzyme activity was linear with respect to extract volume up to 0.5 ml, and with regard to incubation time of up to 60 min.

The effect of the substrate concentration on the rate of reaction is shown in Figure 19. The k_m value was 3.76 mM by the Lineweaver-Burk plot using p-nitrophenyl-β-D-galactose as the substrate in the standard enzyme assay. Calculation of the k_m and V_{max} values was carried out using four different methods and the results are compared in Table 11.

3.1.3.5 α-Mannosidase.

The enzyme parameters of α-mannosidase from malted barley are given in Table 10. It was found that the optimal temperature for enzyme activity was between 45°C and 55°C. After 55°C enzyme activity rapidly decreases up to 65°C. The optimum pH for enzyme activity in this assay system was determined at pH 4.7, although 90% of the optimal activity remained between pH 4.3 and pH 5.3. Under the standard assay conditions the enzyme activity was linear with respect to incubation time of up to 60 min and was also linear with regard to extract volume of up to 0.3 ml.

The effect of substrate concentration on the rate of reaction is shown in Figure 20. The k_m value was calculated at 5.04 mM by the Lineweaver-Burk plot using p-nitrophenyl-α-D-mannose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} values was carried out using four different methods, these values are given in Table 11.

3.1.3.6 β-Mannosidase.

The enzyme parameters of β-mannosidase from malted barley are given in Table 10. The optimal temperature for enzyme activity was at 55°C, although the enzyme was 90% active between the range 45°C

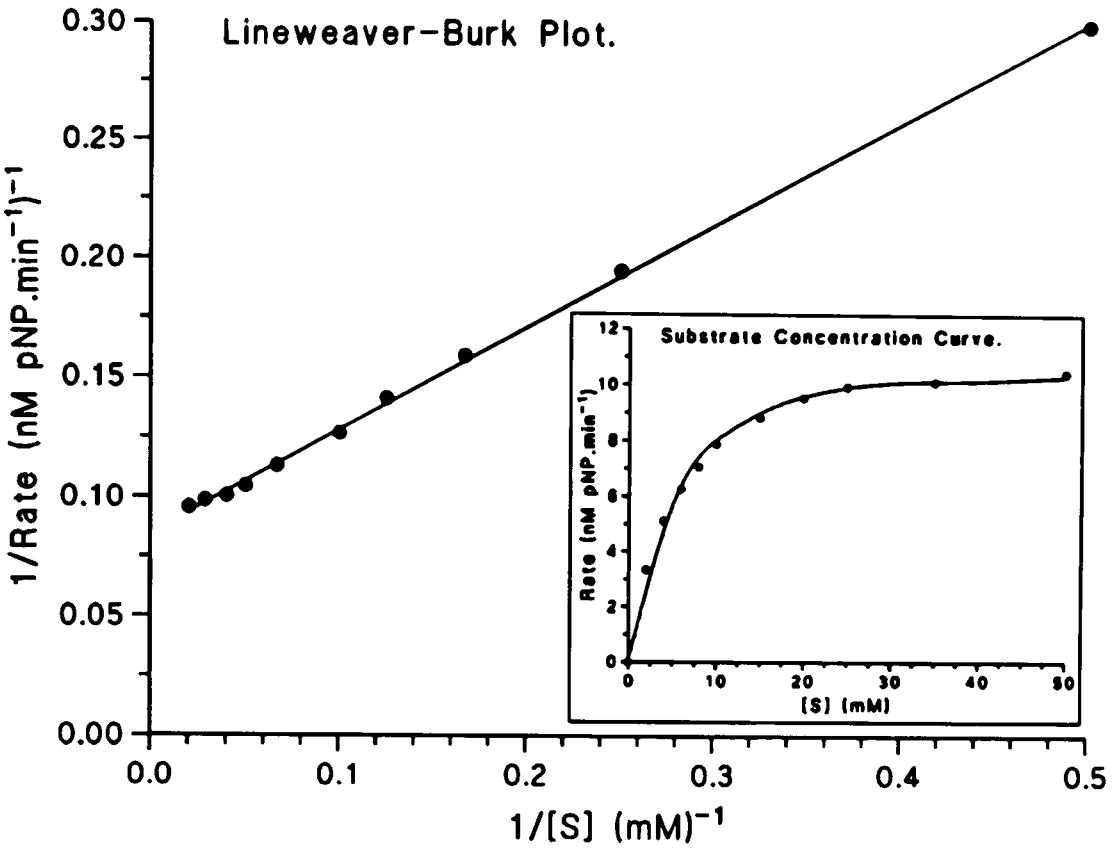


Figure 20. Lineweaver-Burk plot for α-mannosidase using p-nitrophenyl α-D-mannose as the substrate. The insert indicates the substrate concentration curve.

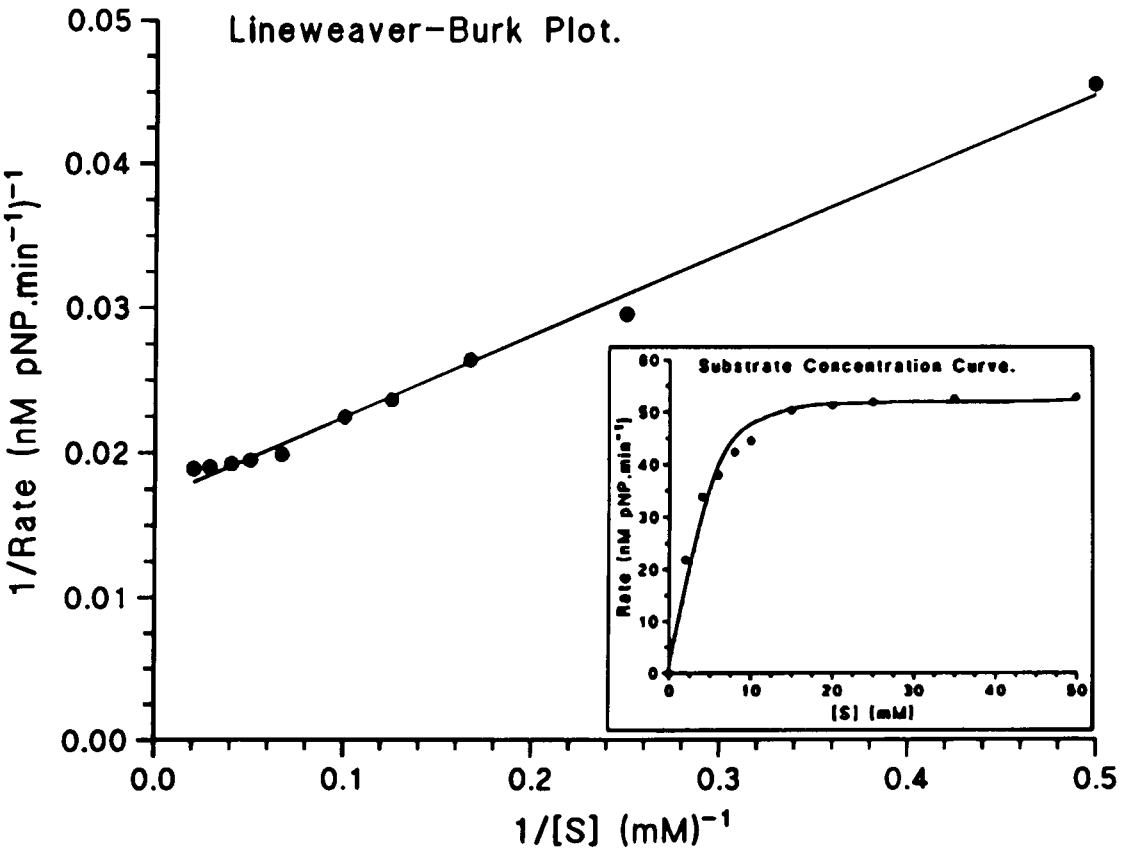


Figure 21. Lineweaver-Burk plot for β-mannosidase using p-nitrophenyl β-D-mannose as the substrate. The insert indicates the substrate concentration curve.

and 65°C. After 65°C there was a rapid loss in enzyme activity. The pH optimum was in the range pH 4.3 to pH 5.6, with all pH values in this range giving a similar enzyme activity. Under the standard assay conditions the enzyme activity was established as linear with respect to extract volume up to 0.4 ml and linear with incubation time up to 60 min.

The effect of substrate concentration on the rate of reaction is shown in Figure 21. The k_m value was 3.34 mM by the Lineweaver-Burk plot using p-nitrophenyl-β-D-mannose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} values was determined using four different methods, these are compared in Table 11.

3.1.3.7 β-N-Acetyl-glucosaminidase.

The enzyme parameters of β-N-acetyl-glucosaminidase from malted barley are given in Table 10. The optimum temperature for enzyme activity was determined at 55°C, with rapid loss of activity after this temperature. The optimal pH for enzyme activity was found to be at pH 4.9, although 90% of the optimal activity remained between pH 4.7 and pH 5.5. Under the standard assay conditions the enzyme activity was linear with respect to extract volume up to 0.5 ml, and with regard to incubation time of up to 60 min.

The effect of substrate concentration on the rate of reaction is shown in Figure 22. The k_m value was determined as 1.54 mM by the Lineweaver-Burk plot using p-nitrophenyl-β-N-acetyl-glucosamine as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} values was carried out using four different methods, the results are given in Table 11.

3.1.3.8 Arabinosidase.

The enzyme parameters of arabinosidase from malted barley are given in Table 10. It was found that the optimum temperature for enzyme activity was 55°C. The optimal pH for enzyme activity was determined at pH 4.6, although enzyme activity was similar throughout

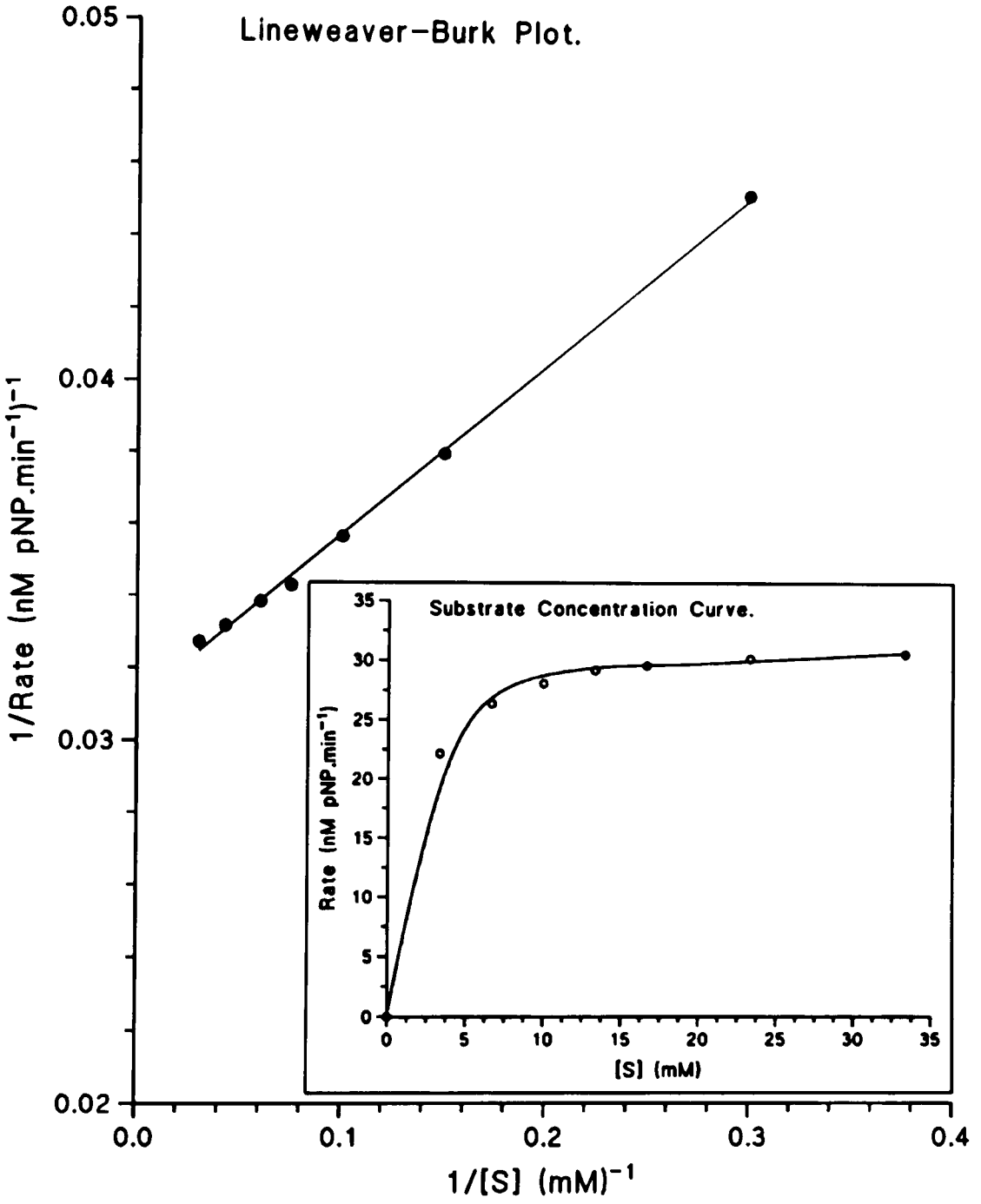


Figure 22. Lineweaver–Burk plot for β–N–acetylglucosaminidase using p–nitrophenyl β–N–acetylglucosamine as the substrate. The insert indicates the substrate concentration curve.

the range pH 4.2 to pH 5.2. Under the standard assay conditions the enzyme activity was linear with respect to extract volume up to 0.5 ml and with regard to incubation time of up to 45 min.

The effect of substrate concentration on the rate of reaction is shown in Figure 23. The k_m value was determined as 48.5 mM by the Lineweaver-Burk plot using p-nitrophenyl-D-arabinose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} values was carried out using four different methods, and these are compared in Table 11.

3.1.3.9 β-Xylosidase.

The enzyme parameters of β-xylosidase from malted barley are given in Table 10. The optimal temperature for this enzyme was determined to be at 55°C with activity decreasing rapidly at higher temperatures. The optimal pH for enzyme activity was pH 4.4, but activity was maintained in the range pH 4.2 to pH 5.0. Under the standard enzyme assay conditions the enzyme activity was linear with regard to incubation time of up to 30 min, and with respect to extract volume up to 0.3 ml.

The effect of substrate concentration on the rate of the reaction is shown in Figure 24. The k_m value was calculated as 4.80 mM by the Lineweaver-Burk plot using p-nitrophenyl-β-D-xylose as the substrate in the standard enzyme assay. Calculation of both the k_m and V_{max} values was carried out using four different methods and these are given in Table 11.

3.1.4 Other Enzyme Groups.

The suitability of other enzyme assays that had already been developed for systems other than malted barley, were also investigated. The two main groups of enzymes studied, were those able to hydrolyze phosphate containing compounds with the release of phosphate and protein hydrolyzing enzymes.

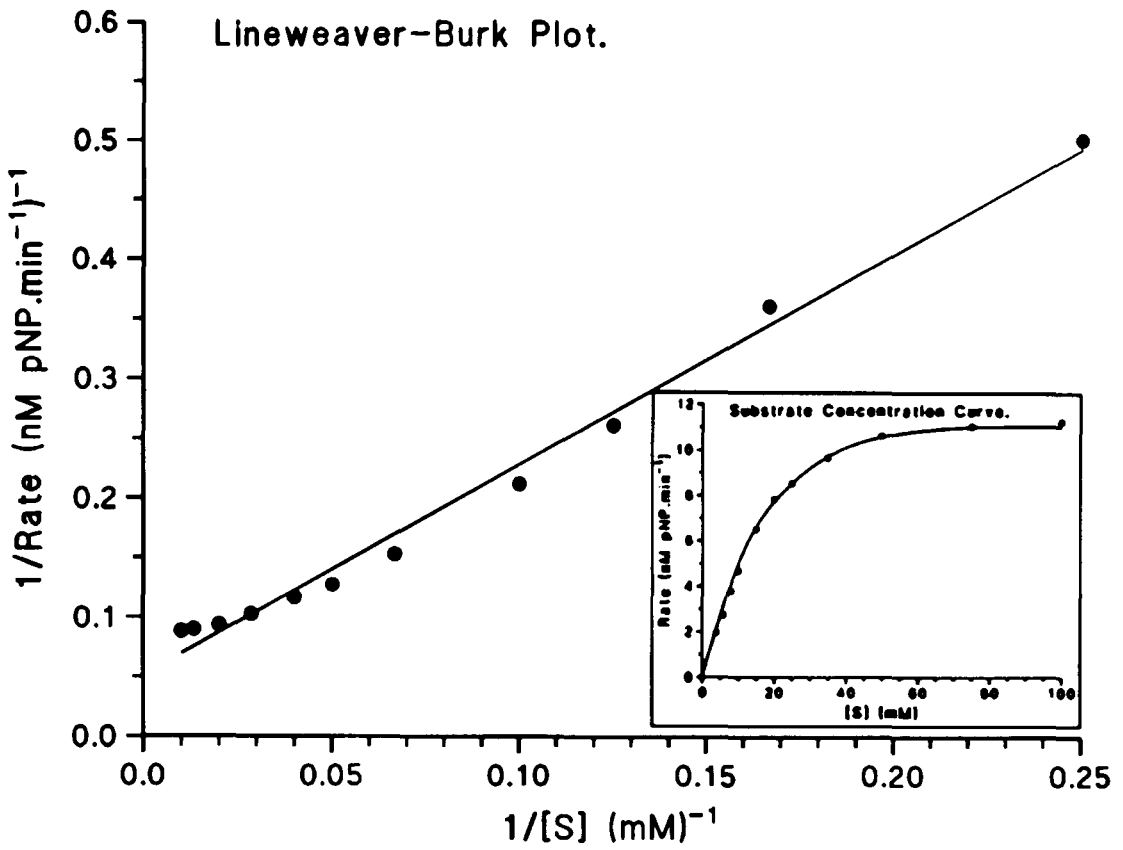


Figure 23. Lineweaver-Burk plot for arabinosidase using p-nitrophenyl arabinose as the substrate. The insert indicates the substrate concentration curve.

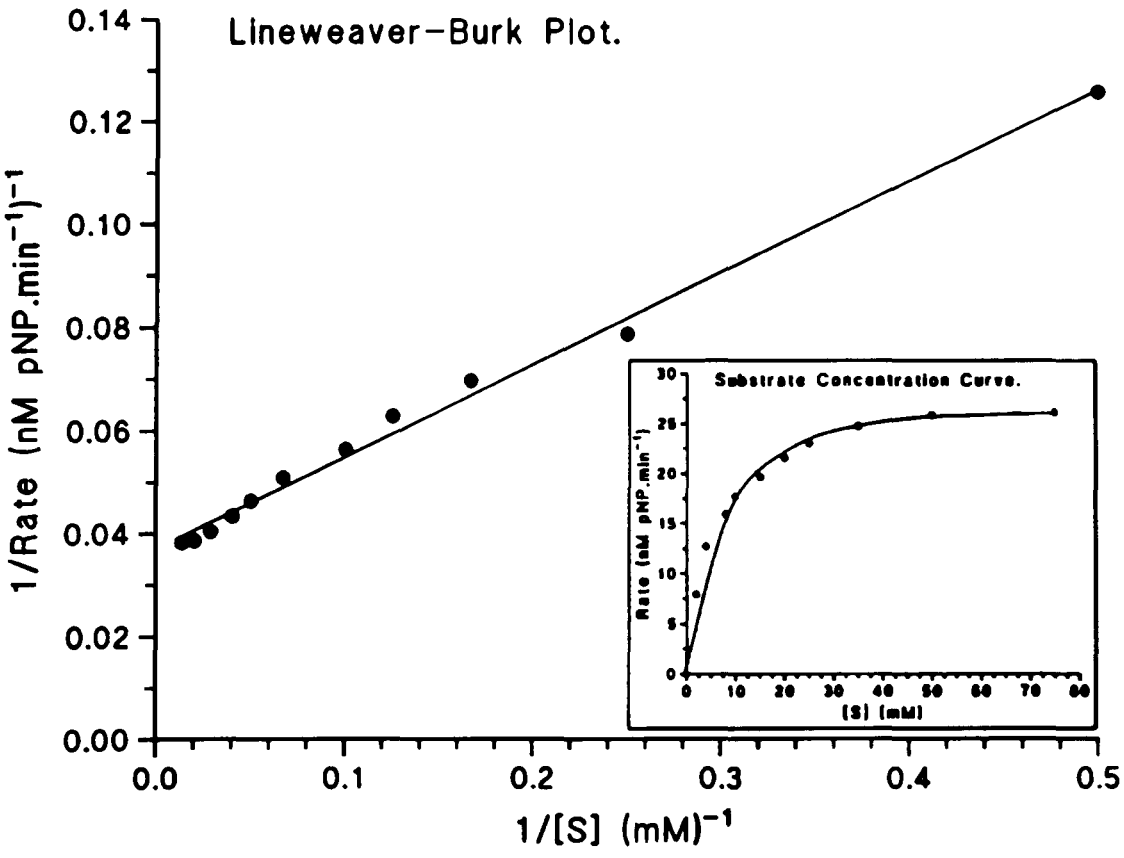


Figure 24. Lineweaver-Burk plot for β-xylosidase using p-nitrophenyl β-xylose as the substrate. The insert indicates the substrate concentration curve.

The enzymes, acid phosphatase and phytase were studied from the former group. It had previously been established, from initial experiments, that malted barley did not have alkaline phosphatase activity. The latter group was divided into those protein hydrolyzing enzymes that were specific for a particular group of protein substrates. These included serine protease, leucine aminopeptidase and carboxypeptidase A type activities. Protease type activity was also studied.

These selected non-carbohydrase enzymes were also found to be present in malted barley, under the assay conditions described previously (Sections 2.2.2.4 to 2.2.2.9).

3.2 Comparison of Amylase Activities From Different Sources.

3.2.1 α-Amylase Activities.

The hydrolysis of starch by α-amylase enzymes varies depending on the origin or preparation source of the α-amylase enzyme. It was therefore decided to study several commercially available α-amylases, comparing their activities using the Phadebas α-amylase assay, as developed for the malted barley enzyme. The substrate specificities of these enzymes were also studied using the p-nitrophenyl maltopentaose and p-nitrophenyl maltoheptaose substrates. The latter two substrates were carried out under similar conditions to the β-amylase assay for the malted barley enzyme, but 1 mM Ca^{2+} was substituted for the 10 mM EDTA. The α-amylase enzymes studied were as follows, *Bacillus licheniformis*, *B. subtilis*, Malted Barley, Porcine Pancreas and Human Saliva.

Buffer compositions are given in Table 4 and the results are presented in Table 12. Under the conditions of the assay used, the α-amylase from *B. licheniformis* was 98% active in buffer 1. Activity was 92% with added EDTA in the buffer system and 97% active in buffer system 4. The activity in buffer systems 5 and 6 was decreased to 85%.

Based on these results *B. licheniformis* α-amylase does not require calcium ions for activity, and EDTA has little effect on the enzyme. The presence of Cu^{2+} ions has only a limited effect on activity, although EDTA plus ascorbic acid induced a slight decrease in activity.

When p-nitrophenyl maltopentaose was used as the substrate it was found that no activity was present. The enzyme readily hydrolyzed p-nitrophenyl maltoheptaose.

α-Amylase from *B. subtilis* was found to have a requirement for calcium ions, since the activity in the buffer system 1 was reduced by 59%. The effects of EDTA, or EDTA in the presence of added ascorbic acid gave almost total inhibition with only 4% of the original activity remaining in each case. The addition of ascorbic acid alone

Table 12. Results from the α-amylase comparison study from different origins with Phadebas tablets as the substrate. Buffers used were 0.1 M Acetate, pH 5.5 with the additions shown. (Asc - Ascorbic Acid, p-NPG5 - p-nitrophenyl maltopentaose, p-NPG7 - p-nitrophenyl maltoheptaose).

Buffer Addition	*Bacillus licheniformis* (EU)	*Bacillus subtilis* (EU)	Malted Barley (EU)	Porcine Pancreas (EU)	Human Saliva (EU)
None	440.5	241.2	14.7	0.0	0.0
Ca^{2+}	448.4	586.0	35.9	41.5	72.5
EDTA	410.7	23.9	0.0	0.0	0.0
Asc + Ca^{2+}	434.7	579.0	55.0	13.4	22.6
Asc + EDTA	384.7	23.2	7.5	0.0	0.4
Cu^{2+}	382.2	563.4	17.0	4.4	6.0
Ca^{2+}, p-NPG5 as substrate	0.0	0.0	3.3	0.6	0.2
Ca^{2+}, p-NPG7 as substrate	9.4	1.1	2.1	1.8	1.0

to the buffer system had no affect the activity, as did the presence of Cu^{2+} ions. No activity was found when p-nitrophenyl maltopentaose was used as substrate. p-Nitrophenyl maltoheptaose was hydrolyzed by the *B. subtilis* α-amylase.

As reported previously, the α-amylase from malted barley has a requirement for added Ca^{2+} for optimum activity. Removal of the Ca^{2+} ions by the chelating agent EDTA, resulted in no activity. Addition of both ascorbic acid and EDTA does not result in total inhibition but activity is reduced by 80%. The addition of Cu^{2+} ions to the buffer has an inhibitory effect on the enzyme, reducing activity by 53%. Activation of the enzyme is found by ascorbic acid in the presence of Ca^{2+}.

Substrate hydrolysis of both p-nitrophenyl maltopentaose and p-nitrophenyl maltoheptaose took place with this α-amylase enzyme. Approximately 50% of the activity of this crude α-amylase preparation consists of β-amylase and utilization of p-nitrophenyl maltopentaose would be expected.

Both of the α-amylase studied from mammalian sources, porcine pancreas and human saliva had very similar activities. Both were dependent on added Ca^{2+} in the buffer solution, as there was no activity in buffer systems 1 and 3. Ascorbic acid addition in the presence of Ca^{2+}, reduces the activity of both enzymes by 60%. Little or no activity was found with buffer system 5, but Cu^{2+} ions reduced activity by approximately 90%.

The porcine pancreas and human saliva α-amylases were found to hydrolyze both of the p-nitrophenyl malto-oligosaccharides substrates. The p-nitrophenyl maltoheptaose was hydrolyzed more readily than the p-nitrophenyl maltopentaose substrate.

3.2.2 β-Amylase Activities.

Purified β-amylases were obtained from two sources, malted barley and sweet potato. The suppliers of the three malt β-amylases declared them to be standardized at 2000° Lintner. They were therefore suitable for the preparation of β-limit dextrin for the determination of α-amylase by the α-Amylase International Method (IoB 1982). Thus they were free from α-amylase activity.

This experiment was carried out using buffer systems with different compositions and also with different substrates. The buffer compositions are given in Table 5, while the results are shown in Table 13.

In the malted barley β-amylases, all three preparations suggested a slight contamination with α-amylase, this was observed from the Phadebas assays. Both sweet potato β-amylase enzymes had quite high levels of activity when using the Phadebas substrate. As expected, all gave activity with the substrate p-nitrophenyl maltoheptaose.

However, hydrolysis of this substrate was approximately 20% of the p-nitrophenyl maltopentaose substrate. The activity on the p-nitrophenyl maltoheptaose substrate was between 44% and 68% of the activity of p-nitrophenyl maltopentaose with the sweet potato β-amylases.

Table 13. Results from the β-amylase comparison study from different origins, with p-nitrophenyl maltopentaose as substrate. Buffers used were 0.1 M Phosphate, pH 7.1 with the additions shown. (Asc - Ascorbic Acid, pNPG7 - p-nitrophenyl maltoheptaose).

Buffer Addition	BDH Malted Barley (EU)	Cloder Malted Barley (EU)	Serva Malted Barley (EU)	BDH Sweet Potato (EU)	BCL Sweet Potato (EU)
None	3.084	2.600	2.812	12.329	11.675
Ca^{2+}	2.939	2.516	2.661	12.274	11.675
EDTA	2.867	2.479	2.522	12.208	11.379
Asc	0.000	0.000	0.000	3.847	2.921
Asc + EDTA	3.886	3.417	3.399	11.923	11.228
Cu^{2+}	0.216	0.065	0.301	4.609	2.376
EDTA, p-NPG7 as substrate	0.498	0.537	0.640	8.426	5.684
Ca^{2+}, Phadebas as substrate	0.750	2.190	0.750	8.200	6.000

The malted barley β-amylases all gave similar results when studied with the six different buffer compositions. As previously reported there were slight differences in activity between buffer systems 1, 2 and 3. No activity was found when ascorbic acid was present in the buffer, but a combination of ascorbic acid and EDTA increased activity by approximately 20%-30%.

The results from the sweet potato β-amylases show that buffer systems 1, 2 and 3 have no effect on the overall activity, *i.e.*, added Ca^{2+} or EDTA has no effect on activity. Ascorbic acid does have an inhibitory effect, but is only found to decrease activity by 69%-75%. Ascorbic acid and EDTA, present in the same buffer system, do not have an activating effect on the sweet potato β-amylases. The presence of Cu^{2+} ions has less of an inhibitory effect than on the malted barley β-amylases.

3.3 Enzyme Activity From Micromalted and Production Malted Barleys.

3.3.1 Micromalted Barley Samples.

Samples of micromalted barleys from the varieties, Triumph, Kym and Golden Promise were kindly given by Moray Firth Maltings, Inverness. These barleys had been processed under different conditions to obtain a range of malt modifications, *i.e.*, under-modified, well-modified and over-modified. In addition, a set of samples at each modification level had been treated with gibberellic acid. This allowed a comparison of enzyme activities in the presence or absence of added gibberellic acid.

These barley varieties were selected because at the time of this particular study Triumph and Golden Promise were the major malting varieties grown in Scotland. Kym was a recent variety, and had gained recognition as a Scottish grown barley suitable for malting. All three are still classified as malting varieties suitable for Scotland, but are now becoming outclassed by other varieties.

Triumph was the mainstay Scottish variety since it was first listed in 1980. It has a short, stiff straw, with a good agronomical yield, but is late in maturing. Ear loss may be a problem at maturity, especially if the harvest is delayed.

Golden Promise is a rather old variety, being first introduced to the Institute of Brewing Recommended List in 1968. Although Golden Promise is an early ripening variety it is extremely prone to mildew and other diseases. This variety produces small grains of good malting quality and has good resistance to head and ear loss when harvested early.

Kym is a variety of medium malting potential, especially in early low ground situations. It is a long, weak strawed barley, with big grains and a good specific weight.

3.3.1.1 Malt Parameters.

Several malt parameters were analyzed from all the micromalted samples. These included fine and coarse hot water extracts, free

α-amino nitrogen and the degree of modification. Single determinations were carried out for the hot water extracts and fine/coarse differences. Free α-amino nitrogen analysis was carried out on single samples, but in duplicate.

From Figure 25, the results from the fine hot water extracts were of a higher value than the corresponding coarse hot water extract. This was evident for each sample irrespective of variety or treatment. The coarse hot water extract with or without gibberellic acid treatment for the varieties Kym and Golden Promise indicated an increase in extract from the under-modified to the over-modified sample. The treatment of Triumph with gibberellic acid also indicated a similar pattern. However, the pattern of results from the Triumph coarse hot water extract without gibberellic acid was different, with the maximum value found with the well-modified sample.

The results of the fine hot water extracts from Triumph and Golden Promise gave similar patterns through the modification range, with and without gibberellic acid treatment. Without gibberellic acid treatment the highest fine hot water extract value was the well-modified sample, followed by the under-modified sample which gave a slightly higher extract than the over-modified sample. When treated with additional gibberellic acid an increase in extract, from the under-modified to the over-modified sample was observed.

The fine extract from the variety Kym displayed a different pattern. Although the well-modified sample, without gibberellic acid treatment gave the higher fine extract in this group of samples, the over-modified extract was higher than the under-modified. Fine extract was greatest with the under-modified sample from the gibberellic acid treated series, followed by the over-modified sample and then the well-modified sample. The fine and coarse hot water extracts from both groups, *i.e.*, with or without gibberellic acid, showed that the variety Triumph had the greatest extract followed by Golden Promise and then the variety Kym.

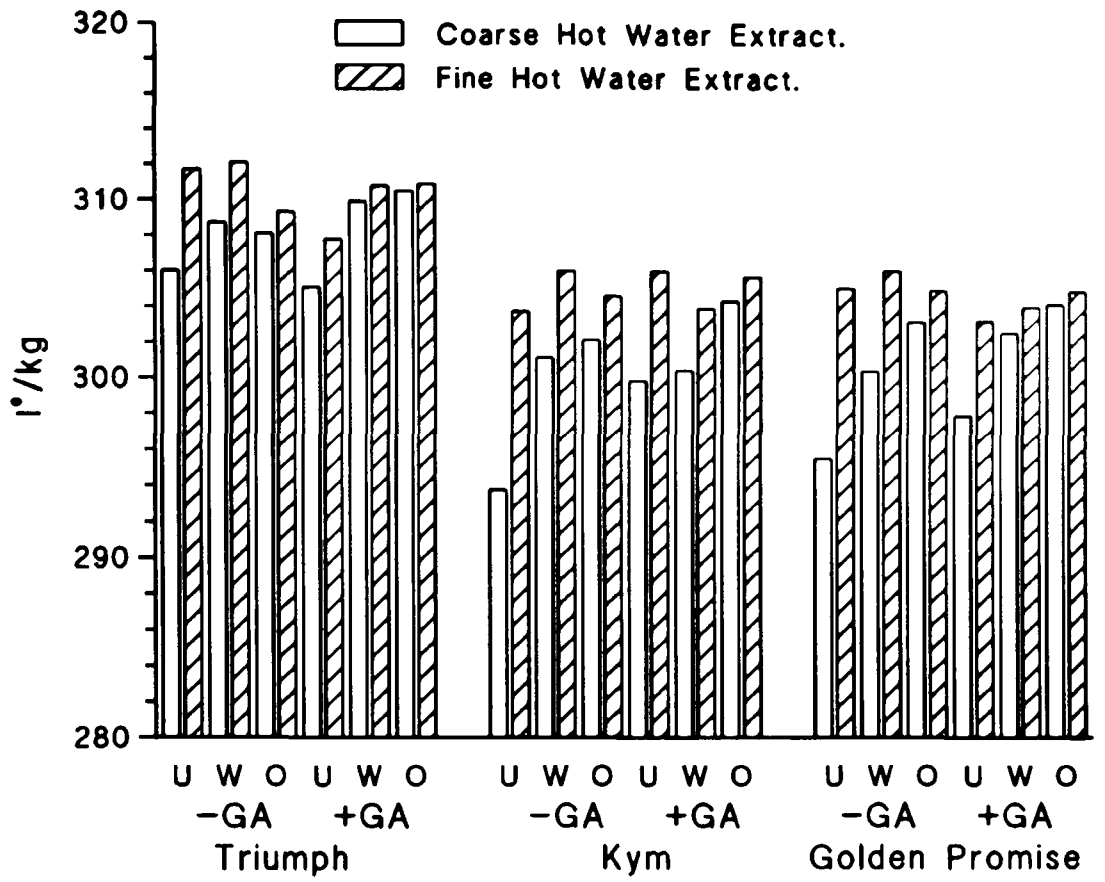


Figure 25. Extract values from coarse and fine hot water extracts from micromalted varieties under different malting regimes.

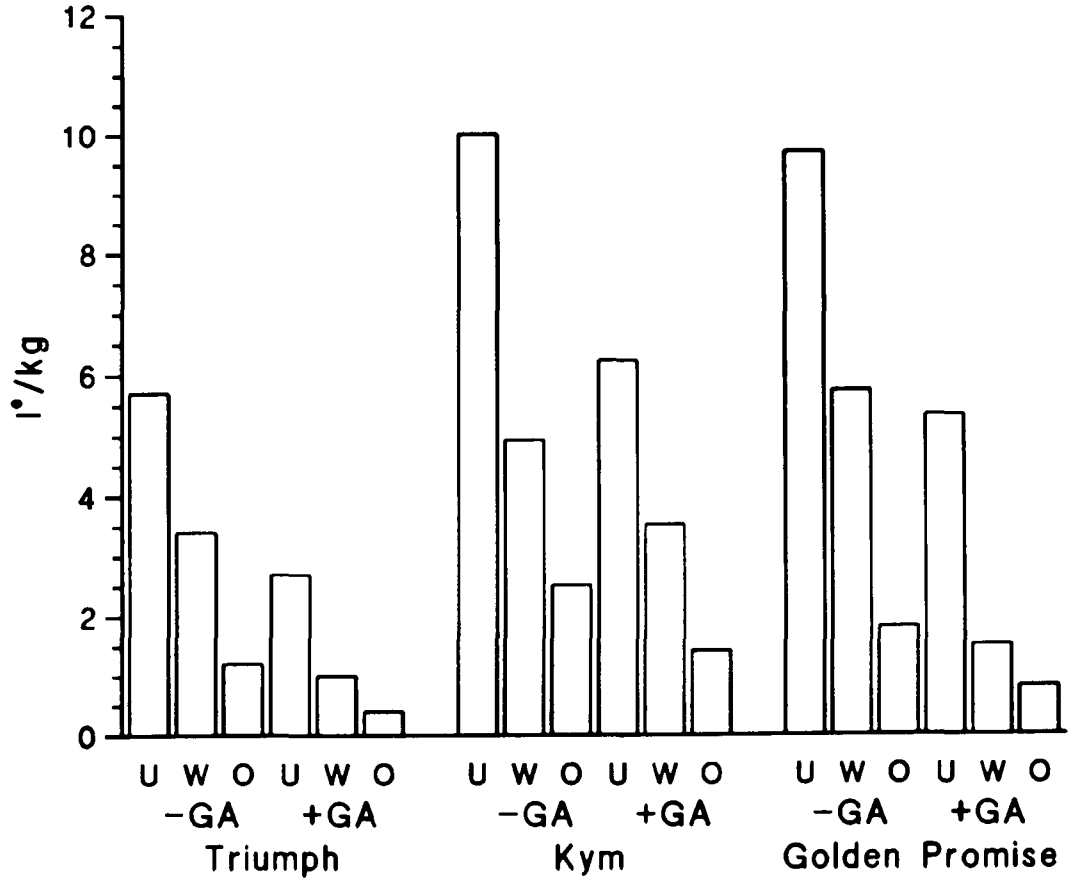


Figure 26. Fine/Coarse differences from the hot water extracts from the micromalted varieties under different malting regimes.

Fine/coarse difference analysis is a parameter used in deciding the degree of modification of a malted barley. The greater the fine/coarse difference, the more under-modified the malt. If the result is below a certain value then the malt may be categorized as over-modified. From Figure 26, the degree of modification of each sample can be determined. The results for Kym and Golden Promise suggest that the untreated gibberellic acid series is indeed under-, well- and over-modified. This is not so for the Triumph series. The effect of gibberellic acid treatment is emphasized, in that the modification levels were within the well- and over-modified classes. The steeping regime for these samples was identical, and was clearly not effective in achieving a modification range for the Triumph samples. However, the influence of the externally applied gibberellic acid was obvious on all three varieties.

Free α-amino nitrogen levels of the fine and coarse extracts indicates the levels of proteolytic activity, not only during mashing, but also during the germination stages of malting. Figure 27 shows the results obtained. The coarse extract, untreated gibberellic acid samples from the variety Kym gave a decrease in the free α-amino nitrogen level from the under-modified to the over-modified malts. Those from Triumph and Golden Promise peaked with the well modified malt sample, with the under-modified malt being higher than the over-modified sample. The gibberellic acid treated samples peaked with the well-modified malt, and again the under-modified samples were higher in free α-amino nitrogen than the over-modified malts. The fine hot water extracts, followed a similar pattern.

3.3.1.2 Enzyme Activity From Micromalted Barley Samples.

Enzymes were investigated from the micromalted samples, where applicable, under mashing-in conditions of temperature and pH, as determined previously. They were also studied under optimum enzyme activity. When these studies were carried out, the assays for β-amylase

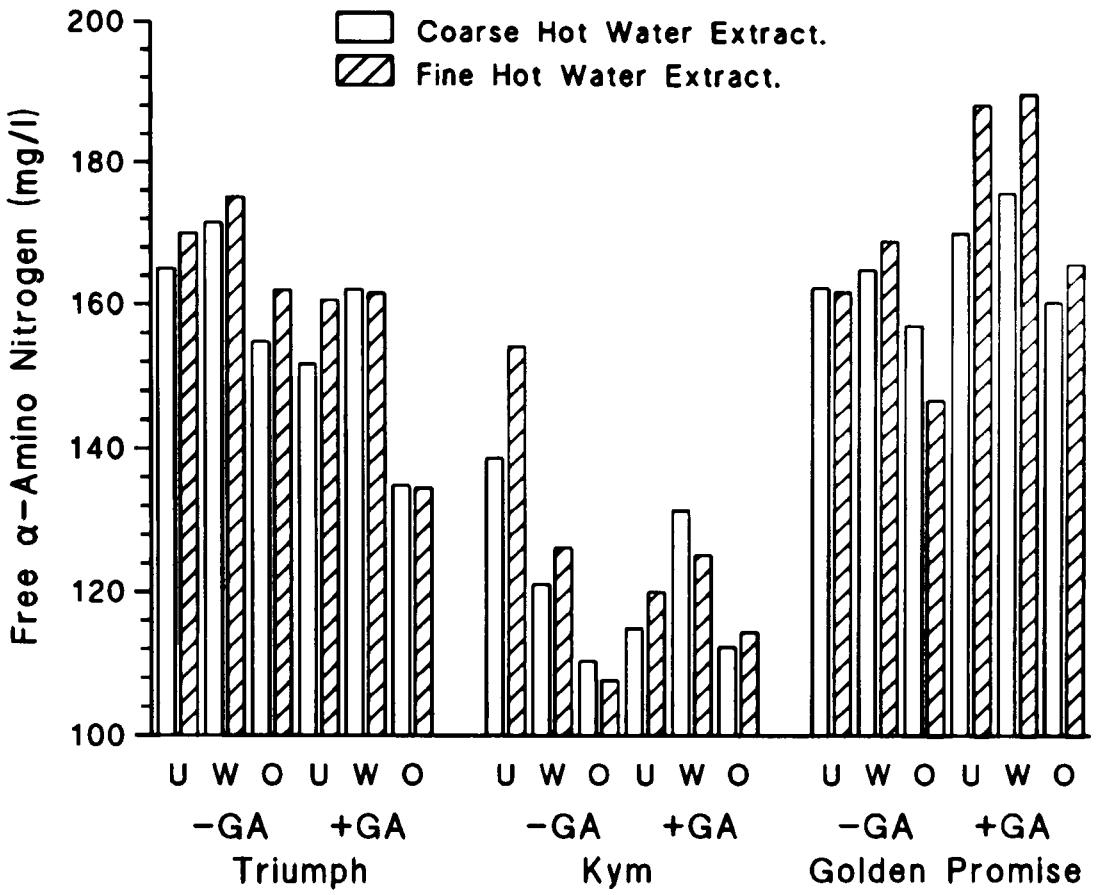


Figure 27. Free α-amino nitrogen levels from the coarse and fine hot water extracts from micromalted varieties under different malting regimes. (Pooled S.D. for Coarse = 7.201 and for Fine = 5.453)

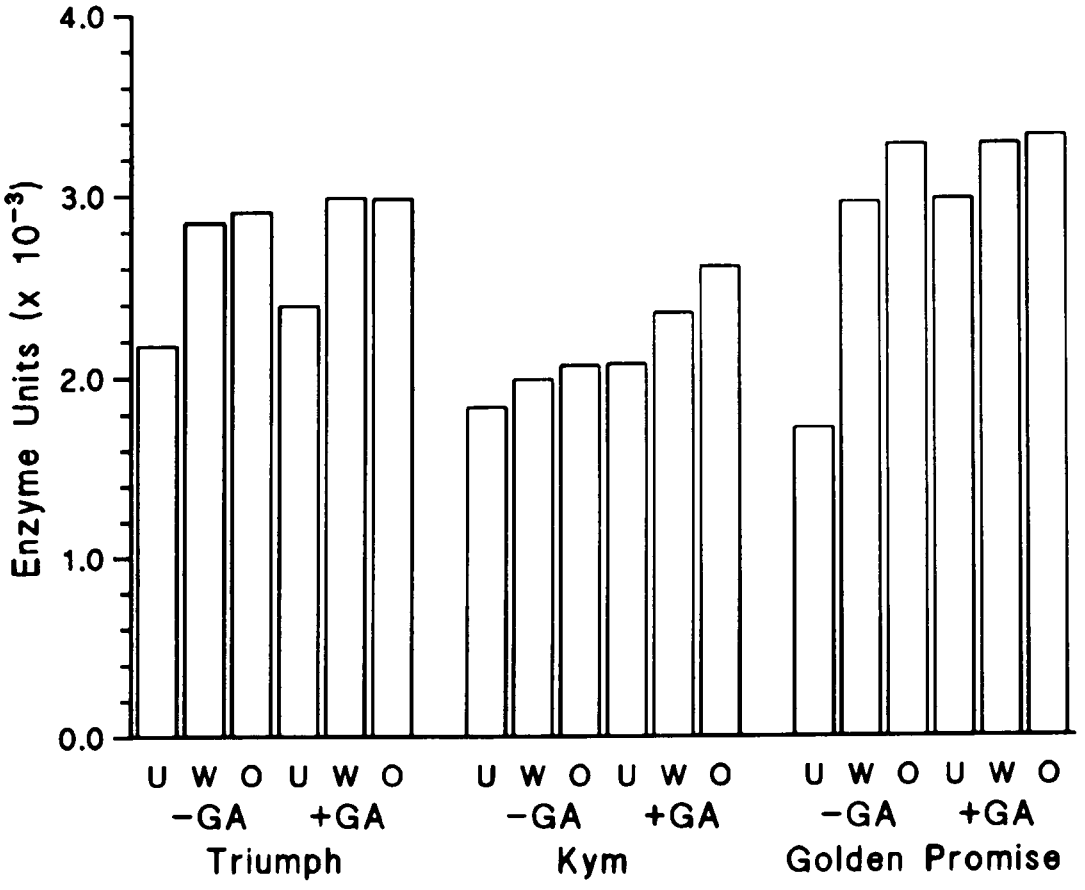


Figure 28. α-Amylase activity from the micromalted varieties under different malting regimes. (Pooled S.D. = 204.1).

and the general proteolytic activity had not been fully developed. It was not possible to carry out the studies on these two enzyme activities later due to inadequate sample sizes.

All enzyme analyses, for mashing-in and optimal conditions, were carried out on two samples from the same extract. Each sample was carried out in duplicate.

Amylases.

α-Amylase activities for the three malt varieties are given in Figure 28. Enzyme activities for the samples without gibberellic acid treatment suggest that the over-modified sample from all three varieties had the highest activity. The under-modified samples gave the lowest activity, whereas the well-modified malt had a slightly lower activity for Triumph (0.059 E.U.) and Kym (0.078 E.U.) than the over-modified malt. The difference between the well-modified and over-modified samples for Golden Promise was 0.321 E.U. The levels of α-amylase activity was greatest from the Golden Promise malt followed by Triumph and then by Kym.

α-Amylase activity was greatest in the samples with additional gibberellic acid. A similar pattern was identified from the micromalted malts with gibberellic acid treatment, the under-modified malt giving the lowest activity followed by the well-modified malt, and the over-modified malt the highest α-amylase activity. Activity differences were largest between the under-modified samples of the two groups from each variety. The level of α-amylase activity was highest from Golden Promise malt and lowest from Kym.

Minor Carbohydrases.

Under optimum conditions for the enzyme α-glucosidase, activity increased throughout the range of modification levels (Figure 29). This was observed with or without gibberellic acid treatment. When the two groups of malts from the same variety were compared, the Triumph malt samples with gibberellic acid treatment had greater α-glucosidase activity than those samples without gibberellic acid

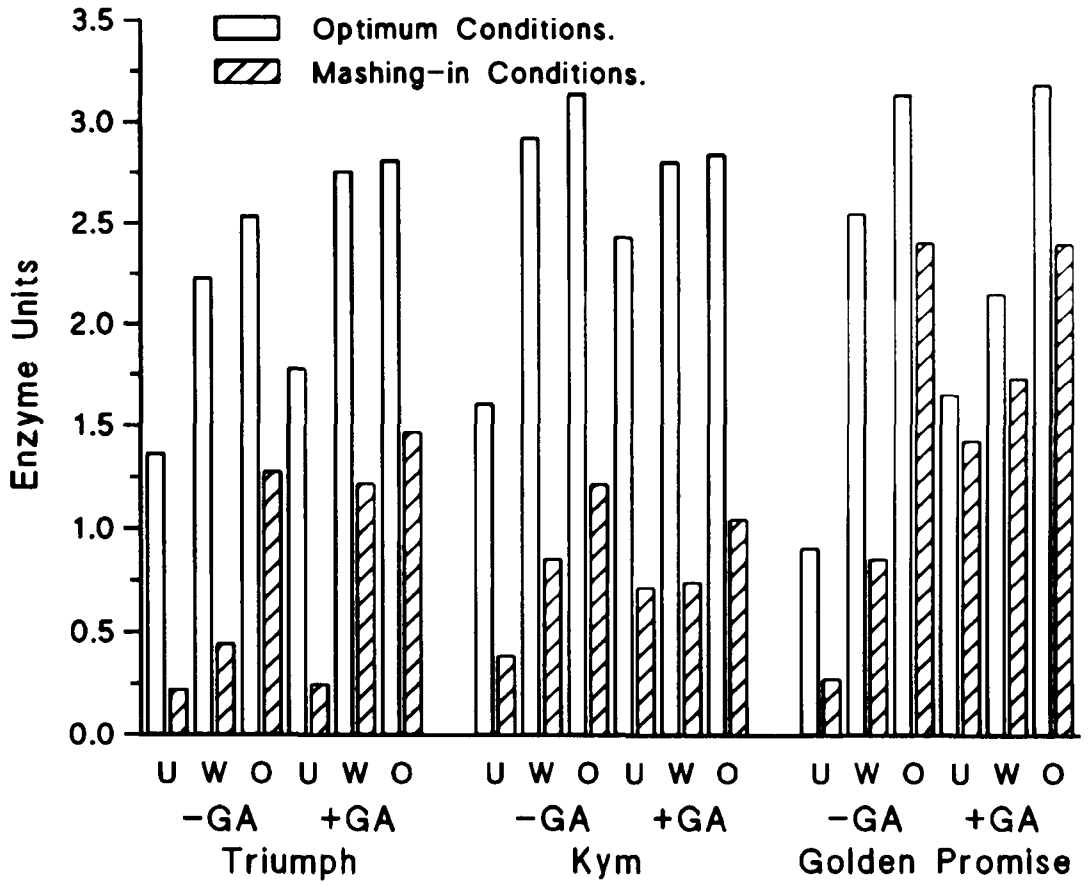


Figure 29. α-Glucosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 0.116 and for Mashing-in = 0.027)

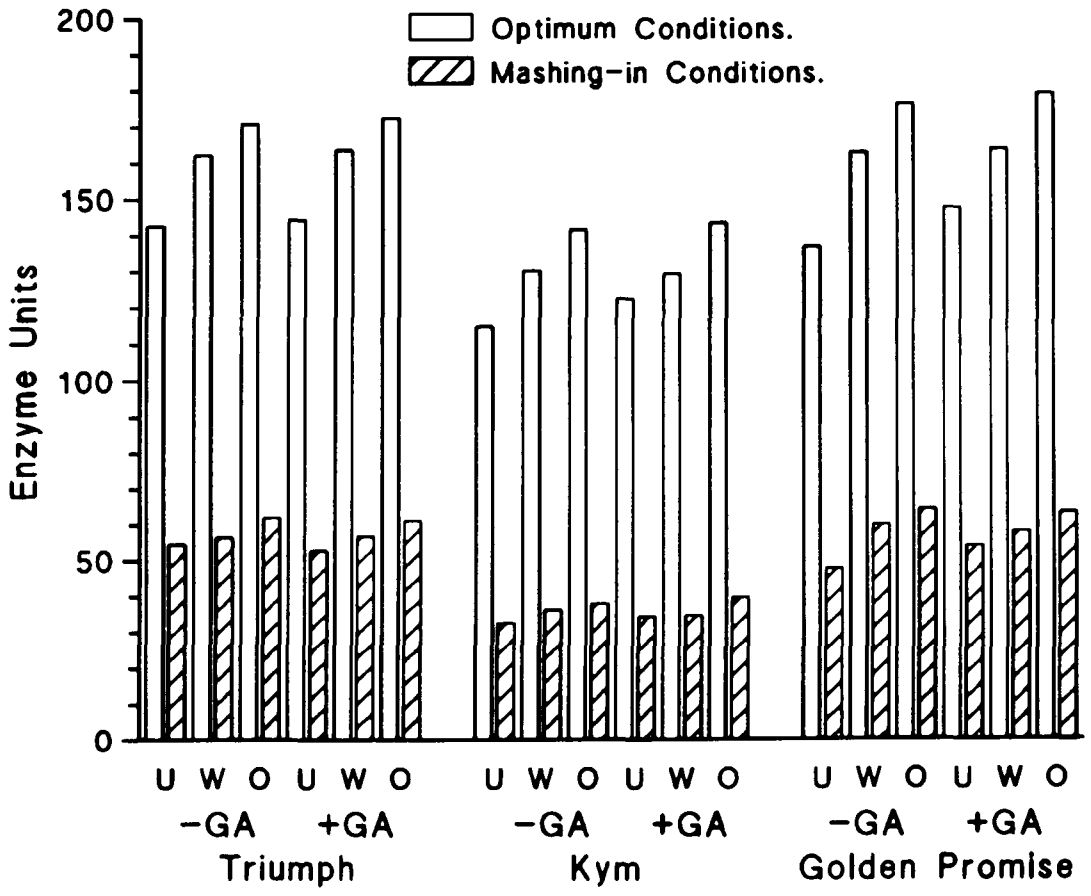


Figure 30. β-Glucosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. For Optimum = 9.182 and for Mashinig-in = 1.444)

treatment. The activity of the well-modified and over-modified gibberellic acid treated samples were decreased in comparison to no gibberellic acid treatment for the variety Kym. While the well-modified malt from the variety Golden Promise, had a lower activity with gibberellic acid treatment. Similar patterns to these results were obtained when α-glucosidase activity was determined under mashing-in conditions. However, the well-modified sample without gibberellic acid treatment was lower than with gibberellic acid treatment for the variety Golden Promise.

Enzyme activity for each malt sample was highest under optimum enzyme conditions. The variety Kym gave higher α-glucosidase activity for the under-modified and well-modified samples with or without gibberellic acid treatment. Golden Promise gave the greatest activity from the over-modified malts. When studied under mashing-in conditions the variety Golden Promise had the highest activity for well-modified and over-modified samples, with and without gibberellic acid treatment and for the under-modified malts with added gibberellic acid. The highest α-glucosidase activity without gibberellic acid treatment for the under-modified malts was shown by the variety Kym. Under optimal conditions, gibberellic acid treatment of Triumph, Kym and Golden Promise under-modified and over-modified malts gave increased α-glucosidase activity when compared to the non-treated malt samples.

Studies of the enzyme β-glucosidase under optimal enzyme conditions gave similar activity patterns within each malt variety, either with or without gibberellic acid treatment (Figure 30). β-Glucosidase activity increased with increasing malt modification. Well-modified and over-modified samples from the same variety showed little or no enhancement of activity with gibberellic acid treatment. A slight increase in activity was detected between the non-treated and treated gibberellic acid under-modified malt samples. The pattern of activity was similar using the mashing-in enzyme conditions.

However, a slight decrease in activity was noticed between the under-modified and well-modified malt samples with and without gibberellic acid treatment for the variety Triumph. A similar situation was found with the well modified malt sample from the variety Kym.

Under both sets of assay conditions for β-glucosidase, the variety Golden Promise gave the highest activity for the gibberellic acid treated samples. This was true for the well-modified and over-modified malt samples without gibberellic acid treatment. β-Glucosidase activity for the under-modified malt without gibberellic acid treatment was highest from the variety Triumph. The variety Kym had the lowest β-glucosidase activity from the three compared varieties. From the results in Figure 30 gibberellic acid treatment does not appear to have an influence on the activity levels of the enzyme β-glucosidase.

The activities of α-galactosidase from the varieties Kym and Golden Promise gave similar patterns of activity for the under-modified, well-modified and over-modified malts with and without gibberellic acid treatment, under optimum and mashing-in assay conditions (Figure 31). In both treatments the α-galactosidase activity from Kym was greater than that from Golden Promise. Levels of activity, from the variety Triumph, under optimum assay conditions without gibberellic acid, suggested that the activity was highest with the well-modified sample and lowest with the under-modified malt. However, with gibberellic acid treatment the under-modified sample was highest, with the lowest α-galactosidase activity from the well-modified malt. The Triumph malt samples, under mashing-in conditions, indicated an increase in activity from the under-modified to the over-modified for both sets of treatments.

The variety Triumph gave the highest α-galactosidase activity under mashing-in conditions, although the activities were reduced by 30% from the optimum assay conditions. The activity was highest in Triumph, followed by Kym and finally by Golden Promise. Under optimum assay conditions Kym gave the greatest activity for the

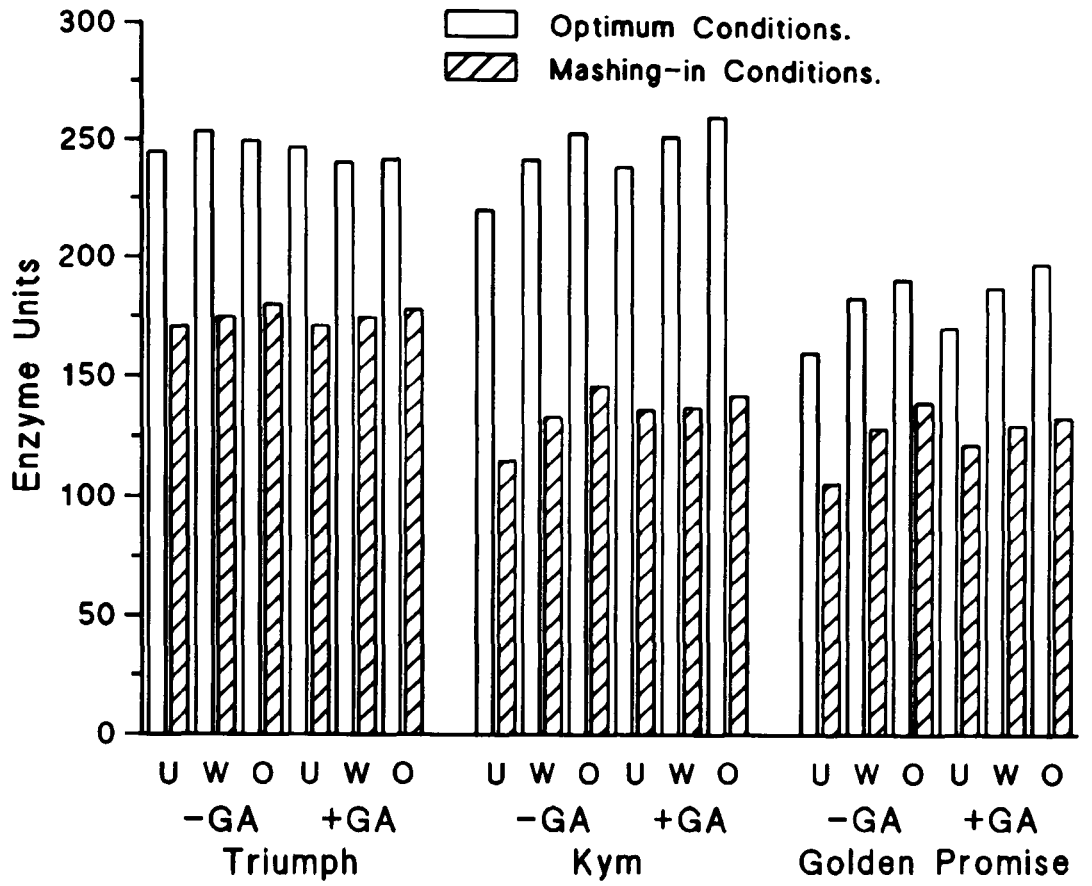


Figure 31. α-Galactosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 5.932 and for Mashing-in = 3.946)

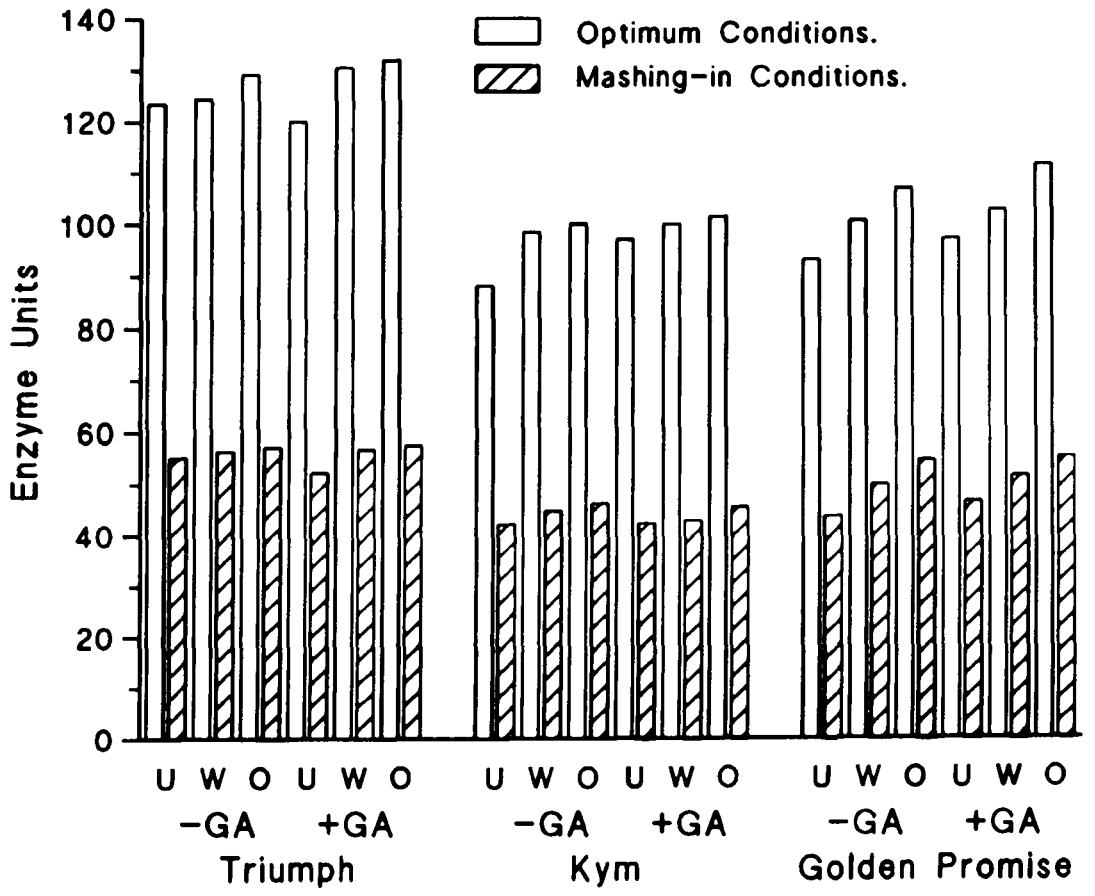


Figure 32. β-Galactosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 3.708 and for Mashing-in = 2.061)

well-modified and over-modified malt samples with gibberellic acid treatment. The highest activity for the under-modified malts was found from the variety Triumph. Kym also gave the highest activity for the over-modified malt without gibberellic acid treatment. Under-modified and well-modified malt samples from Triumph gave the highest activity at these modification levels for the non gibberellic acid treated samples. It would appear that gibberellic acid treatment, under optimal assay conditions induced α-galactosidase activity at all modification levels in Kym and Golden Promise, but only at the under-modified level from the variety Triumph. The effects of gibberellic acid treatment on the over-modified malt under mashing-in conditions from Triumph, Kym and Golden Promise suggest a slight decrease in activity.

Under both sets of assay conditions the activity of β-galactosidase was found to increase from the over-modified to the under-modified samples with or without gibberellic acid treatment for the three varieties (Figure 32). The enzyme activity from the variety Triumph was greatest under both sets of assay conditions, with or without gibberellic acid treatment, followed by Golden Promise and then Kym. Overall, gibberellic acid slightly stimulated the levels of β-galactosidase activity, except with the under-modified samples from the variety Triumph.

The enzyme α-mannosidase under both sets of assay conditions followed a similar pattern of enzyme activity (Figure 33). An increase in activity was detected from the under-modified to the over-modified malt samples. This was true for both sets of malt samples, either with or without gibberellic acid treatment. The levels of activity decreased by approximately 30%-40% between optimum assay conditions and the conditions for mashing-in. For the gibberellic acid malts, under optimum assay conditions, the activity was highest with Golden Promise for the well- and over-modified samples. The highest activity for the under-modified gibberellic acid treated malts was found with Triumph.

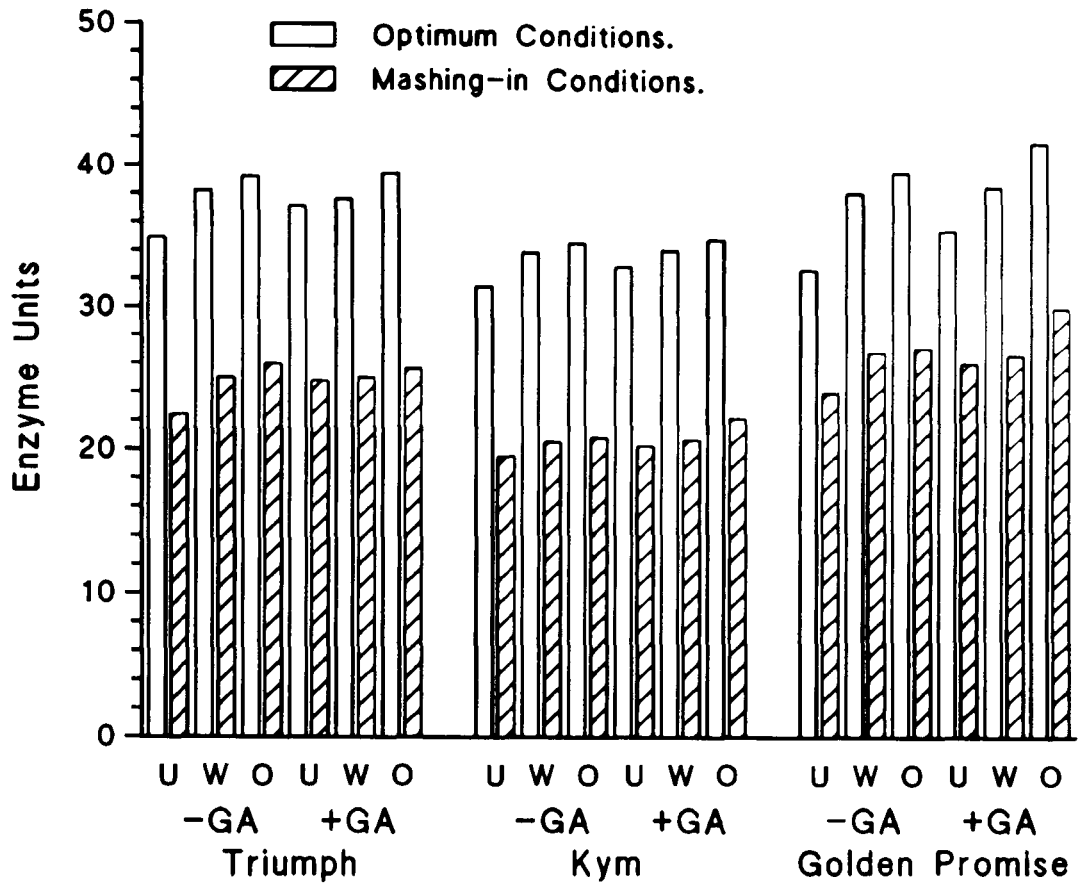


Figure 33. α-Mannosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 1.810 and for Mashing-in = 1.835)

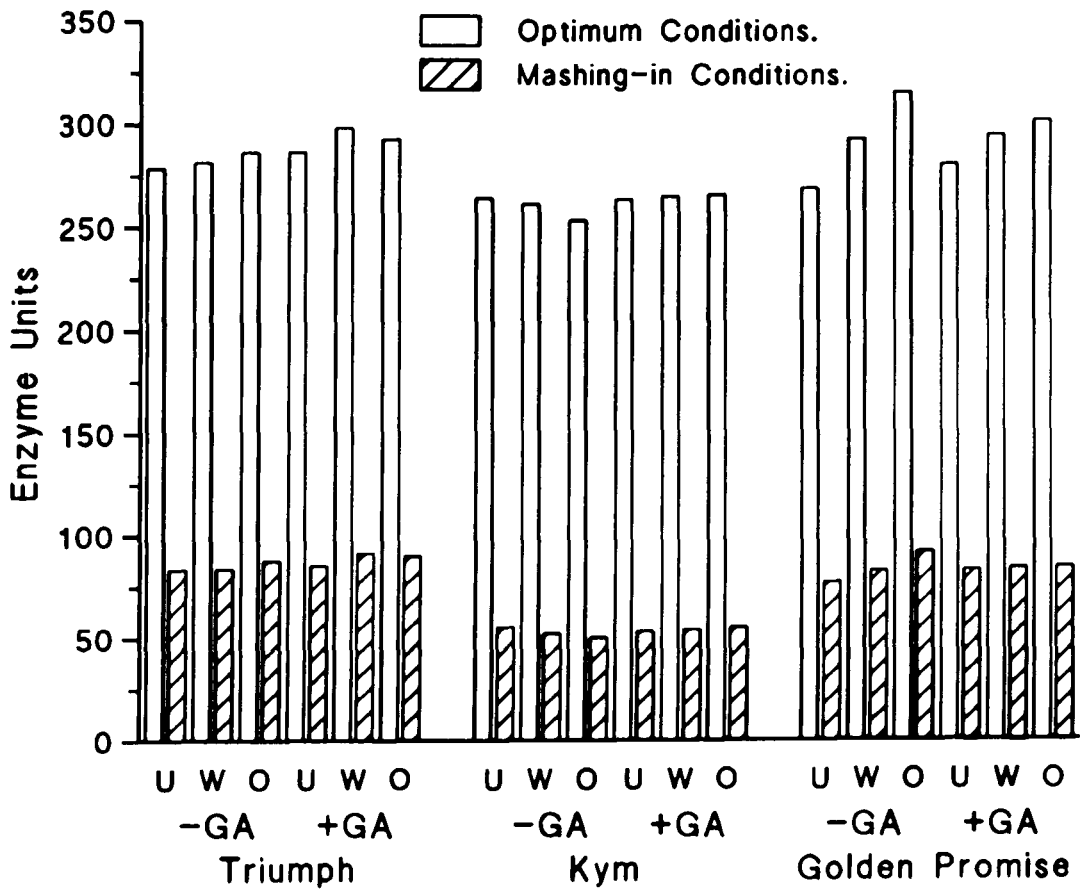


Figure 34. β-Mannosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 6.125 and for Mashing-in = 3.467)

A similar situation was found with the malt samples without gibberellic acid. Under mashing-in conditions Golden Promise gave the highest activity for the three modification levels, with both treatments. From these results it would appear that gibberellic acid treatment was slight but was a factor in enzyme stimulation, especially with the under-modified malts.

From Figure 34 activity levels for the enzyme β-mannosidase follow no set pattern between the varieties. However, a pattern can be detected within the individual varieties under both sets of enzyme assay conditions. The malt samples from Triumph, without gibberellic acid treatment, increased by 7.80 E.U. under optimal conditions and by 4.34 E.U. under mashing-in conditions with increasing modification level. However, when the malt samples are treated with gibberellic acid, they peak with the well-modified malt sample. With the variety Kym a decrease in activity was detected, as the modification level increased without gibberellic acid treatment. The opposite effect was found with gibberellic acid treatment, with activity levels increasing slightly with increasing levels of modification. The enzyme activity from the variety Golden Promise increased steadily with increasing modification, with or without gibberellic acid treatment. With both sets of assay conditions, the gibberellic acid treated samples generally gave increased levels of activity over the non gibberellic acid treated samples.

The level of β-mannosidase activity was lowest from the variety Kym for both treatments and all modification levels. Enzyme activities from the Triumph and Golden Promise samples were highest, depending on the modification level and treatment used. Gibberellic acid treatment of the varieties Triumph and Kym would appear to stimulate enzyme activity. However, with the over-modified Golden Promise malt samples activity was decreased, and slightly increased with the under-modified malt samples. The well-modified malt samples were similar within treatments from Golden Promise.

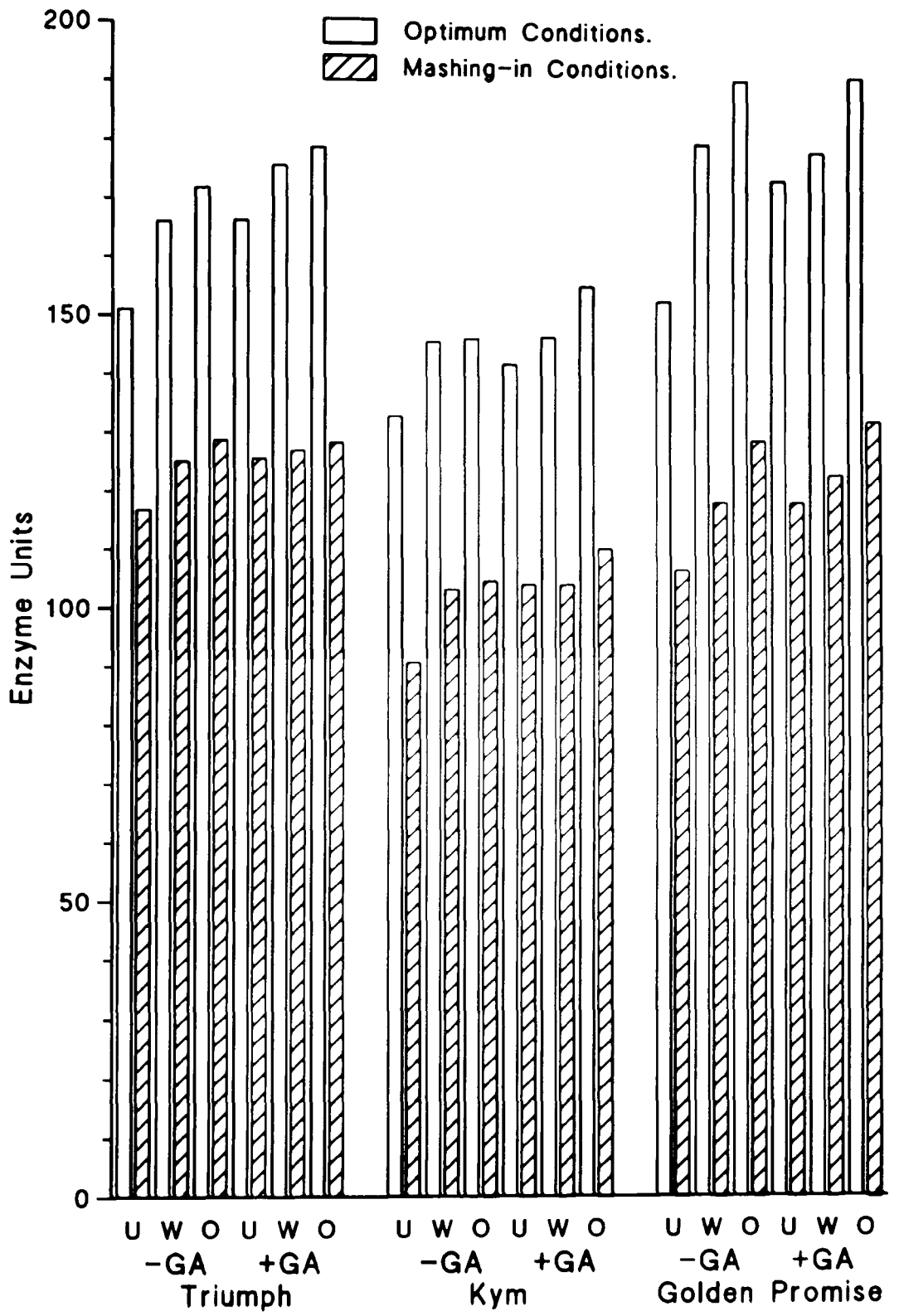


Figure 35. N-Acetlyglucosaminidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 4.386 and for Mashing-in = 4.873)

Figure 35 indicates N-acetyl-glucosaminidase activity levels for both sets of assay conditions. An increase in enzyme activity with increasing modification was observed with or without gibberellic acid treatment. Gibberellic acid stimulation was predominantly found with the under-modified malt samples. Under mashing-in conditions N-acetyl-glucosaminidase activity was greatest from the variety Triumph for the under-modified and well-modified malts. The over-modified enzyme levels were highest with Golden Promise. For optimum enzyme assay conditions the Golden Promise enzyme levels were highest in all samples.

Optimum assay conditions for the enzyme arabinosidase (Figure 36), show that the pattern of enzyme activity was similar from all three varieties. The variety Triumph gave the highest activity for both under-modified and over-modified malt samples from non-gibberellic acid and gibberellic acid treated micromalted samples. The activity of this enzyme increased throughout the modification levels with both treatments for all three varieties. The activity pattern of arabinosidase through the modification levels from the mashing-in assay conditions suggest an increase in activity from under-modified to over-modified. The activity of arabinosidase was highest from the variety Golden Promise for all samples for the mashing-in assay conditions.

The effect of gibberellic acid treatment on arabinosidase from the optimum assay conditions appeared to be very slight, except for the under-modified malt samples. However, with mashing-in assay conditions, gibberellic acid stimulated the range of modification levels for Triumph. For the varieties Kym and Golden Promise, although there was an appreciable increase in enzyme activity with the under-modified malt samples, a decrease in enzyme activity was observed with the well-modified and over-modified samples.

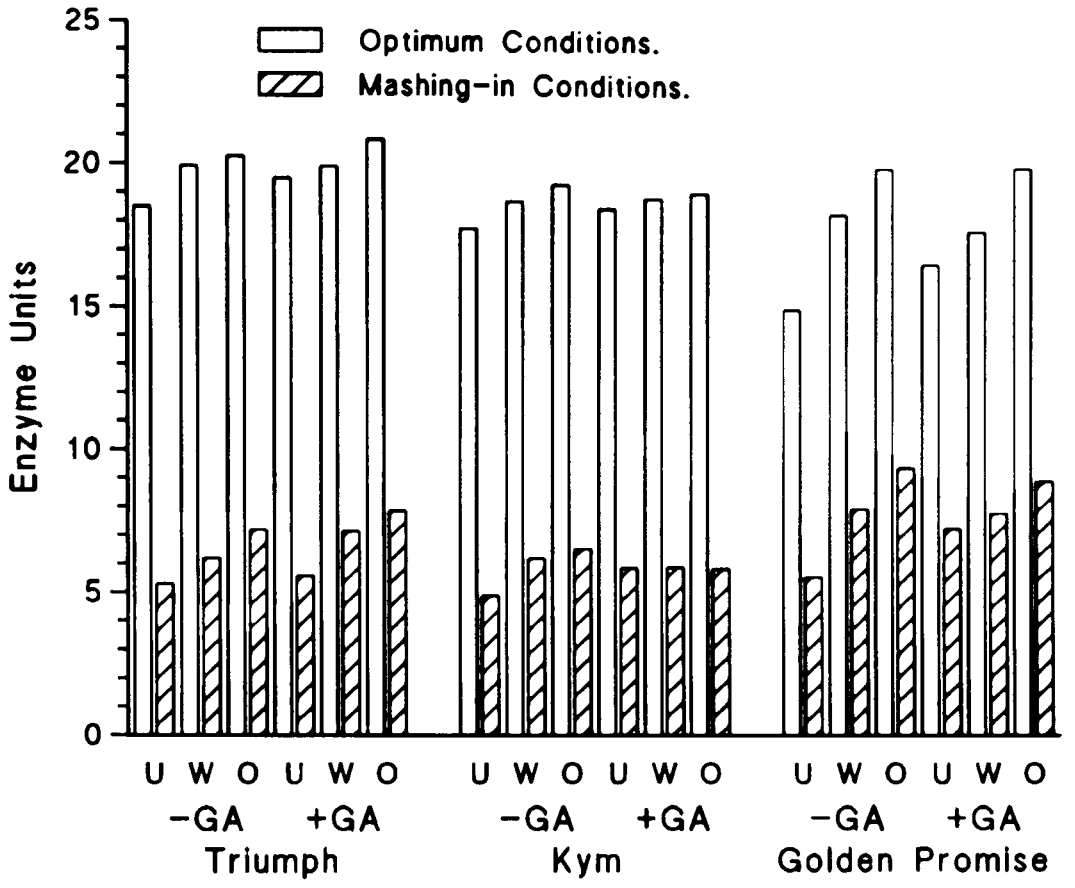


Figure 36. Arabinodase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 1.960 and for Mashing-in = 0.415)

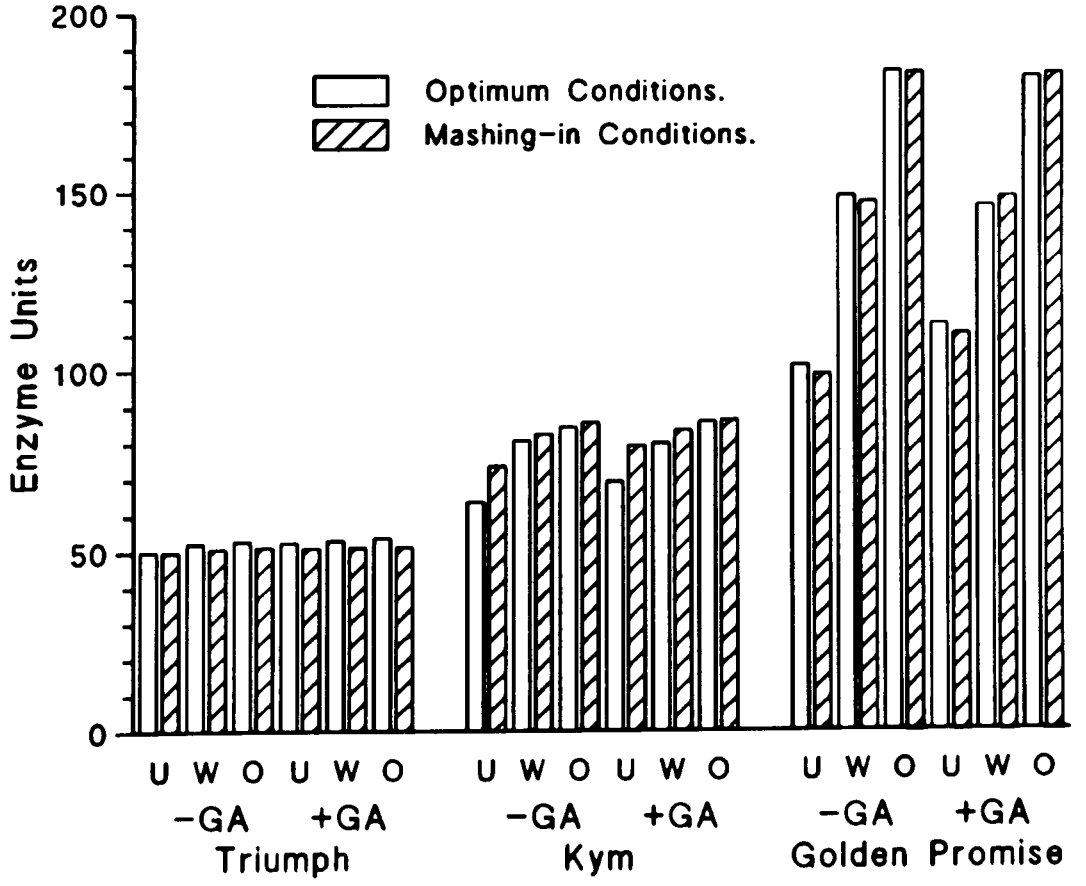


Figure 37. β-Xylosidase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 3.288 and for Mashing-in = 3.670)

Activity of the enzyme β-xylosidase was greatest from the variety Golden Promise, with or without gibberellic acid treatment, at all modification levels (Figure 37). Lowest β-xylosidase activity was from the Triumph malt samples at all modification levels. Activity of the enzyme for the Triumph samples gave a slight increase throughout the modification range for both treatments. The same pattern was also observed for Kym. The increase in β-xylosidase activity from the variety Golden Promise was larger between the modification levels. Gibberellic acid addition, gave no appreciable increase in enzyme activity in all three varieties under optimum or mashing-in assay conditions, except the under-modified samples where a slight increase was detected.

Phosphatases.

Acid phosphatase activity under both sets of assay conditions was greatest for the variety Triumph, and least for the variety Golden Promise (Figure 38). An increasing pattern of acid phosphatase activity was observed throughout the modification levels from each variety with or without gibberellic acid treatment. Gibberellic acid treatment of the malt gave no visible stimulation of the acid phosphatase activity from all three varieties, except the under-modified malt samples from the variety Kym.

Phytase activity from the three malted barley varieties gave a similar pattern to that of acid phosphatase for the gibberellic acid treated malt samples, Figure 39. The activity from Golden Promise was higher than that of Kym in all the modification levels. Activity from the Triumph malt samples without gibberellic acid treatment, peaked with the well-modified malt sample. The levels of activity for the under-modified and over-modified Kym malt samples without gibberellic acid treatment gave a similar result, the well-modified malt sample gave the highest activity for this treatment. Again, gibberellic acid treatment of the malt samples gave no increase in the activity of phytase.

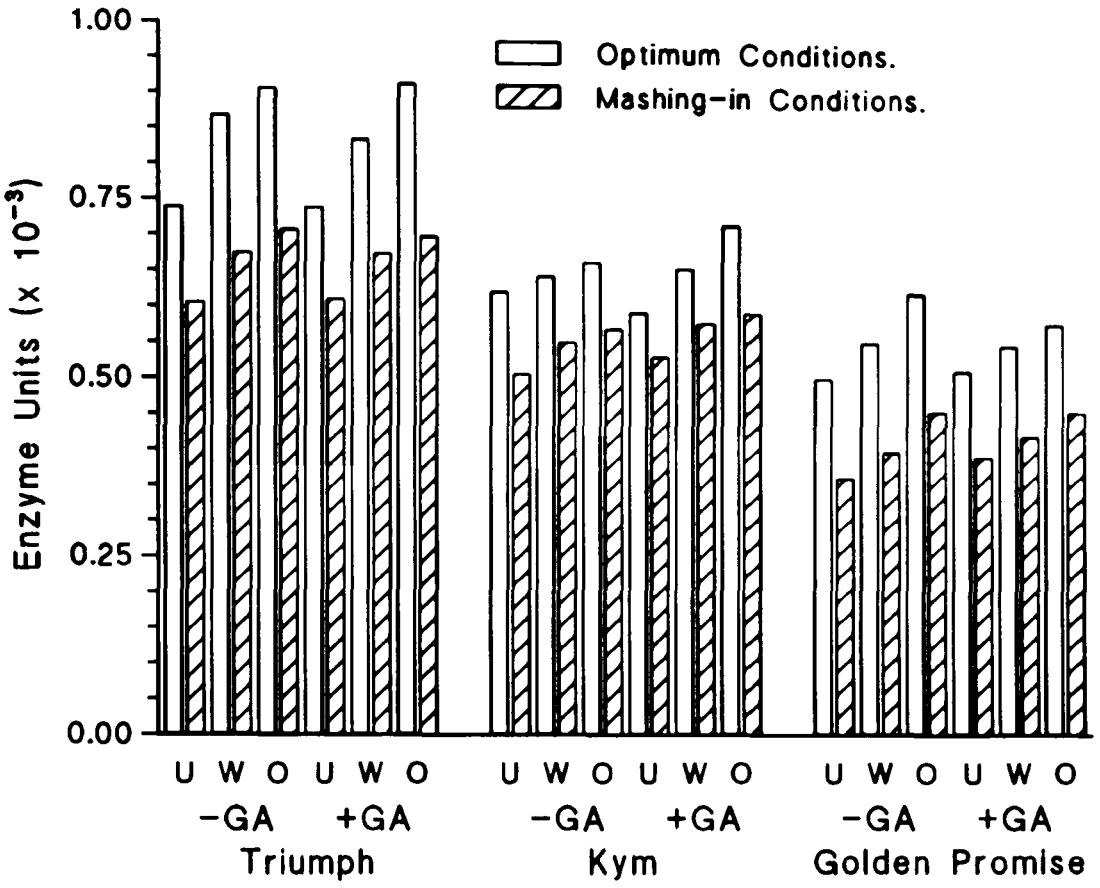


Figure 38. Acid phosphatase activity from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 56.901 and for Mashing-in = 43.630)

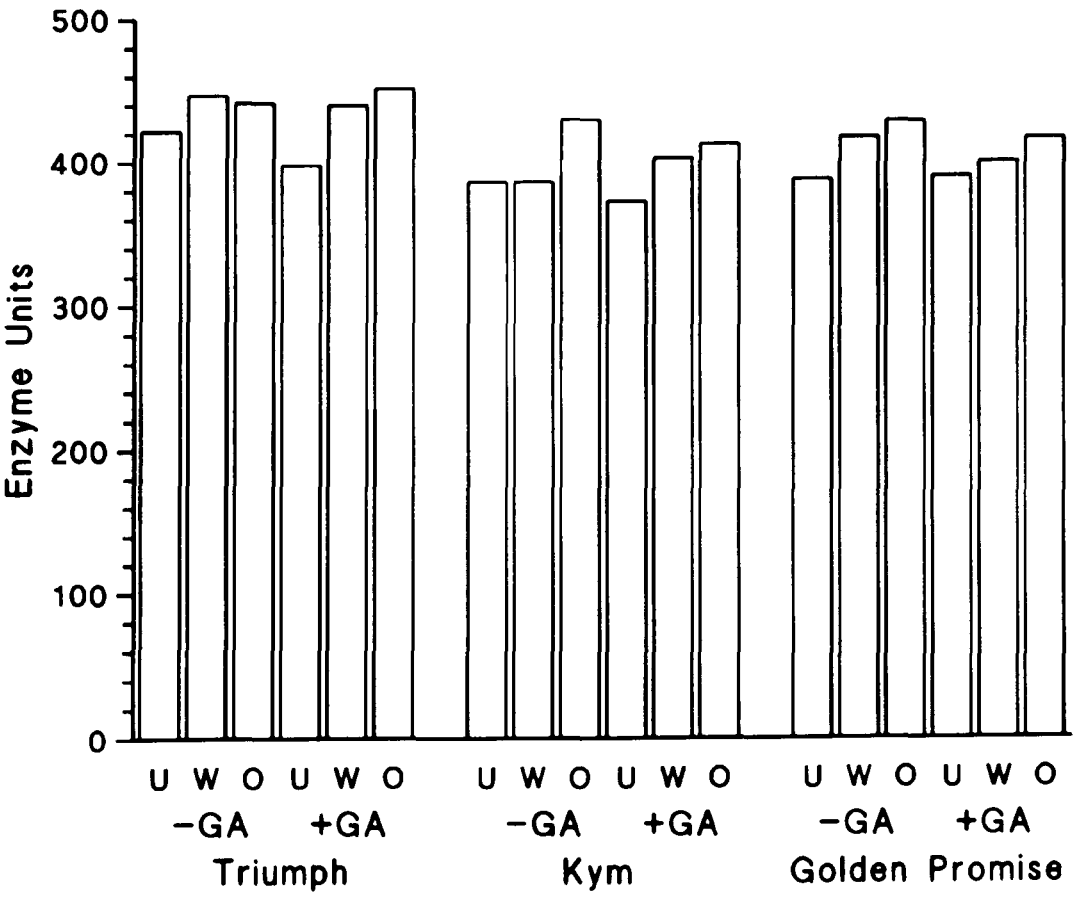


Figure 39. Phytase activity, optimum assay conditions, from the micromalted varieties under different malting regimes. (Pooled S.D. for Optimum = 48.576)

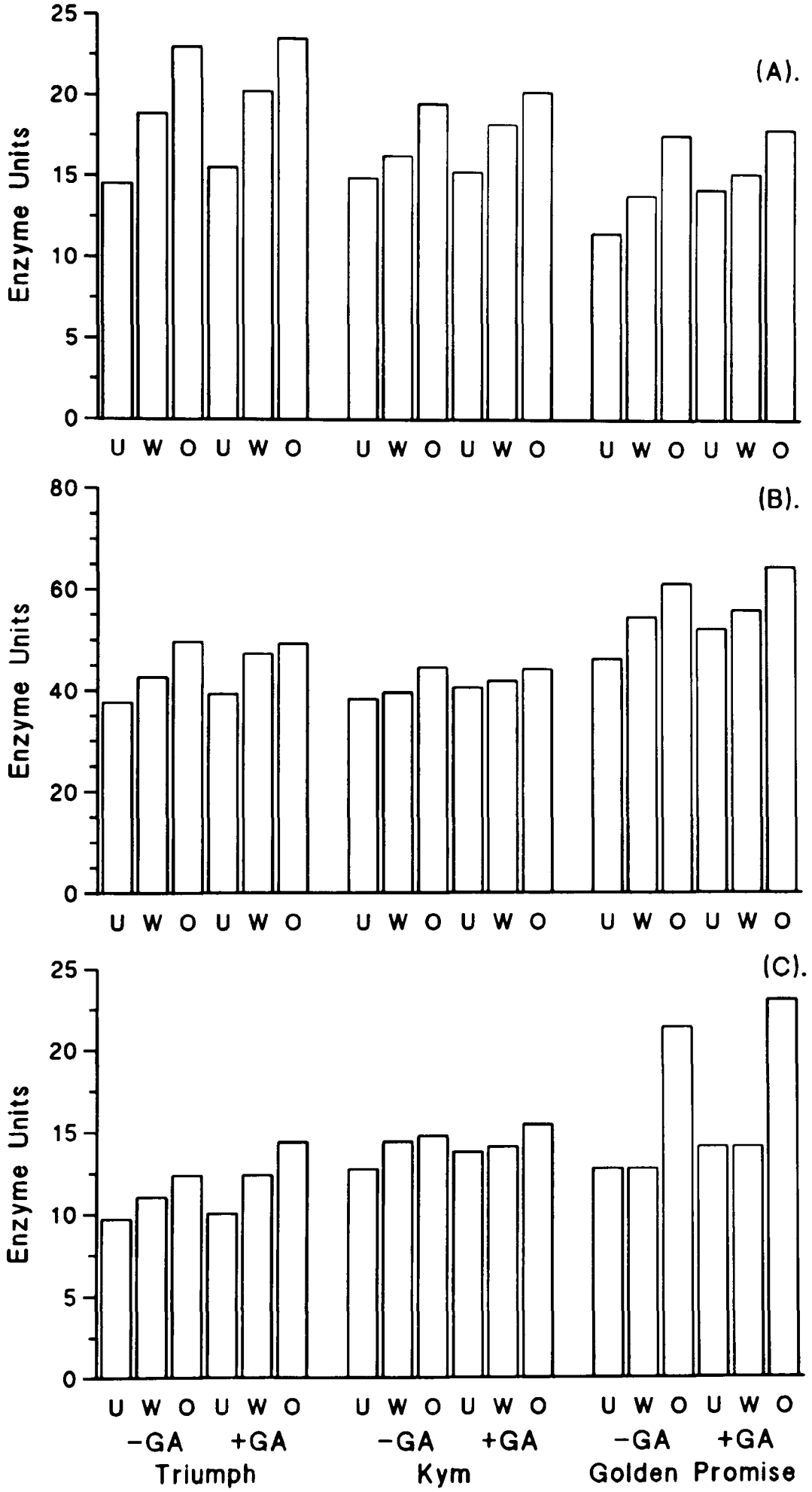


Figure 40. Activity of serine protease (A), leucine aminopeptidase (B) and carboxypeptidase A (C) type enzymes for optimum assay conditions from the micromalted varieties under different micromalting regimes. (Pooled S.D. for (A) = 1.380, for (B) = 2.102 and for (C) = 1.639)

Proteases.

Serine protease type activity was greatest from the variety Triumph, with the activity from Kym slightly higher than that of Golden Promise. The results are given in Figure 40. An increase in activity was observed from the under-modified to the over-modified malt samples either with or without gibberellic acid treatment. The gibberellic acid treated malt samples gave slightly higher levels of serine protease type activity than the non-gibberellic acid treated samples.

Leucine aminopeptidase type activity followed a similar pattern of activity through the modification levels to the serine protease type activity as shown in Figure 40. The variety with the highest leucine aminopeptidase type activity was Golden Promise. The activity for this group of enzymes was again slightly stimulated, but for the under-modified and well-modified samples with gibberellic acid treatment. Gibberellic acid treatment of the over-modified malt produced a decrease in activity when compared to the non gibberellic acid treated malts for Triumph and Kym. An increase in activity was detected with the Golden Promise over-modified, gibberellic acid treated sample.

Carboxypeptidase A type activity also followed a pattern similar to the other protease enzyme activities Figure 40. The activity from this enzyme group was also slightly stimulated by gibberellic acid treatment. Enzyme activity from the variety Kym was highest for the under-modified and well-modified malts, but Golden Promise had the highest activity for the over-modified samples. Activity from the Golden Promise under-modified and well-modified malt samples were comparable within treatments, but the gibberellic acid treated samples were slightly higher.

3.3.2 Production Malted Barley Samples.

Samples of malted barley were obtained from the nine Chivas distilleries. These samples were collected from six maltsters and

represented three non-blended varieties, Camargue, Pipkin and Triumph, and two blends Triumph/Halcyon and Triumph/Natasha. All samples were deemed representative of the malt being received at the distilleries during September - December 1988. Each sample was within the Chivas Brothers' malt specification.

This specification ensured that malt produced for the Company conformed with specified analytical parameters. The essential functions of this specification were to ensure that no processing problems were encountered within the Distilleries optimization of extract and spirit quality was of the required standard.

All of the 126 samples were analyzed for α-amylase, β-amylase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, N-acetyl-glucosaminidase, arabinosidase and β-xylosidase. Due to the number of samples, statistical analysis was carried out on certain groups. These groups consisted of Triumph samples from all six maltsters, Triumph samples from three maltsters using a larger range of samples. All the varieties (single and blended), and malted barley received from Maltster B, but comparing Pipkin, Triumph/Natasha and Triumph.

The statistical analysis carried out to decide if the results from the varieties or maltsters were significantly different was one-way analysis of variance. However, this was only the first step in the statistical analysis. The aim of these analyses was to decide which maltsters/varieties gave significantly higher levels of enzyme activities. The approach adopted for the second part of the analysis was that of Duncan's Multiple Comparison Test (Caulcutt and Boddy 1983).

So as to ensure the validity of the statistical analysis the results within a group were drawn at random. Cochran's test was performed on the population standard deviations (Caulcutt and Boddy 1983), and it was established that the population standard deviations were all below the table values at the 95% significance level within the groups.

3.3.2.1 Effects of Maltster.

Using the variety Triumph, the results from all six maltsters were analyzed using the one-way analysis of variance technique. For all enzymes except β-galactosidase, arabinosidase and β-xylosidase no significant difference was detected between maltsters. A similar result was obtained when Maltsters A, B and F were analyzed, but using a larger sample size. However, the following enzyme activities were significantly different, β-galactosidase, β-mannosidase, N-acetyl-glucosaminidase, arabinosidase and β-xylosidase.

By using Duncan's Multiple Comparison Test, the enzymic activity from the maltsters can be judged to be significantly different or similar. As the maltsters are ranked in terms of ascending average enzyme activity, those maltsters giving the highest enzyme activity can be determined. Results are given in Table 14 for Maltsters A to F supplying the variety Triumph. Those maltsters who show no significant difference are underlined.

For α-amylase, no significant differences were detected between Maltsters C, E and F and also between A and D. However both groups were significantly different from each other and from Maltster B. β-Amylase activity levels from the variety Triumph indicated no significant difference, at the 95% level, between Maltsters B, C and D in one group and A, D, E and F. No significant difference between Maltsters B, C, E and F was detected for α-glucosidase, while Maltsters A and D were not significantly different at the 95% level from each other, but were from the others. For β-glucosidase, there was a significant difference at the 95% level between pairs of maltsters and at the upper range of the β-glucosidase activity there was no significant difference at the 95% level between Maltsters B and E. With α-galactosidase, only Maltsters A and E were significantly different at the 95% level from the others. For β-galactosidase, Maltsters F and B were significantly different at the 95% level from the others. Only Maltster C displays any significant difference at the 95% level from

Table 14. Duncan's Multiple Comparison Test for the variety Triumph from six different Maltsters.

α-Amylase.

Maltster	A	D	E	C	F	B
Mean	6328.4	6374.6	7077.1	7087.4	7096.1	7655.2

β-Amylase.

Maltster	F	E	A	D	C	B
Mean	10493.7	10808.1	11319.4	12271.3	13255.8	13856.5

α-Glucosidase.

Maltster	D	A	C	F	B	E
Mean	0.866	0.927	1.144	1.201	1.207	1.275

β-Glucosidase.

Maltster	A	F	D	C	B	E
Mean	37.655	38.754	40.475	41.176	42.163	43.515

α-Galactosidase.

Maltster	A	C	D	B	F	E
Mean	56.289	64.585	65.175	66.086	66.384	71.697

β-Galactosidase.

Maltster	E	A	C	D	F	B
Mean	34.377	34.422	35.040	36.170	39.509	43.738

α-Mannosidase.

Maltster	C	D	A	F	E	B
Mean	11.415	12.893	12.896	12.964	13.494	13.984

β-Mannosidase.

Maltster	D	A	F	E	C	B
Mean	89.516	92.908	94.353	98.223	98.984	111.465

N-Acetyl-glucosaminidase.

Maltster	A	C	D	F	E	B
Mean	51.574	55.435	57.510	59.582	62.642	63.124

Arabinosidase.

Maltster	A	D	C	E	F	B
Mean	5.853	5.978	6.002	6.083	6.703	7.643

β-Xylosidase.

Maltster	A	E	F	C	D	B
Mean	28.074	33.660	34.350	34.572	36.187	44.436

the other Maltsters for the enzyme α-mannosidase. The enzyme β-mannosidase shows an unusual pattern of no significant difference at the 95% level. Maltsters A, D and F, Maltsters A, E and F and a third group containing Maltsters C, E and F show no significant difference at the 95% level within the group but difference between them. Maltster B was significantly different at the 95% level from all groups. For the enzyme N-acetyl-glucosaminidase no significant difference at the 95% level was found within the following groups, C and D, D and F, E and F and B and F, but there was difference between groups. Maltsters B and F gave significant difference at the 95% level with the other Maltsters for arabinosidase. For the enzyme β-xylosidase no significant difference at the 95% level between the Maltsters C, D, E and F.

By carrying out Duncan's Multiple Comparison Test on Maltsters A, B and F in the second one-way analysis of variance, a similar pattern emerged (Table 15). However, a slightly higher proportion of the enzyme activities gave no significant difference at the 95% level between Maltsters. Maltster B was significantly different at the 95% level for most enzyme activities. Again, a crude ranking of these Maltsters suggested that Maltster B gave the highest enzyme activity for all the enzymes studied, followed by Maltster F and then by Maltster A.

3.3.2.2 Effects of Malted Barley Variety or Blend.

During the period of sampling, Maltster B was supplying malted barley from the varieties Pipkin and Triumph, and from a blend of Triumph/Natasha. Statistical analysis, previously suggested that Maltster B gave the highest average enzyme activity when using the variety Triumph. The standard deviation from this Maltster was not significantly different at the 95% level from the other five maltsters. It was, therefore, assumed that this maltster would malt barley varieties consistently. Any differences would therefore be due to the effects of the variety or blend.

Table 15. Duncan's Multiple Comparison Test for the variety Triumph from three different Maltsters.

α-Amylase.

Maltster	A	F	B
Mean	6698.5	7000.9	8180.4

β-Amylase.

Maltster	F	A	B
Mean	10202.8	11447.0	11868.9

α-Glucosidase.

Maltster	A	F	B
Mean	0.923	1.104	1.193

β-Glucosidase.

Maltster	A	F	B
Mean	39.180	39.329	43.072

α-Galactosidase.

Maltster	A	F	B
Mean	58.049	65.198	65.605

β-Galactosidase.

Maltster	A	F	B
Mean	36.546	36.969	44.064

α-Mannosidase.

Maltster	A	F	B
Mean	12.195	12.596	14.309

β-Mannosidase.

Maltster	F	A	B
Mean	94.225	95.469	111.833

N-Acetyl-glucosaminidase.

Maltster	A	F	B
Mean	52.607	57.135	63.034

Arabinosidase.

Maltster	A	F	B
Mean	6.049	6.293	7.891

β-Xylosidase.

Maltster	A	F	B
Mean	29.588	33.650	44.487

Table 16. Duncan's Multiple Comparison Test for the varieties Pipkin (A), Triumph/Natasha (B) and Triumph (C) supplied by Maltster B.

α-Amylase.

Variety	C	B	A
Mean	7957.4	8000.0	9958.4

β-Amylase.

Variety	B	C	A
Mean	11510.7	13166.6	17891.8

α-Glucosidase.

Variety	B	C	A
Mean	1.006	1.068	1.192

β-Glucosidase.

Variety	B	C	A
Mean	42.760	43.839	57.394

α-Galactosidase.

Variety	C	B	A
Mean	65.400	64.464	96.413

β-Galactosidase.

Variety	B	C	A
Mean	42.932	44.074	45.154

α-Mannosidase.

Variety	B	C	A
Mean	14.508	15.105	24.438

β-Mannosidase.

Variety	B	C	A
Mean	111.459	114.661	130.587

N-Acetyl-glucosaminidase.

Variety	B	C	A
Mean	62.399	65.236	103.421

Arabinosidase.

Variety	B	C	A
Mean	7.710	7.874	8.988

β-Xylosidase.

Variety	B	C	A
Mean	44.045	44.510	66.679

One-way analysis of variance showed a significant difference at the 95% level in the enzyme activities, except α-glucosidase, β-galactosidase and β-mannosidase, for all enzymes between varieties and blends. Duncan's Multiple Comparison Test, (Table 16), showed that Pipkin was significantly different at the 95% level from Triumph and Triumph/Natasha for all enzyme activities, except α-glucosidase and β-galactosidase. No significant difference at the 95% level was detected in the three varieties when the results for these two enzymes were studied. Overall the average enzyme activities from Triumph/Natasha were slightly higher than Triumph, with one-way analysis of variance suggesting no significant difference at the 95% level being found between the two except N-acetyl-glucosaminidase activity. The average enzyme activity from Pipkin was higher than the others in all enzymes studied.

Malted barley from the varieties Camargue, Pipkin and Triumph and from the blends Triumph/Halcyon and Triumph/Natasha were also analyzed using the statistical methods. However, these were not from a single maltster, and any effect due to maltsters may influence the overall results.

The one-way analysis of variance suggested a significant difference at the 95% level in all enzyme activities between the varieties and blended varieties except α-glucosidase and β-galactosidase. The variety with the highest enzyme activity was Pipkin, in all but α-glucosidase (Table 17). However, there was no significant difference at the 95% level in activities between this variety, and Camargue and Triumph/Halcyon for α-glucosidase, as showed by Duncan's Multiple Comparison Test. Triumph, Triumph/Halcyon and Triumph/Natasha were not significantly different at the 95% level to each other in 7 of the 11 enzymes. Camargue was supplied by Maltster C and could not be ranked successfully with the other varieties due to the erratic nature of its performance in the Duncan's Multiple Comparison Tests.

Table 17. Duncan's Multiple Comparison Test for all varieties from all six Maltsters. (A - Camargue, B - Pipkin, C - Triumph/Halycon, D - Triumph/Natasha, E - Triumph)

α-Amylase.

Variety	C	E	D	A	B
Mean	6175.7	7120.1	8006.0	9491.7	10030.1

β-Amylase.

Variety	A	C	D	E	B
Mean	8541.8	10535.4	11891.6	12159.1	17780.4

α-Glucosidase.

Variety	D	E	A	B	C
Mean	0.960	1.071	1.152	1.247	1.432

β-Glucosidase.

Variety	A	D	E	C	B
Mean	36.287	41.814	42.422	44.321	57.993

α-Galactosidase.

Variety	D	E	C	A	B
Mean	65.132	66.923	69.595	69.814	99.310

β-Galactosidase.

Variety	C	E	A	D	B
Mean	36.123	37.432	38.694	41.960	45.487

α-Mannosidase.

Variety	E	A	C	D	B
Mean	13.612	13.914	14.818	14.873	24.975

β-Mannosidase.

Variety	A	E	C	D	B
Mean	76.922	100.229	101.191	111.100	129.244

N-Acetyl-glucosaminidase.

Variety	A	E	D	C	B
Mean	56.046	59.629	62.182	65.617	105.861

Arabinosidase.

Variety	A	E	C	D	B
Mean	6.339	6.599	6.671	7.623	9.005

β-Xylosidase.

Variety	C	E	A	D	B
Mean	35.143	35.609	42.343	44.320	69.242

3.4 Enzyme Activities In The Scotch Malt Whisky Process.

The activities of malted barley enzymes are important in the production of the wort to be used by the yeast during the fermentation stage of Scotch Malt Whisky production. During the mashing process, the large polymers are broken down to their respective monomeric or oligomeric constituents. The wort produced is then cooled to a suitable temperature for yeast pitching and then fermented. These processes can be divided into two main sections, the first involves the mashing procedure, with its associated wort draining and the second, is fermentation itself.

To obtain a complete overview of the enzyme activities, the carbohydrases were studied under optimal conditions for malted barley enzymes. Activities were also studied under the conditions found in the mash liquor, wort or wash at the time of sampling. This required the determination of several parameters for each process, and included pH, temperature, specific gravity, yeast viability, biomass, free amino nitrogen, protein and phosphate. The development of some major products of fermentation were also followed. These products of fermentation were not necessarily from a yeast fermentation, since bacterial fermentations may also be present.

3.4.1 Mashing.

The mashing processes employed in the production of Scotch Malt Whisky was explained in the introduction. The mash, which was studied was carried out under normal distillery procedures by an experienced distillery operator. It was therefore a typical mash for this distillery.

Protein and phosphate analyses were carried out in duplicate on single samples. Soluble free α-amino nitrogen levels were determined in triplicate from a single sample. Enzyme analyses, for amylases, minor carbohydrases, arabinosidase, β-xylosidase and acid phosphatase, were carried out on two samples, in duplicate. The remaining enzymes were assayed in duplicate from a single sample.

3.4.1.1 Process Variables.

To obtain results for the optimal and mash tun conditions for the enzyme activities, it was necessary to carry out a series of initial observations on the samples before storage. These observations ascertained the temperature and pH of the sample in the mash tun, before removing a sample from the main bulk of the mash. Other factors studied, included free amino nitrogen in the mash liquor, the soluble protein content and the amount of free phosphate at the time of sampling. Results of these parameters are given in Figures 41 and 42 (Table 20, Appendix II).

The First Water.

Mashing-in of the grist and mashing liquor was achieved at a temperature of 64.5°C to 65.5°C. During the 42 min of mashing-in, the temperature of the mash entering the mash tun was predominantly 65.5°C. The pH of the mash, during mashing-in, was pH 5.54. During mashing-in, the temperature in the mash tun was 64.5°C with a pH of 5.48. Once mashing-in was completed the mash was mixed by raking for two revolutions. Samples were removed from the same region of the mash tun during the study. After raking the temperature in the mash tun was found to have dropped to 63.0°C and the pH had fallen to pH 5.39. Through-out the remainder of the resting and draining stages of the first water both the temperature and pH were found to decrease. The temperature after 145 min (to the end of first water draining) fell to 60.5°C and the pH to pH 5.28. The samples were prepared as in Section 2.2.9.1.

The initial level of soluble protein was in the order of 1.415 mg/ml, but was reduced to 0.832 mg/ml in the mash tun during mashing-in. A similar reduction was observed with the free α-amino nitrogen. After raking, both the protein concentration and free α-amino nitrogen levels were increased over the mash tun levels during mashing-in. Both were relatively constant during the remainder of the first water period.

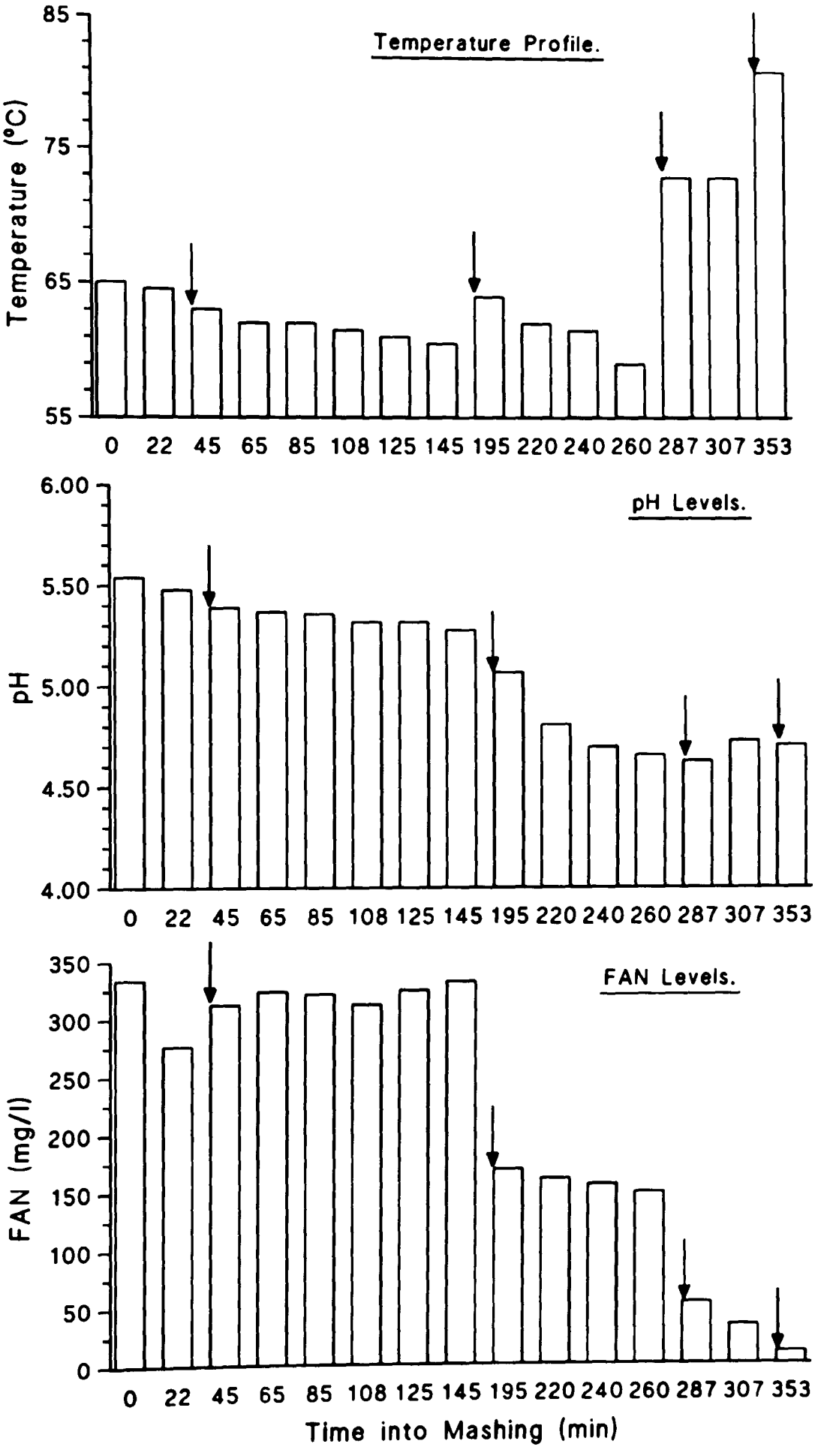
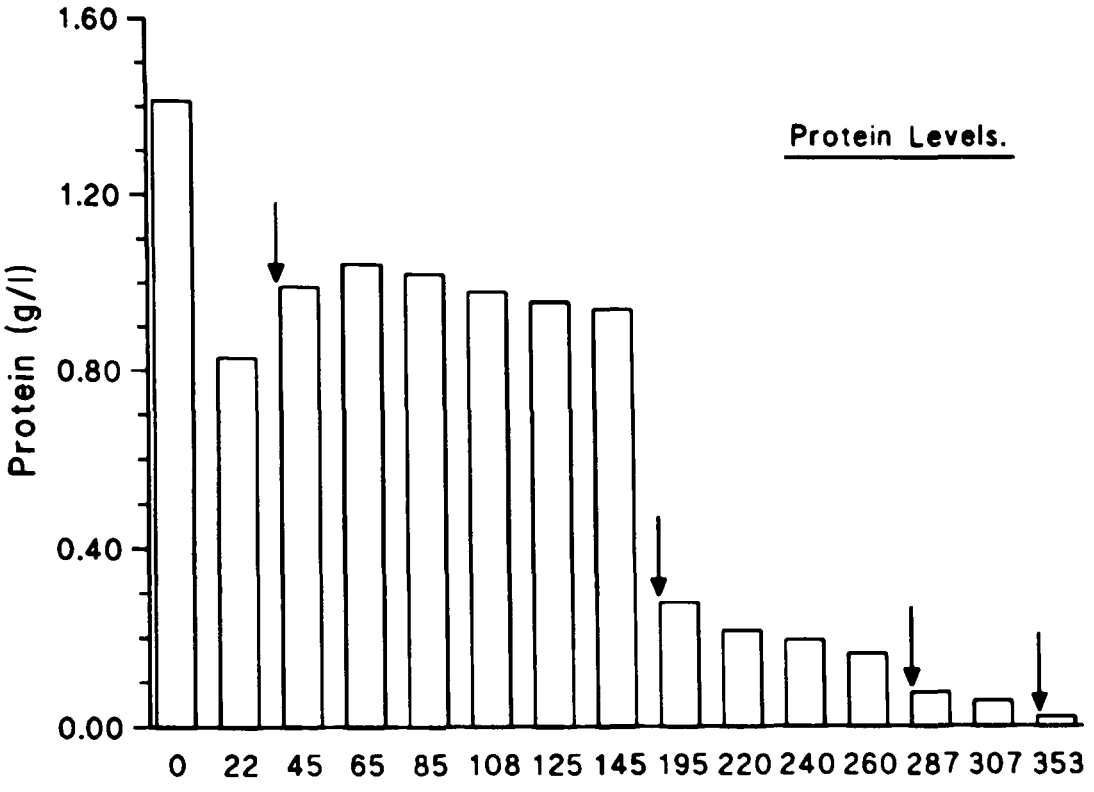


Figure 41. Measurements of temperature, pH and free α-amino nitrogen (FAN) (Pooled S.D. = 13.298) during the mashing process. Raking after water addition shown by arrows.



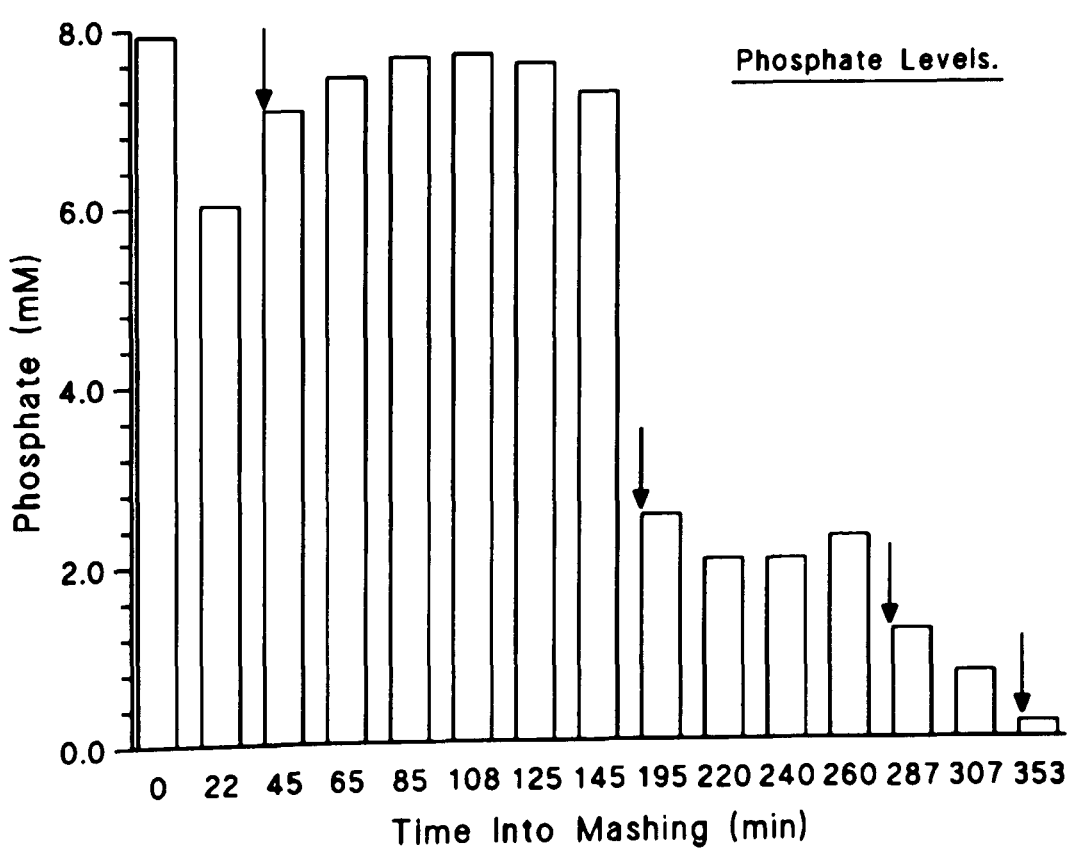


Figure 42. Measurement of protein (Pooled S.D. = 0.063) and phosphate (Pooled S.D. = 0.190) levels during the mashing process. Raking after water addition shown by arrows.

The concentration of phosphate ions during the first water period was relatively consistent during the resting and draining stages. However, a substantial difference was observed between the initial spout mashing-in level of 7.93 mM and the concentration in the mash tun after 22 min mashing-in, 6.03 mM. A similar pattern was found with the first water protein levels.

The Second Water.

Through-out the second water period the temperature of the mash bed was low, since the second water temperature at the mashing spout was 72°C. Monitoring of the mash bed temperatures from subsequent mashes (70.5 to 73.0 °C) indicated these temperatures to be correct. These low second water temperatures may not be typical within the industry as a whole, but were those being used in the distillery at the time. After one revolution of the mash tun rakes, the temperature of the mash bed was 64.0°C, and this decreased to 59.0°C over a period of 65 min. The pH of the mash decreased during this period from pH 5.07 to pH 4.66. During the period of 215 min, from first water raking to the end of second water draining, the temperature reduction in the mash tun was 4.0°C. The reduction in the pH over the same period was found to be 0.73 of a pH unit.

The levels of soluble free α-amino nitrogen, protein and phosphate levels were quite significantly reduced between the mashing-in and fourth water values, as seen in Figures 41 and 42. All are probably affected by the length of time the mash was in the mash tun and by the temperatures used during mashing. During the period the mash bed was in the mash tun, many proteins may have been precipitated out of solution, as were the polyphosphates and other phosphate containing moieties. Many enzymes responsible for protein degradation and those for phosphate release will have been denatured or removed from the mash in the wort.

Sparging Waters.

Both sparging waters are for the leaching of any molecules not removed by the previous two waters. Little, if any enzyme activity will be present at the elevated temperatures and low pH of these waters. This was evident from the low concentrations of free α-amino nitrogen, protein and phosphate present in the liquor. Most of the material made available from the sparge waters will be obtained from the husk and non-starchy endosperm parts of the malt.

3.4.1.2 Enzyme Activities During Mashing.

All enzymes investigated during the mashing process were studied, where applicable, under the temperature and pH of the mash at the time of sampling. The enzyme activities were also studied at the optimum conditions for the malt enzymes.

Amylases.

Under the conditions of mashing, it was observed from Figure 43 that α-amylase activity was relatively stable during mashing-in and in the mash tun up to 22 min. Thereafter, a steady decrease in activity was observed to the end of the first water. Activity of α-amylase was greatly reduced during the second water, only 19% after raking compared to the end of the first water draining. The activity pattern for the enzyme under optimum conditions was similar to the mashing conditions, during the first two waters. However, an increase in activity was detected after first water raking. The decrease in activity between the two waters was approximately 78%. Under both sets of assay conditions no α-amylase activity was found during the sparge waters.

The activity of β-amylase was determined under optimum conditions (see Figure 44) and the bulk of the activity was during mashing-in, immediately after grist and liquor mixing. Activity decreases by 92% between mashing-in and the mash resting in the mash tun for 22 min from the commencement of mashing-in. At the end of the mashing-in period and after raking, the activity increased slightly when compared

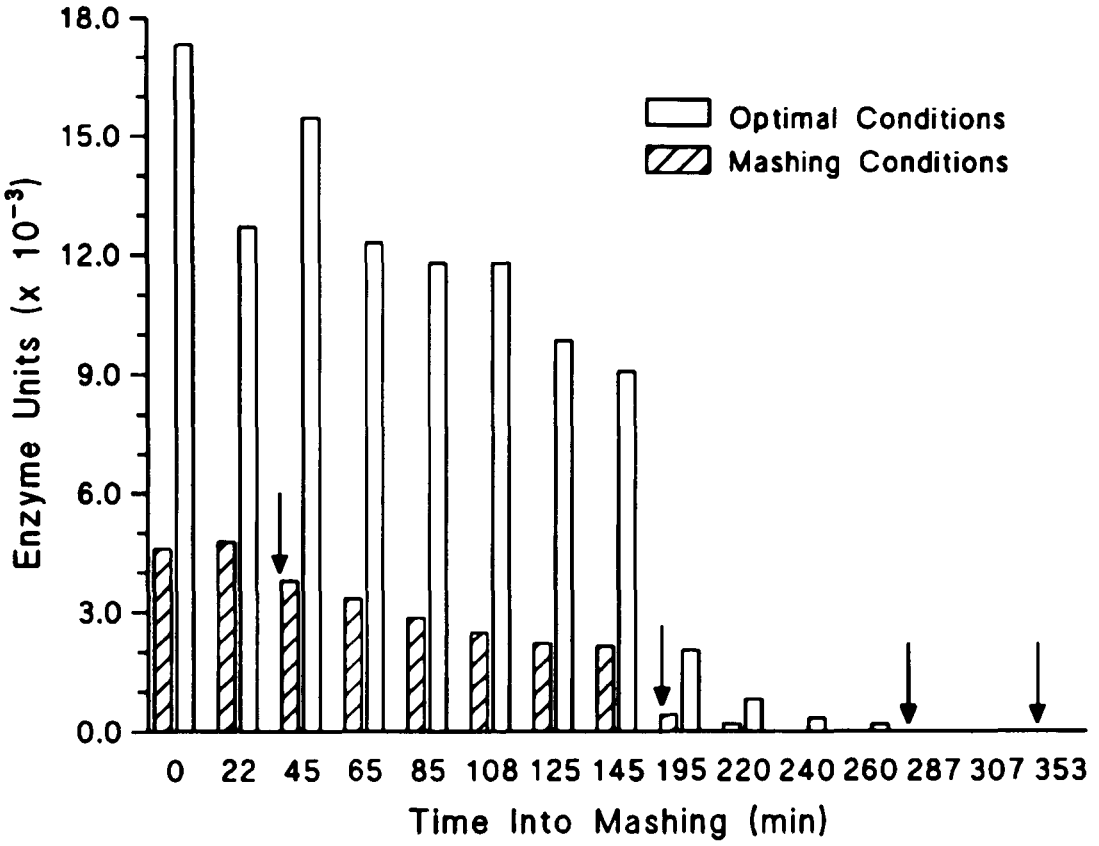


Figure 43. α-Amylase activity under mashing (Pooled S.D. = 37.552) and optimal (Pooled S.D. = 247.57) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

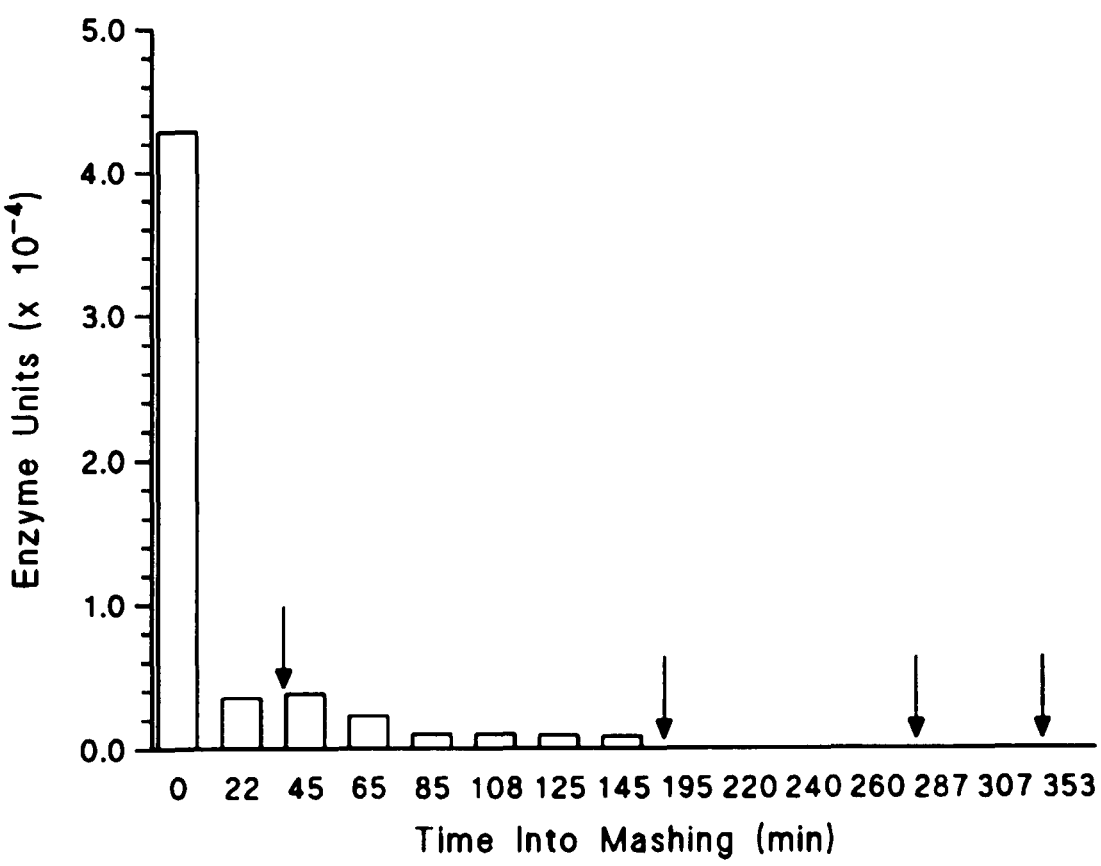


Figure 44. β-Amylase activity under optimal (Pooled S.D. = 285.32) conditions during the mashing process. Raking after water addition shown by arrows.

to the 22 min sample. However, after 85 min in the mash tun activity was very low, being only 2% of the mashing-in activity. A very slight activity, (16 E.U.) was detected during the second water, but this was a fraction of the initial activity. No β-Amylase activity was present during the third and fourth waters.

Minor Carbohydrases.

The detailed levels of activity for the minor carbohydrase and phosphatase enzymes are given in Tables 21 and 22. Each enzyme was studied under mash tun conditions in addition to the optimum conditions for that enzyme.

An overall decrease in α-glucosidase activity, under mash tun conditions (Figure 45), during the first waters was observed. Activity during mashing-in increased slightly after 22 min in the mash tun but after raking activity decreased. Optimum conditions gave a decrease in activity in the mash tun during mashing-in but increased after raking, activity then decreased until the end of the first water. Activity in the second water under both sets of conditions continued to decrease from the level found in the first water. No activity under both sets of conditions was found in the third and fourth waters.

The activity of β-glucosidase (Figure 46) during the mashing process followed a similar pattern for both sets of assay conditions. The bulk of the activity was found during mashing-in, activity in the mash tun after 22 min decreased by 85.5% under mash tun conditions and by 79.5% for optimum conditions. Activity continued to decrease throughout the duration of the first and second waters and no activity was detected in the third and fourth waters.

The activity from both sets of assay conditions for α-galactosidase, (Figure 47), followed similar patterns, with the optimum activity greater than the mash tun activity. Activity was greatest during mashing-in and steadily decreased to 85 min, then it increased to

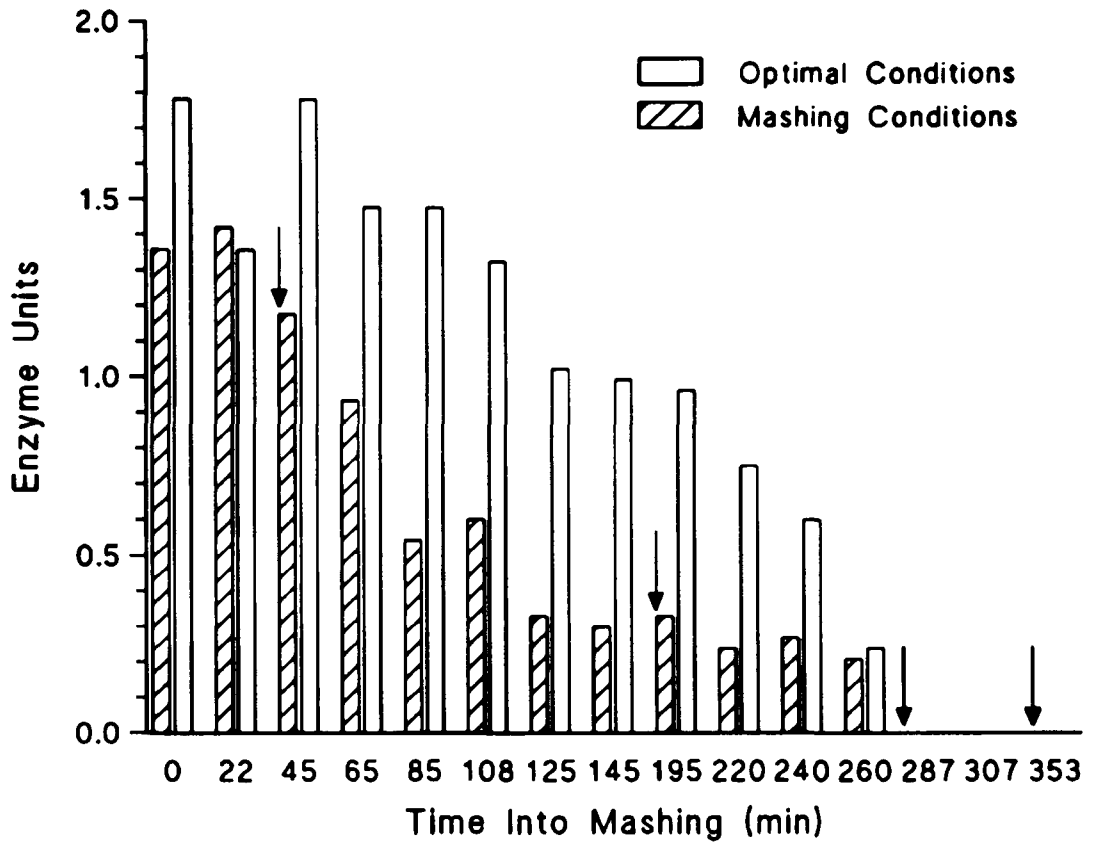


Figure 45. α-Glucosidase activity under mashing (Pooled S.D. = 0.074) and optimal (Pooled S.D. = 0.100) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

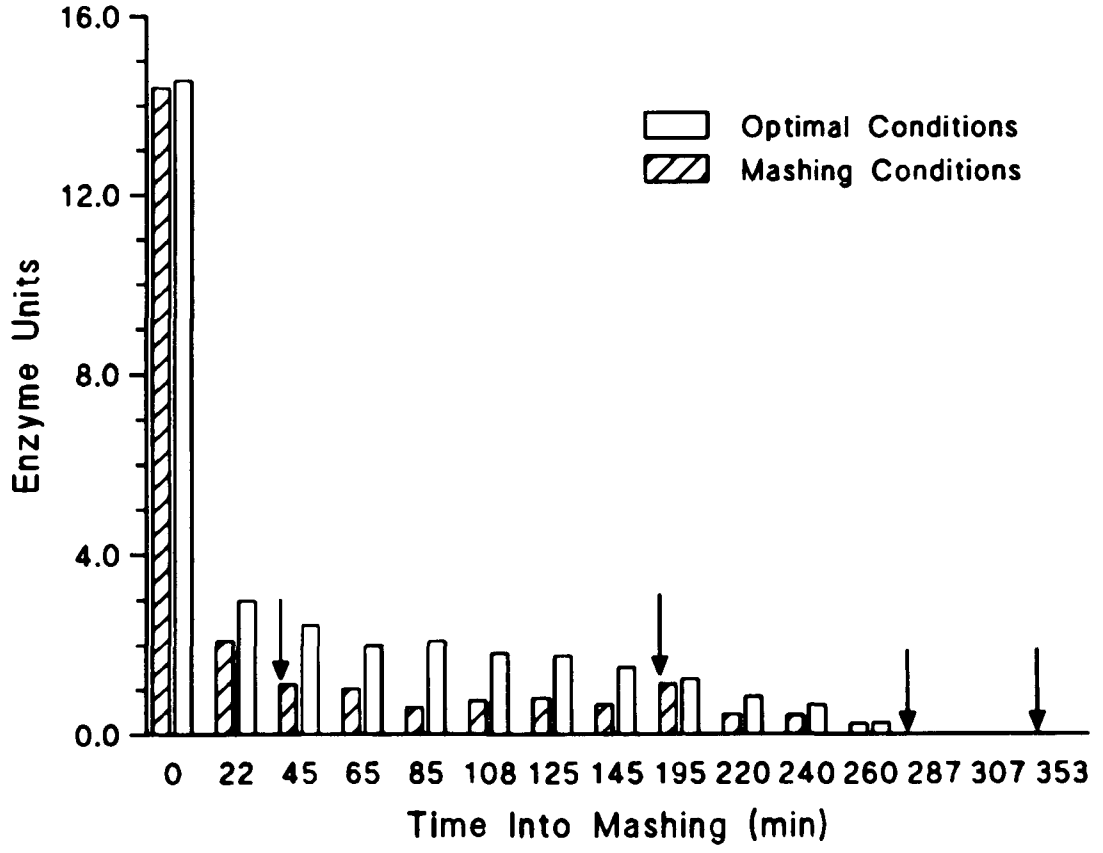


Figure 46. β-Glucosidase activity under mashing (Pooled S.D. = 0.064) and optimal (Pooled S.D. = 0.118) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

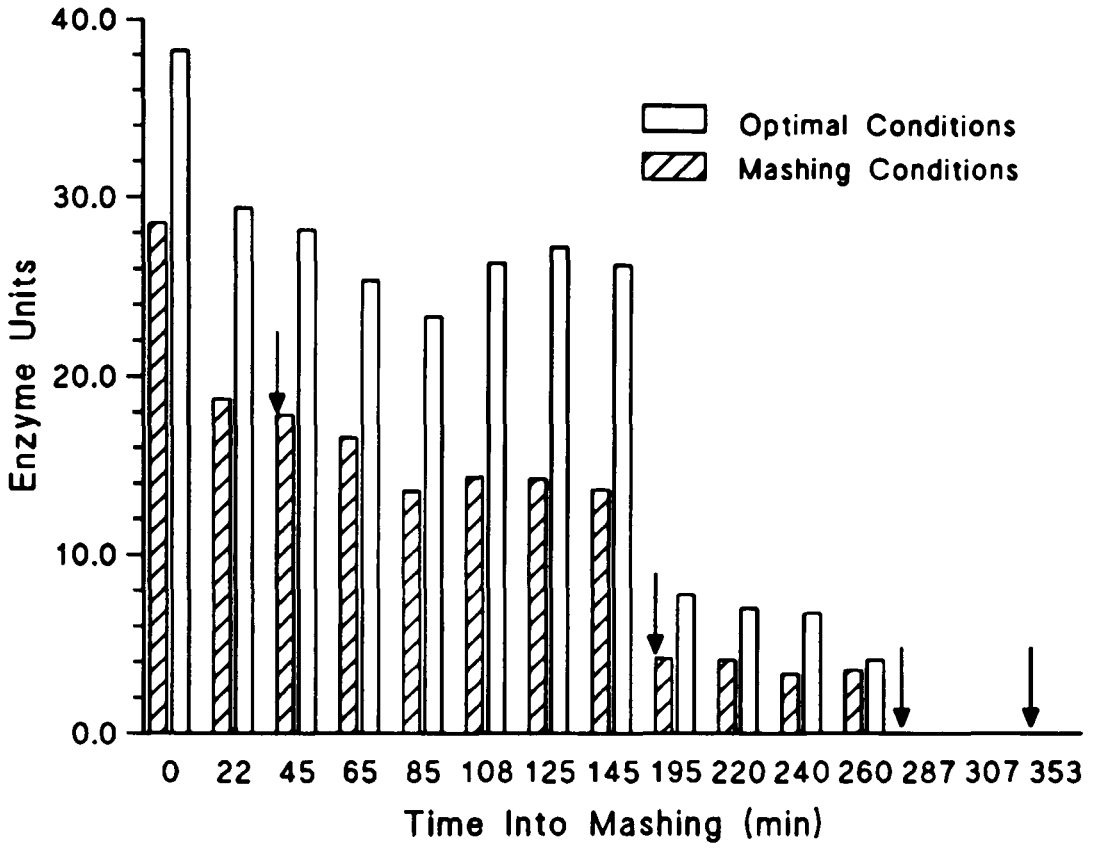


Figure 47. α-Galactosidase activity under mashing (Pooled S.D. = 0.207) and optimal (Pooled S.D. = 0.217) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

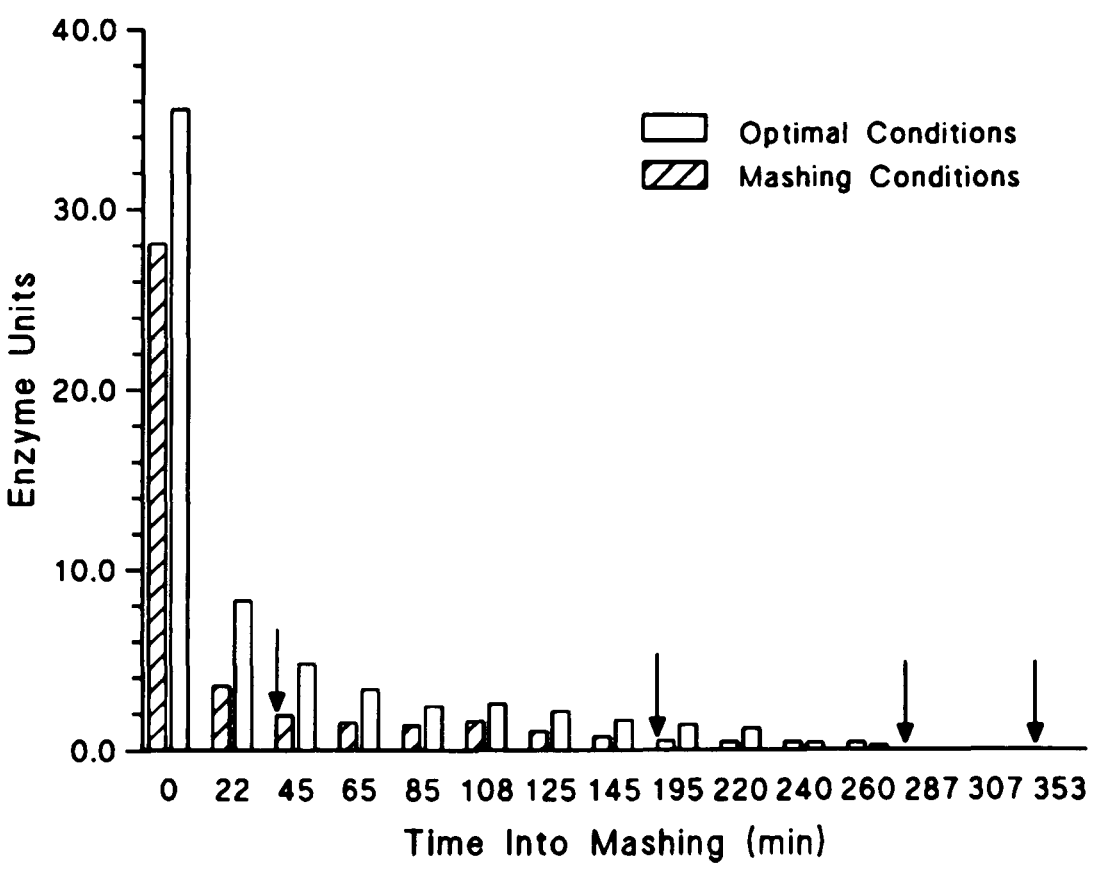


Figure 48. β-Galactosidase activity under mashing (Pooled S.D. = 0.094) and optimal (Pooled S.D. = 0.162) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

the end of the first water. Initial second water activity was decreased by 60% under both sets of assay conditions. No activity was detected in the sparging waters.

β-Galactosidase activity (Figure 48) was greatest during the mashing-in process. Activity then decreased by 87.4% under mash tun conditions and by 76.8% for the optimum conditions after 22 min into the mashing process. Thereafter, activity decreased until the end of the second water. No activity was found in the third and fourth waters.

Activity from both sets of assay conditions for the α-mannosidase enzyme (Figure 49) were very similar. A decrease in activity throughout the first and second waters was observed, with no activity in the sparge waters. Initial activity, especially under optimum conditions, was quite high during mashing-in, but activity fell rapidly thereafter. β-Mannosidase (Figure 50) also was more active during mashing-in. After first water raking the activity had been reduced considerably and at the end of the second water only a very slight level of activity was detected. There was no activity of this enzyme during the third and fourth waters.

Activity of N-acetylglucosaminidase (Figure 51) was found to follow a similar pattern to α-mannosidase, however activity fell rapidly after mashing-in, especially from the optimum assay conditions. Optimum and mash tun activities, in the mash tun, were quite similar and decreased throughout the period of mashing until the sparge waters. No activity was detected in the sparges.

The activity of α-arabinosidase (Figure 52) followed a different pattern. After high levels of activity during mashing-in, the activity was reduced by approximately 35-45% after mash tun raking. Under optimum enzyme conditions, activity began to increase until 108 min and then fell to the end of first water draining. However, under mashing conditions, activity began to rise gradually. During the

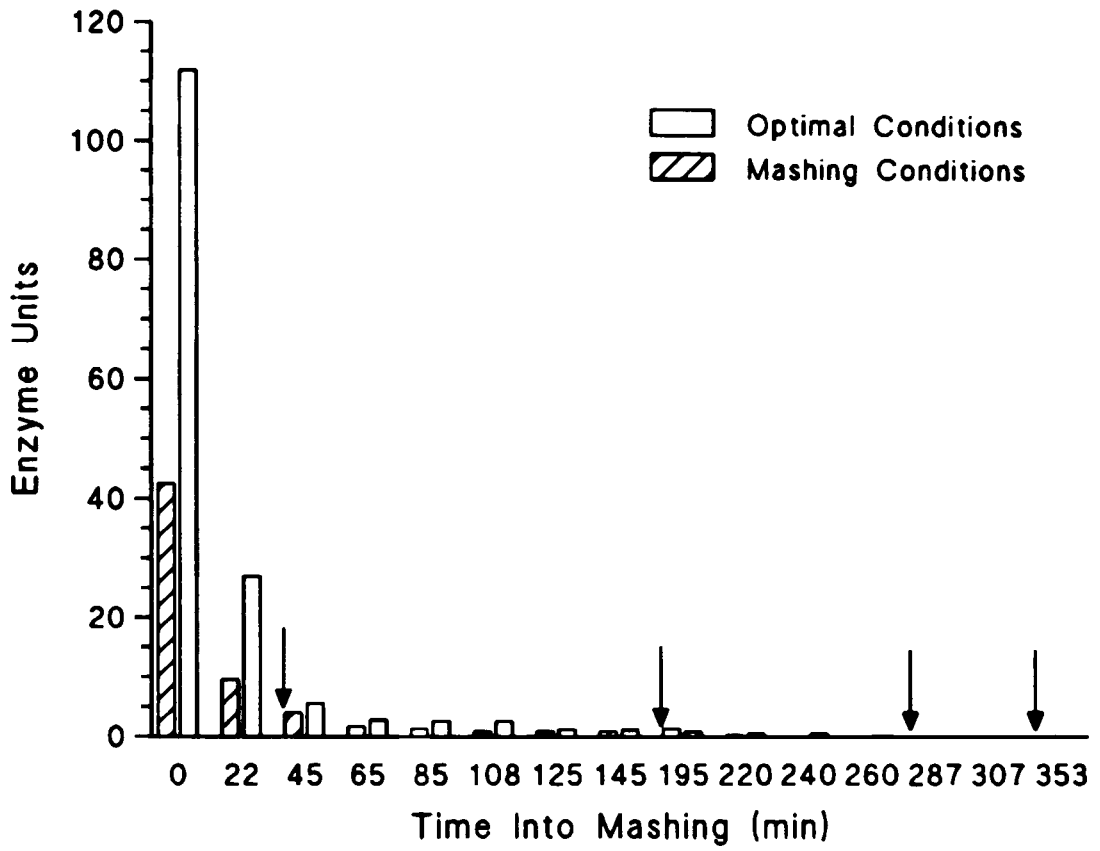


Figure 49. α-Mannosidase activity under mashing (Pooled S.D. = 0.377) and optimal (Pooled S.D. = 0.240) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

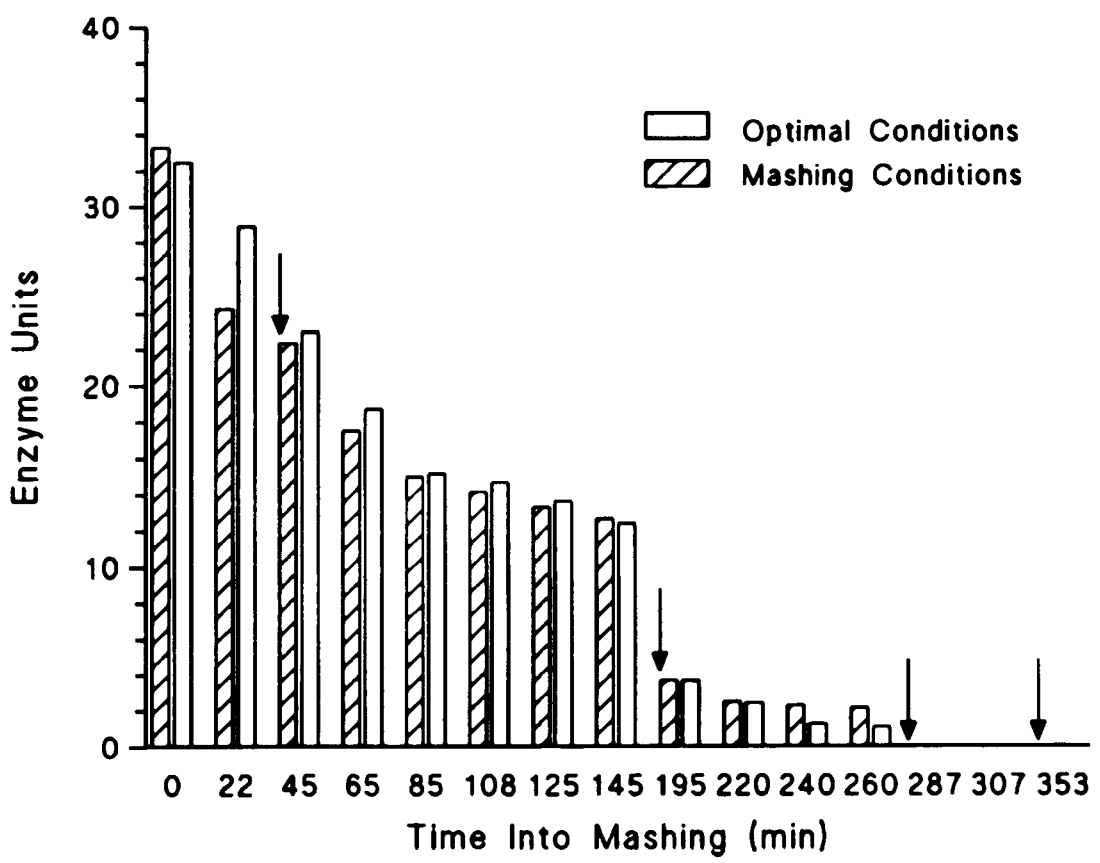


Figure 50. β-Mannosidase activity under mashing (Pooled S.D. = 0.120) and optimal (Pooled S.D. = 0.123) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

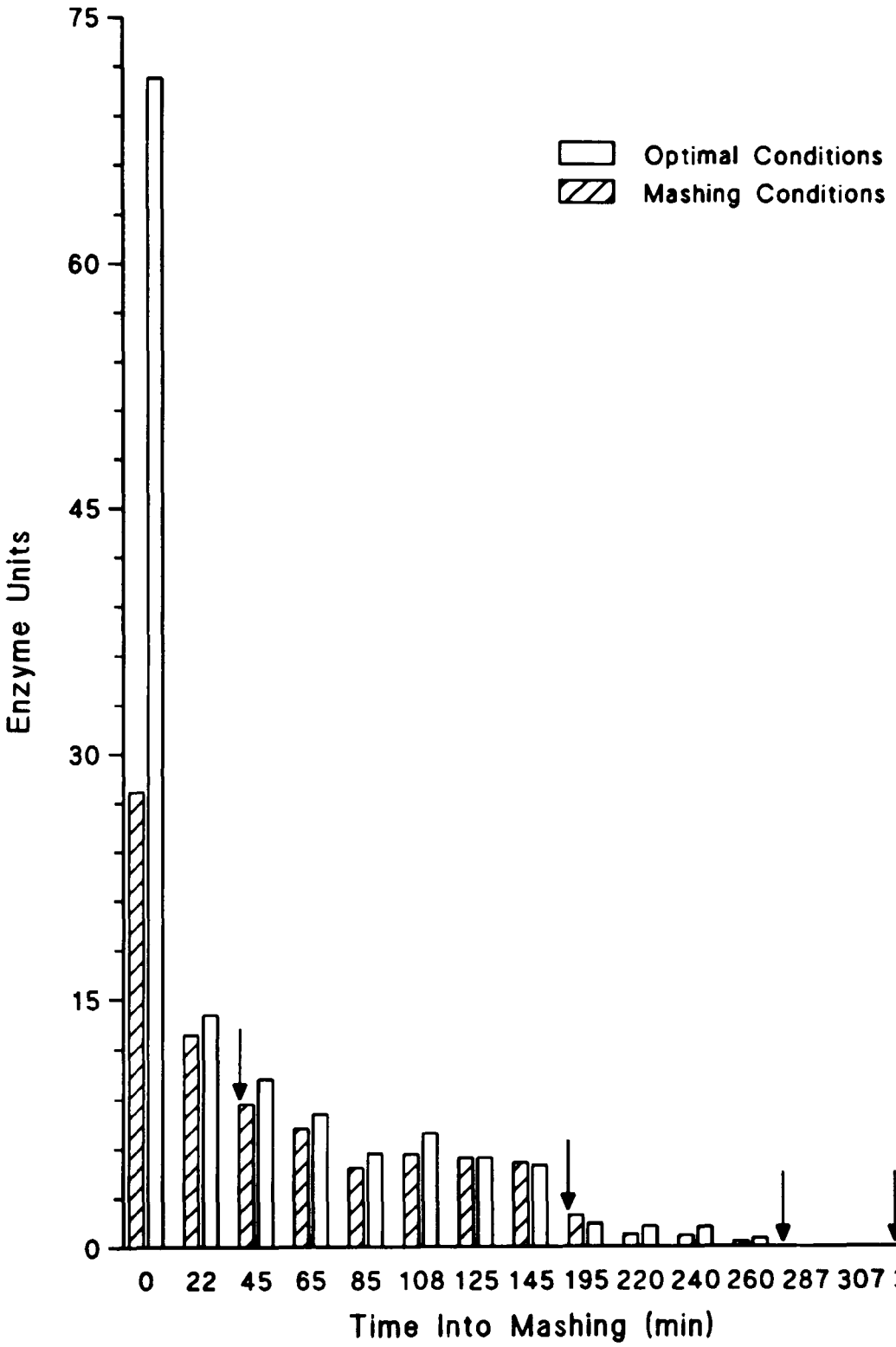


Figure 51. N-Acetyl-glucosaminidase activity under mashing (Pooled S.D. = 0.161) and optimal (Pooled S.D. = 0.239) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

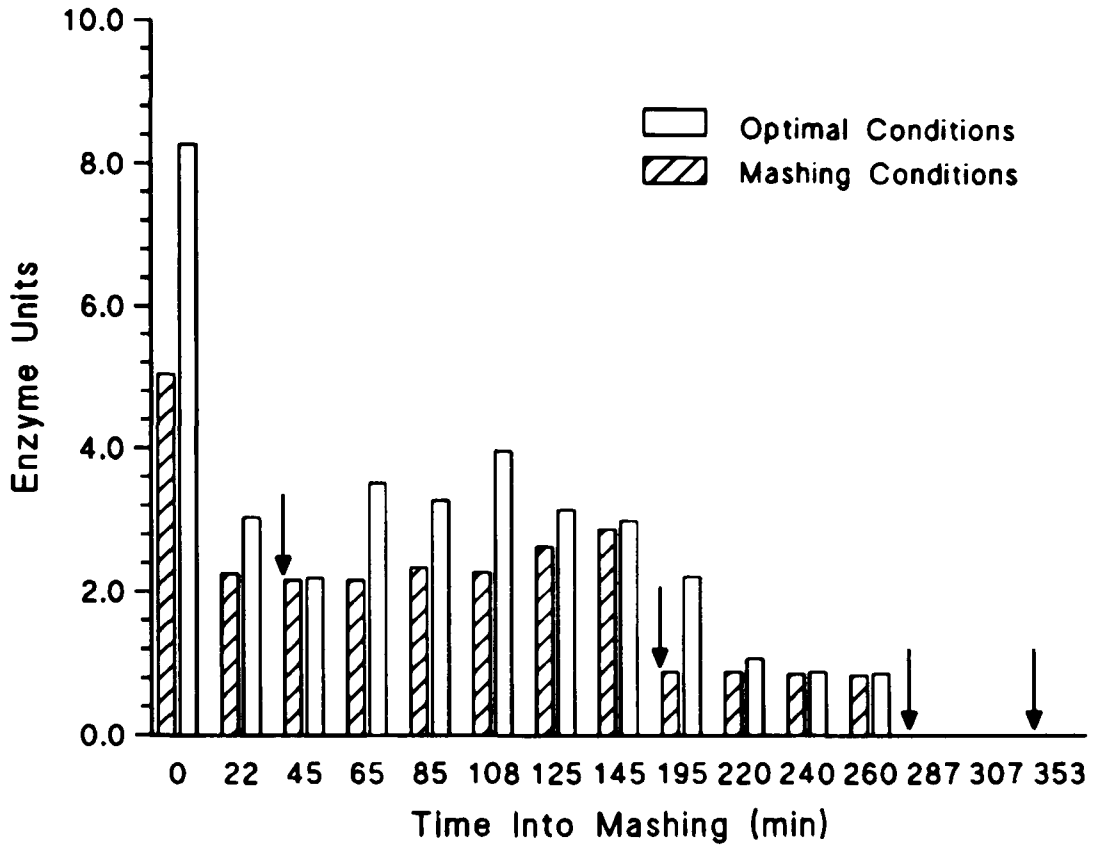


Figure 52. Arabinosidase activity under mashing (Pooled S.D. = 0.101) and optimal (Pooled S.D. = 0.122) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

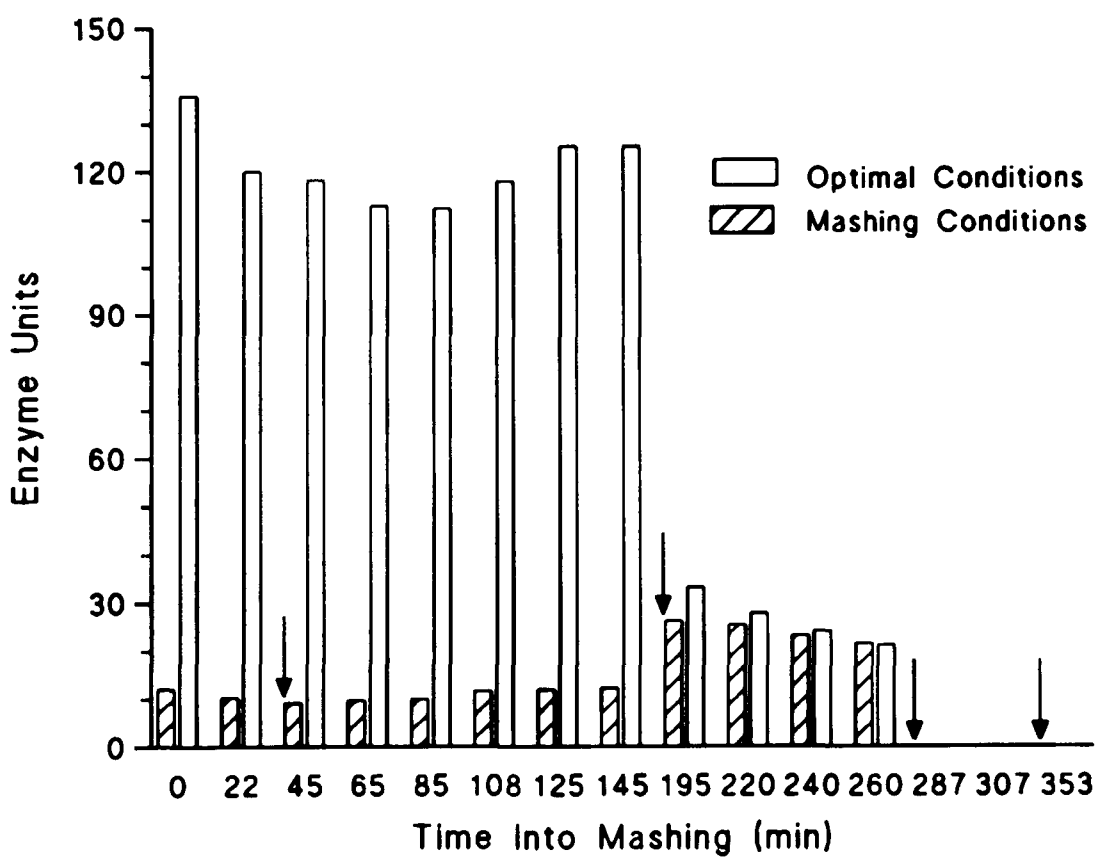


Figure 53. β-Xylosidase activity under mashing (Pooled S.D. = 0.300) and optimal (Pooled S.D. = 0.223) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

second water the activity was relatively stable for the mash tun conditions assay, but decreased over this period for the optimal conditions assay. No activity was observed in the sparging waters.

β-Xylosidase activity (Figure 53) under optimum conditions decreased to 85 min and then increased steadily to 92% of the initial mashing-in activity at the end of first water draining. Under mash tun conditions the activity of β-Xylosidase was relatively constant during the first water. Second water activity decreased by 75% from the initial mashing-in activity and gradually decreased. The second water activity, under mash tun conditions, followed a similar pattern but, the activity increased by approximately 115%. No β-Xylosidase activity was detected during the third and fourth waters.

Phosphatases.

The activity of the enzyme acid phosphatase, (Figure 54), was greatest during mashing-in, and from this point was reduced during the first and second waters. No activity was detected in the sparge waters. Both sets of assay conditions were similar. The mashing time coarse activity of the phytase enzyme was only studied under the optimum assay conditions (Figure 55, Table 23, Appendix II). Activity was greatest after mashing-in and was reduced after mash tun raking. However, activity increased from mash tun raking up to 85 min in the mash tun before decreasing to the end of the first water. Activity during the second water remained relatively constant at approximately the same level as at the end of the first water. No activity was found during the third and fourth sparging waters.

Proteinases and Proteases.

The levels of activity for the general proteinase activity and proteases studied are detailed in Table 23. Each assay was carried out under optimal conditions only.

General proteinase type activity (Figure 56) was relatively constant during mashing-in and up to 65 min into the mash. Thereafter activity began to decline until the end of the first water. Once raking

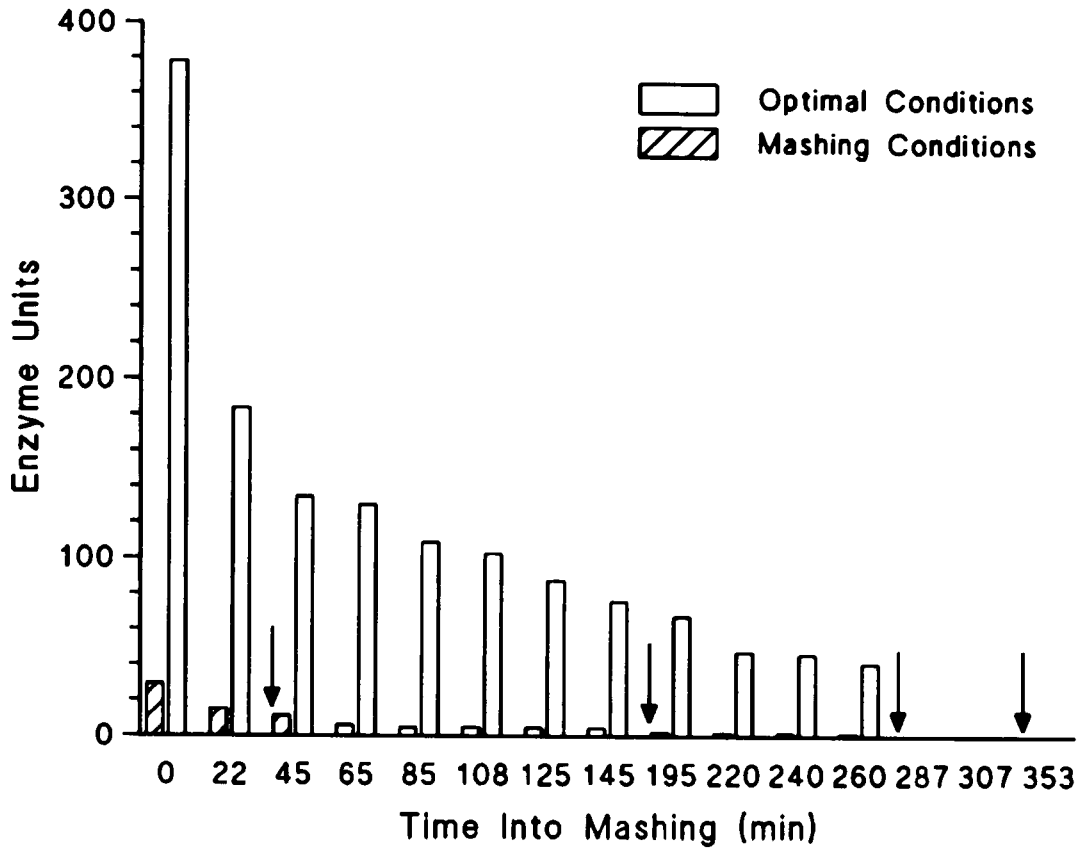


Figure 54. Acid Phosphatase activity under mashing (Pooled S.D. = 0.221) and optimal (Pooled S.D. = 0.244) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

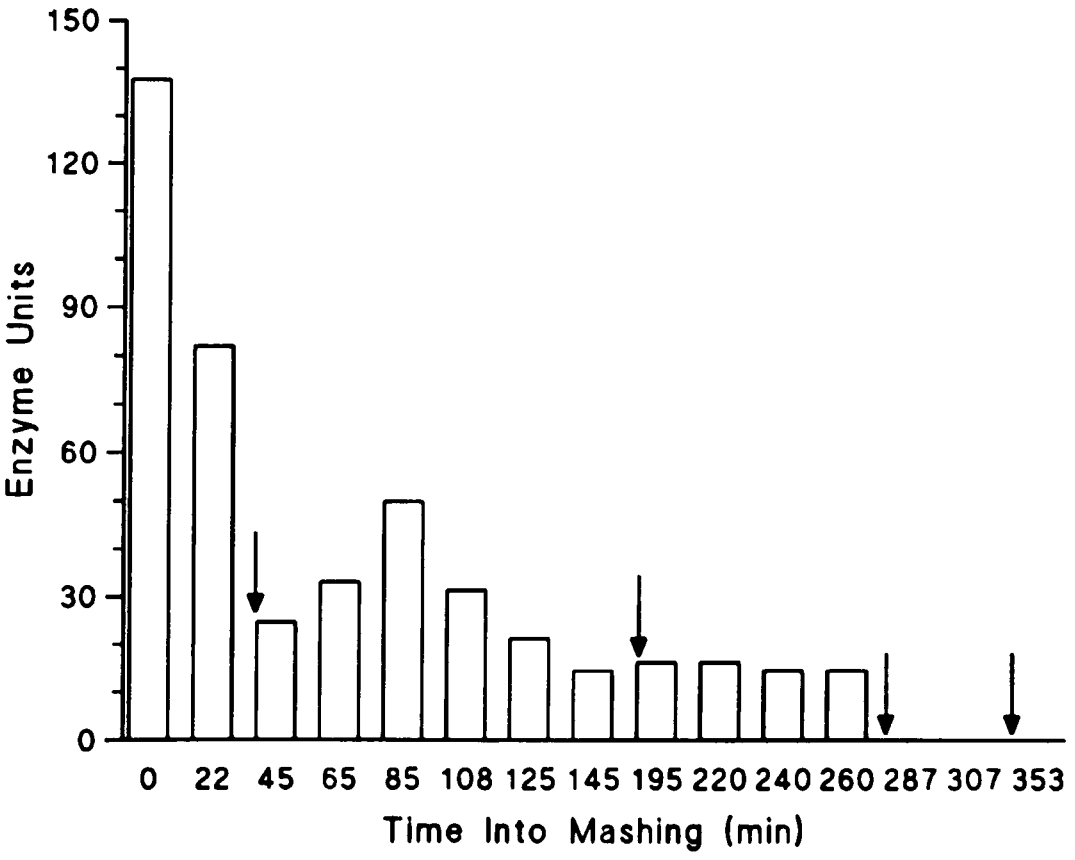


Figure 55. Phytase activity under optimal (Pooled S.D. = 0.378) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

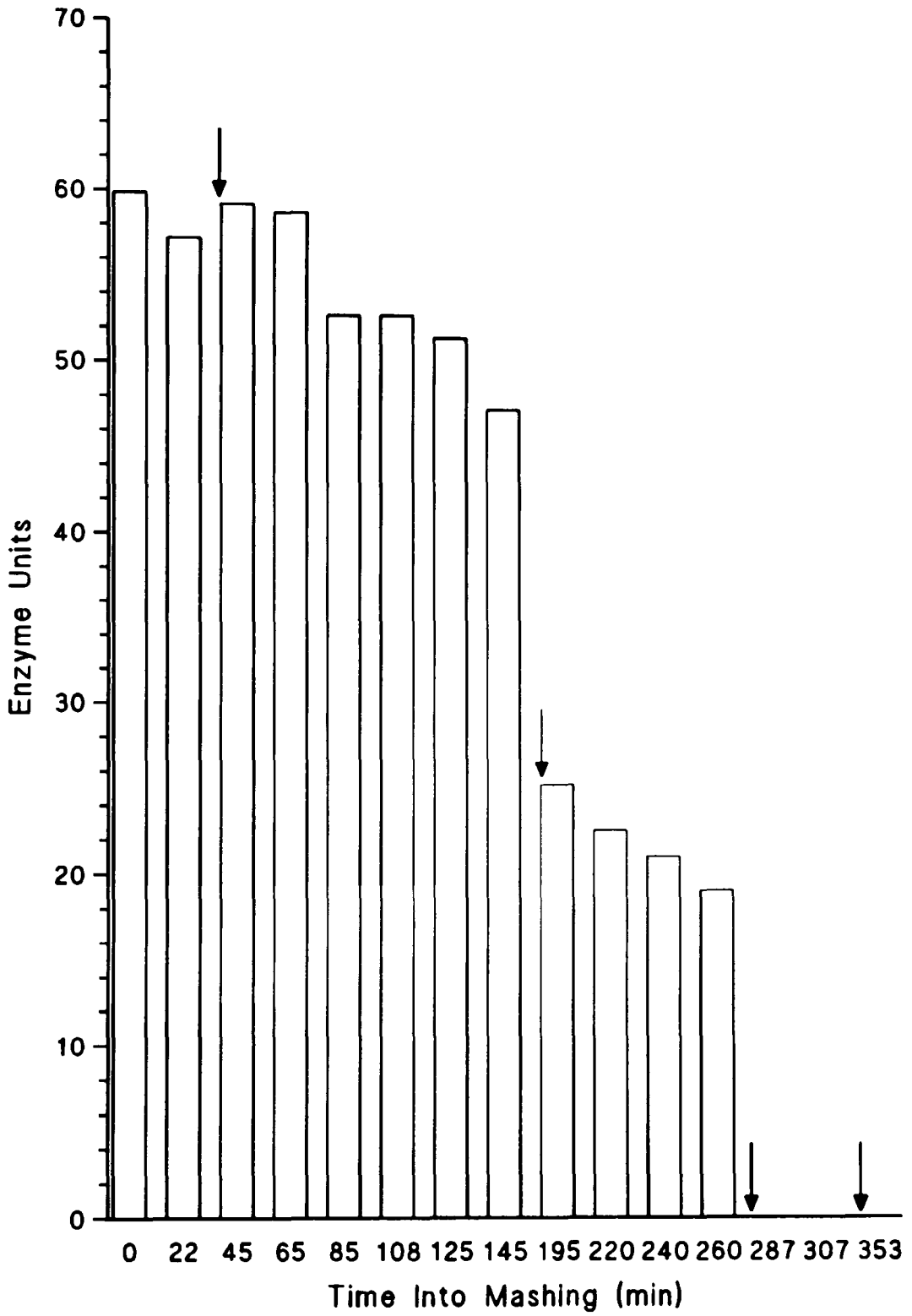


Figure 56. General proteinase activity under optimal (Pooled S.D. = 0.836) enzyme conditions during the mashing process. Raking after water addition shown by arrows.

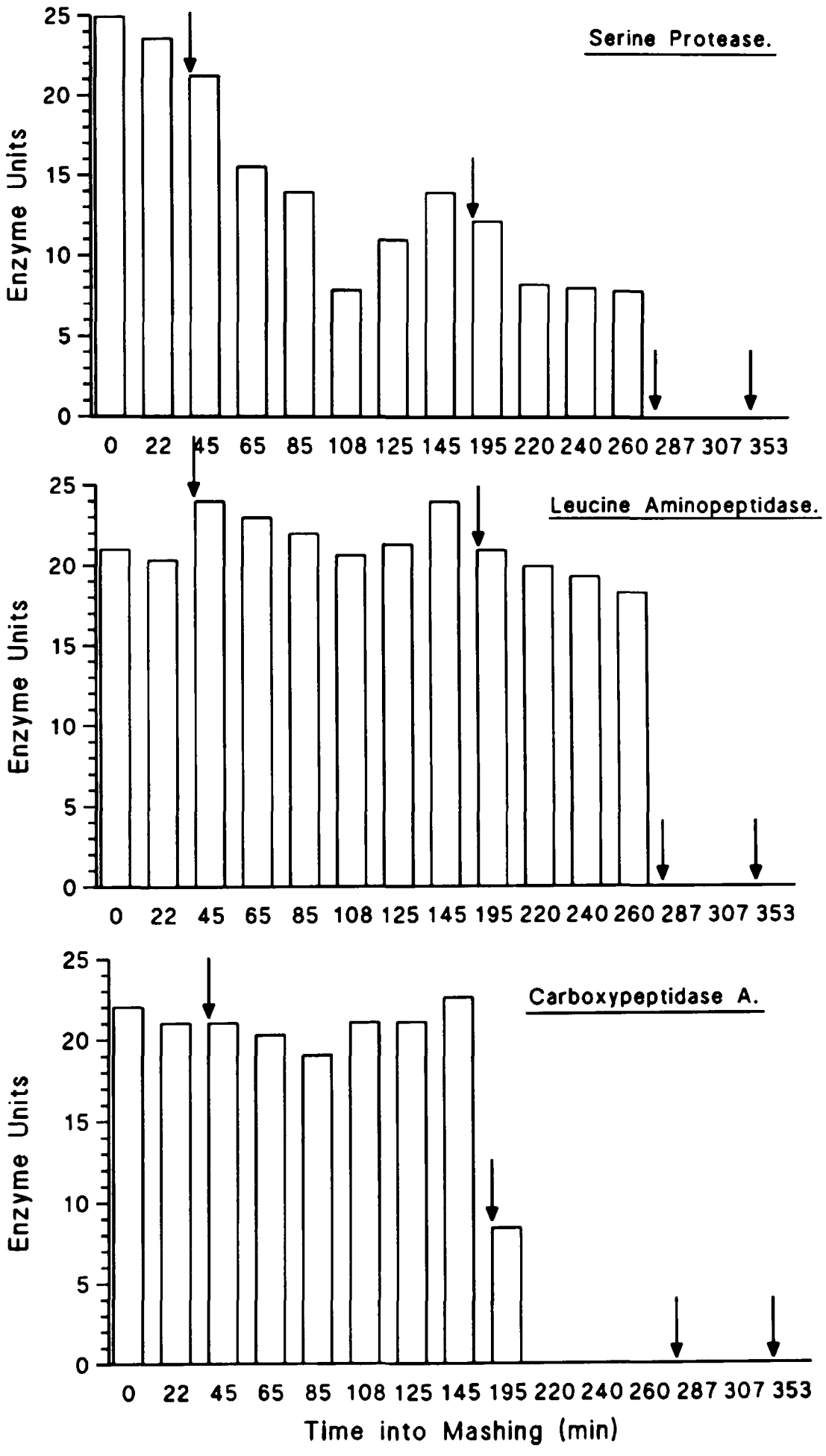


Figure 57. Serine Protease (Pooled S.D. = 0.803), Leucine Aminopeptidase (Pooled S.D. = 0.780) and Carboxypeptidase A (Pooled S.D. = 0.199) activities under optimal enzyme conditions during the mashing process. Raking after water addition shown by arrows.

was completed, after second water application, general proteinase type activity was found to have decreased by 47% from the end of the first water. A decrease in activity was observed during the period of the second water, and no activity detected during the sparging waters.

The levels of serine protease type activity (Figure 57) decreased from mashing-in until 108 min into the first water. From this time until the end of the first water activity increased. Activity was reduced by 13% from the end of the first water and after second water raking, activity was further reduced until the end of the second water.

Leucine aminopeptidase type activity (Figure 57) rose after first water raking, once mashing-in was completed. Activity fell slightly up to 108 min into the mash and then began to increase until the end of the first water. Although second water activity levels were not greatly reduced from the end of the first water activity, an overall decrease in activity was observed during the second water.

Carboxypeptidase A type activity (Figure 57) was relatively stable through-out the first water, with a very slight decrease in activity from mashing-in up to 85 min into mashing. Activity then rose to its maximum level at the end of the first water. Although a reduced activity was present at the beginning of second water raking, no further activity was detected during this water.

No levels of serine protease, leucine aminopeptidase or carboxypeptidase type activities were detected during the duration of the third and fourth waters.

3.4.2 Wort Draining.

The mash from which the wort draining was studied, was carried out under normal distillery procedures by an experienced distillery operator. All parameters studied were similar to those during the

mashing process, *i.e.*, all mashing-in temperatures and hot liquor temperatures were the same, the malt was supplied by the same maltster and was of the same barley variety.

The process variables of protein, phosphate and soluble free α-amino nitrogen levels were determined in duplicate from a single sample. Enzyme analyses, for amylases, minor carbohydrases, arabinosidase, β-xylosidase and acid phosphatase, were carried out on two samples, in duplicate. The remaining enzymes were assayed in duplicate from a single sample.

3.4.2.1 Process Variables.

It was necessary to carry out initial observations on the wort collected, to find the wort conditions for enzyme activity in later experiments. Those parameters observed were the pH and temperature of the wort before it entered the washback, along with the specific gravity. Further analyses were carried out on the wort and included the free amino nitrogen, phosphate and protein concentrations. The results for these six parameters are given in Figures 58 and 59 (Table 24, Appendix II).

During first water draining from the mash tun the temperature of the wort remained at a constant 20°C. This was also the temperature of the wort in the washback at the end of first water draining. A similar result was obtained at the end of second water draining with the temperature of the wort to, and in, the washback maintaining a constant 20°C.

The pH of the wort from the mash tun when draining commenced was pH 5.14. The pH then rose to a maximum of 5.32 between 50 and 70 min into the first water draining before falling to pH 5.32 at the end of first water draining. The pH of the wort in the washback, at the end of first water draining was 5.11. During the second water draining, there was a decrease in wort pH from pH 5.25 to pH 4.92, with a final washback pH of 5.02.

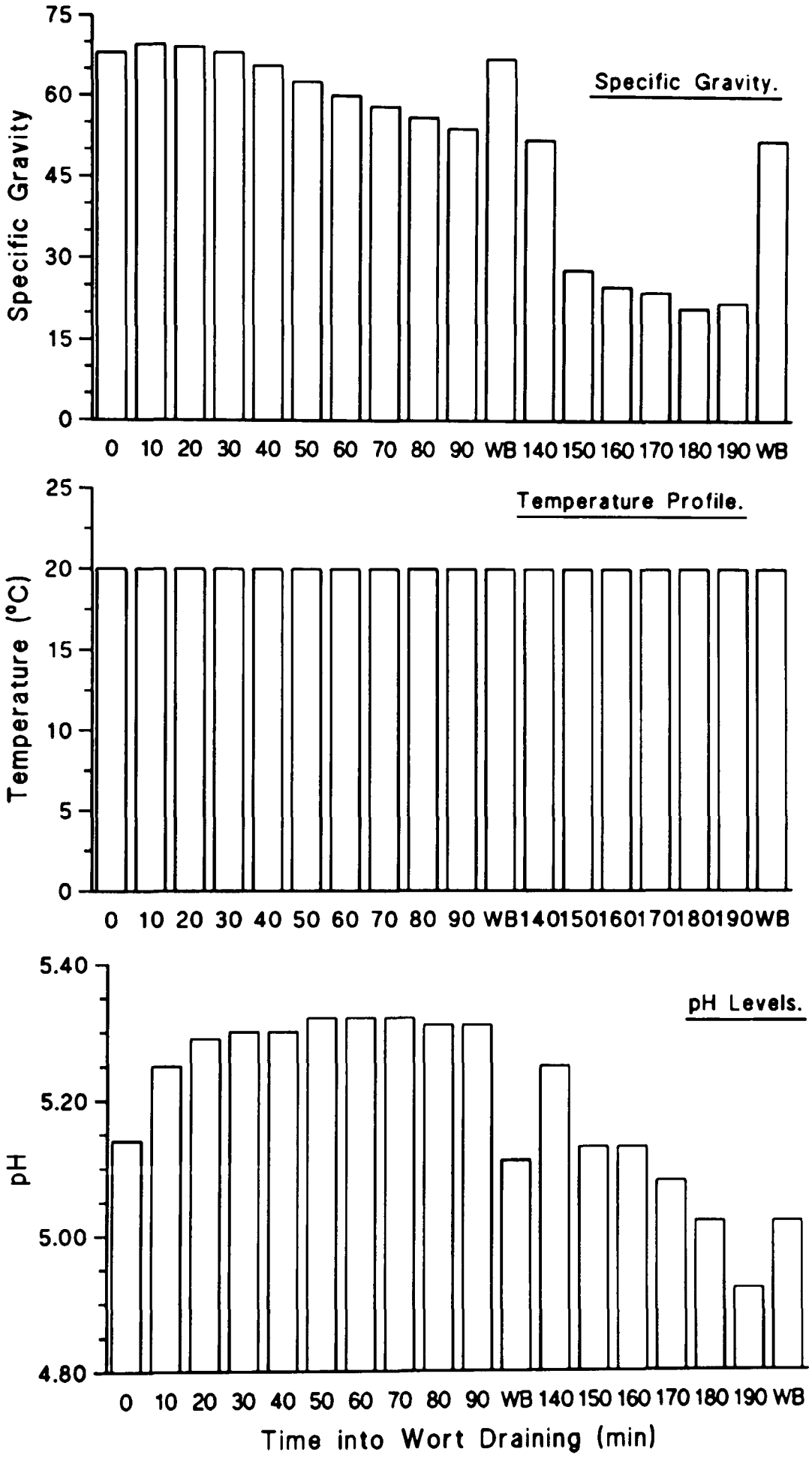


Figure 58. Measurement of specific gravity, temperature and pH variables during the wort draining process. Washback samples were removed at the end of each draining.

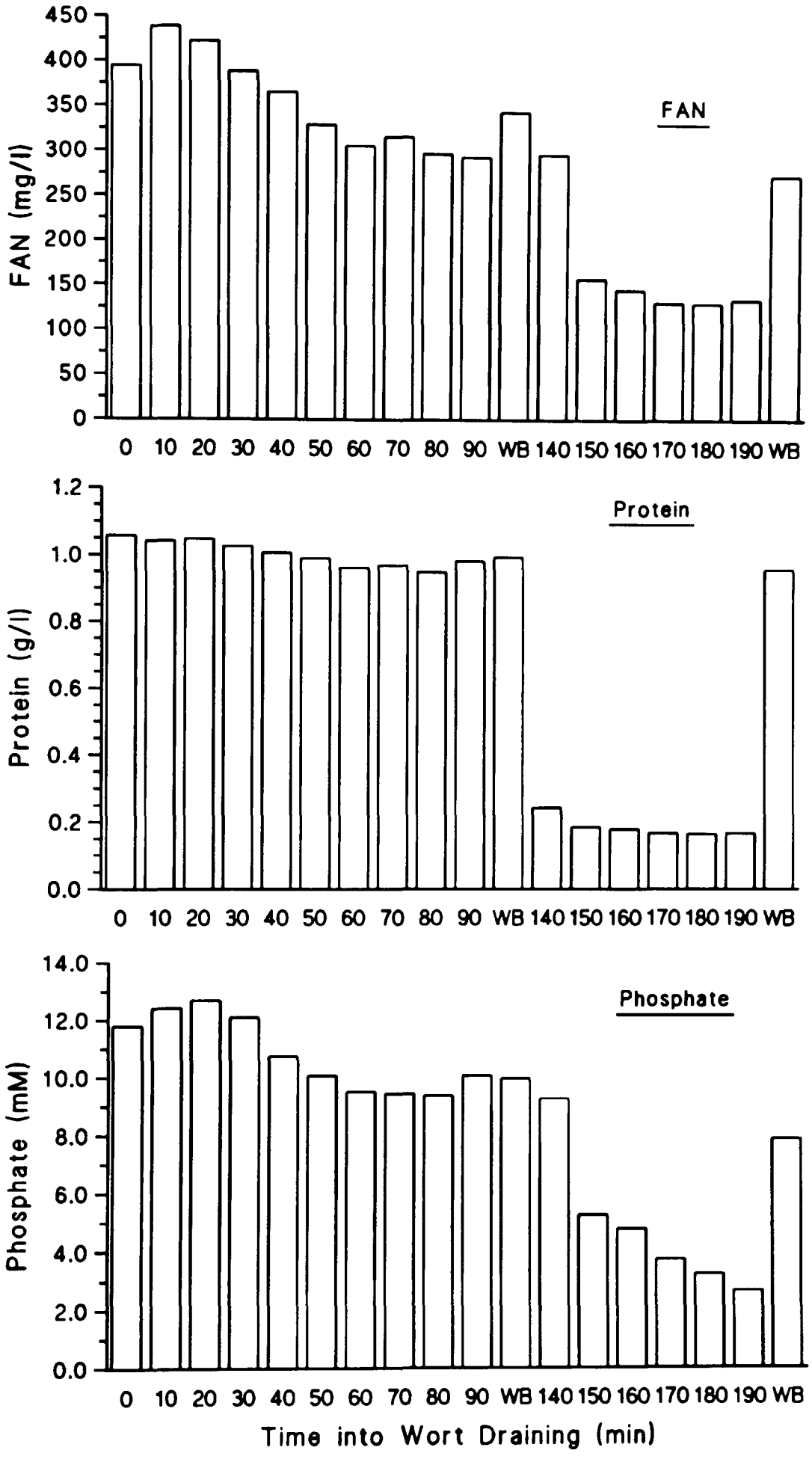


Figure 59. Measurement of phosphate (Pooled S.D. = 0.071), protein (Pooled S.D. = 0.038) and Free α-amino Nitrogen (FAN) (Pooled S.D. = 4.213) variables during the wort draining process. Washback samples were removed at the end of each draining.

The parameter that is used to monitor aspects of efficiency in the distillery is specific gravity. By following this parameter during wort draining a concentration gradient is found throughout the period of first and second water draining. Specific gravity is taken as the amount of fermentable sugars present, but encompasses all components of the wort, including substrates available to the yeast during fermentation. The specific gravity is therefore dependent, not only on the malt mashed, but also on the ratio of grist to mashing liquor. The original gravities of the washbacks in a distillery can therefore be selected within a reasonably close band. Many metabolites studied, especially total carbohydrate, free amino nitrogen and phosphate, show very similar patterns to the specific gravity results. The bulk of the metabolites are released into the wort during first water draining and during the second water. Thereafter, subsequent waters will provide metabolites that are leached from the mash or by enzymes not inactivated by the second water temperature.

Protein levels were found to follow a similar pattern to the specific gravity results, however the extent of the decrease in protein concentration during first water draining was not pronounced. Enzymic protein will be distributed heterogeneously within the wort in the mash tun, as would the non-enzymic protein. The effects of the mash bed as a filter also may be partially responsible for the more uniform concentration of protein throughout the first water draining period. The concentration of protein during the second water draining period was reduced by approximately 75%, compared to the first water protein concentrations.

The pitching of the yeast into the washback was carried out after 7 min of first water draining. This enabled the fermentation to proceed as quickly as possible, but will affect the various parameters studied, due to yeast metabolism, and also the secondary conversion of malt enzymes in the wort.

3.4.2.2 Enzyme Activities During Wort Draining.

All enzymes investigated during the first and second water, wort draining were done so, where applicable, under the pH and temperature of the wort when sampled. They were also assayed under the optimal conditions for the malted barley enzymes, determined previously.

Amylases.

During wort draining from the first water a steady decline in α-amylase activity was observed. A similar pattern was also found during second water draining. These results are shown in Figure 60. The activities for wort draining conditions and under mash tun conditions were comparable, although the activity of the α-amylase enzyme was greatly reduced under wort draining conditions. The residual activity was only 5-6% of the activity established under optimal conditions. This was found for all samples drawn.

The activity of β-amylase was only studied under optimal conditions, see Figure 61. Activity was found to decrease throughout the wort draining of both waters. Wort draining commenced approximately 60 min into the first water mashing cycle, and after 15 min following second water raking. The activity of both α-amylase and β-amylase in the drained wort was similar to that from the mashing process at the equivalent times. The dramatic decline in β-amylase activity was not observed during wort draining since this occurred during mashing-in.

The activity for both enzymes in the washback under optimal conditions are detailed in Table 26, (Appendix II). The activity decreased by approximately 30% for both between the end of first water draining and second water draining. α-Amylase activity under wort draining conditions was reduced by 43% between the two waters in the washback. This larger decrease in activity may be due to the differences in the pH of the wort in the washbacks between the two sampling times.

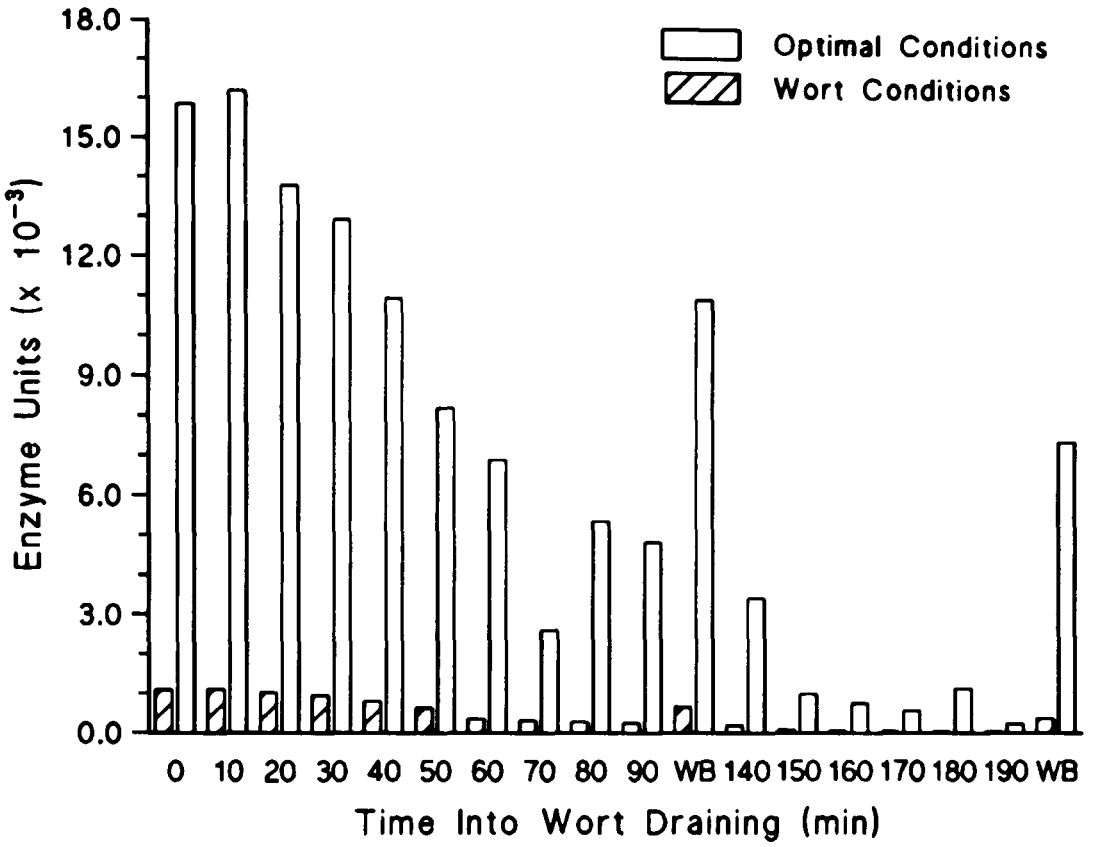


Figure 60. α–Amylase activity under wort (Pooled S.D. = 22.72) and optimal (Pooled S.D. = 200.05) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

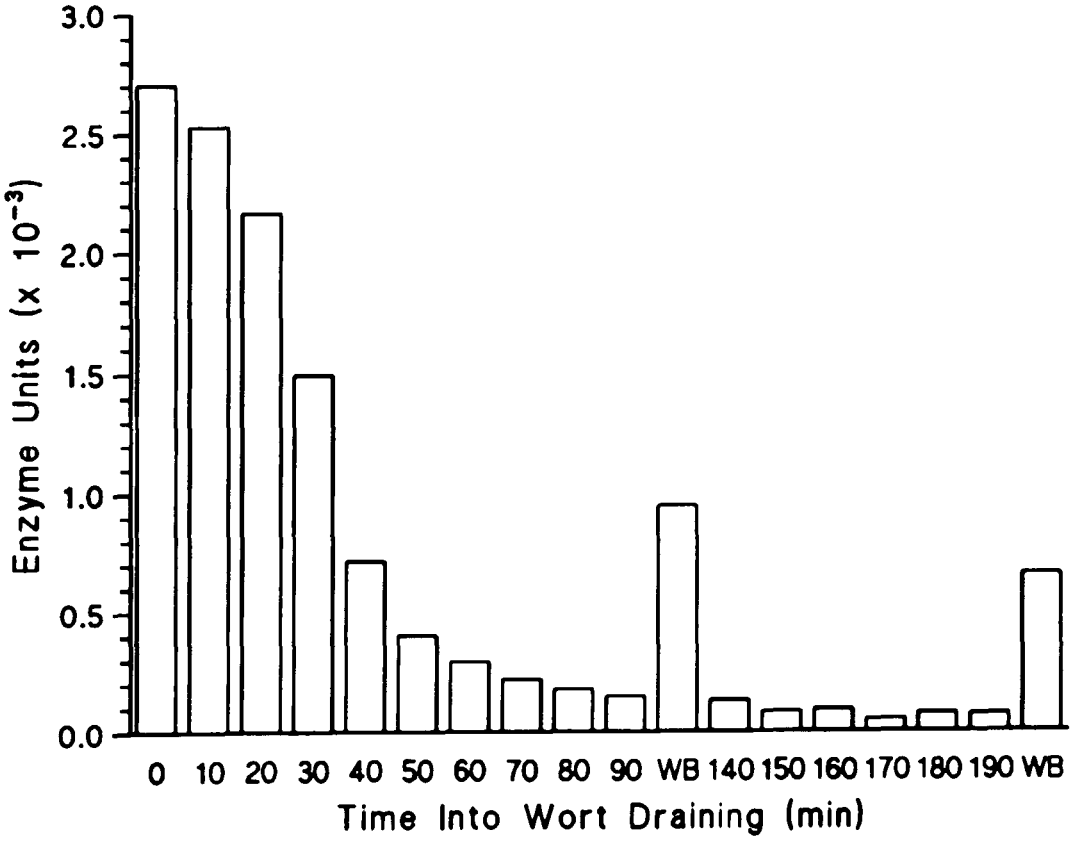


Figure 61. β–Amylase activity under optimal (Pooled S.D. = 13.29) conditions during the wort draining process. Washback samples were removed at the end of each draining.

Minor Carbohydrases.

The results for the minor carbohydrases and phosphatases are detailed in Table 25 and 26, (Appendix II).

The activity of α-glucosidase (Figure 62) for wort conditions during the first water draining exhibited a decrease up to 60 min. Between 60 to 90 min the levels began to rise from 3% of the initial activity at 0 min to 30% of the initial activity. Enzyme levels decreased during the 50 min period of second water draining. A similar pattern was obtained when the optimal enzyme conditions were studied. The minimum level of activity, under optimal conditions, was found after 70 min during first water draining, and was 6% of the initial activity at 0 min. At the end of first water draining α-glucosidase activity had risen to 25% of the initial activity. Activities for both sets of assay conditions for α-glucosidase had decreased between 30 and 40% in the washback between the end of first water and second water draining.

β-Glucosidase activity (Figure 63) during first water draining, under the wort assay conditions, fell to zero enzyme units after 70 min, thereafter, activity increased to 31% of the initial activity at 0 min at 90 min. Activity fell throughout the period of second water draining and no activity was found after 40 min. Under optimal enzyme assay conditions, there was an increase in activity to 20 min into the first water draining, but it then decreased until the end of the first water. Second water activity decreased throughout the draining period. β-Glucosidase activity, under both sets of assay conditions, in the washback, at the end of both draining periods was reasonably high, in comparison to the levels during draining.

The activity of α-galactosidase under wort draining conditions of pH and temperature, Figure 64, fell during the first 50 min into draining. Thereafter, a steady increase in activity until the end of the first water draining was observed. Second water draining activity decreased throughout the period of this water. Optimal assay

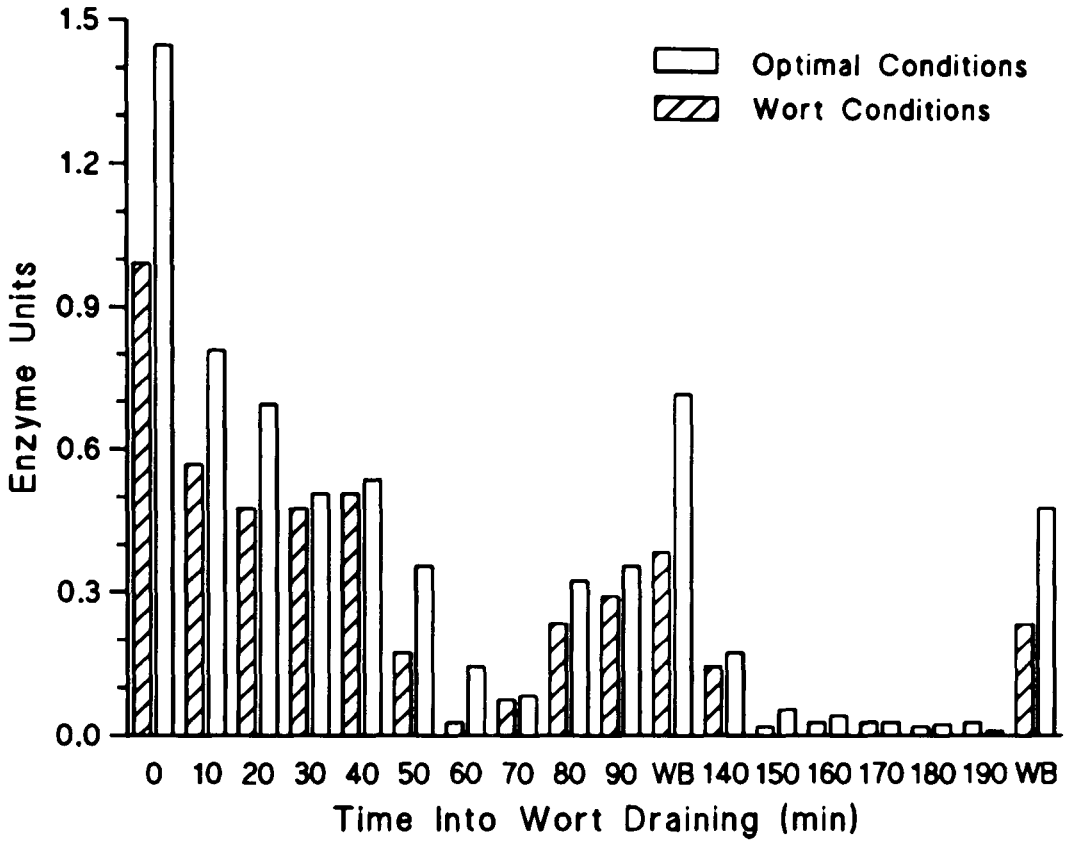


Figure 62. α-Glucosidase activity under wort (Pooled S.D. = 0.037) and optimal (Pooled S.D. = 0.029) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

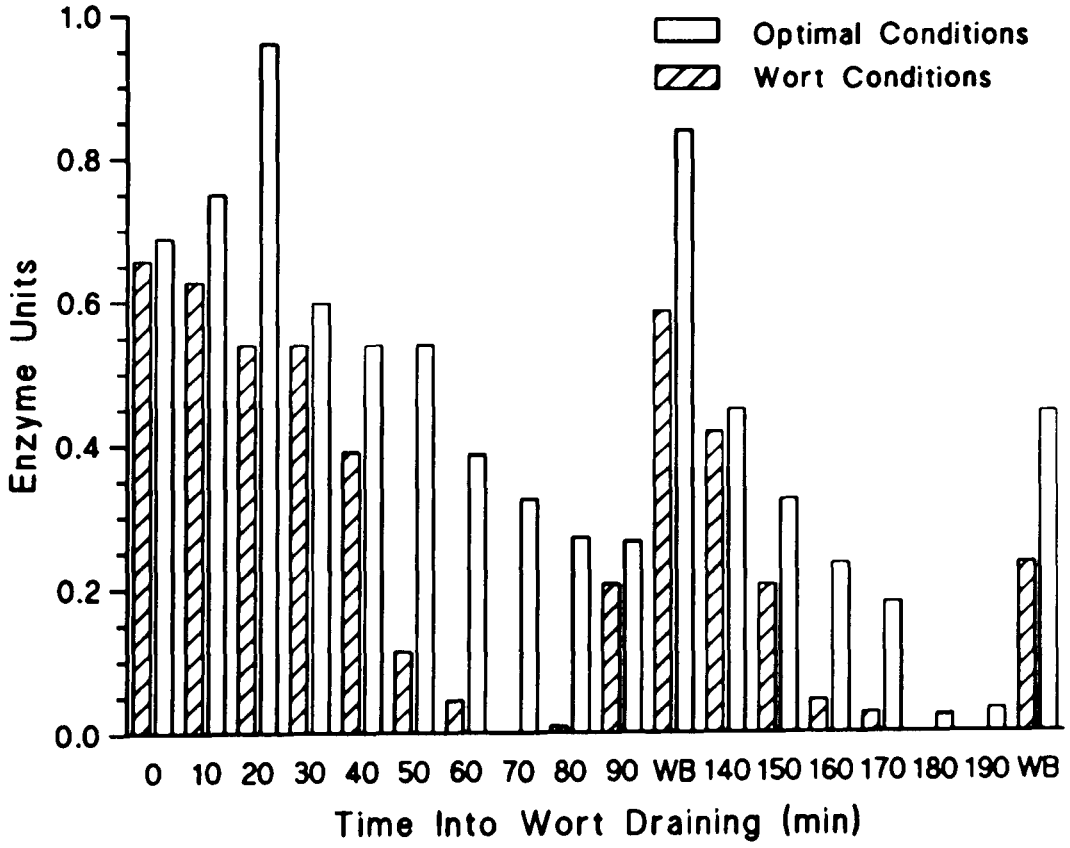


Figure 63. β-Glucosidase activity under wort (Pooled S.D. = 0.013) and optimal (Pooled S.D. = 0.022) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

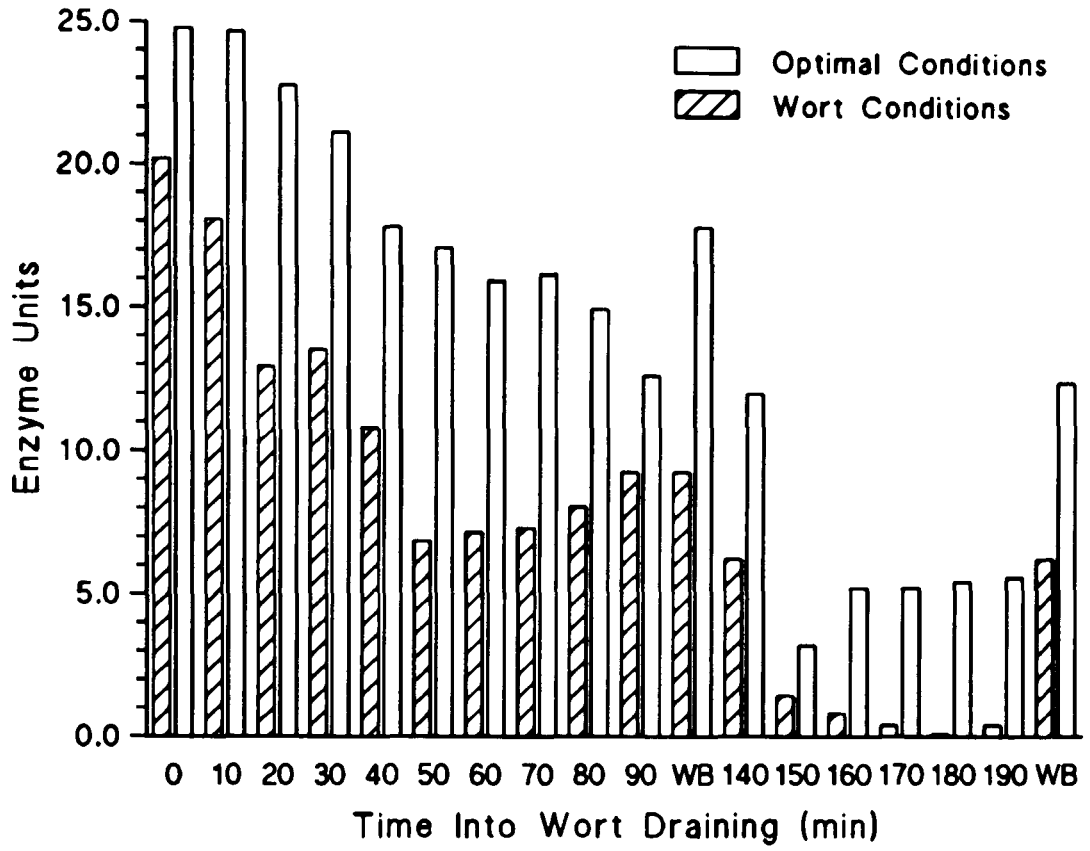


Figure 64. α-Galactosidase activity under wort (Pooled S.D. = 0.943) and optimal (Pooled S.D. = 0.264) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

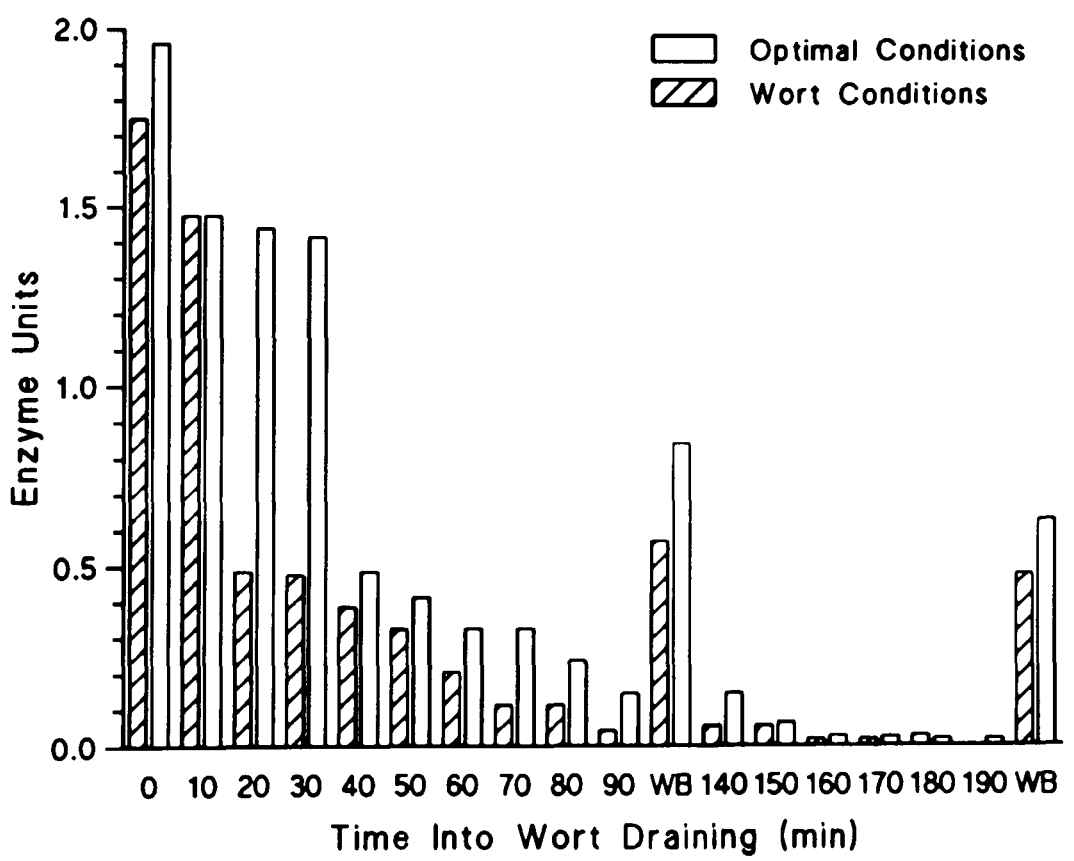


Figure 65. β-Galactosidase activity under wort (Pooled S.D. = 0.021) and optimal (Pooled S.D. = 0.034) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

conditions of the enzyme gave a steady decrease in activity through the first water, followed by a further decline up to 20 min into the second water draining. Thereafter, activity began to rise until the end of second water draining. The activity of α-galactosidase in the washback at the end of both waters was comparable to the activity during draining.

β-Galactosidase activity, Figure 65, under both sets of enzyme conditions was found to decrease throughout both first and second waters. Activity in the washback once the wort draining was completed was approximately 30% of the initial activity at 0 min. This was the case for both sets of enzyme conditions.

The activities of α-mannosidase (Figure 66), and β-mannosidase (Figure 67), both followed similar patterns with initial activities of the optimal assay conditions quite high. These activities then declined up to 80 min into draining. A slight increase in activity was observed from this time until the end of first water draining. The activity patterns for both enzymes under wort assay conditions were also comparable. After an initial decrease in activity up to 20 min the levels of enzyme activity remained relatively constant throughout first water draining.

Activity during the second water draining was only seen to any extent with α-mannosidase under optimal conditions. Little or no activity was detected after 20 min draining of the second water under wort assay conditions with α-mannosidase or β-mannosidase. Only a slight activity was observed for β-mannosidase under optimal conditions from the second water draining period. Washback levels of activity were much greater for α-mannosidase than for β-mannosidase under both sets of enzyme assay conditions.

The pattern of N-acetylglucosaminidase was similar for both sets of assay conditions (Figure 68). Activity decreased throughout the total draining period. The enzyme activity under wort conditions was

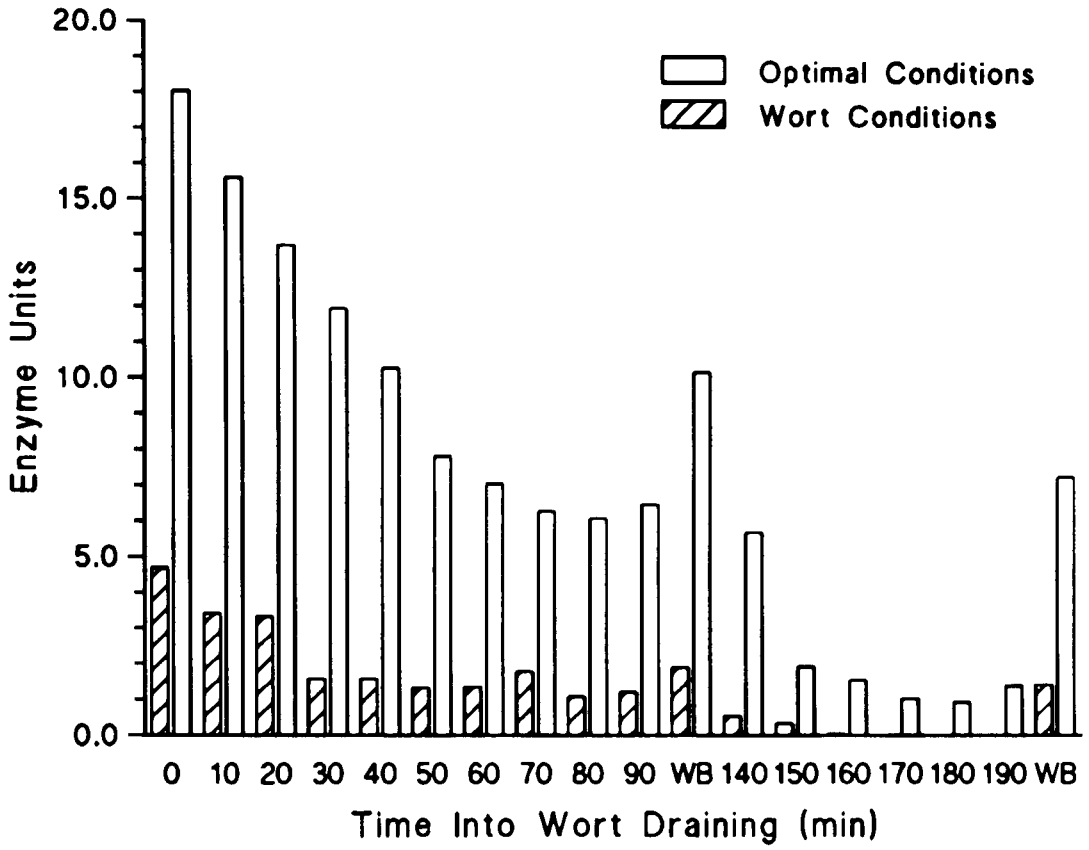


Figure 66. α-Mannosidase activity under wort (Pooled S.D. = 0.095) and optimal (Pooled S.D. = 0.267) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

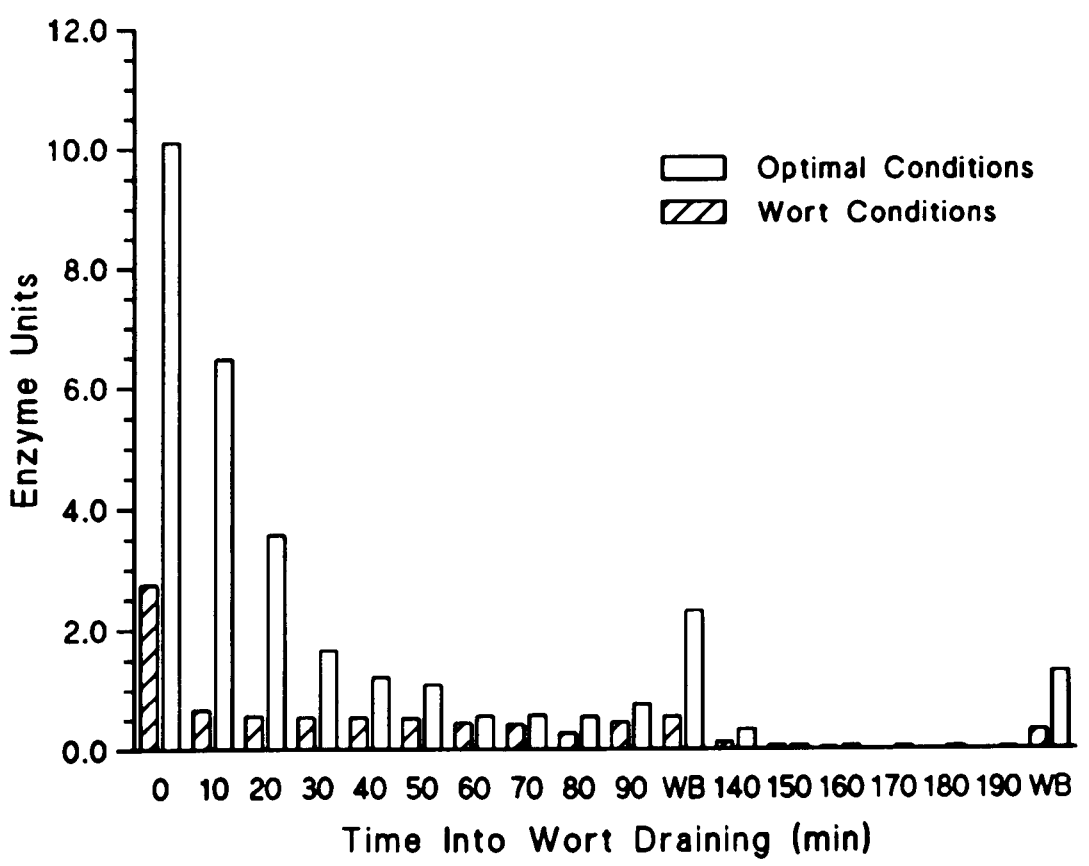


Figure 67. β-Mannosidase activity under wort (Pooled S.D. = 0.034) and optimal (Pooled S.D. = 0.060) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

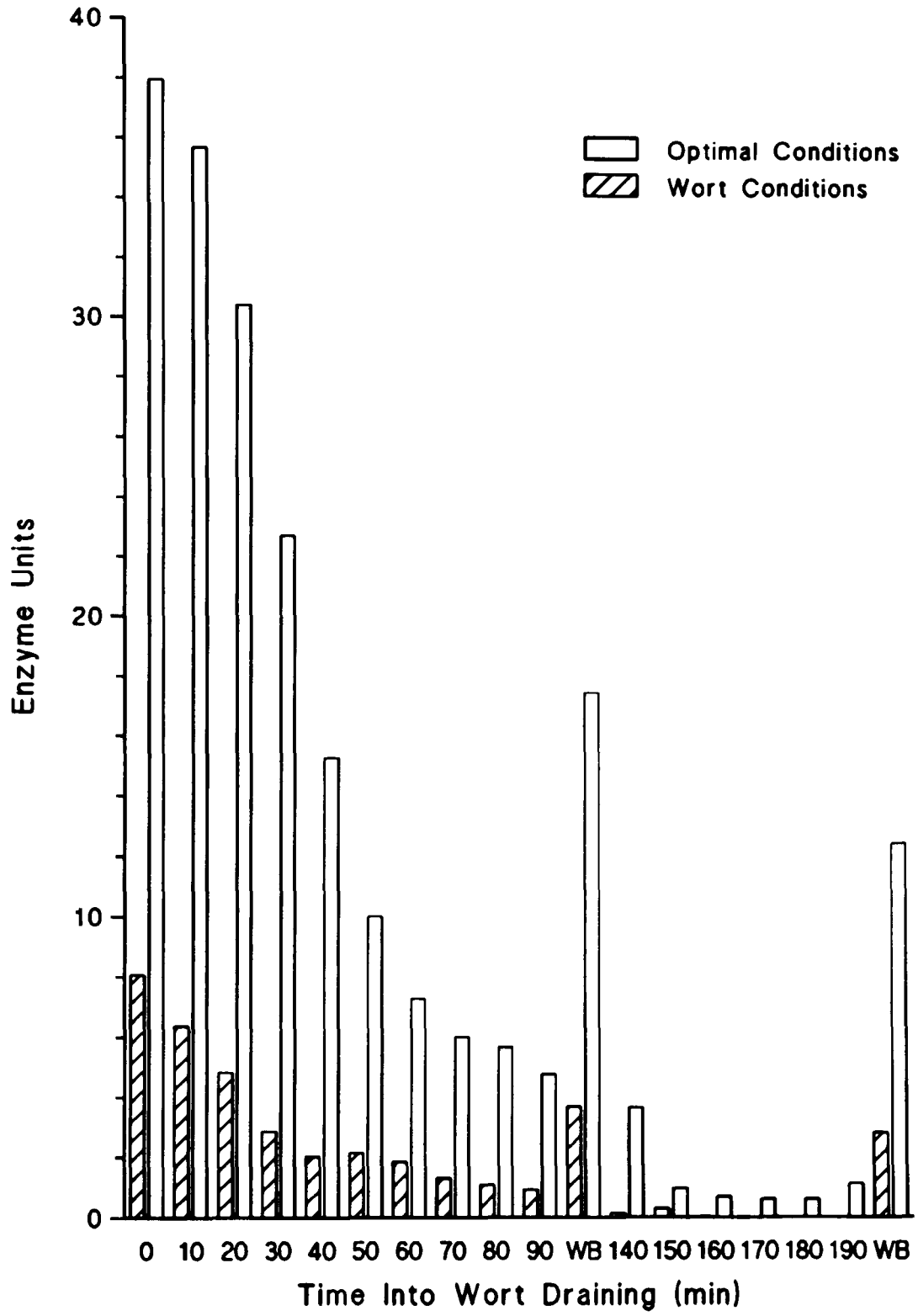


Figure 68. N-Acetyl-glucosaminidase activity under wort (Pooled S.D. = 0.069 and optimal (Pooled S.D. = 0.309) enzyme conditions during optimal enzyme conditions during the wort draining process.

approximately 25% of the optimal enzyme activity in all samples. Activity levels after the completion of second water draining in the washbacks were quite high for both sets of enzyme conditions.

Activity of arabinosidase (Figure 69), was relatively constant throughout the first and second water draining, although the activities in the second water were lower for the wort enzyme conditions. Enzyme activity in the washback after the second water draining under wort enzyme conditions was much reduced when compared to the end of the first water draining period.

After an initial increase in activity of β-xylosidase, under optimum enzyme conditions, (Figure 70), the activity decreased steadily until the end of the second water draining. Under wort assay conditions of pH and temperature the activity of β-xylosidase declined up to 30 min. The activity remained relatively steady from this time up to the end of first water draining. A decrease in activity was found during the second water draining for both sets of assay conditions. Activity in the washback, after second water draining, under wort assay conditions was relatively high, approximately 80% of the initial first water draining activity once draining had been completed. These high results in the washback may be due to the very high levels of activity found under optimum assay conditions, which are approximately 90% higher than the wort assay conditions activity.

Phosphatases.

The activity of acid phosphatase was found to decline steadily under both sets of enzyme assay conditions during the total period of wort draining (Figure 71). The levels of activity of phytase (Figure 72) were studied under optimal assay conditions. Activity was greatest during the initial stages of wort draining but then decreased up to 40 min into first water draining. Between 40 to 70 min the activity increased slightly before decreasing towards the end of the first water draining period. Activity during the second water draining decreased between 10 to 50 min.

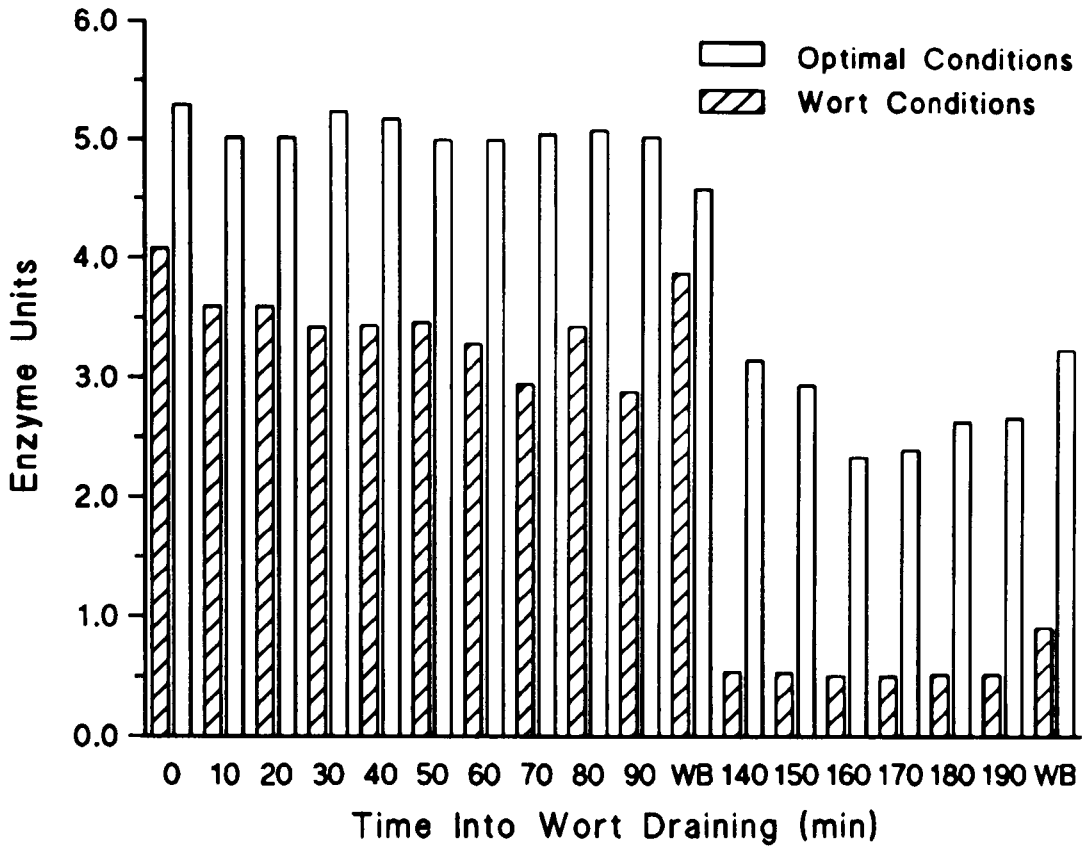


Figure 69. Arabinosidase activity under wort (Pooled S.D. = 0.199) and optimal (Pooled S.D. = 0.309) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

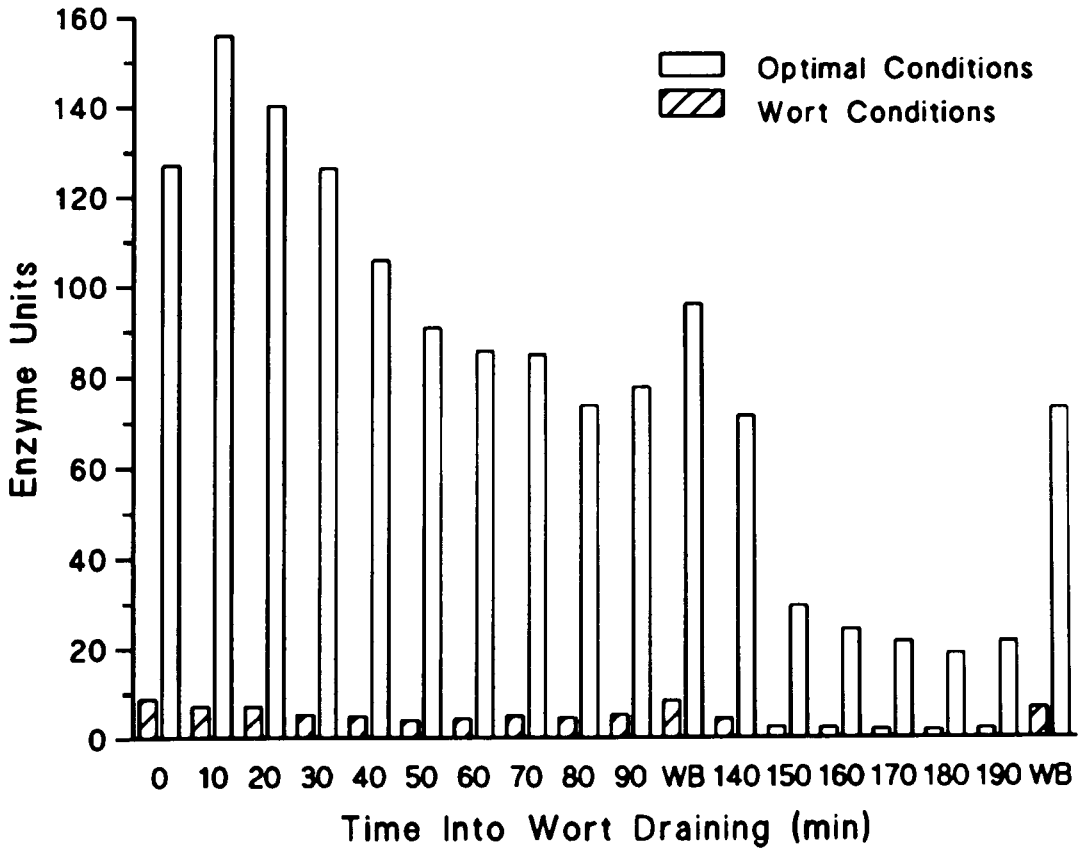


Figure 70. β-Xylosidase activity under wort (Pooled S.D. = 0.163) and optimal (Pooled S.D. = 1.038) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

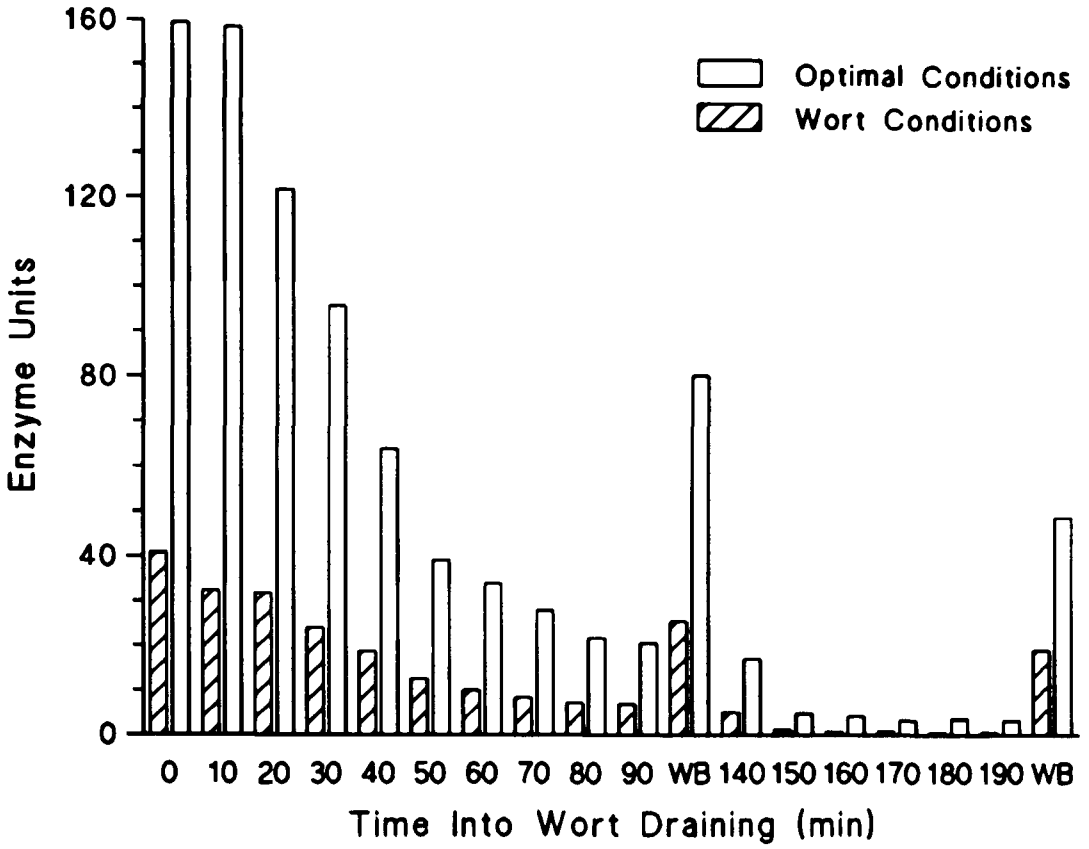


Figure 71. Acid phosphatase activity under wort (Pooled S.D. = 0.289) and optimal (Pooled S.D. = 0.958) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

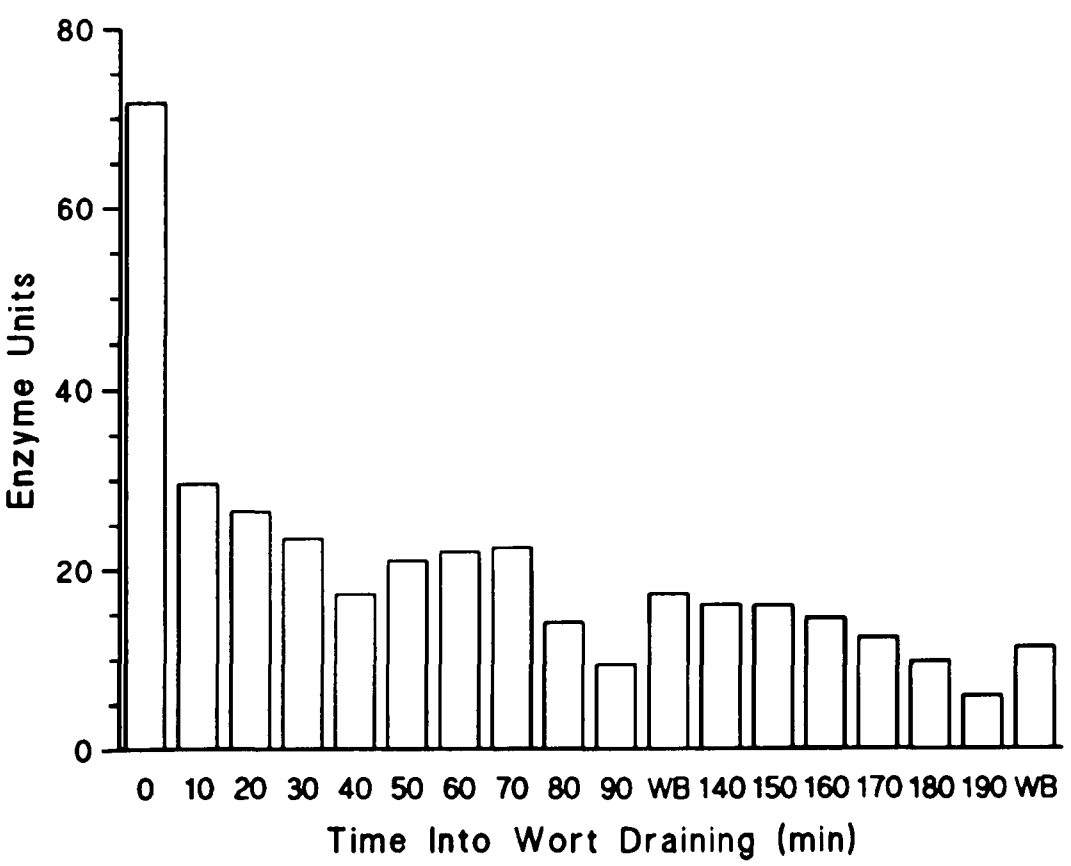


Figure 72. Phytase activity under optimal (Pooled S.D. = 0.738) enzyme conditions during wort draining. Washback samples were removed at the end of each water.

Proteinases and Proteases.

General proteinase type activity and the activities of the proteases studied are detailed in Table 27 (Appendix II). Each of the enzyme types studied were done so under optimal assay conditions only.

General proteinase type activity increased slightly over the initial 20 min period of wort draining (Figure 73). For the remainder of the first water draining, and during the second water draining activity decreased. A substantial activity remained in the washback after both waters had been drained from the mash tun.

The levels of serine protease type activity (Figure 74) were present mainly during first water draining. Activity rose to a peak between 50 to 60 min before decreasing to 80 min. An increase in activity was found at 90 min during first water draining. The serine protease type activity decreased during second water draining. Activity in the washback after the second water had drained suggested a slight decrease from the level after first water draining.

Leucine aminopeptidase type activity was found to increase slightly during the first 60 min of first water draining, (Figure 74). After this period the activity remained quite constant during the final period of first water draining and the whole of the second water. Activity was found to increase after second water draining in the washback.

Carboxypeptidase A type activity was observed from Figure 74 to decrease throughout the period of draining. Activity dropped significantly after 50 min draining of the first water. The carboxy-peptidase A type activity was still shown to be present in the washback after second water draining, although the activity was determined under optimal assay conditions.

3.4.3 Fermentation.

Having obtained the wort from the malted barley grist, through the mashing and wort draining processes, it was now possible to initiate the fermentation process. This process is carried out by

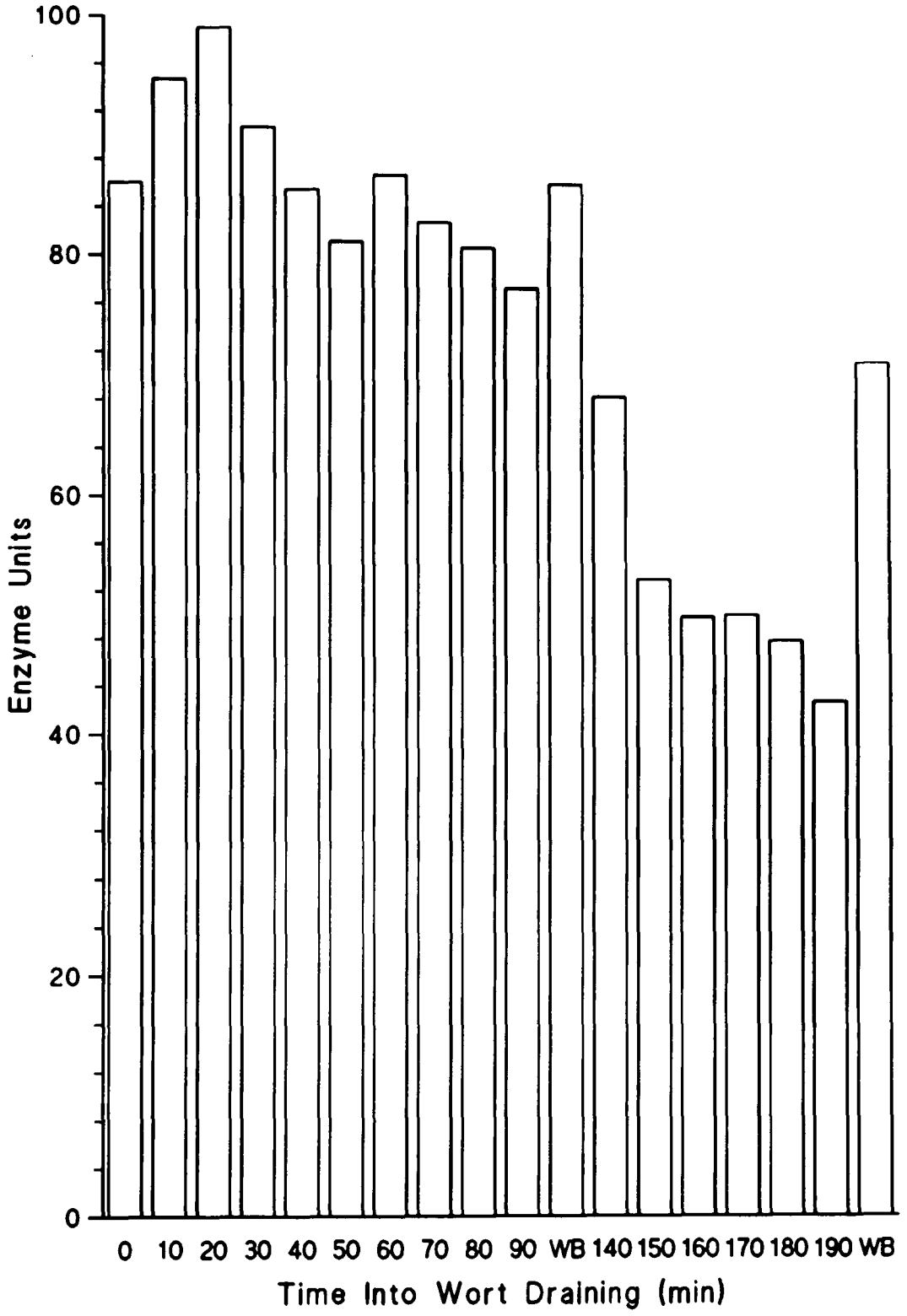


Figure 73. General proteinase activity under optimal (Pooled S.D. = 1.570) enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

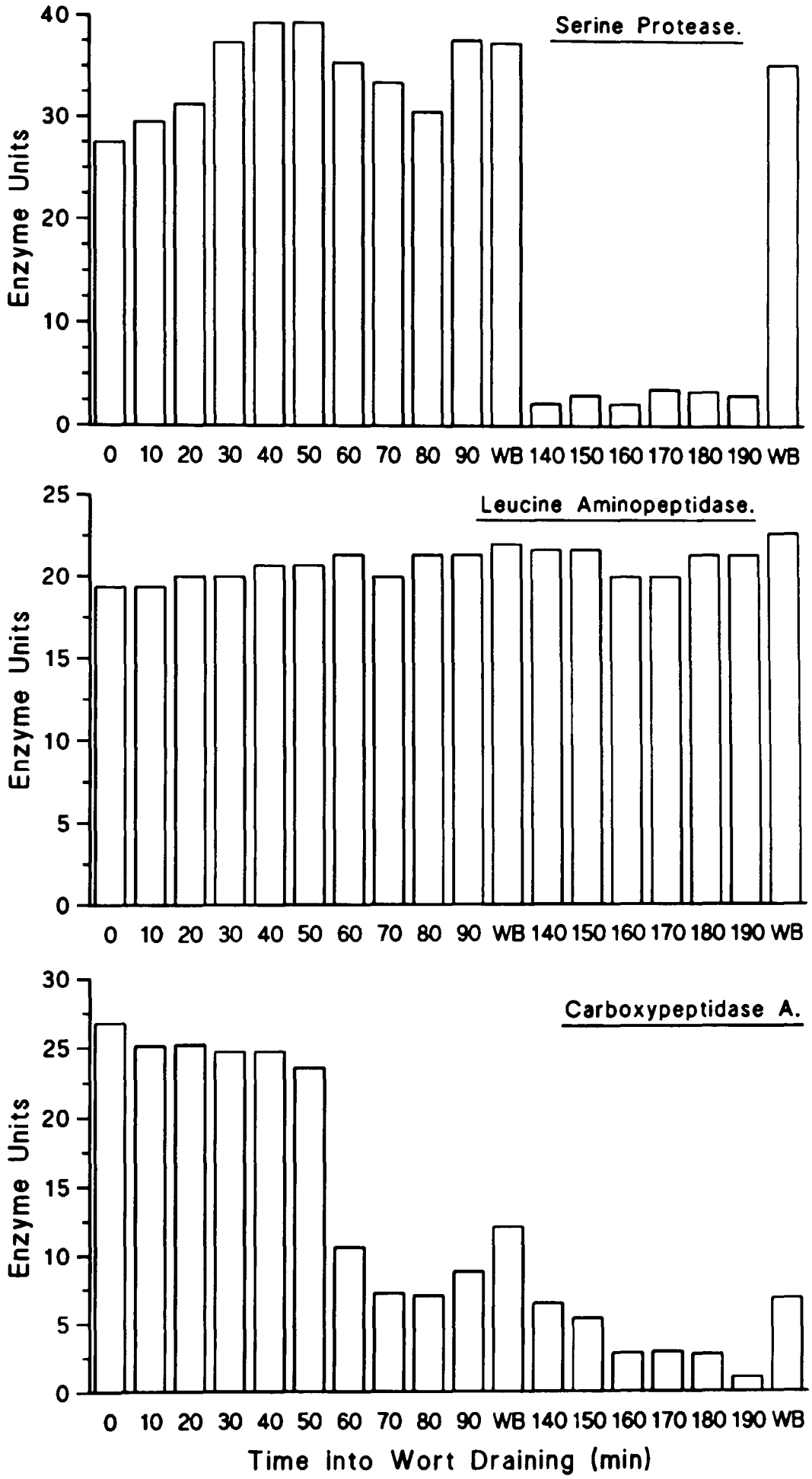


Figure 74. Serine protease (Pooled S.D. = 1.158), leucine amino-peptidase (Pooled S.D. = 0.968) and carboxypeptidase A (Pooled S.D. 0.720) activities under optimal enzyme conditions during wort draining. Washback samples were removed at the end of each draining.

pitching a quantity of yeast into the washback once the temperature of the wort in the washback has equilibrated to the required pitching temperature. As stated in the previous section on wort draining this temperature is approximately 20°C to 22°C, and takes place after about 7 min into the first water draining. The fermentation of the yeast on the malted barley wort commences within a very short lag phase. Distillery practice is to declare or set, *i.e.*, zero hours for fermentation, the washback once the second water has completely drained to the washback.

Because of the practical difficulties of monitoring a single washback, a series of four washbacks was studied through-out the fermentation time coarse. Each fermentation was mashed with the same malted barley variety from a single maltster, under normal distillery procedures, by experienced distillery operators. All primary distilling yeast was from the same batch and secondary strains were from the same supplier with the corresponding batch number. Each washback was set within 8 hr of each other and comparisons were made between washbacks at 12 and 24 hr. Although only the results from the washback sequence were reported, results at each of these times were within a 95% confidence interval. The washbacks were inoculated with yeast once the temperature of the wort in the washback had stabilized. Fermentation began almost immediately, but 0 hr was deemed to be when the washback was set.

The process variables of yeast viability, biomass, protein, phosphate and soluble free α-amino nitrogen levels were determined in duplicate from a single sample. Succinic acid, lactic acid, acetic acid, ethanol and glycerol results were obtained in duplicate from two single samples. Enzyme analyses, for amylases, minor carbohydrases, arabinosidase, β-xylosidase and acid phosphatase, were carried out on two samples, in duplicate. The remaining enzymes were assayed in duplicate from a single sample.

3.4.3.1 Process Variables.

It was necessary to carry out initial observations on the fermenting wort or wash to find the washback conditions for enzyme activities in the washback. These again consisted of temperature and pH. Further washback parameters were studied and included specific gravity, yeast viability, wash solids, free α-amino nitrogen, protein and phosphate concentration. In addition the major products of yeast and bacterial fermentation were measured to enable a more comprehensive study of fermentation as carried out in the production of Scotch Malt Whisky. The major products of yeast and bacterial fermentation studied were ethanol, glycerol, succinic acid, lactic acid and acetic acid.

From the results in Figure 75, (Table 28, Appendix II) the washback temperature at the beginning of fermentation was 21°C. This increase in temperature in comparison to the wort draining temperature was due to a cooler ambient temperature. The fermentation rate is governed by several factors, and one of these is the initial temperature of the fermentation. Washback temperature remained constant at 21°C during the first six hours of fermentation and then rose steadily to 30°C after 22 hr. Thereafter, the temperature increased gradually to 32°C at the end of fermentation. The pH profile of the washbacks studied gave a drop in pH from pH 4.92 at the beginning of fermentation to pH 4.02 after 20 hr. A rise in pH was then observed to 4.33 after 42 hr. At the end of fermentation (46 hr) the pH was 4.27. If the fully fermented wash had been left over a longer period the pH would have fallen further. Studies in this laboratory have shown that the pH decline after approximately 40–48 hr is due to an increase in the bacterial population of the washback. Sometimes these bacterial fermentations after the main yeast fermentation are used to enhance certain types of whisky, *i.e.*, American sour mash whiskey.

It was possible to monitor the fermentation efficiencies by following the decrease in gravity during fermentation. From Figure 75, Specific Gravity, no fermentation problems were encountered with any of the

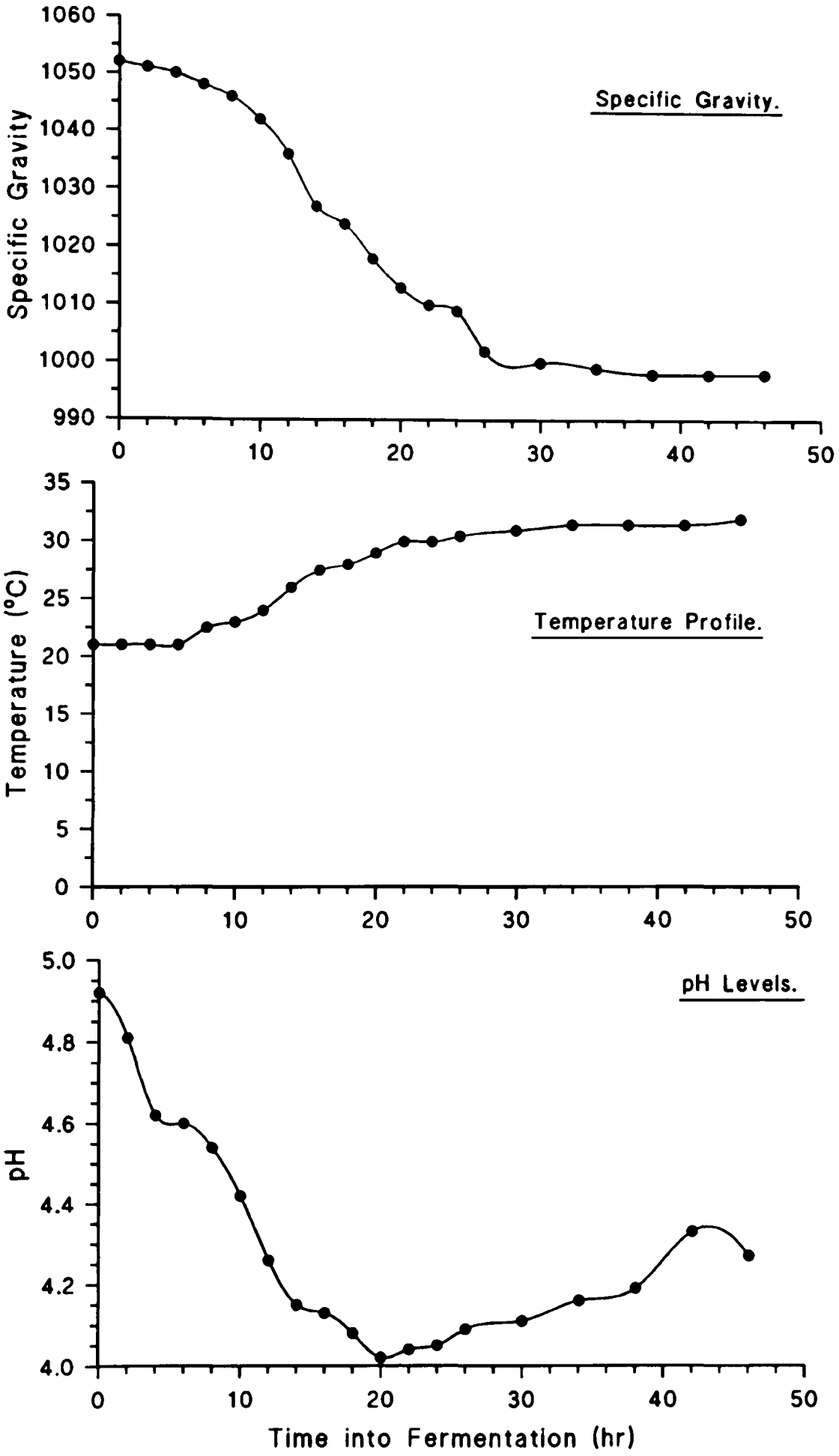


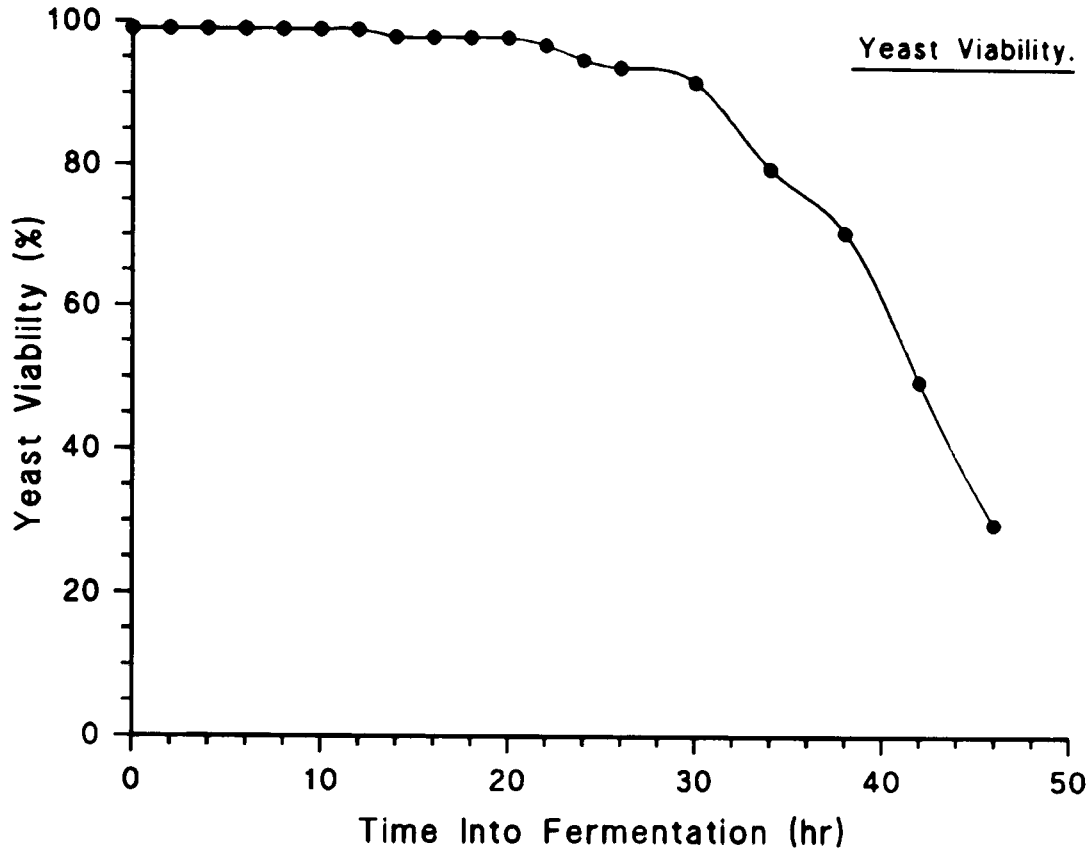
Figure 75. Measurement of specific gravity, temperature and pH variables during the fermentation process.

washbacks studied. Yeast viability, Figure 76, remained above 90% until just after 30 hr and then fell to 30% viable at the end of fermentation. This result was expected during this type of distillery fermentation as was the biomass. The biomass weights rose steadily to peak between 22-38 hr before a decrease was observed (Figure 76).

Soluble free α-amino nitrogen content, Figure 77, indicated the overall utilization of amino acids by the yeast during fermentation. A sharp decrease in the soluble free α-amino nitrogen content to 14 hr was followed by an inactive period of amino acid uptake to 30 hr. The soluble free α-amino nitrogen content in the fermenting wash began to increase thereafter. The decrease in soluble free α-amino nitrogen content with its subsequent increase after 30 hr followed an inverse pattern to the wash biomass results.

The protein concentration of the wash was determined throughout the fermentation, Figure 77, and a decrease over the initial 10 hr period was observed. During the next 20 hr period, a peak in protein concentration in the fermenting wash was seen at 20 hr. After 30 hr another smaller rise in protein levels was observed before the level declined towards the end of fermentation. This pattern in the soluble protein content probably reflected the different biological systems found within the fermentation at different times. The initial decrease may be seen as the degradation of the malted barley proteins, with probably yeast proteins being released into the fermentation media up to 30 hr. Once yeast lysis occurs, yeast protein will increase in the wash may then be used by the bacterial population present in the wash.

Phosphate levels, Figure 77, decreased rapidly over the first 12 hr of the fermentation, from 24 hr until the end of fermentation an overall increase was observed. Phosphates from the malted barley have a buffering effect on the mashing enzymes, but are also required for yeast growth.



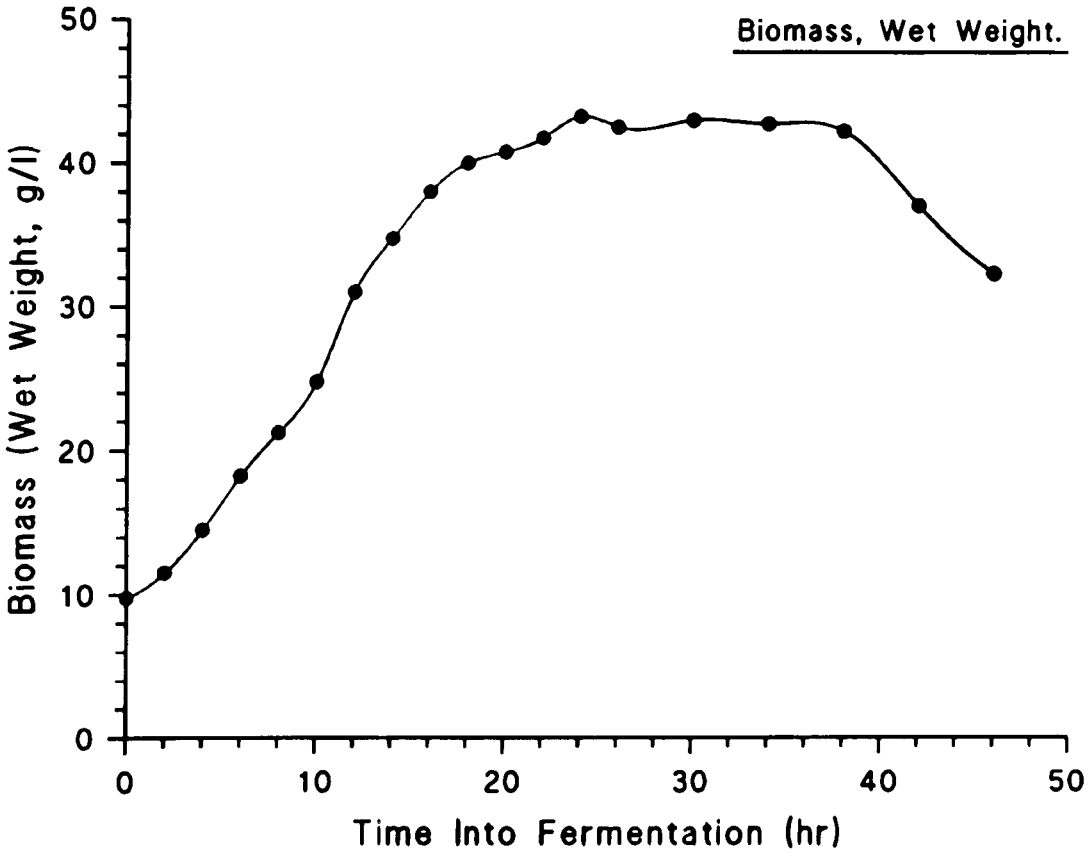


Figure 76. Measurment of biomass (wet weight) (Pooled S.D. = 0.788) and yeast viability (Pooled S.D. = 1.428)variables during the fermentation process.

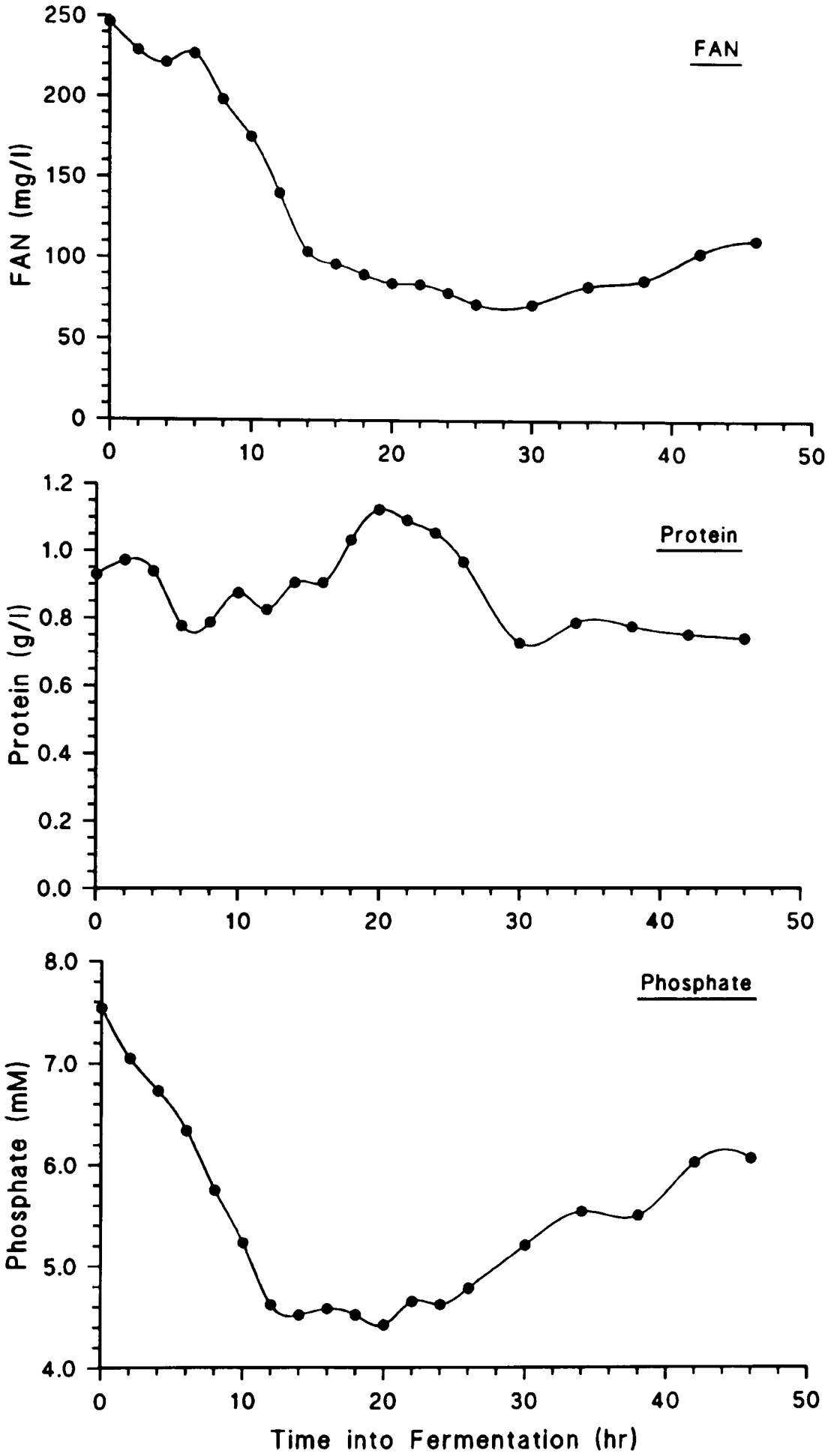


Figure 77. Measurement of Free α-amino nitrogen (FAN) (Pooled S.D. = 1.663), protein (Pooled S.D. = 0.047) and phosphate (Pooled S.D. = 0.096) variables during the fermentation process.

The major products of anaerobic fermentation by yeast are ethanol and CO_2, with the minor fermentation products being glycerol and to a certain extent succinic acid. During fermentation of the malted barley wort, the concentrations of ethanol, glycerol and succinic acid all increase steadily before reaching a plateau. However, the succinic acid levels decreased between 18 and 30 hr before rising towards the end of the fermentation. This effect on succinic acid is probably due to bacterial metabolism. The results of these end products are given in Figures 78 and 79 as detailed in Table 29, (Appendix II).

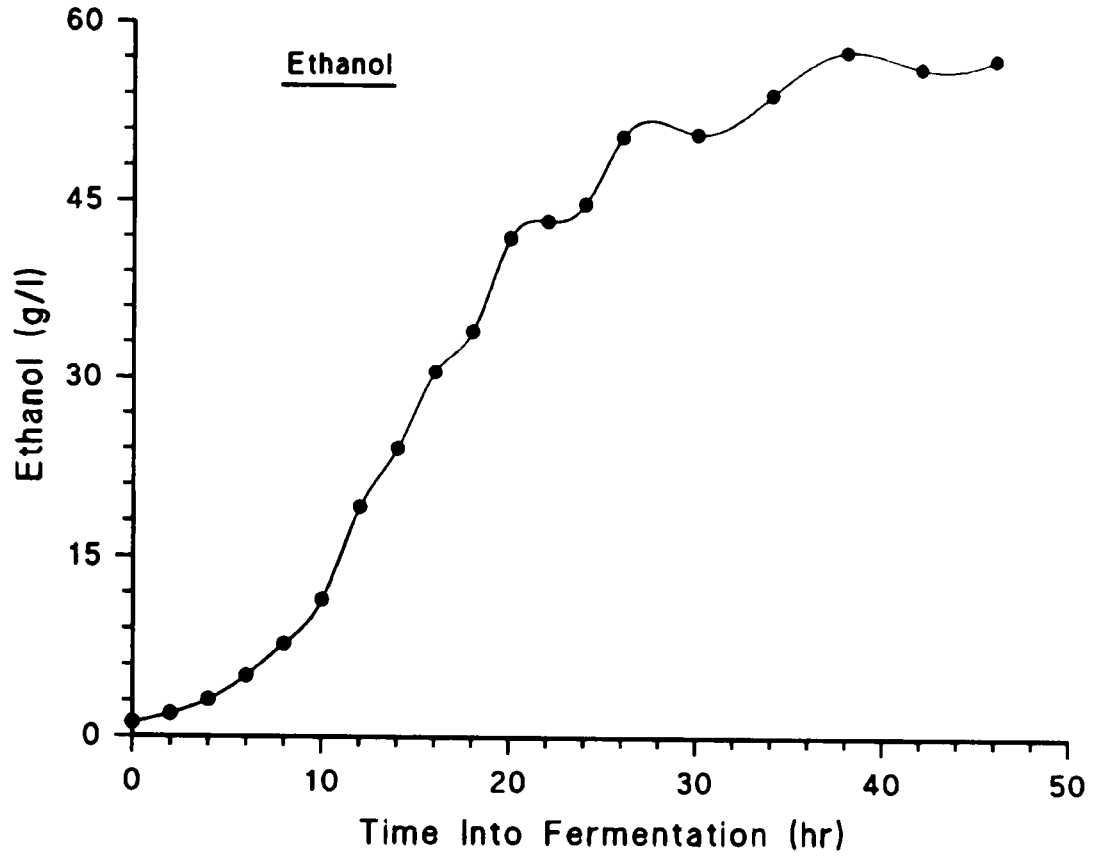
Besides yeast fermentation end products, there are also the products from bacterial metabolism. Since the most common distillery associated bacteria are the *Lactobacillus* and *Acetobacter*, the levels of lactic acid and acetic acid were monitored throughout the fermentation. Both these organic acids may be excreted from yeast. From Figure 79, the concentrations of lactic acid and acetic acid began to rise rapidly towards the end of active yeast fermentation. Thus suggesting a bacterial fermentation was proceeding within the bulk of the wash.

3.4.3.2 Enzyme Activities During Fermentation.

Enzyme activity was carried out under fermentation conditions of pH and temperature where applicable, and under optimum enzyme conditions. Optimal enzyme conditions, as stated previously, are for the malted barley enzymes.

Amylases.

During fermentation conditions the activity of α-amylase decreased by 263 E.U. after the first 12 hr of fermentation. After this period the activity of α-amylase remained relatively low (5-14 E.U.), although there was a very slight increase between 30 hr and the end of fermentation. Under optimal α-amylase conditions the pattern of activity was very similar to that found under fermentation conditions. However, the level of activity is 15 to 25 times higher under the optimal conditions. This is illustrated in Figure 80.



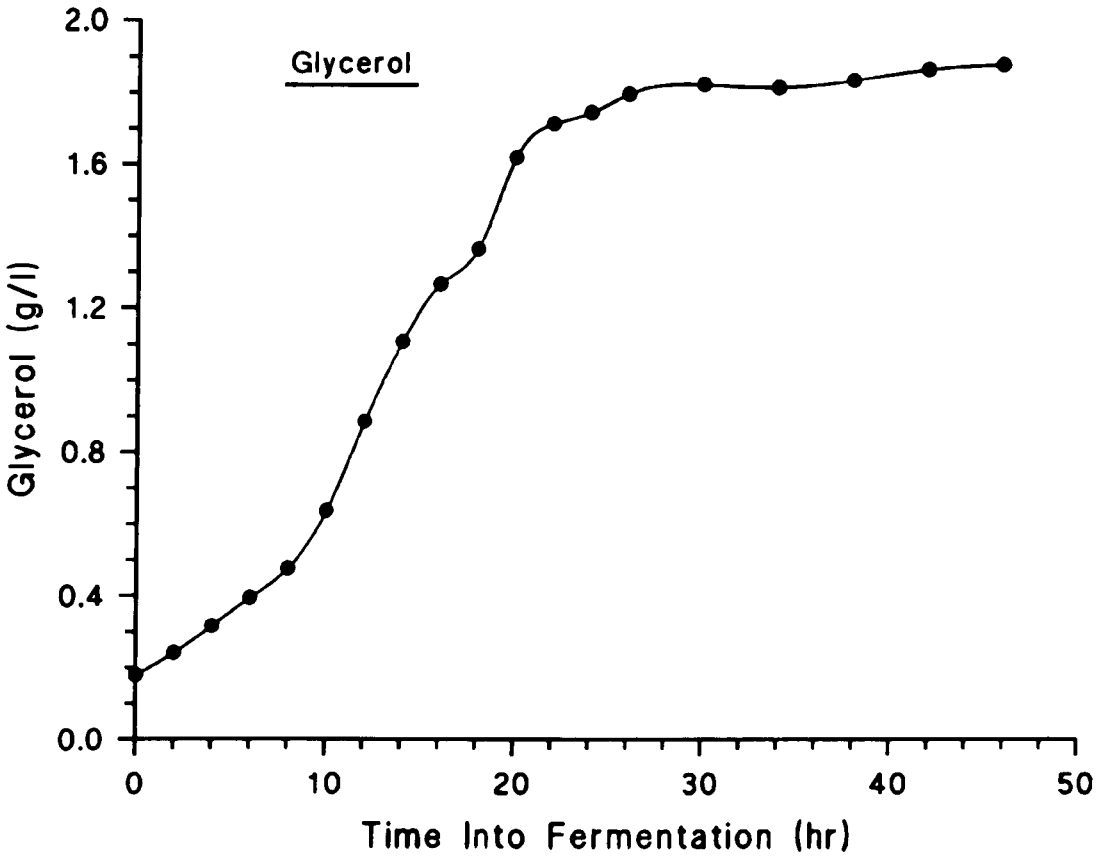


Figure 78. Measurment of ethanol (Pooled S.D. = 0.071) and glycerol (Pooled S.D. = 0.011) concentrations during the fermentation process.

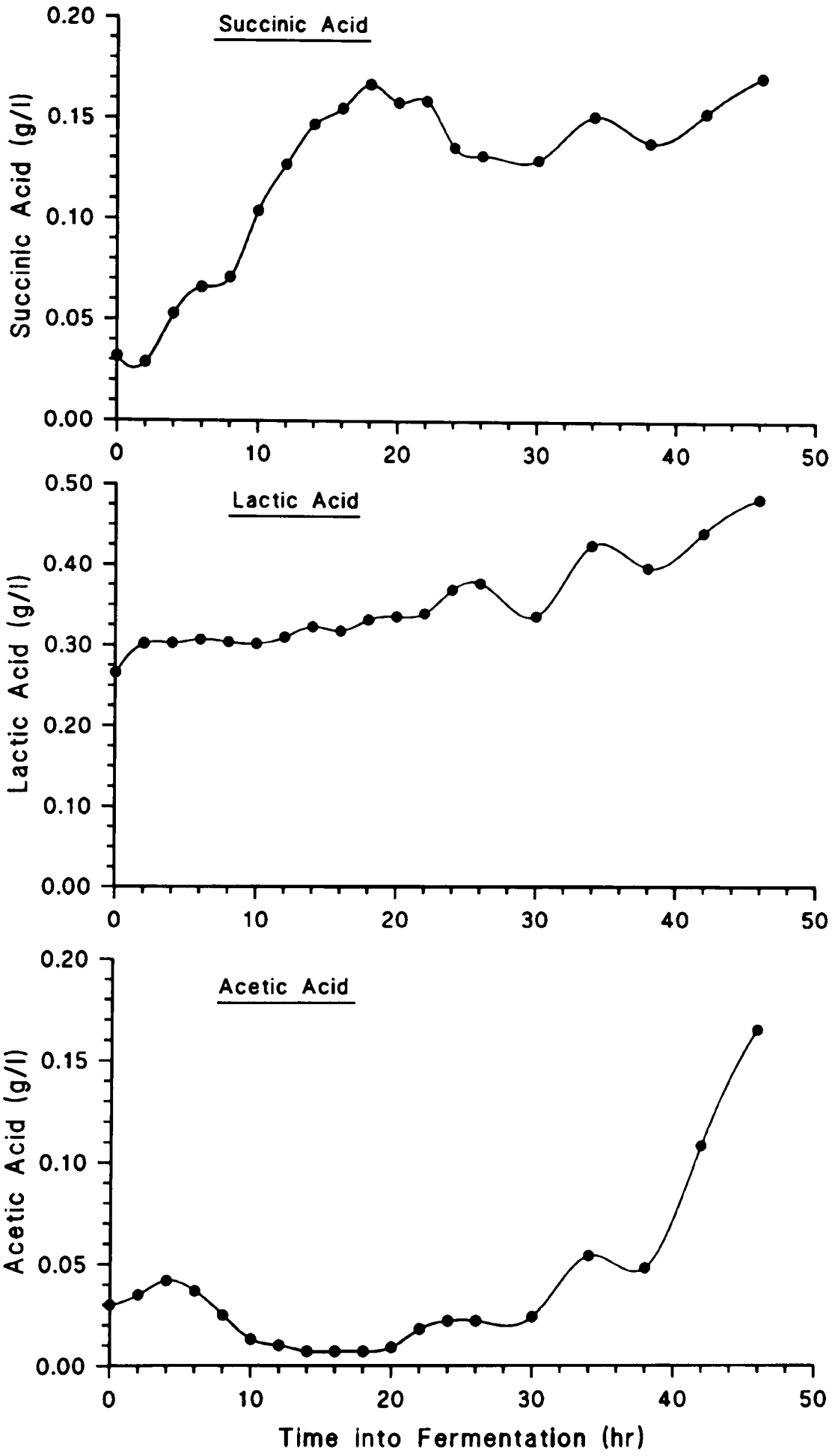


Figure 79. Measurement of succinic acid (Pooled S.D. = 0.004), lactic acid (Pooled S.D. = 0.007) and acetic acid (Pooled S.D. = 0.003) concentraations during the fermentation process.

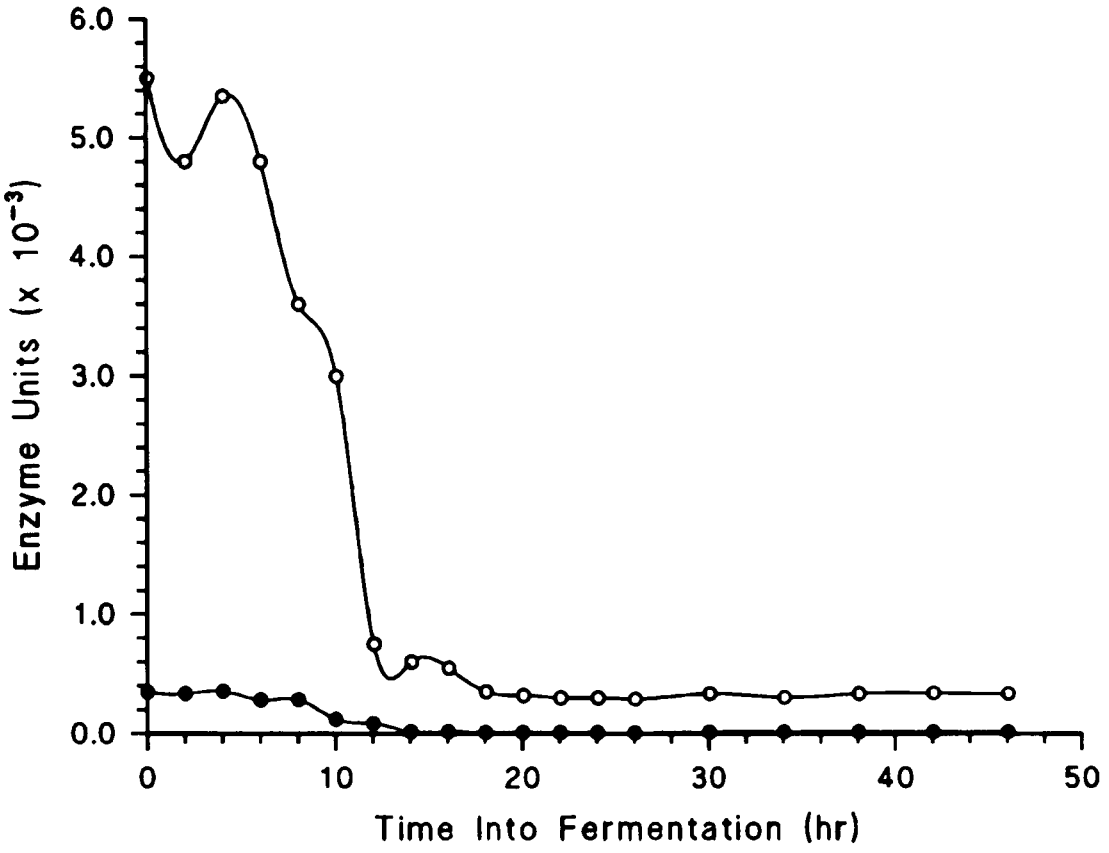


Figure 80. α-Amylase activity under fermentation (●-●) (Pooled S.D. = 2.48) and optimal (o-o) (Pooled S.D. = 48.95) conditions during the fermentation process.

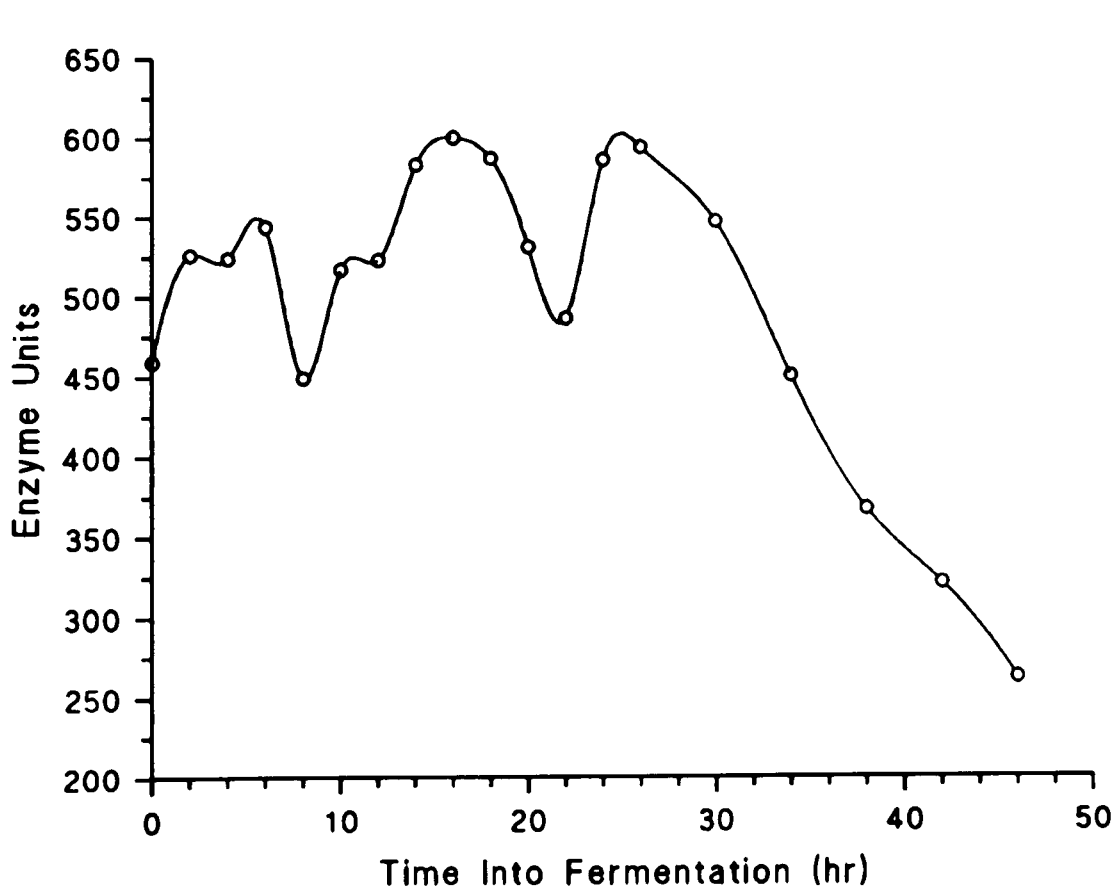


Figure 81. β-Amylase activity under optimal (Pooled S.D. = 8.214) conditions during the fermentation process.

α-Amylase activity in the fermenting wash, from these results, can therefore be attributed to the malted barley enzymes, but under fermentation conditions was minimal. Although the slight rise in activity towards the end of fermentation may be due to microbial α-amylase.

The activity of β-amylase during fermentation is given in Figure 81. Most of the activity was probably attributable to the malted barley β-amylase since the relative activity remained constant, 500 - 600 E.U., during the first 26 hr of fermentation. The activity of β-amylase at the beginning of the fermentation was reduced by 85 fold when compared with the activity during mashing-in. This dramatic decrease suggests that the activity in the fermenting wash would be at a minimum.

Minor Carbohydrases.

The results for the minor carbohydrases during fermentation are detailed in Tables 30 and 31 in Appendix II.

Under optimal enzyme conditions, Figure 82, α-glucosidase activity was found in three distinct peaks during the fermentation period. The highest activity was found after 16 hr into the fermentation with other peaks of activity at 4, 24 and 34 hr. Activity levels observed under fermentation assay conditions, Figure 82, gave activity peaks at 16, 24 and 30 hr. The greatest activity was observed at 16 hr into fermentation. α-Glucosidase under both sets of assay conditions gave a similar profile throughout fermentation, but the activity from the fermentation assay conditions was lower.

The activity of the β-glucosidase enzyme was observed as four distinct areas during the fermentation (Figure 83). Under fermentation and optimal assay conditions, peaks of activity were observed at 6, 12, 18 and 26 hr, with a further peak at 2 hr under optimal assay conditions. The activity from optimal assay conditions was highest during the initial part of the fermentation, activity was decreased

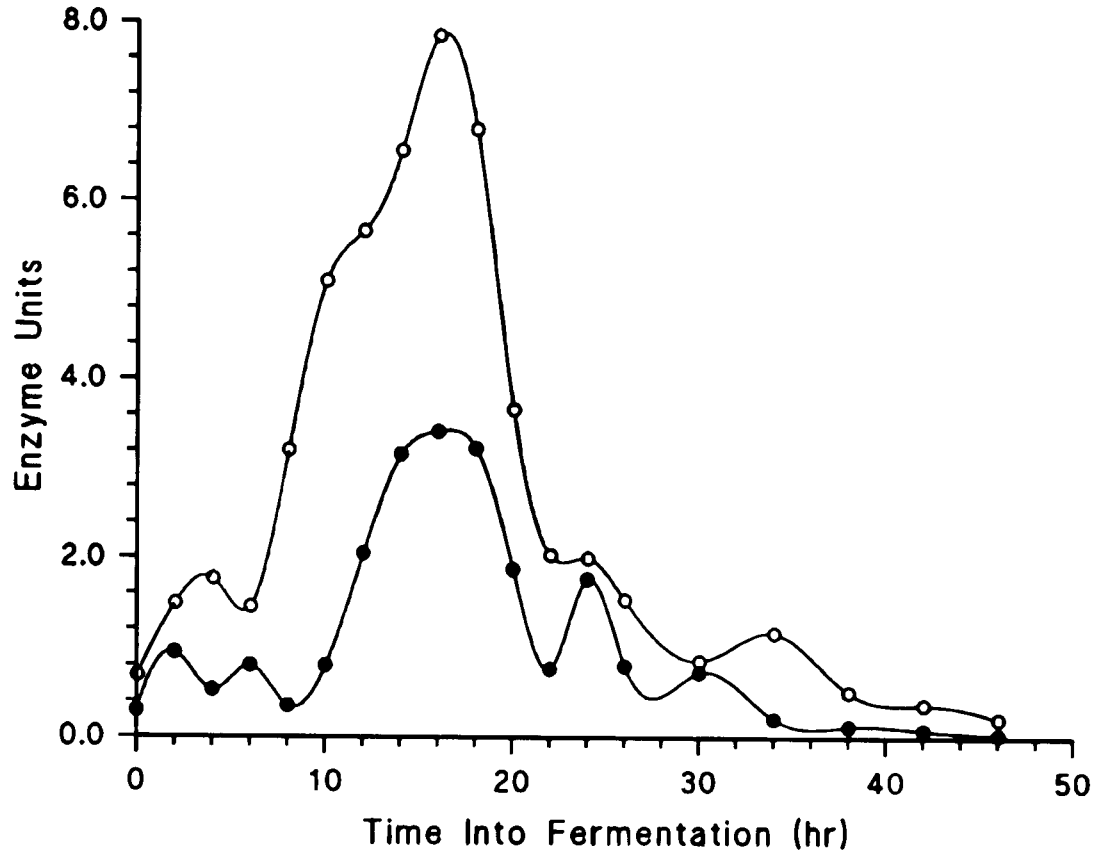


Figure 82. α-Glucosidase activity under fermentation (●-●) (Pooled S.D. = 0.051) and optimal (o-o) (Pooled S.D. = 0.048 conditions during the fermentation process.

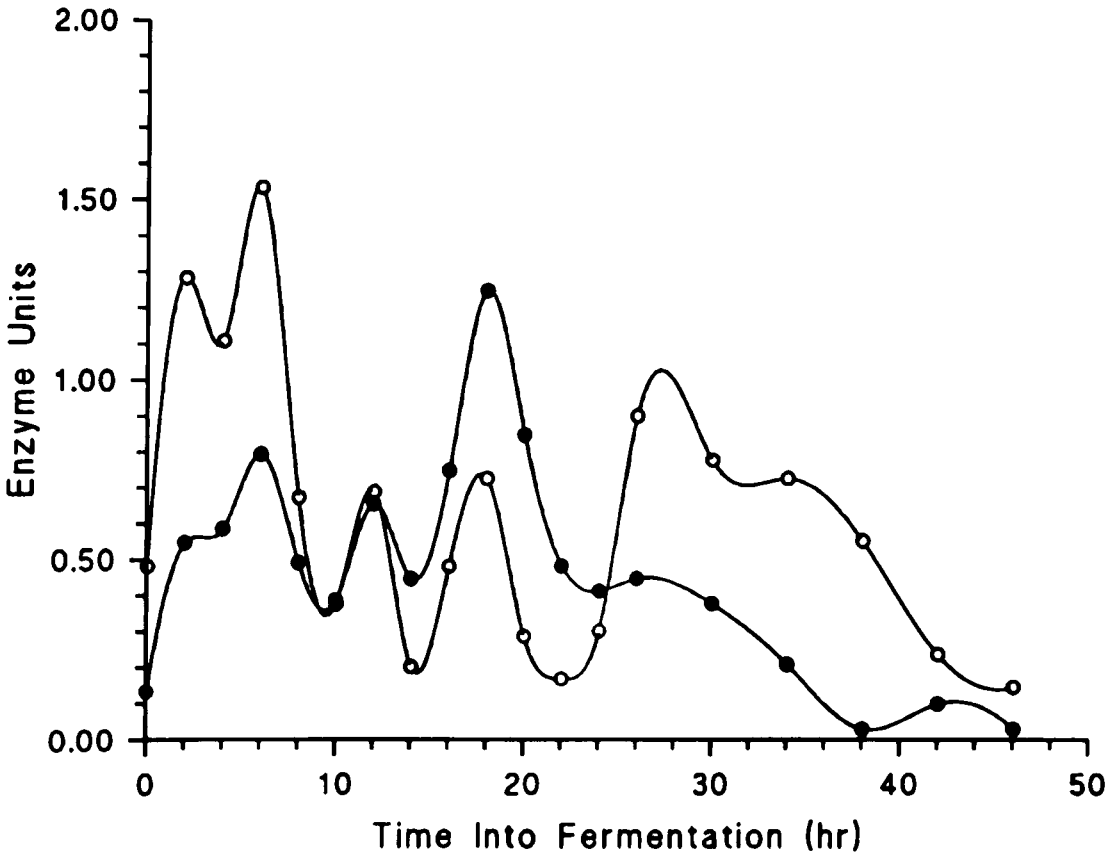


Figure 83. β-Glucosidase activity under fermentation (●-●) (Pooled S.D. = 0.052) and optimal (o-o) (Pooled S.D. = 0.085) conditions during the fermentation process.

during the middle section, but again increased during the latter stages of fermentation. When fermentation assay conditions were studied, activity was greatest during the middle part of fermentation.

α-Galactosidase and β-galactosidase activities (Figures 84 and 85) were found to follow a different pattern from the glucosidases. The activity of both enzymes, under the fermentation assay conditions, follow a similar pattern, both having initial peaks of activity at the beginning of fermentation. However, in both cases activity peaks at 18 hr but does not decline until after 24 hr. A peak of activity is observed towards the end of fermentation at 42 hr. The activities of the enzymes under optimal assay conditions also follow this pattern, but do not decline until after 30 hr. This type of enzyme activity pattern during the middle period of fermentation could be caused by the yeast enzymes being more stable in the fermentation environment, or less prone to microbial proteinases.

α-Mannosidase activity, under both sets of assay conditions, decreased until 16 hr (Figure 86). Thereafter, a slight increase in activity, up to 20 hr was observed. An increase in activity, with optimal assay conditions was detected towards the end of the fermentation. In contrast the activity of the β-mannosidase (Figure 87) followed a similar pattern to the glucosidase activities. Both assay conditions gave quite high levels of activity during the initial period of the fermentation. During the middle section of the fermentation the activities were similar, with a peak of activity at 20 hr. A different β-mannosidase enzyme was present during this part of the fermentation than found during the initial period of fermentation. Assay conditions may not be optimal for this enzyme during this period, due to very similar levels of activity from both assays. There was a further rise in activity towards the end of fermentation at 42 hr.

N-Acetylglucosaminidase activity under fermentation conditions indicated two small peaks of activity during the first 8 hr of fermentation, with another two small peaks between 10 and 20 hr (Figure

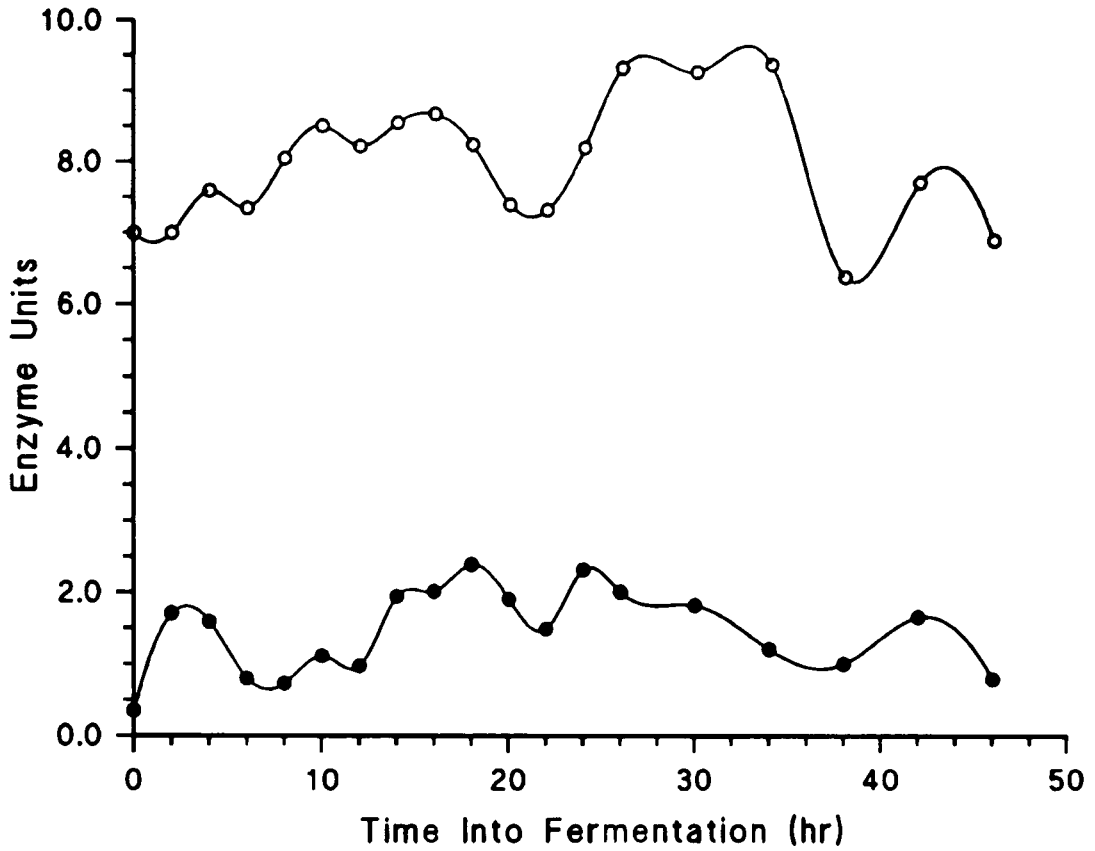


Figure 84. α-Galactosidase activity under fermentation (●-●) (Pooled S.D. = 0.099) and optimal (o-o) (Pooled S.D. = 0.145) conditions during the fermentation process.

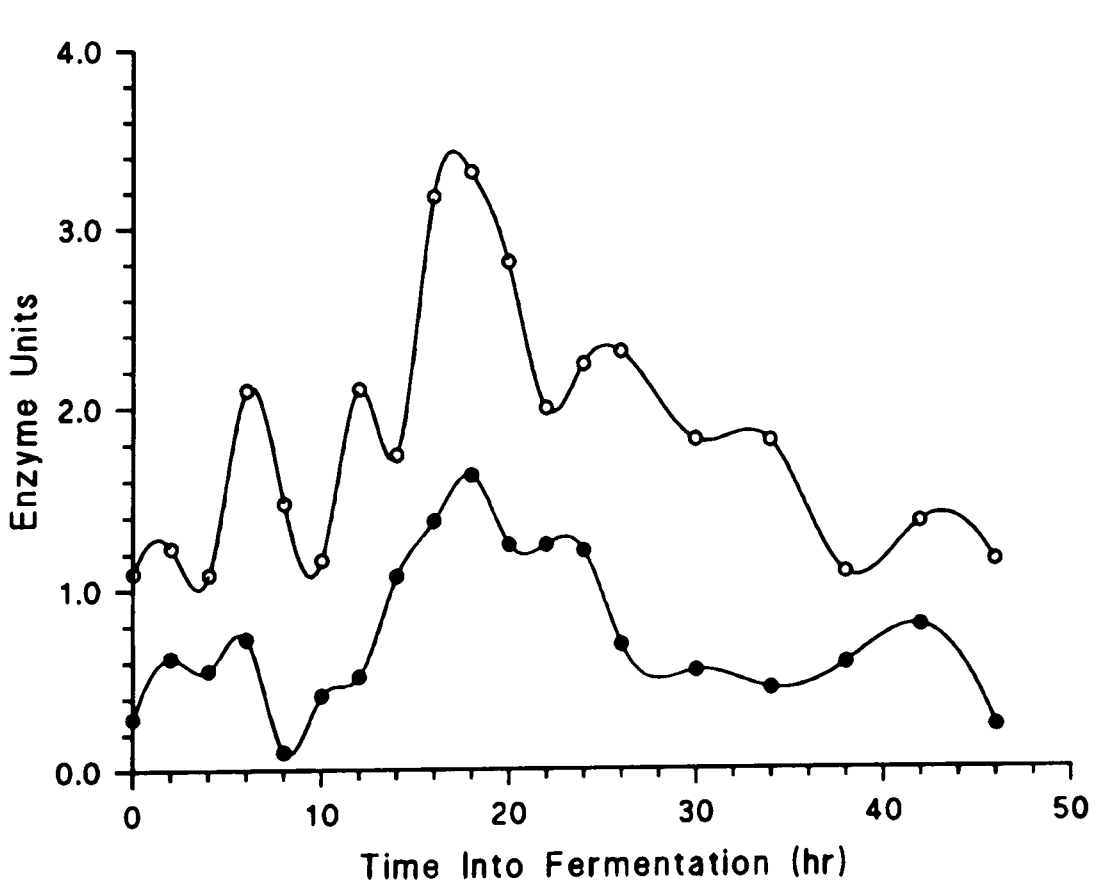


Figure 85. β-Galactosidase activity under fermentation (●-●) (Pooled S.D. = 0.056) and optimal (o-o) (Pooled S.D. 0.127) conditions during the fermentation process.

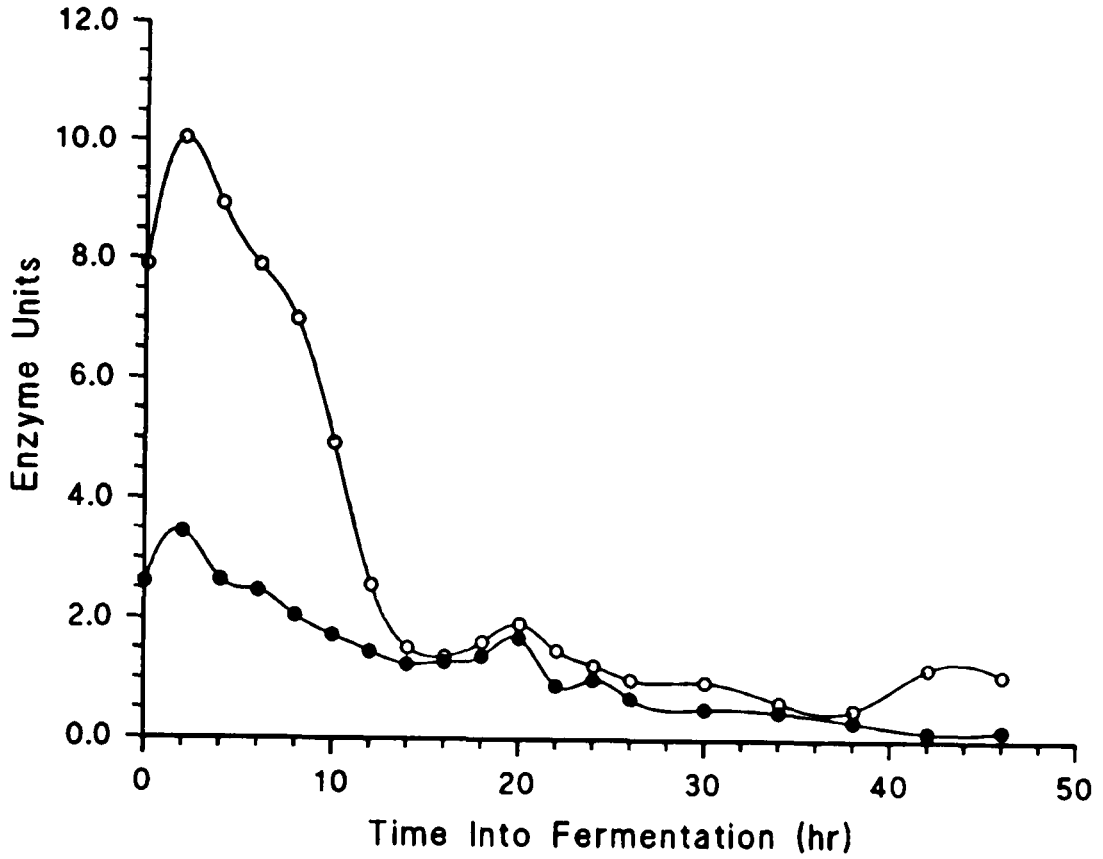


Figure 86. α-Mannosidase activity under fermentation (●-●) (Pooled S.D. = 0.097) and optimal (o-o) (Pooled S.D. = 0.128) conditions during the fermentation process.

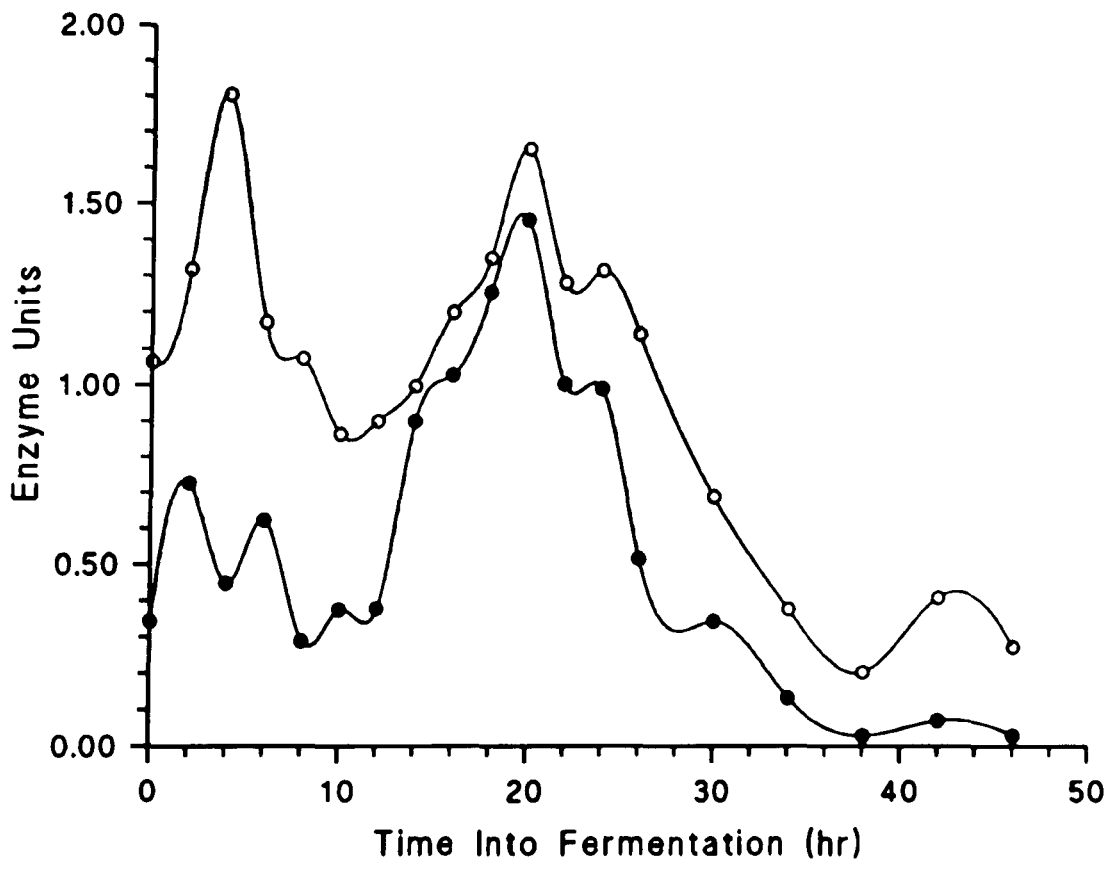


Figure 87. β-Mannosidase activity under fermentation (●-●) (Pooled S.D. = 0.034) and optimal (o-o) (Pooled S.D. = 0.064) conditions during the fermentation process.

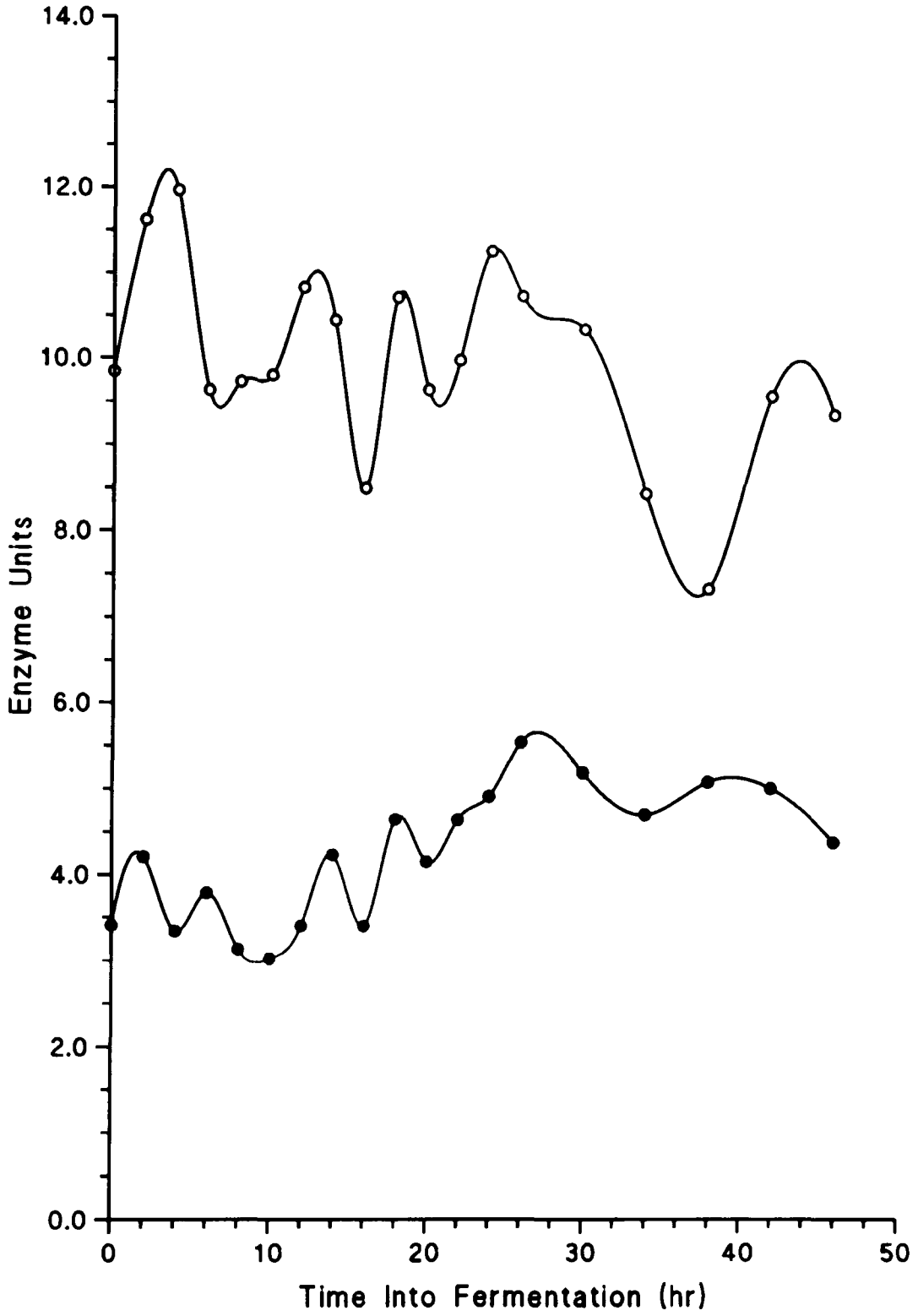


Figure 88. N-Acetyl-glucosaminidase activity under fermentation (●-●) (Pooled S.D. = 0.152) and optimal (o-o) (Pooled S.D. = 0.209) conditions during the fermentation process.

88). Thereafter, activity increased during the remainder of the fermentation. Under optimal assay conditions N-acetylglucosaminidase activity displays a similar pattern, but with the highest activity during the first 6 hr of fermentation. The activity pattern of N-acetylglucosaminidase was similar to that of α-galactosidase during fermentation.

Arabinosidase, Figure 89, activity under both assay conditions was greatest during the middle section of fermentation. After 12 hr, the activities from both sets of assay conditions were similar. However, a large peak of activity was detected 20 hr into the fermentation under optimal assay conditions. This would suggest an arabinosidase activity associated with the yeast.

The enzyme activity profile of β-xylosidase, Figure 90, under optimal conditions rose slightly at the beginning of fermentation and then decreased until 16 hr. Thereafter, the enzyme activity increased to approximately 50% of the initial activity after a further 4 hr and remained at this level until the end of fermentation. Enzyme activity under fermentation assay conditions increased gradually until 24 hr. β-Xylosidase activity steadily increased until the end of fermentation, where it was 234% higher than the initial activity.

Phosphatases.

Acid phosphatase under both sets of assay conditions decreases gradually throughout the period of fermentation (Figure 91). Activity declines by 82% under fermentation conditions and by 91% when the optimal assay conditions were studied.

Phytase activity under optimal assay conditions (Figure 92) was observed to follow a similar pattern to the proposed three enzyme population system. Major activity peaks are found after 4, 16 and 30 hr. The latter activity at 30 hr was spread throughout the latter stages of the fermentation from 20 to 46 hr.

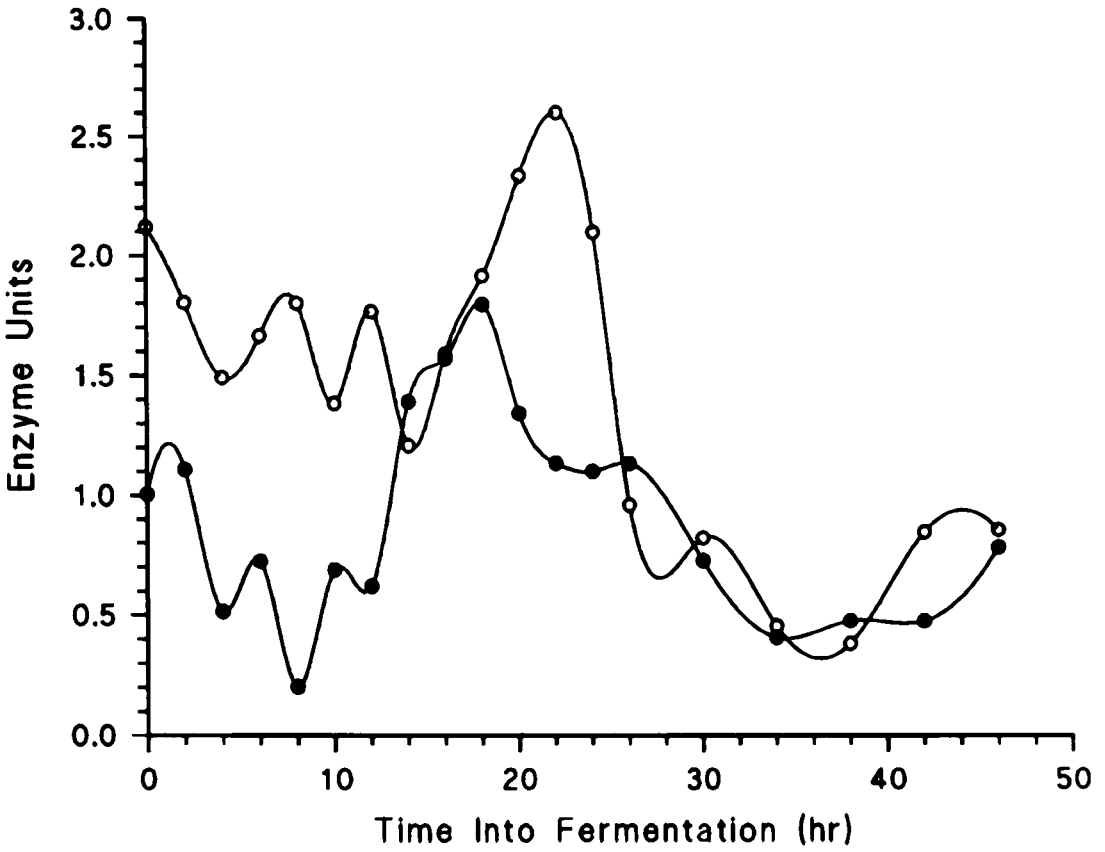


Figure 89. Arabinosidase activity under fermentation (●–●) (Pooled S.D. = 0.050) and optimal (o–o) (Pooled S.D. = 0.084) conditions during the fermentation process.

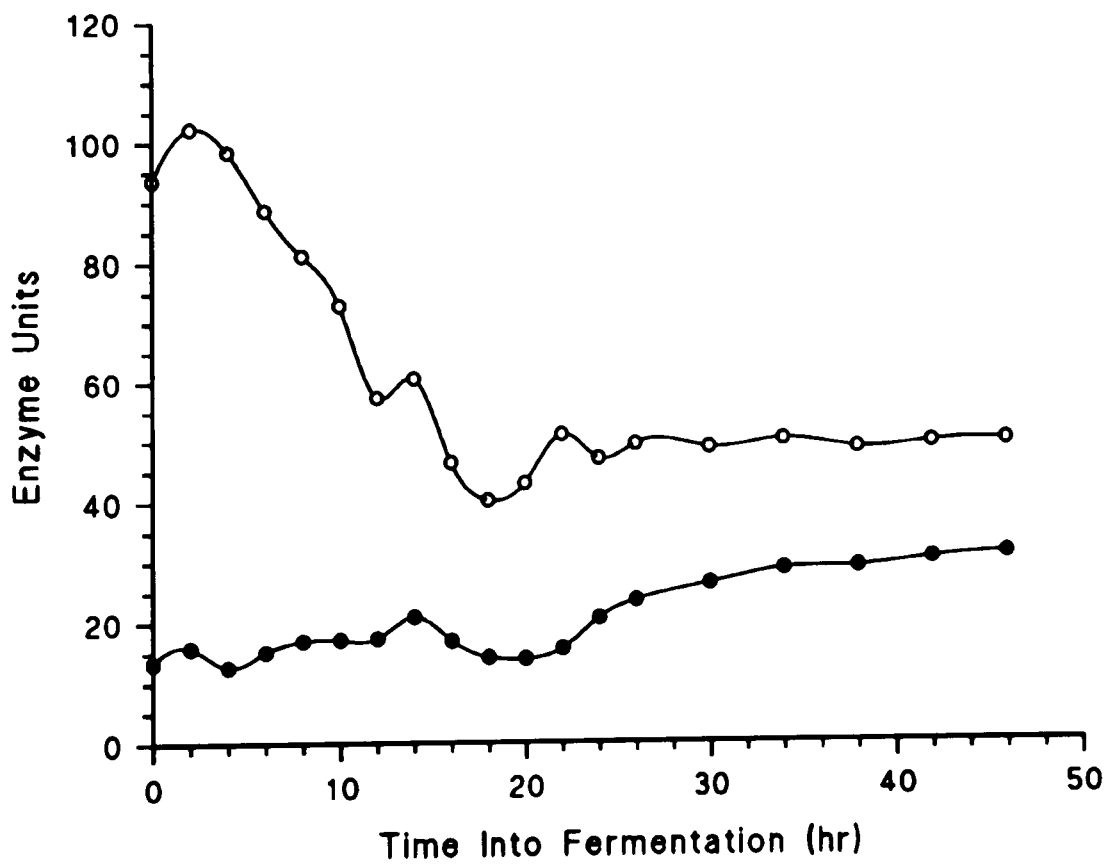


Figure 90. β–Xylosidase activity under fermentation (●–●) (Pooled S.D. = 0.257) and optimal (o–o) (Pooled S.D. = 1.089) conditions during the fermentation process.

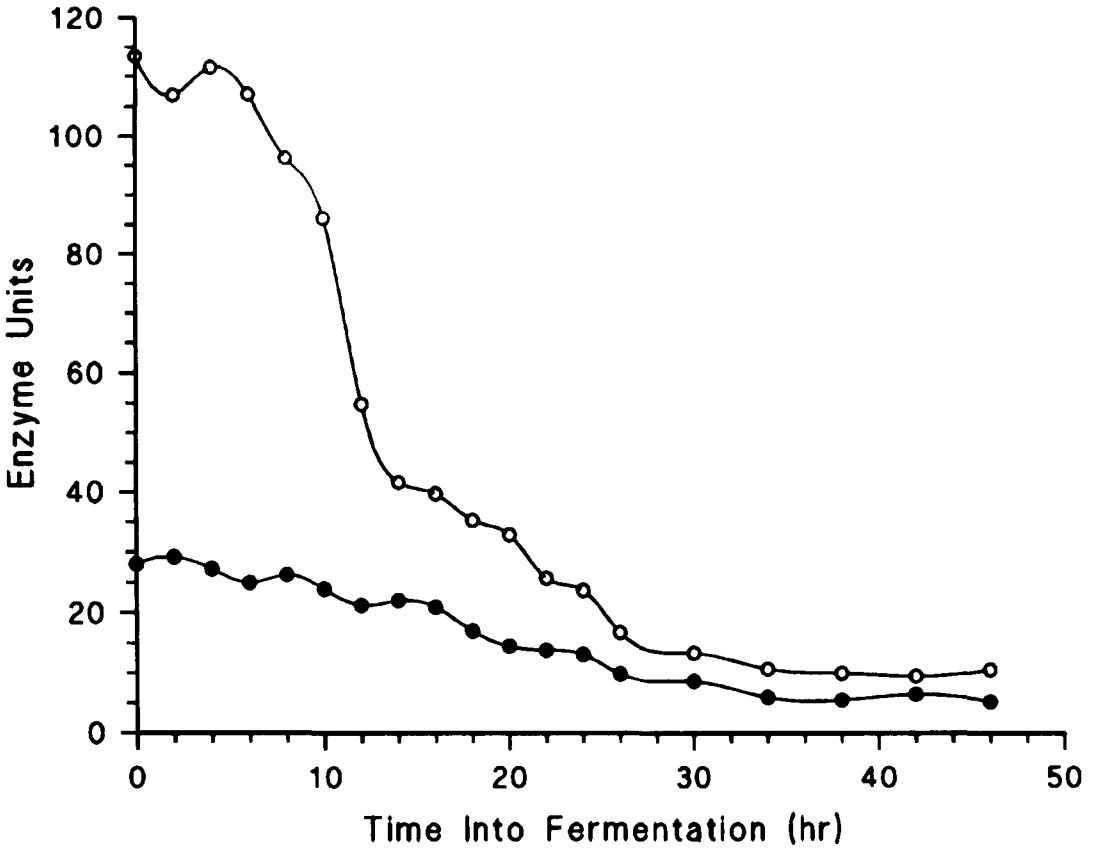


Figure 91. Acid phosphatase activity under fermentation (●–●) (Pooled S.D. = 0.381) and optimal (o–o) (Pooled S.D. = 0.752) conditions during the fermentation process.

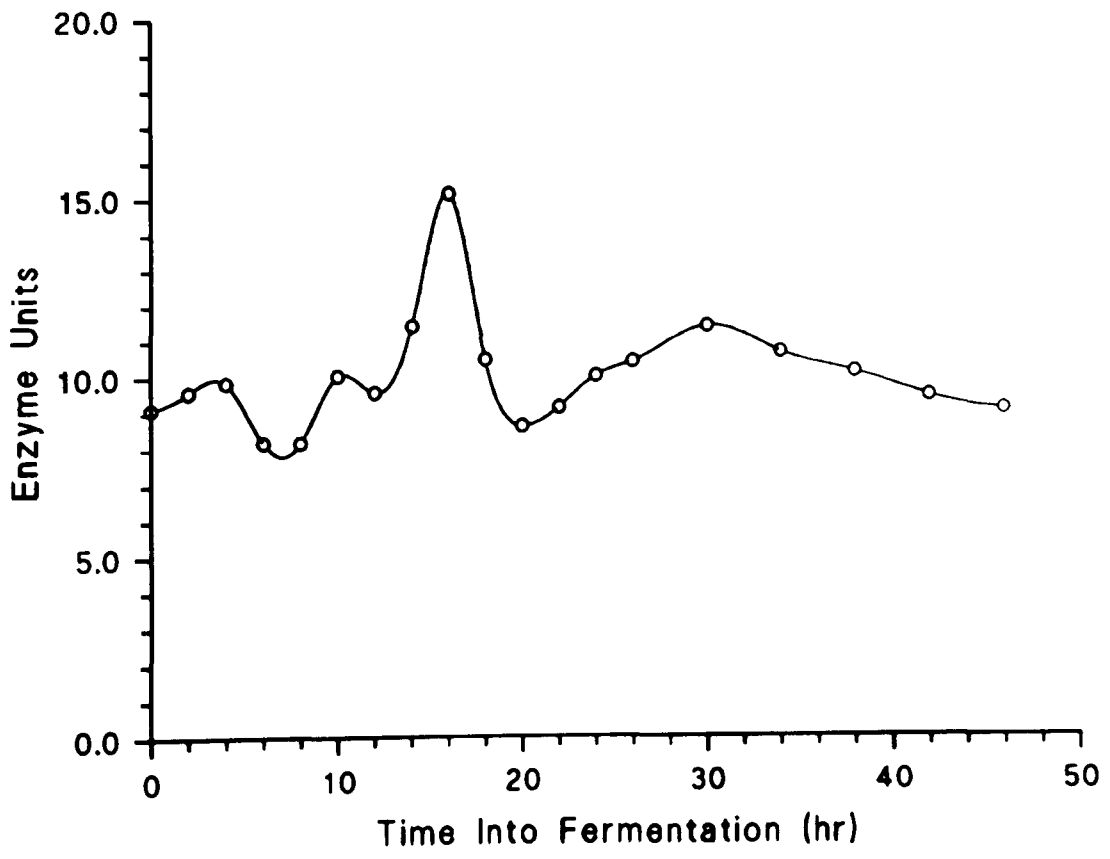


Figure 92. Phytase activity under optimal (Pooled S.D. = 0.183) conditions during the fermentation process.

Proteinases and Proteases.

The activity levels from the general proteinase type activity (Figure 93) can be divided into three distinct groups with peaks of activity at 4, 18 and 46 hr.

The activities of the proteases studied are given in Figure 94. It was observed that the largest peak during the study of the serine protease activity occurred during the initial stages of fermentation. Activity during the remainder of the fermentation was also present but at lower levels. With the leucine aminopeptidase activity, the major part of the activity was found during the middle period of the fermentation. Carboxypeptidase A activity was most prominent during the latter stages of fermentation.

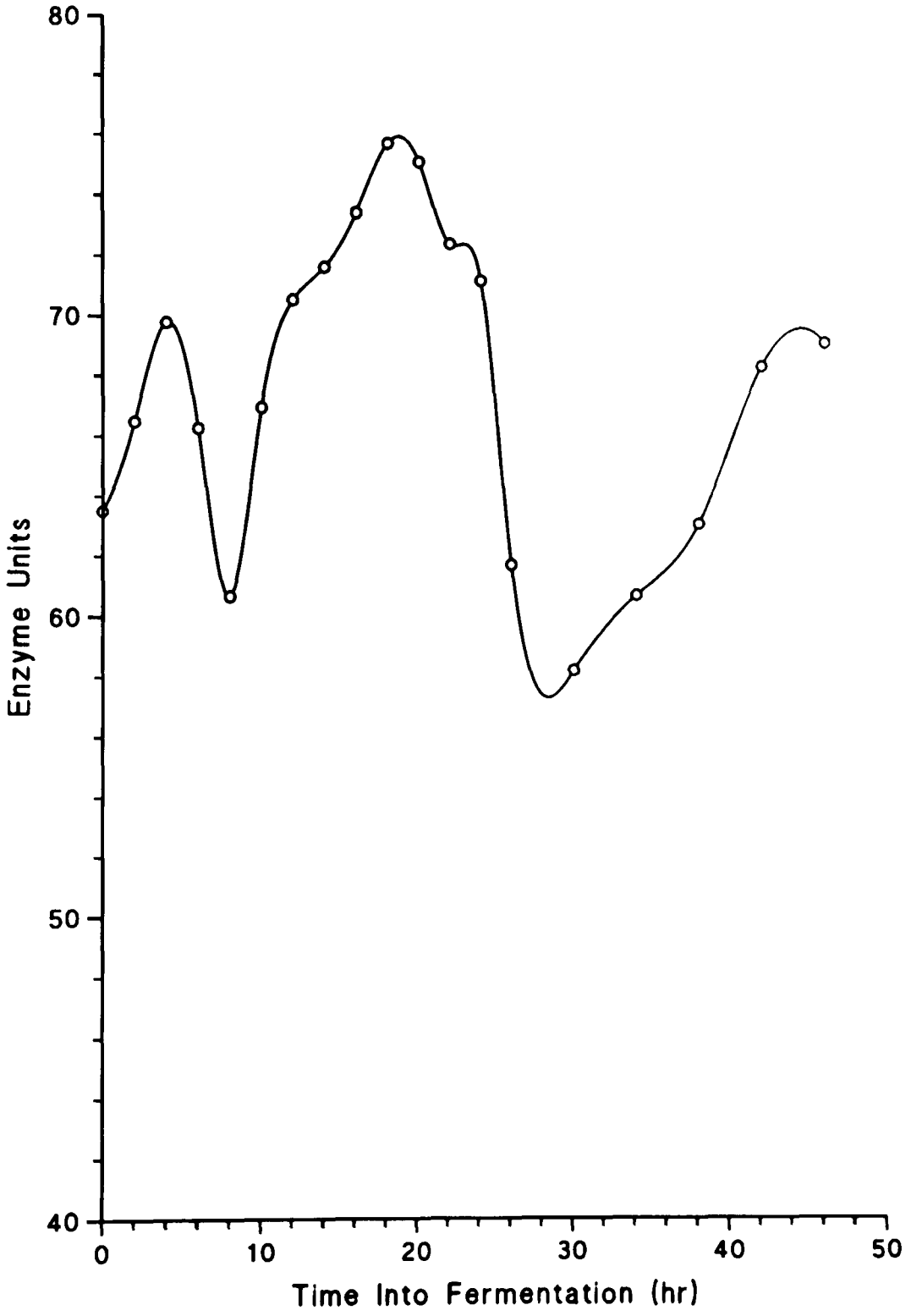


Figure 93. General proteinase type activity under optimal (Pooled S.D. = 0.884) enzyme conditions during the fermentation process.

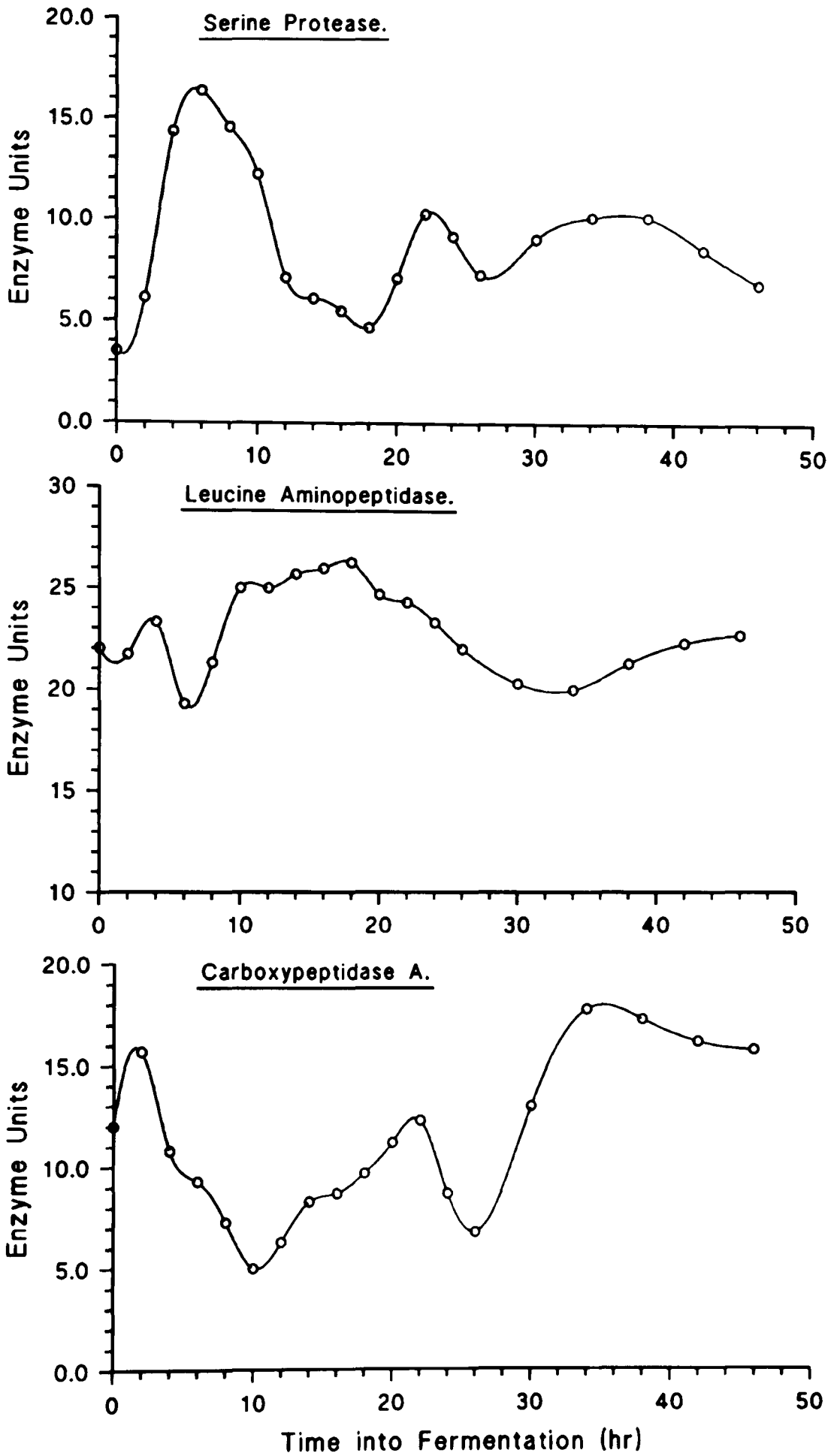


Figure 94. Serine protease, (Pooled S.D. = 0.525) leucine aminopeptidase (Pooled S.D. = 0.757) and carboxypeptidase A (Pooled S.D. = 0.862) activities under optimal enzyme conditions during the fermentation process.

3.5 Carbohydrate Concentrations During Scotch Malt Whisky Processes.

The activity of the amylase enzymes on the malted barley starch are responsible for the production of the fermentable sugars and oligosaccharides in the wort. It is the production of these carbohydrates during the mashing, wort draining and initial stages of fermentation, and the utilization of them during fermentation that is responsible for yeast metabolism. Fermentable sugars are those that are easily utilized by the yeast during fermentation and include glucose, maltose and maltotriose. The non-fermentable sugars, oligosaccharides and starch, should also be studied since their degradation produces fermentable sugars through the activity of the amylases.

3.5.1 Mashing.

From Figures 44 and 45 it was observed that the activities of amylases during the mashing process were mainly found during mashing-in and throughout the first mashing water. During mashing-in, the grist and mashing liquor are rapidly mixed. This eases gelatinization of the starch particles in the grist, allowing immediate access for the malted barley amylases on the starch grains. The activity of the enzymes at this stage is very high, and starch hydrolysis is very rapid. However, due to the elevated temperatures used during mashing-in, the β-amylase enzymes were rapidly inactivated. The levels of fermentable sugars, especially maltose will be rapidly produced during mashing-in. Figures 95 and 96 show the levels of the different carbohydrate fractions during the mashing process.

Carbohydrate analysis was carried out using an HPLC assay, and as can be expected from such a procedure the results were easily quantified. During mashing-in 84.5% of the carbohydrate that was quantified was fermentable sugars, (see Table 33, Appendix II), with approximately 80.0 g/l of maltose and 14.3 g/l of glucose and 15.5

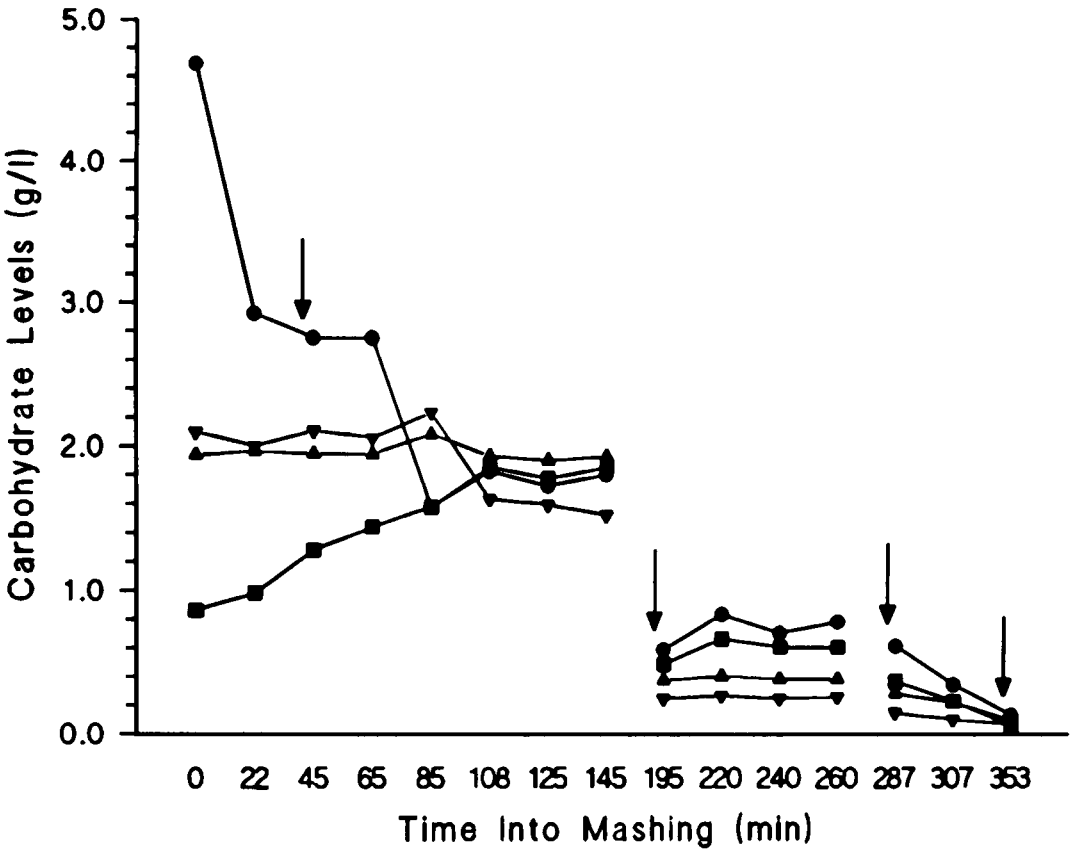


Figure 95. Maltotetraose (●–●) (Pooled S.D. = 0.095), maltopentaose (■–■) (Pooled S.D. = 0.055), maltohexaose (▲–▲) (Pooled S.D. = 0.045) and maltoheptaose (▼–▼) (Pooled S.D. = 0.032) concentrations during the mashing process. Raking after water addition shown by arrows.

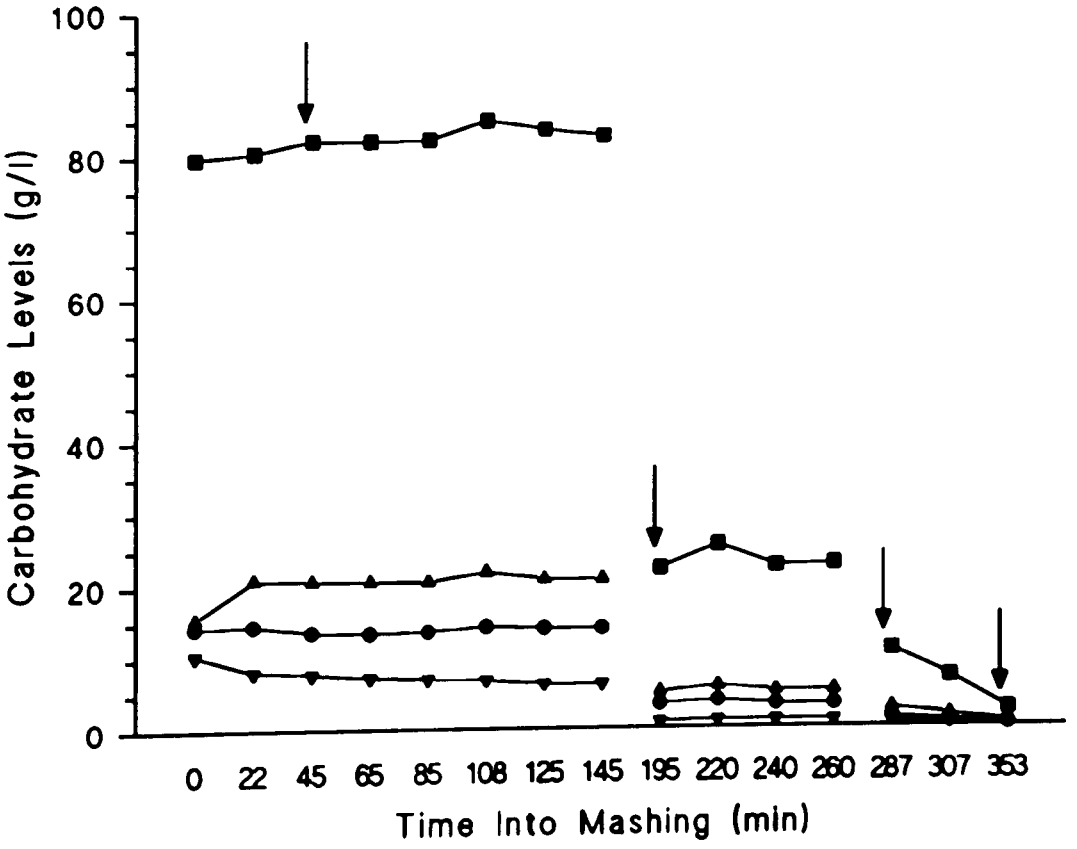


Figure 96. Glucose (●–●) (Pooled S.D. = 0.118), maltose (■–■) (Pooled S.D. = 0.903), maltotriose (▲–▲) (Pooled S.D. = 0.152) and starch (▼–▼) (Pooled S.D. = 0.173) concentrations during the mashing process. Raking after water addition shown by arrows.

g/l of maltotriose. Oligosaccharides larger than maltodecanaose and starch account for 10.5 g/l of the total carbohydrate at the time of mashing-in.

During the first water period, several changes in the carbohydrate profiles were distinguishable by HPLC. The increase in the fermentable sugar fraction was due to increases in maltose by 3.5 g/l and maltotriose by 5.4 g/l. This overall increase in the fermentable sugars, during the first water period was 8.70 g/l. The levels of starch and larger oligosaccharides at the end of the first water period, in the mash tun, was reduced by 4.3 g/l.

The profiles of oligosaccharides, maltotetraose to maltoheptaose, also varied during first water draining in the mash tun (Figure 95).The maltotetraose concentration decreased by 2.87 g/l, maltopentaose increased by 1.00 g/l and maltoheptaose was reduced by only 0.57 g/l. Although the concentration of maltohexaose varied slightly during first water draining, there was no overall effect in its concentration, and it remained at 1.94 g/l at the end of the first water draining.

During the second water draining the levels of individual carbohydrates, quantified by HPLC analysis, were similar after raking and at the end of the draining period. However, an increase in the carbohydrates was detected 15 min into the second water. This increase may be explained by the short resting time in the mash tun before draining commences. Fermentable sugars were present in larger concentrations in the second water, starting at 92.4% of the total carbohydrate and falling to 91.3% at the end of draining.

The concentration of carbohydrate obtained from the third and fourth waters in the mash tun were low. The fermentable sugars made up 87% and 83% of the totals respectively. The carbohydrates obtained from these waters would be used in the subsequent mash. The total carbohydrate profile from all samples taken during the mashing process is shown in Figure 97.

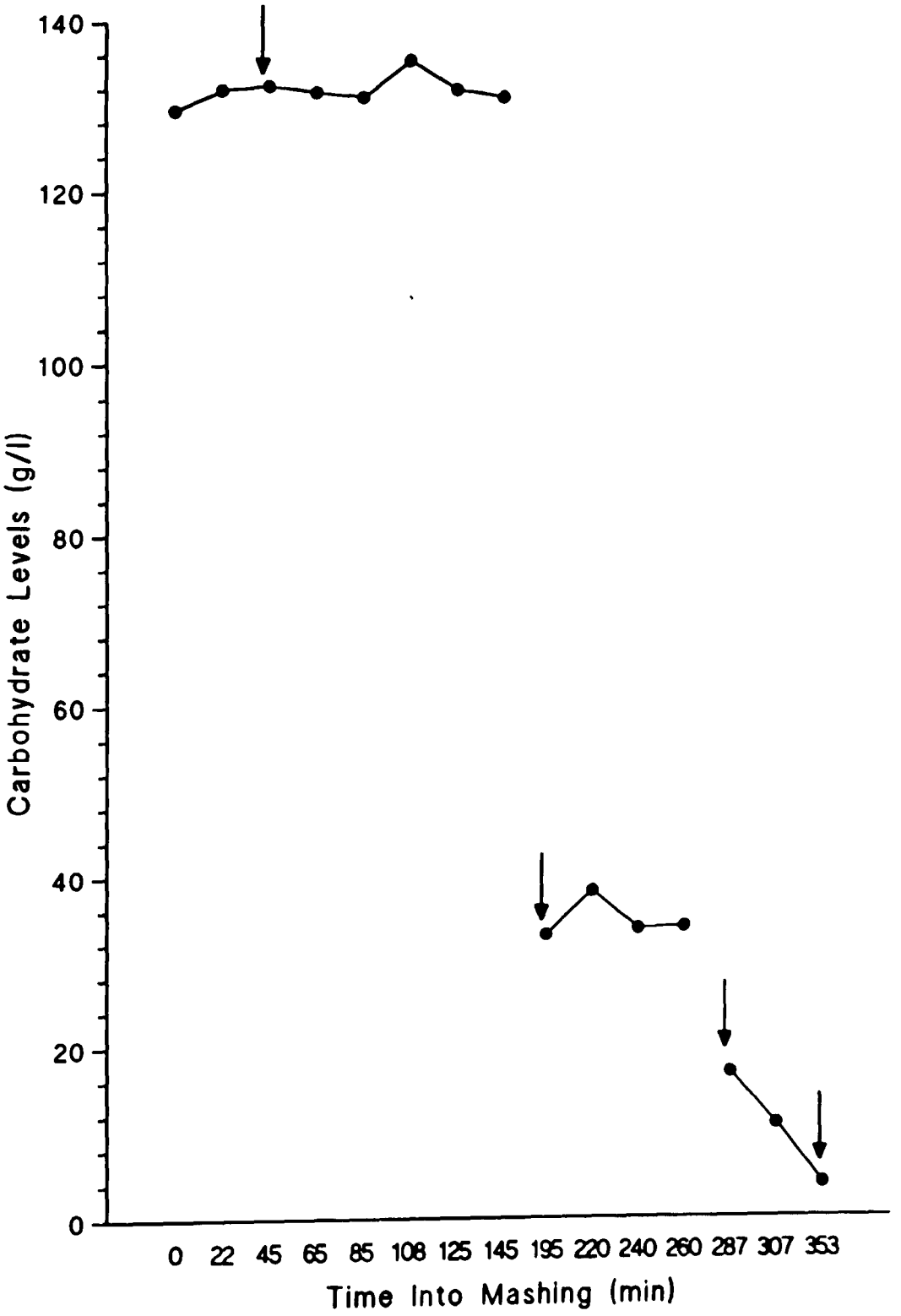


Figure 97. Total soluble carbohydrate (Pooled S.D. = 1.039) concentration during the mashing process. Raking after water addition shown by arrows.

3.5.2 Wort Draining.

During mashing the carbohydrate profiles were followed in a mash tun environment. However, in the wort draining part of the process it is the carbohydrate profile of the cooled wort, after draining from the mash tun before it enters the washback that is being studied. The mashing and mash tun draining processes are therefore of importance when the results of this study are being interpreted. Before mashing-in it is normal practice to fill the false bottom of the mash tun with mashing liquor. This prevents air locking once draining commences. The first runnings of the wort from the mash tun would be expected to be dilute in wort carbohydrate, as shown in Figures 98 and 99. The highest total wort carbohydrate was not however detected until 20 min into first water draining (Figure 100).

A carbohydrate gradient was present during the period of first water draining, Figures 98 and 99, from 20 min into draining. The concentrations of starch, maltoheptaose, maltohexaose, maltopentaose and maltotetraose decreased from 10 min into first water draining until draining was completed. It was observed that maltotriose, maltose and glucose followed a similar pattern, but the decrease in concentration began after 20 min. As expected, the total carbohydrate profile also followed a similar pattern. When the percentage of fermentable sugars to the total carbohydrate was calculated, an increase from 0 min to 40 min was found. This was followed by a 0.8% decrease in fermentable sugars over the next 20 min period before levelling out at 87.8% until the first water draining was complete.

The concentration of the maltotetraose component behaved differently to the other carbohydrates. Maltotetraose, decreased in concentration to 1.88 g/l from the initiation of draining up to 50 min. Thereafter the level of maltotetraose increased steadily up to 80 min into draining before falling slightly to 3.68 g/l. Carbohydrate levels

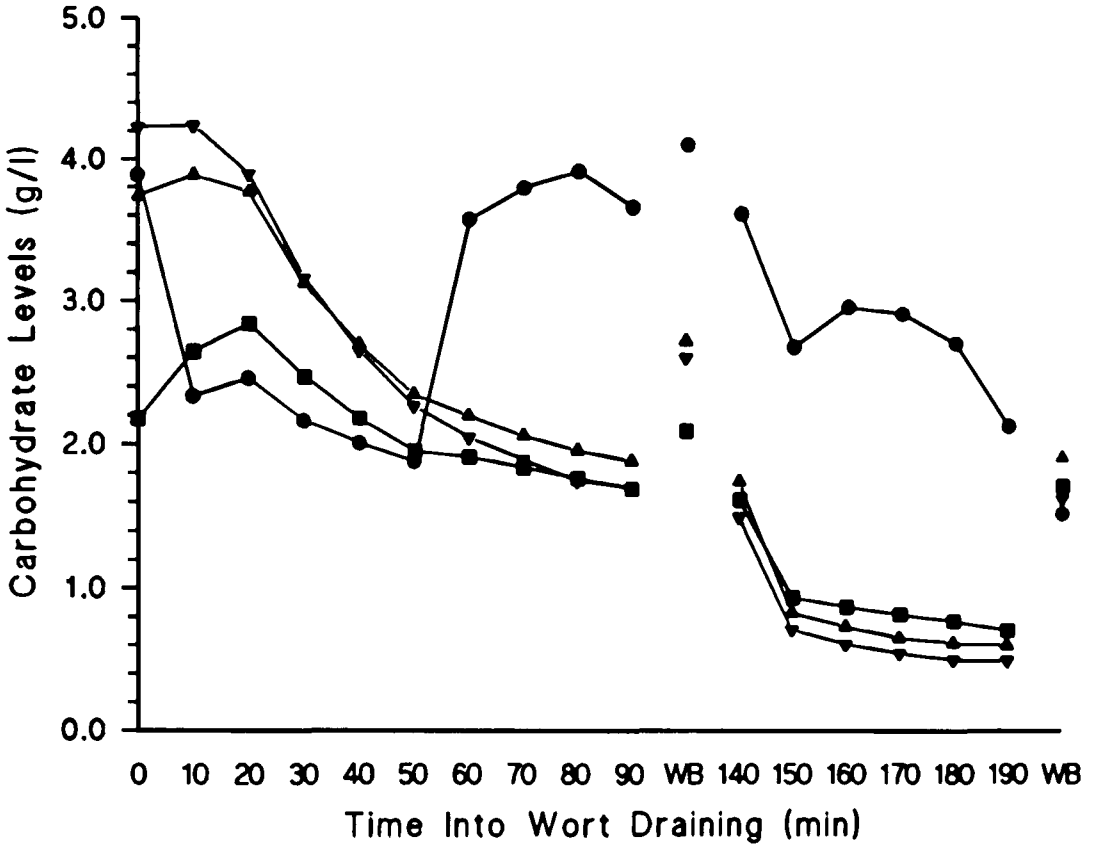


Figure 98. Maltotetraose (●–●) (Pooled S.D. = 0.062), maltopentaose (■–■) (Pooled S.D. = 0.073), maltohexaose (▲–▲) (Pooled S.D. = 0.061) and maltoheptaose (▼–▼) (Pooled S.D. = 0.074) levels during wort draining. Washback samples were removed at the end of each draining.

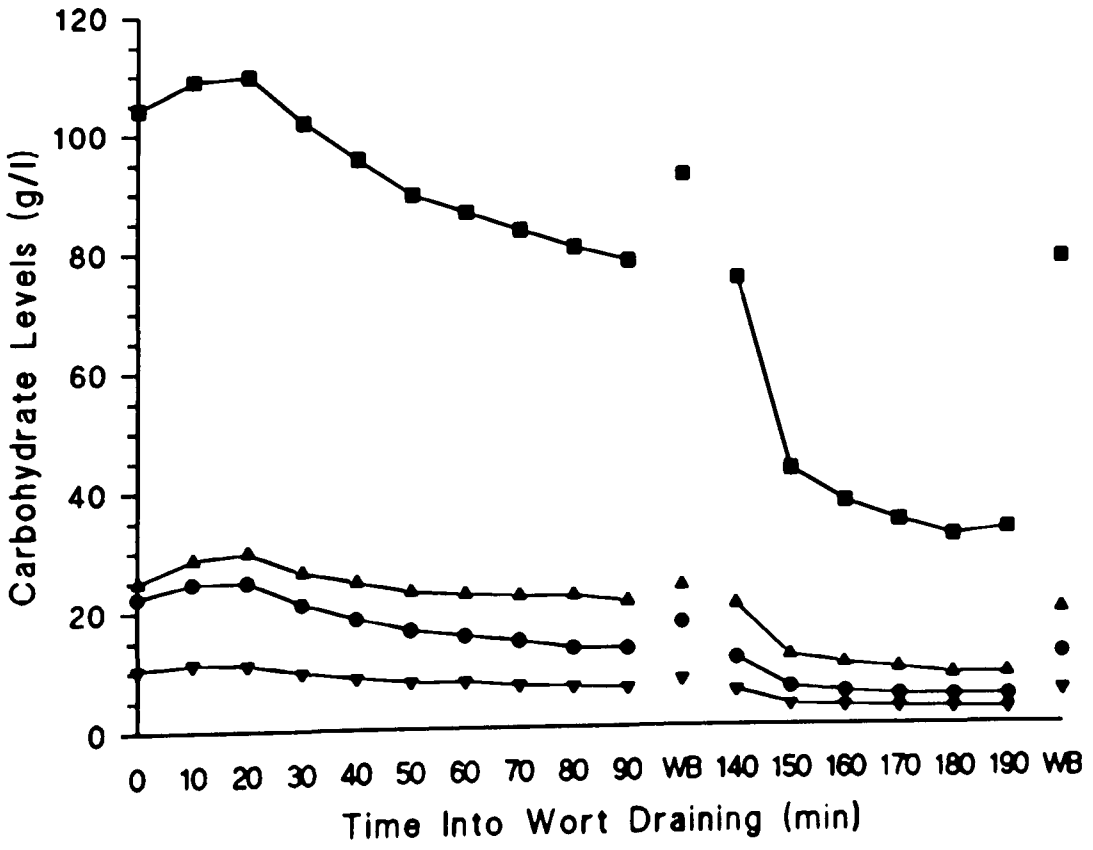


Figure 99. Glucose (●–●) (Pooled S.D. = 0.857), maltose (■–■) (Pooled S.D. = 01.698), maltotriose (▲–▲) (Pooled S.D. = 0.083) and starch (▼–▼) (Pooled S.D. = 0.083) levels during wort draining. Washback samples were removed at the end of each fermentation.

at the end of draining were similar to those after 40 min draining, however this did not apply to maltotetraose. The concentration of maltotetraose was highest in the washback after first water draining was completed. The pattern of the maltotetraose concentration may be caused by α-amylase activity on larger oligosaccharides that are hydrolyzed to give maltotetraose and other carbohydrates, larger than maltoheptaose. This pattern was not however observed during the mash tun studies.

Wort draining of the second water gave higher levels of all carbohydrates at 0 min. This was probably a result of wort from the false bottom and pipe-work that had not drained during first water draining. All carbohydrates decreased in concentration as the second water draining progressed, however a slight increase in maltose occurred from the last sample from the second water draining.

The final carbohydrate profile in the washback after all wort has been drained is given in Figure 100.

3.5.3 Fermentation.

Carbohydrate profiles during the fermentation process followed a similar pattern for each individual carbohydrate, as did the total carbohydrate (Figures 101, 102 and 103). This type of study also demonstrates the continued activity of the malted barley amylase enzymes during the initial stages of fermentation. Results for this process are tabulated in Table 35, (Appendix II).

When the total carbohydrate profile was compared with the specific gravity results (Figure 75), both follow a very similar pattern. The percentage of the fermentable sugars in the total carbohydrate, decreased slightly during the first 3 hr of fermentation, but then increase by 1.9%. From this point until 12 hr into the fermentation, the decrease in fermentable sugars was slight, only 2.8%. Thereafter this decrease was more dramatic and at the end of the fermentation only 31.8% of the total carbohydrate was fermentable sugars.

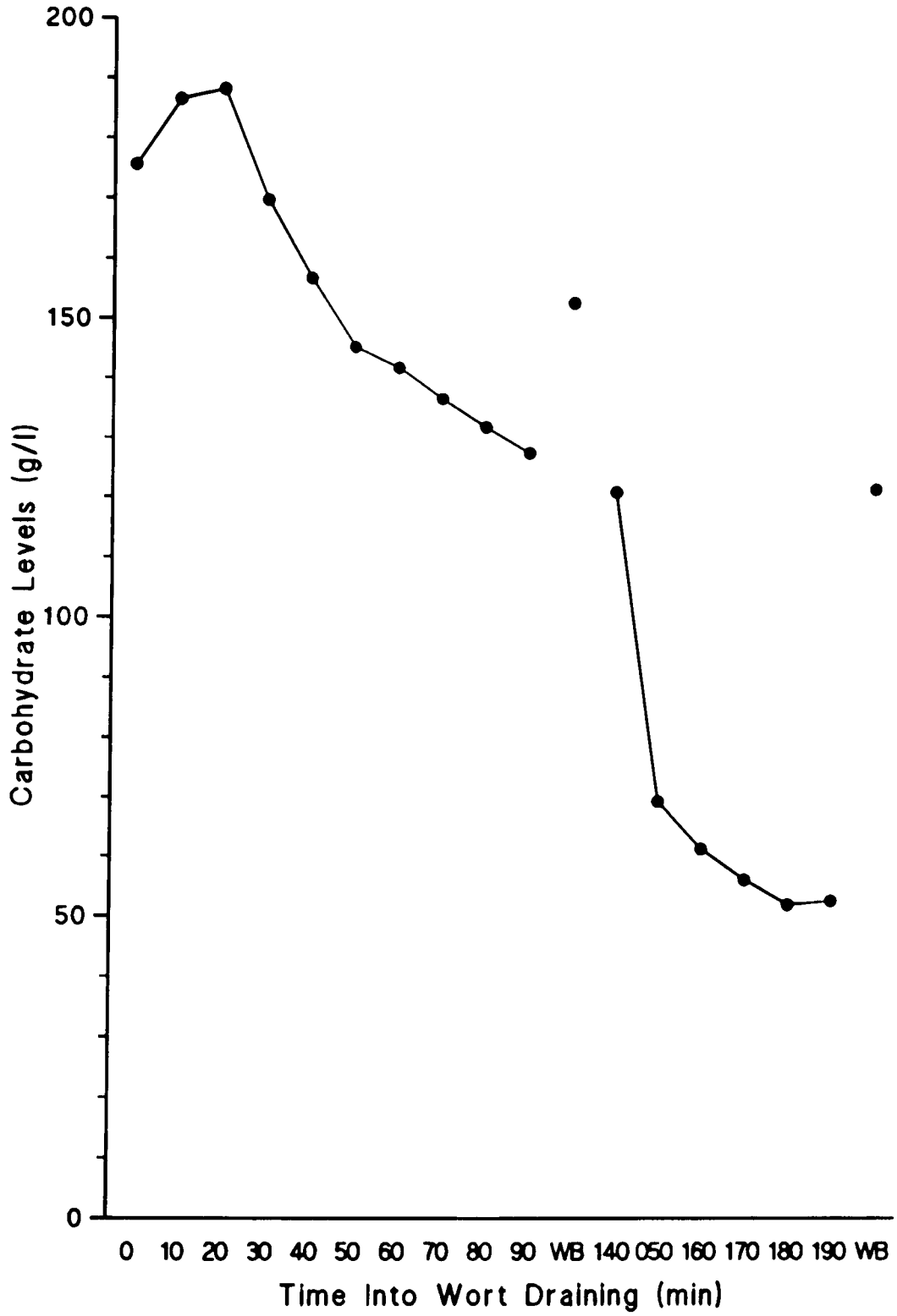


Figure 100. Total soluble carbohydrate (Pooled S.D. = 2.229) levels during wort draining. Washback samples were removed at the end of each draining.

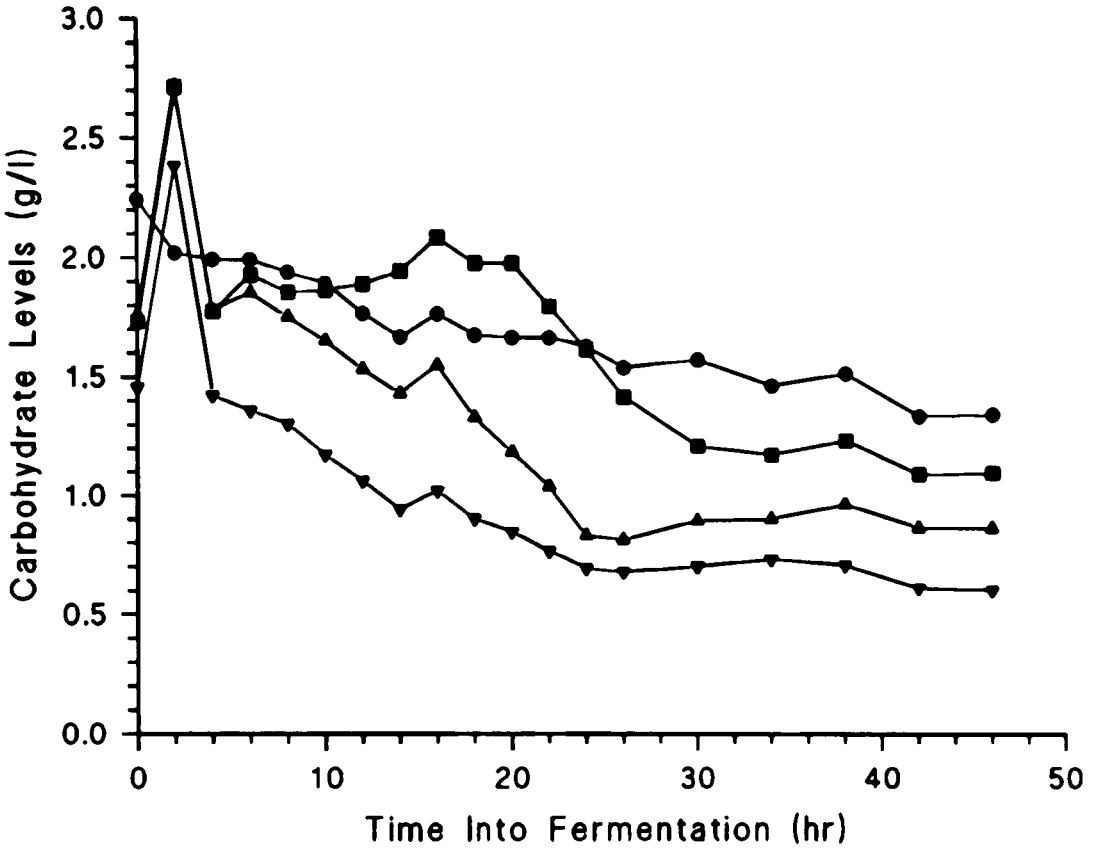


Figure 101. Maltotetraose (●–●) (Pooled S.D. = 0.096), maltopentaose (■–■) (Pooled S.D. = 0.090), maltohexaose (▲–▲) (Pooled S.D. = 0.098) and maltoheptaose (▼–▼) (Pooled S.D. = 0.078) levels during fermentation.

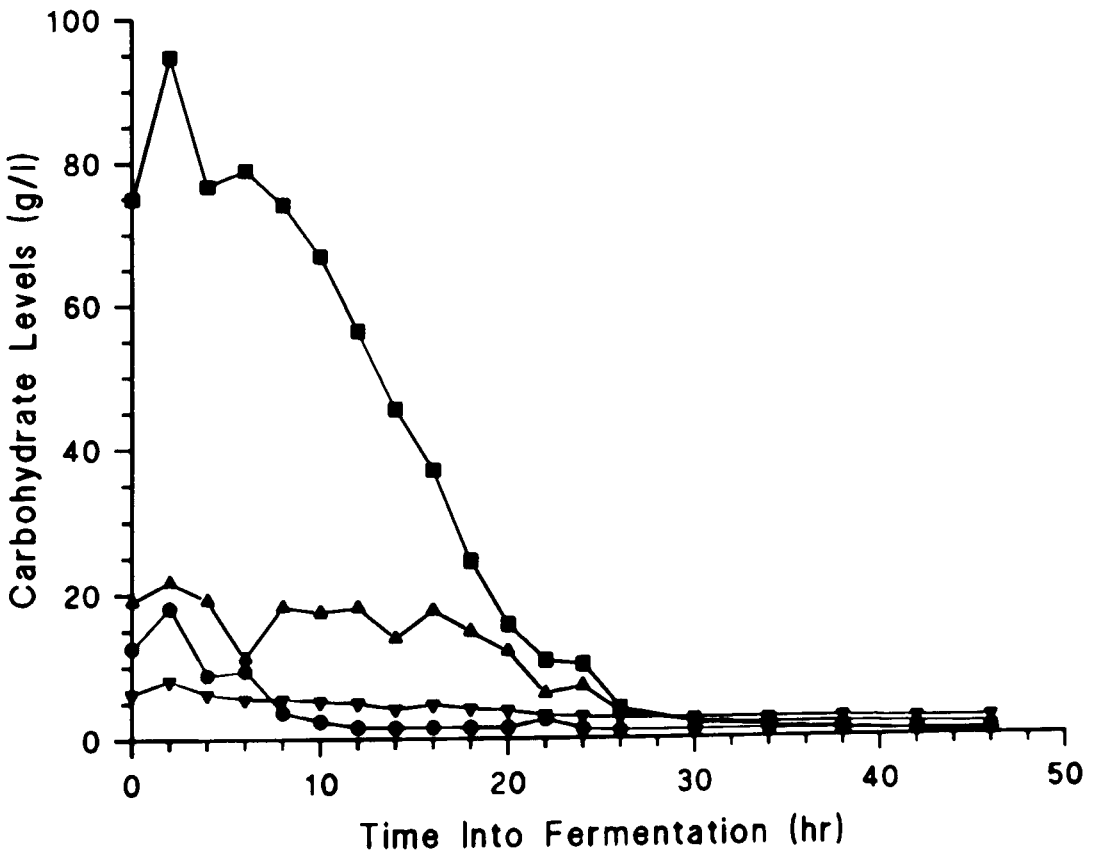


Figure 102. Glucose (●–●) (Pooled S.D. = 0.213), maltose (■–■) (Pooled S.D. = 0.673), maltotriose (▲–▲) (Pooled S.D. = 0.282) and starch (▼–▼) (Pooled S.D. = 0.144) levels during fermentation.

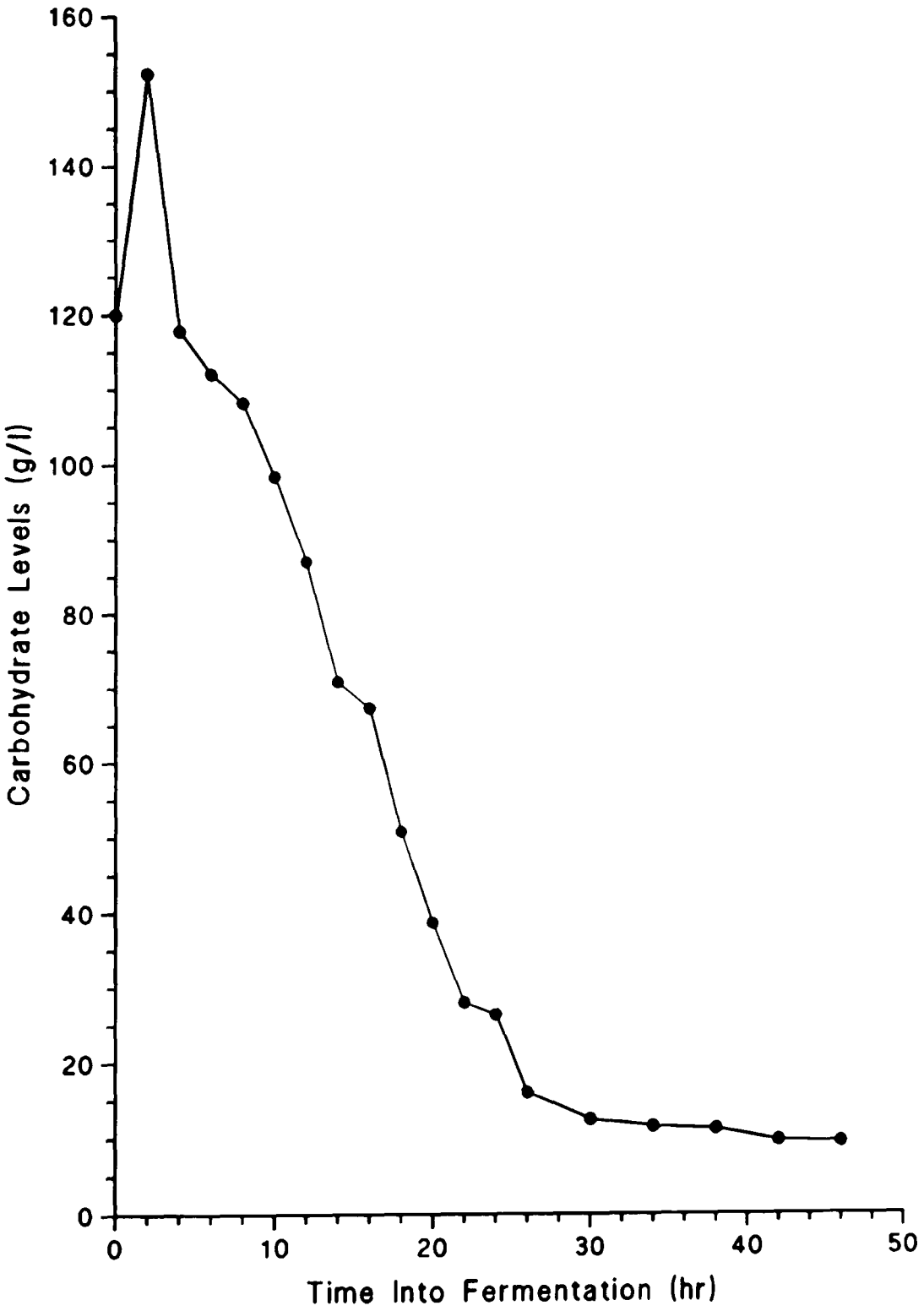


Figure 103. Total soluble carbohydrate (Pooled S.D. = 1.038) levels in the wash during the fermentation process.

Maltotetraose was found to follow a near linear decrease, but utilization of this component was slow. During the 46 hr of fermentation only 0.88 g/l of the maltotetraose was utilized. Maltopentaose gave an initial increase in concentration of 0.98 g/l, presumably due to malted barley enzyme activity, but this fell rapidly to the initial level after 4 hr. However, from 4 hr to 20 hr a slight increase in maltopentaose concentration was detected before a decrease took place with a subsequent levelling off in concentration to 1.20 g/l after 30 hr. Maltohexaose, maltoheptaose and starch followed a similar pattern to each other, and are used throughout the first 26 hr of fermentation.

4 DISCUSSION.

4.1 Assay Method Development.

There is a requirement for specific substrates in order to assay for specific enzyme activities in a complex mixture of enzymes, as found in a malted barley extract. Most of the assays developed for cereal amylases, require the enzyme to be purified or partially purified, due to the non-specific substrates used. In many of these assays the substrate is invariably starch. Such assays can be tedious, vary depending on the starch substrate used and due to assay conditions (Asp 1990). Interference by other starch degrading enzymes may cause other problems if purified enzymes are not used. Some of these difficulties may be overcome by using chromatogenic starch (Kennedy *et. al.* 1988) or malto-saccharide substrates (McCleary and Sheehan 1987).

The use of the Phadebas test tablet was suitable for the assay of α-amylase from a malted barley extract (see Section 3.1.1.). The action of α-amylase on the water insoluble, cross linked starch polymer of the Phadebas test tablet releases a water soluble blue dye. The absorption at 620 nm of the blue solution was a function of the α-amylase activity. This was suggested through the series of experiments for substrate utilization, and also from enzyme activity under different buffer conditions. By using a heat inactivation step, it was possible to demonstrate that the α-amylase from malted barley had a requirement for malto-oligosaccharides of six glucose units or higher.

The high, non-heat treated activity of the enzyme preparation on p-nitrophenyl maltotetraose and p-nitrophenyl maltopentaose implied that other amylolytic enzymes were hydrolyzing these substrates. This could have been due to the α-glucosidase used as the coupling enzyme in the reaction for the release of p-nitrophenol from the degradation products of the α-amylase activity. Another possible enzyme was β-amylase, present in the crude enzyme preparation. No

activity was detected from the heat treated preparation when p-nitrophenyl maltotetraose and p-nitrophenyl maltopentaose were used as substrates. Malted barley α-amylase was only able to use p-nitrophenyl substrates greater than p-nitrophenyl maltohexaose. This is in general agreement with MacGregor and MacGregor (1985). The yeast α-glucosidase did not use p-nitrophenyl maltopentaose and only p-nitrophenyl maltotetraose to a limited degree. Activity on the p-nitrophenyl maltotetraose and p-nitrophenyl maltopentaose substrates was therefore due to β-amylase.

Previously reported assays for the determination of β-amylase had been developed using non-specific reducing sugar assays with starch as the substrate. These methods were limited for the quantitative determination of β-amylase in complex mixtures of carbohydrate hydrolyzing enzymes. As a result, these assays were measuring total saccharifying activity. The work of Mathewson and Seabourn (1983) showed the suitability of p-nitrophenyl malto-oligosaccharides for the determination of β-amylase activity, but the method was not ideal.

The method of Mathewson and Seabourn was used as the basis for the determination of β-amylase activity (see Section 3.1.2.). The preferred substrate of β-amylase under the conditions of the assay was p-nitrophenyl maltopentaose. This substrate was not hydrolyzed by malted barley α-amylase, nor the yeast α-glucosidase which was used as a coupling enzyme in the assay. This was demonstrated by a series of experiments using different buffer conditions.

Figure 104 summarizes the activity of both α-amylase and β-amylase from a crude malted barley extract using their respective assays, but with additions to the buffer systems as outlined in Table 3. The α-amylase assays were also carried out using the 0.1 M Phosphate buffer, pH 7.1 since this activity was minimized when assaying for β-amylase activity. α-Amylase activity from malted barley was dependent on added Ca^{2+} ions in the buffer system for maximum

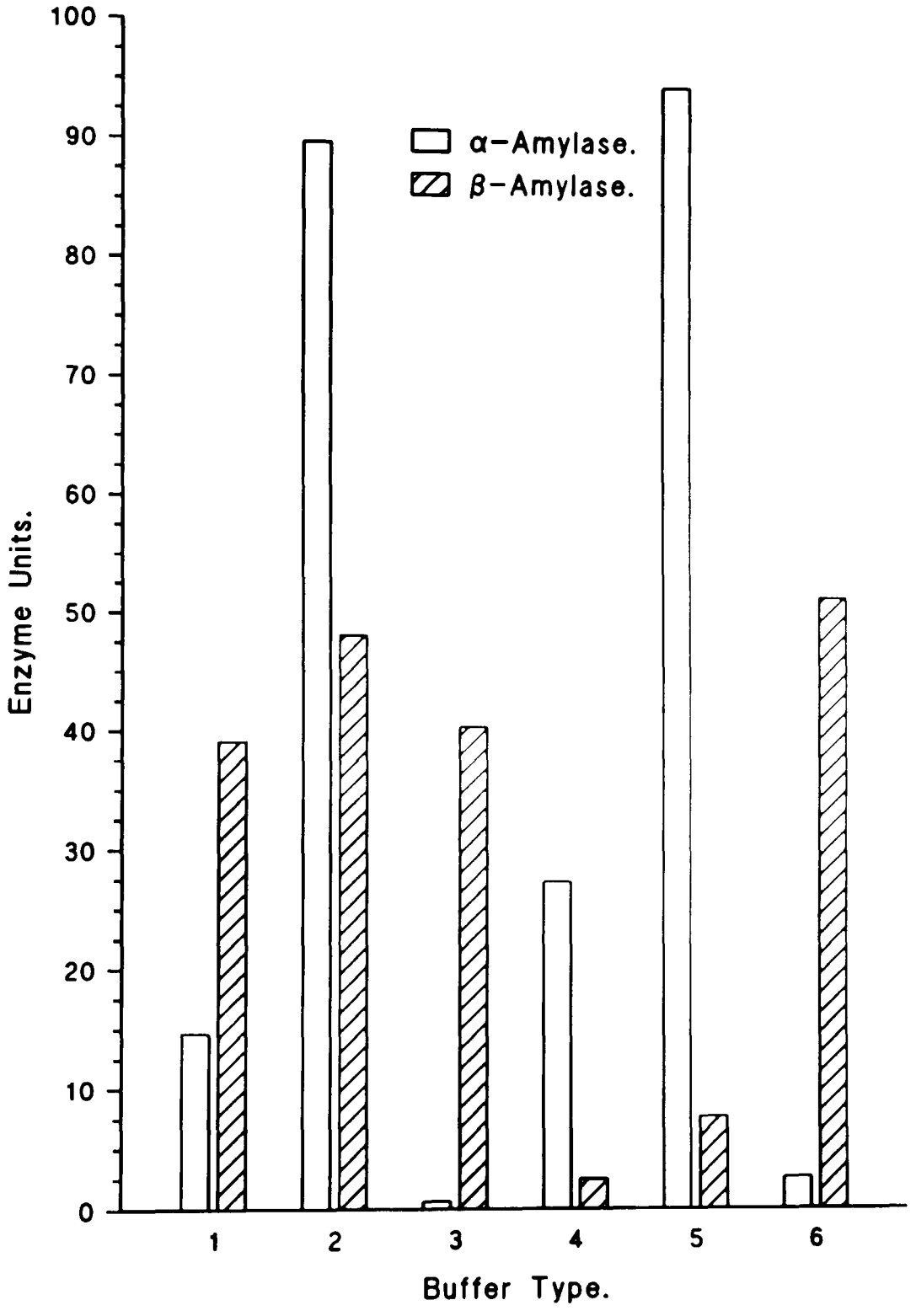


Figure 104. Activation and inhibition of the crude α–amylase enzyme using the Phadebas assay system and the crude β–amylase enzyme using the 4–nitrophenyl maltopentaose assay system. Buffer type 1 – 0.1 M Phosphate, pH 7.1, 2 – As 1 with 1mM Ca^{2+}, 3 – As 1 with 10 mM EDTA, 4 – As 1 with 10mM Ascorbate, 5 – As 4 with 1mM Ca^{2+}, 6 – As 4 with 10mM EDTA.

activity. EDTA addition inhibited α-amylase under these assay conditions. β-Amylase activity was unaffected in the presence of Ca^{2+} ions or EDTA, but was inhibited in the presence of ascorbic acid this inhibition was overcome using EDTA.

The mechanism of ascorbic acid inhibition on β-amylase is through a copper catalyzed oxidation of the ascorbic acid to form an ascorbate free radical. This free radical is then attracted and bound to the readily available disulphide groups of cysteine and methionine side chains within the β-amylase molecule. Ascorbic acid inhibition of the β-amylase molecule is non-competitive (Baker & Smiley 1985) suggesting that the disulphide groups are not at the active site. Activation of the enzyme by the chelating group EDTA in the presence of ascorbic acid may be due to conformational changes within the β-amylase molecule. EDTA either prevents the formation of complexes of the ascorbic acid derivative, copper ion and the disulphide groups of the β-amylase molecule or disrupts them. This may make the active site of the enzyme more susceptible to the substrate molecule.

During the preparation of this thesis, a similar assay for the determination of β-amylase was published (McCleary & Codd 1989). However, the assay of McCleary & Codd used a 0.1 M maleate buffer, pH 6.2 with EDTA addition at a concentration of 1 mM. The yeast α-glucosidase was incorporated into the substrate and was also at saturation levels. A further difference was that the temperature of incubation was only 40°C in the McCleary & Codd method.

Although α-amylase and β-amylase are the major starch hydrolyzing enzymes of malted barley, other enzymes are present in the malt that will contribute to the degradation of starch and other carbohydrates. The use of p-nitrophenol labelled substrates would enable many carbohydrases to be assayed in the complex mixture found in a malted barley extract. Many of these enzymes have already

been characterized from other fields of research but few have been studied from distilling malt, nor through the processes involved in the production of Scotch Malt Whisky.

Assays were modified for use with a malted barley extract and the final assay parameters for each enzyme is given in Table 10. At the time of this study the malted barley of choice, within the industry, was Triumph. All enzyme parameters were established using this variety. During enzyme method development, it was assumed that the enzyme parameters would be the same for each different variety. Although this assumption was probably valid there may be factors that affect the individual varieties, *i.e.*, geographical area, environmental conditions during barley development, malting conditions, malting plant.

The results indicated that the developed or modified assays were applicable for the malted barley enzymes under study. Although those assays for α-glucosidase and arabinosidase were not entirely optimum for the chosen conditions due to the high k_m value (Table 11). In general the more tightly a substrate binds to the enzyme the lower the value of k_m.

The work of Mitchell and Newman (1972) and Houston *et al* (1974) mainly characterized the α- and β-mannosidases. Assay conditions from both studies were different to those developed in this work, as was the calculation for p-nitrophenol concentration. Calculation by Mitchell and co-workers was by the Beer-Lambert Law at 420 nm, whereas the standard curve method was used in this study.

These assays were suitable for studying the enzymes in the complex mixture of hydrolases in a malted barley extract. They would therefore be suitable for studying enzyme activities and patterns in the wort and wash from the Scotch Malt Whisky processes of mashing, wort draining and fermentation. Although the assays for α-glucosidase and arabinosidase were not ideal, the specific nature of the substrates

made them suitable. The phosphatase, proteinase and peptidase assays were also appropriate for investigating these enzymes during the Scotch Malt Whisky processes.

4.2 Amylase Activities From Different Sources.

The assays for amylase activities were developed for use with malted barley enzymes, however, they were applicable for the determination of amylases from other sources. From the experiments carried out, the α-amylases from different sources gave different activities depending on the buffer system used.

The α-amylase from *B. licheniformis* does not have a requirement for calcium ions unlike the enzyme from *B. subtilis*. Ascorbic acid has little effect on the activity from *B. subtilis*, but does have a slight inhibitory effect on *B. licheniformis* α-amylase. Copper inhibition affects the *B. licheniformis* enzyme to a greater extent. The enzymes from both sources show no activity when p-nitrophenyl maltopentaose was used as substrate, but a slight activity was noted with p-nitrophenyl maltoheptaose, this was in agreement with Ohnishi *et.al.* 1990. However, this activity was minimal.

The mammalian α-amylases show that they have an absolute requirement for calcium ions, and are inhibited to a certain degree by ascorbic acid and copper ions. The porcine pancreas enzyme gave a slight activity when both p-nitrophenyl maltopentaose and p-nitrophenyl maltoheptaose were used as substrate. No activity was present using p-nitrophenyl maltopentaose as substrate with the human saliva α-amylase, but a slight activity was detected with p-nitrophenyl maltoheptaose.

Amylolytic activity from malted barley, although not having an absolute requirement for calcium ions, requires added calcium ions in the assay buffer solution to achieve its optimal activity. Ascorbic acid has an activation effect in the presence of calcium ions, but the reverse was found with added EDTA. A similar mechanism to that of β-amylase, as discused earlier, may be present. Since the malted

barley α-amylase used for this study was a crude preparation, activity on the p-nitrophenyl maltopentaose and p-nitrophenyl maltoheptaose substrates was expected.

α-Amylase activities from the bacterial, plant and mammalian sources were not similar using the basic malted barley assay conditions. Although, this was based on only results from different buffer compositions and not on pH and temperature conditions. The review by Marschall *et al* (1982) indicates the differences in temperature stability between the plant and bacterial enzymes. While the work of Ceska *et al* (1969) shows the conditions required for mammalian α-amylases. However, all the α-amylases from each group gave activity when the malted barley α-amylase assay conditions were used.

As with the α-amylases from different sources, it was evident (Table 13) that β-amylases from malted barley and sweet potato gave different responses to activating and inhibitory compounds.

Two sources of β-amylases were compared, malted barley and sweet potato. All the preparations were from supposedly purified β-amylase but, all gave activity with the Phadebas α-amylase assay, this indicated residual α-amylase activity.

Since the malted barley β-amylases were obtained as standardized at 2000° Lintner, they should have been suitable for the preparation of β limit dextrin for the determination of α-amylase by the α-amylase International Method (IoB 1982). Obviously they were not, due to the activity observed using the Phadebas assay. This method should now be considered for replacement, with an assay employing a specific substrate for α-amylase.

Comparison of the two sources of β-amylases show very similar activities in all six buffer systems. The level of activity within the groups was also similar, as expected. Greatest activity was found with no additions to the basic buffer system, but the activity with added EDTA was lower than with added calcium ions.

4.3 Enzyme Activities From Micromalted Barleys.

All samples from each malt modification, with or without gibberellic acid, were micromalted under a similar malting regime. Malt parameters were studied to ensure the range of modifications for each variety were accurate. Although the use of added gibberellic acid in the production of distilling malt is prohibited, comparisons of the affects of this naturally occurring plant hormone (Palmer 1974) can be made between modifications. Gibberellic acid accelerates the synthesis and/or release of enzymes into the endosperm to degrade endospermic material. The range of modification of the micro-malted samples were increased by those samples treated with added gibberellic acid.

From the coarse and fine hot water extracts (Figure 25), Triumph gave the highest extracts with or without gibberellic acid, followed by Golden Promise and then Kym. Fine hot water extracts were always higher than the coarse extracts. The full extract potential is normally released from the fine grind. The fineness of the grind ensures that all available starch will be made soluble and therefore, available for amylolytic conversion during the laboratory mash. This may explain why the under-modified samples gave higher extracts from Triumph and Golden Promise without gibberellic acid, when compared to the over-modified malt samples from the same varieties. Starch, in under-modified malt, was not as readily solubilized as in the over-modified malt. The difference between the two hot water extracts indicates the degree of modification of the malt.

The effect of the degree of modification was evident from the fine-coarse difference results (Figure 26). With all three varieties, a reduction in the degree of modification takes place from under-modified to the over-modified malts. This reduction is not so pronounced with the variety Triumph, since the under-modified sample was well-modified. This may be explained in that the malting regime was not suitable for this variety. Application of added gibberellic acid, accelerated the rate of modification during malting. The effect

of this is to reduce malting times. From Figure 26 a well-modified malt without gibberellic acid treatment is equivalent to the under-modified malt, with gibberellic acid treatment. If application of additional gibberellic acid were allowed, malt production for the Scotch Malt Whisky process could be carried out using a four day steeping and germination cycle instead of five days. Gibberellic acid is therefore seen as an activating agent during malting (Paleg 1960a, Palmer 1974).

Soluble free α-amino nitrogen is important during the malting, mashing and fermentation processes of the Scotch Malt Whisky production since yeast growth is ultimately dependent on it (Berry and Ramsey 1983). Storage proteins, laid down in the barley during grain development are utilized for seedling growth during germination. The amino acids are used for enzymic protein and structural proteins. Levels of free α-amino nitrogen would be expected to follow the pattern of protein modification that peaks with the well-modified malt sample. This was the case for Triumph and Golden Promise, with or without gibberellic acid treatment, and for the variety Kym with gibberellic acid treatment. However, the micromalted samples without gibberellic acid treatment from Kym gave an unexpected result. These samples gave decreasing free α-amino nitrogen values through-out the modification range. This may have been due to non-ideal micromalting conditions or inaccuracies during the preparation of the laboratory extracts. Although the levels of free α-amino nitrogen in Triumph and Golden Promise (see Section 3.3.1.1.) were similar to those of Lie (1973), those from the variety Kym were decreased

A malted barley extract was prepared and enzyme activities were studied under optimal conditions and under the conditions during laboratory mashing, *i.e.*, temperature at 65°C, and the pH of the final hot water extract. The majority of the carbohydrase enzymes from three varieties, with or without gibberellic acid treatment, gave similar patterns of activity (see Section 3.3.1.2.). This was basically an

increase in activity over the modification range from the under-modified to the over-modified malt samples. This pattern would be expected, since longer germination times will cause an increased concentration of hydrolytic enzymes. These enzymes are necessary to sustain respiration and allow the hydrolysis of the endosperm reserves.

α-Glucosidase activity from the variety Triumph does not follow the normal pattern of activity. Without gibberellic acid treatment the enzyme activity was decreased for the over-modified malt sample. For the gibberellic acid treated samples, the activity decreases through-out the modification range. Another enzyme that does not follow the normal pattern is β-mannosidase. With this enzyme it is the gibberellic acid treated malt samples from Triumph and the samples from Kym without gibberellic acid treatment that show the highest activities. The activity of the former, decreases after the malt is well-modified, whereas the activity decreases from the under-modified to the over-modified samples from the variety Kym. With the β-mannosidase enzyme, under mashing conditions, activity was affected as for β-mannosidase under optimal conditions. These results may express varietial differences in the activities of the enzymes during germination. It may also suggest that the enzymes are only active during these stages of development, and after any requirement for them they are inhibited or degraded.

Well-modified malt is required for the production of Scotch Malt Whisky (see Section 1.2.3.). Although all the enzymes required by the barley grains during germination are present in excess, varietial differences in enzyme levels were present between the well-modified samples. The variety Golden Promise contained the highest levels of enzyme activity for α-amylase, β-glucosidase, α-mannosidase, β-mannosidase, N-acetylglucosaminidase and β-xylosidase. In all but β-xylosidase, Golden Promise was followed by the variety Triumph and then Kym. Triumph gave the highest activity with α-galactosidase,

β-galactosidase and arabinosidase, while the activity levels of α-glucosidase was greatest with Kym. A similar pattern was followed with gibberellic acid treatment, except that the varieties Triumph and Kym were reversed for the enzyme α-galactosidase, *i.e.*, activity was highest with Kym. Overall it would appear that the variety Golden Promise contained the highest levels of carbohydrase activity followed by Triumph and then Kym.

Activity levels of all carbohydrases under the laboratory extract conditions suggested very similar patterns. There was an increase in activity through the modification range. Gibberellic acid stimulation showed a very slight increase in enzyme activity for most of the well-modified and over-modified samples. Under-modified malt samples gave a noticeable increase in enzyme activity with gibberellic acid treatment. From all the enzymes studied, except for β-xylosidase, the activities under laboratory extract conditions were lower than under optimal enzyme conditions. The β-xylosidase activity under both sets of enzyme conditions were similar.

Gibberellic acid stimulation was evident in almost all carbohydrase enzymes studied, from the three varieties. For some enzymes this stimulation was slight, but was more evident between the under-modified samples. Externally applied gibberellic acid, a practice not permissible with distilling malt, will therefore increase the rate of endosperm modification. This will give a well-modified malt in approximately the same time as an under-modified malt without gibberellic acid treatment.

The activities of acid phosphatase under optimal assay conditions increase from the under-modified to the over-modified malt samples. Overall, no stimulation was detected with gibberellic acid treatment, except with the over-modified sample from the variety Kym. Similar results were observed under laboratory extract conditions. Phytase activity, under optimal assay conditions, gave an increase in activity through the modification ranges, except for the Kym and Triumph

samples without gibberellic acid treatment. In both these cases, activity peaked with the well-modified sample. Gibberellic acid treatment did not stimulate phytase activity. Variety activity levels for both enzymes suggest greatest activity from Triumph, then Kym followed by Golden Promise.

Serine protease, leucine aminopeptidase and carboxypeptidase A type activities were all stimulated by gibberellic acid addition. All three enzyme activities increased throughout the modification range, except for the under-modified and well-modified samples from the variety Golden Promise for carboxypeptidase A. Carboxypeptidase A activities from this variety were identical with and without gibberellic acid treatment.

From the results discused, it appears that enzyme activity may vary with the barley variety. However, other factors may influence the level of an enzyme within the grain. Consideration also should be given to agronomical factors, geographic and climatic parameters, malting conditions (both equipment and regimes) and possibly mashing conditions (both equipment and regimes).

4.4 Statistical Analysis of Production Malted Barley Enzyme Results.

During the time of this study (see Section 3.3.2.1.), six different maltsters were suppling malted barley of the variety Triumph. Overall results suggested that Maltster B demonstrated the highest average enzyme activity for the enzymes studied, except α-glucosidase, β-glucosidase and α-galactosidase. However, for α-glucosidase and β-glucosidase activities Maltster B was not significantly different from Maltster E, who gave the highest activity for these two enzymes. From Table 14, a crude ranking of the enzyme activities from all Maltsters was possible. The overall activities show that Maltsters B and E consistently achieved the highest enzyme activity, followed by Maltsters C and F and finally Maltsters A and D.

A further statistical study of one Maltster from each of the three groups was undertaken to determine if an increased sample size affected the statistical significant difference between Maltsters. Again Maltster B was significantly different from Maltsters A and F in seven of the eleven enzymes studied. The order of overall enzyme activity for the Maltsters was unchanged. By increasing the number of samples analyzed it became obvious that the significant difference between Maltsters A and F decreased. Only activity from α-galactosidase giving significant difference between these Maltsters.

Maltster B supplied malt from the varieties Pipkin and Triumph and a blend of Triumph/Natasha during the period of this analysis. Duncan's Multiple Comparison Test suggested that the variety Pipkin was significantly different from the other two groups of samples. In addition, Pipkin also gave the highest level of overall enzyme activity. It was assumed that Maltster B malted the barleys consistently, and no significant difference would be due to the effects of malting.

The varieties and blends Camargue, Pipkin, Triumph, Triumph/Halcyon and Triumph/Natasha were analyzed using Duncan's Multiple Comparison Test. Since these were not from a single Maltster, effects due to Maltsters may influence the results. Pipkin was again the variety with the higher overall enzyme activity, and was significantly different for nine of the enzymes studied. As expected, the variety Triumph and the blends in which Triumph was a proportion were not significantly different in most of the enzyme activities. Since the percentage of each variety in the blends was not known, variation could be expected. The variety Camargue, is descended from Triumph, and does not show significant difference from Triumph or the Triumph blends. Camargue gave the lowest activity levels overall for all the varieties and blends studied.

The statistical significant difference between Maltsters may be attributed to several factors. The major parameters for these differences would include malting regimes and differences in malting

equipment. Although Triumph was the major variety of this study, the effects of geographical, cultivation and agronomical methods also may influence the levels of enzyme activity. It may be significant that Pipkin is a winter barley, while the other groups being compared were composed of spring varieties.

4.5 Enzyme Activity Within the Scotch Malt Whisky Process.

Since the Scotch Malt Whisky process does not include a wort boiling stage, nor the use of sterile fermentations, malted barley enzymes survive into the fermentation. Any micro-organisms, associated with the malt, which are able to survive the mashing temperatures, may also be viable when they enter the fermentation. The presence of micro-organisms on the washbacks, which have survived washback sanitation, may prevail into the fermentation. Bacteria from the yeast preparations added to the fermentation may also result in contamination. Airborne micro-organisms can infect the wort or wash. Because of these infections, there may be three or more different enzyme systems associated with the wort or wash. The enzyme assays employed in the analysis of the three processes were those developed for the malted barley enzymes and may not be optimum for yeasts and other micro-organisms.

This study of enzyme activities during the process of Scotch Malt Whisky production was carried out on normal distillery mashes and fermentations. A certain amount of variation could therefore be expected in the results. This was especially true for the fermentation section of the study, since four individual washbacks were sampled to obtain a comprehensive time course of the fermentation process. The four washbacks that were sampled were filled from successive mashes.

4.5.1 Mashing.

The discussion on enzyme activity through-out mashing, will centre on mashing-in, and first and second water drainings within

the mash tun environment. No enzyme activity was detected in the sparge waters. The temperatures of the third and fourth waters probably would denature enzymic protein.

During the period the mash was resting in the mash tun, and through-out first and second water draining (see Section 3.4.1.1.) temperature and pH levels progressively decreased. However, after the addition of the second water, the temperature in the mash tun increased and remained at a higher level. Within the mash tun the temperature and pH parameters are very important. Besides the alteration of pH with temperature, salts within the mashing liquor and malt may contribute to the lowering of the pH. This is especially true with calcium ions, which will complex with phosphates and other compounds, *e.g.*, peptides and proteins. Such interactions allow the release of hydrogen ions with a fall in pH. A pH equilibrium was reached within the mash after 45 min. This may be achieved via the calcium-phosphate complexes, and by peptides and amino acids. Levels of free α-amino nitrogen and phosphate (Figures 41 and 42) would appear to suggest this. The release of protein into the mash liquor may also be dependent on the pH of the mash. The decreases in protein concentration towards the end of mashing may also be attributed to the complex nature of the mashing system, due to the pH/phosphate relationship, with the resultant precipitation of protein complexes.

α-Amylase was active through-out the first water, converting gelatinized starch to oligosaccharides of various lengths. The activity of β-amylase was more limited, (see Figure 44) in that the main proportion of activity was only present during the mashing-in stage. This was in general agreement with Muller (1991). The activities of both enzymes were highest during mashing-in, and this was probably due to environmental stabilization during hydration. Mashing liquor temperatures are greater than the temperature of the mix from the mash spout. However, the micro-environment of the enzymes will

ensure that the amylases, especially β-amylase, are not completely denatured during mashing-in. The temperature in the mash tun declines during the first water and α-amylase activity will be affected by this factor, since the optimal temperature for α-amylase was 65°C. Although α-amylase activity under mashing conditions is reduced from the optimal enzyme conditions, the amount of active enzyme present, allows almost complete saccharification of the malted barley starch. β-Amylase was quickly denatured due to the mash tun temperatures, the temperature of mashing is well in excess of the β-amylase optimal temperature for activity, the bulk of maltose formation takes place during mashing-in. The concentration of maltose rose slightly during the period of first water draining (Figure 96).

The purpose of the mashing process is to extract the soluble material from the malted barley grist. The major hydrolysis is that of starch breakdown by α- and β-amylases. To achieve starch solubilization, relatively high temperatures, *i.e.*, 63-66°C must be used. These temperatures favour the activity of α-amylase, but β-amylase activity quickly declines once the mash is in the mash tun. It is normal practice for a mash to rest for a period in the mash tun, once it is mashed in. In some cases this can be up to 30 min. One of the main aims of this rest is to allow complete saccharification of the starch. From the results in Figures 43 and 44, it would appear that this resting period is not required, or should be kept to a minimum.

Activity from the enzymes α-glucosidase, α-galactosidase and β-mannosidase closely followed a similar pattern to that of α-amylase activity as found during the mashing process. However, the decrease in activity between the first and second mashing waters was not so pronounced as with α-amylase. For α-glucosidase, the difference between optimal and mashing conditions was less than that of α-amylase, likewise for α-galactosidase. Activity of α-galactosidase began to increase towards the latter third of the first water under

both sets of assay conditions. This may have been due to the temperature and/or pH environment of the mash, during this period, being more suitable to this enzyme. A gradual decrease in β-mannosidase activities were observed through-out the period of the first water, with activity levels from optimal and mashing assay conditions being very similar.

β-Glucosidase, β-galactosidase and α-mannosidase activities were similar to that of β-amylase. The initial activities during mashing-in and during the resting period were relatively high when compared to the rest of the mash. The enzyme N-acetylglucosaminidase, also suggested this pattern, but the activity during the first water resting period was similar to that after 45 min. Arabinosidase and β-xylosidase, did not follow the patterns of the other carbohydrase enzyme activities. β-Xylosidase activity under optimum conditions was relatively stable through-out the first water cycle, but activity under mashing conditions was greatly reduced. Arabinosidase activity was also quite stable, but increased as the first water progressed. Activity for arabinosidase decreased during the second water. The activity of β-xylosidase under mashing conditions for the second water increased over the first water levels, but the optimum condition activities decreased. This result cannot be readily explained since the temperature in the mash tun was similar to the temperature during the initial stages of first water draining, but the pH within the mash was between 5.0 and 4.6.

The minor carbohydrases play a very limited role in the hydrolysis of starch. However, their actions may have a number of effects. Action on non-starch polysaccharides may lead to a decrease in wort viscosity, *i.e.*, arabinosidase. These enzymes may also be responsible for the production of some of the ribose sugars necessary for yeast nucleotide synthesis. Amino sugars may be required for yeast cell structures, other monosaccharides may be incorporated into yeast carbohydrate and polysaccharides. There is also a possibility that monosaccharides

may be transformed to glucose once they have entered the yeast cell. The actions of α- and β-glucosidase will lead to increased glucose levels. These increases may be small, but all will lead to increasing spirit yields.

Acid phosphatase and phytase are two enzymes responsible for the release of inorganic phosphate into the mashing liquor during the mashing cycle. Acid phosphatase under the optimal conditions gave relatively high activity when compared to the mashing conditions, but activity was reduced through the mashing cycle. The enzyme activity pattern of phytase, under optimal enzyme conditions, was similar to the release of inorganic phosphate into the mashing liquor. Activity peaked at 85 min into the mashing cycle, while the inorganic phosphate levels were also highest at this time. Inorganic phosphate release would appear to follow closely the activity of both acid phosphatase and phytase. Inhibition of the enzyme activities may be occurring when the required inorganic phosphate levels are achieved, especially during first water draining.

Through-out the period of first water draining general proteinase type activity was seen to decrease, although the levels of activity remained relatively high. Serine protease type activity went through a minimum at 108 min into the mashing cycle, as did leucine amino-peptidase and carboxypeptidase A type activities, although the latter two were only to a limited extent. The pattern of protease and peptidase enzyme activity followed a similar pattern to that of the free α-amino nitrogen levels (Figure 41). However, there was no similarity between the enzyme patterns and protein levels in the mashing liquor.

The actions of the phosphatases and proteinases were discussed previously, and they may play a supporting role to the carbohydrases, in that they maintain the environmental conditions within the mash

tun. In maintaining these conditions as long as possible during the mashing process, carbohydrate and polysaccharide hydrolyzing enzymes will remain active for longer periods.

This study was carried out on an infusion type mashing system, using the operating procedures of a particular Chivas Brothers distillery. Other distilling companies use different mashing systems and/or operating procedures. Those systems using similar procedures to the one studied, *i.e.*, infusion and semi-lauter or lauter, would probably give a similar type of wort to that analyzed. However, differences in operating procedures may lead to different wort compositions (Lenz 1989). Temperatures during mashing-in may be lower or higher giving poorer mashing efficiencies, giving non-optimum amylase activities. Lower temperatures may enhance the activity of minor carbohydrases, phosphatases and proteases. The deeper mashing beds found in infusion and semi-lauter systems may provide a more stable environment for the hydrolases than the shallow lauter systems.

Wort quality from other systems (see Section 1.1.) may be poorer than the infusion type systems and continued enzyme activity may not be as high. This would be due to the inadequate stabilization of the enzymes during mashing. A similar effect can be seen when a mash is taken into an infusion mash tun too thinly, *i.e.*, very high liquor to grist ratio. Alternatively, a low liquor to grist ratio may also lead to poor mashing efficiencies.

Wort composition and quality is extremely important to the distiller, since it has a major impact on his final product. The traditional infusion or modified lauter mashing systems produce the composition and quality which the distiller requires. The more modern systems may not give either, but this may be due to their inability to provide the correct environment for the complex mixture of hydrolases found in the mash tun.

4.5.2 Wort Draining.

The draining of the wort from the mash tun to the washback can be considered as an integral part of the mashing process. The wort obtained from the mash tun is drained through the mash bed and rapidly cooled to 20°C to 22°C. The cooling of the wort has two functions, first to enable yeast pitching into the wort, in the washback, as quickly as possible, allowing the fermentation to proceed rapidly. The second, which is a consequence of the first, is to prevent excessive heat denaturation of the malt enzymes that may be active during the fermentation stage.

Wort draining commenced after a 20 min resting period in the mash tun, the first sample from this study would therefore be equivalent to the 65 min sample from the mashing cycle. The temperature of the wort, at the sampling point, remained constant at 20°C through-out the draining cycle. Wort pH also remained quite constant (pH 5.29 to pH 5.32). As expected, there was a specific gravity gradient over the period of first water draining. Free α-amino nitrogen, protein and phosphate levels remained similar, or slightly above the mashing cycle study levels. The pattern of these three variables was also similar to those found during the mashing cycle study.

The amylase and carbohydrase enzymes studies during wort draining suggest that all follow similar patterns, with comparable levels of activity to the mashing cycle studies. This was not totally unexpected, since only the draining of the wort through the mash bed had taken place. However, the enzyme activities under the conditions of wort draining were not entirely similar between the two studies. This could be due to the lower temperature of the wort when the samples were taken. A similar situation was also observed with acid phosphatase and phytase.

General proteinase and the peptidase activities during wort draining were found to differ from those observed during the mashing cycle. The pattern of the general proteinase type activity did not differ greatly. But a decrease in activity during first and second water wort draining was observed. During first water wort draining, the levels of activity for serine protease type activities were also increased, but a different pattern was observed, with activity peaking at 50 min into wort draining. Activity during second water wort draining was decreased in comparison to that during the mashing cycle. Leucine aminopeptidase type activity was slightly decreased but remained at a constant level through-out the total period of wort draining. Carboxypeptidase A type activity was similar between the two studies up to 60 min into wort draining, activity then fell, but activity was detected during second water wort draining. This was in contrast to the mashing cycle where no activity was found from second water mash liquor samples. These results may suggest that the environment from which the samples were drawn does affect the activity of the protease/peptidase enzymes. All classes of protein hydrolytic enzymes studied, except carboxypeptidase A, tend to be more stable from the wort samples. During the draining of the mash, enzymes may be released more readily from the mash bed if they are associated with protective environment of the draff particles. Proteinase and peptidase enzymes may be released in this way.

Probably one of the biggest differences between brewing and distilling takes place during wort draining. The wort is not boiled, on the contrary it is cooled. Thus, those enzymes surviving mashing, along with the associated microflora of the malt enter the fermentation vessel. These surviving enzymes continue to hydrolyze their substrates, increasing the levels of metabolites available to the yeast during fermentation.

4.5.3 Fermentation.

The fermentation can be divided into three major stages, as seen from the biomass time course results (Figure 76). The first stage encompasses active yeast growth, the second the stationary stage and the final phase is of yeast decline and bacterial growth. Wort composition also affects the fermentation, and this is governed by the quality of the malt and mashing procedures employed.

The active growth stage of the yeast was relatively short slowing down 14 - 18 hr into the fermentation (Figure 76), this was probably due to limitations in the availability of soluble free α-amino nitrogen (Berry and Ramsey 1983). From Figure 77 soluble free α-amino nitrogen levels were at a minimum after 14 to 18 hr in the fermentation studied. During growth heat is generated, and since the washbacks are not under temperature control the fermentations were set at a temperature of 21°C. This was slightly higher than during the wort draining study but setting temperatures vary depending on the ambient temperature. By adjusting the setting temperature, the fermentation will achieve the desired final temperature.

Alcohol production was completed in about 30-34 hr (Figure 75). By the end of alcohol production the specific gravity fell to about 1000. The decline in specific gravity is a good indicator of fermentation rate and any problems associated with fermentation. Another indication of how the fermentation is progressing can be observed by monitoring the pH of the wash. During the active growth stage there is little bacterial growth. (This may not always be the case, especially if malt and/or yeast quality and/or plant sanitation is not satisfactory.) During the stationary phase of the fermentation, a limited bacterial population will develop, mainly Lactobacilli. Once fermentation was complete the yeast will begin to autolyze, releasing nutrients into the wash. Yeast autolysis will give a further drop in the specific gravity, but the bacterial population will increase dramatically at this point. A rapid increase in wort pH was due to acid production from

bacterial activity, and towards the end of fermentation the pH again fell. The effects of bacterial activity on pH towards the end of the fermentation are demonstrated in Figure 79. By observing the levels of succinic acid, lactic acid and acetic acid during fermentation the concentrations begin to rise once the yeast has reached the stationary stage. Once yeast decline began, the levels of the three organic acids increased dramatically. Succinic acid is a product of yeast fermentation. After the active growing phase the concentration of succinic acid decreased, thereafter, an increase due to yeast autolysis or bacterial fermentation was observed.

The profiles of the individual amylase and carbohydrase enzymes were followed through-out the fermentation time course (see Section 3.4.3.2.). For all, except for α-amylase, β-xylosidase and N-acetylglucosaminidase, the activity within the fermentation time course could be divided into three distinct sections. The first section was between 0-8 hr, the second between 8-24 hr and the final section from 24 hr to the end of fermentation. Within each of these sections a different enzyme system was being observed. Initially the malted barley enzymes would be the predominant activity, and this system was then superseded by the yeast enzymes and finally the bacterial enzyme system would be prevalent. No distinction could be made between yeast extra-cellular or intra-cellular enzymes, but the enzymes released may still be active after autolysis. The time coarse profiles from α-amylase and β-xylosidase are similar to each other, but slightly different from the other enzymes. The initial peak of enzyme activity appeared for a longer period and the bulk of this activity was attributed to the malted barley enzyme system. The yeast and bacterial systems being of much lower activity. N-Acetylglucosaminidase activity suggested the possibility of more than one system during the second section of fermentation. This may be due to an extra-cellular enzyme system before autolysis. A similar pattern may be occurring with β-glucosidase and β-galactosidase. However, more

detailed studies may be necessary to decide this. The analysis of enzyme activity under fermentation conditions closely follows the optimal enzyme assay conditions for all carbohydrases studied.

Acid phosphatase activity followed a similar pattern to that of α-amylase and β-xylosidase. Levels of this enzyme were quite high in the malt and survived mashing and wort draining to a greater extent, as do α-amylase and β-xylosidase, than the others. Because of this, the bulk of acid phosphatase activity during fermentation probably will be from the malted barley enzyme system. Phytase was only studied under optimal conditions, and indicated four peaks of enzyme activity. Two peaks of activity were detected between 8-24 hr and both were probably associated with the yeast enzyme system. A prolonged period of activity was observed during the latter section of fermentation, suggesting that microbial activity of phytase or phytase type activity was widespread.

The general proteinase type activity also indicated that three peaks of enzyme activity were present during fermentation. The other protease and peptidase type activities studied also inferred this. However, serine protease and carboxypeptidase A tended to give higher activities during the first period of fermentation, which extended into the second period. The activities of the three enzyme types revealed that each gave a higher activity in a particular section of the fermentation. The highest activity for serine protease type activity was observed during the first period of fermentation, leucine aminopeptidase type activity was highest during the second period and carboxypeptidase A during the final period. All the protein degrading enzymes are active during the period of the fermentation. Proteolytic activity may be associated with a particular group of enzymes depending on the enzyme system that is more active during that period of the fermentation.

The study of the hydrolytic enzymes within the fermentation from the Scotch Malt Whisky process was very complex. Malt, yeast and bacterial enzymes are active at different stages, or may overlap during the fermentation. The hypothesis that a number of different enzymes systems were present during the fermentation appears to be correct due to the patterns of the individual enzymic activities. During the Brewing process these enzyme systems should not be seen due to the sterility of the wort and equipment used. Thus, only the yeast enzyme system would be prevalent. Since only the assay conditions for malted barley enzymes were used, the levels of activity for the yeast and bacterial enzymes are not quantitative, but give an indication of the patterns of activity.

4.6 Carbohydrate Profiles During Mashing and Fermentation.

The carbohydrate profiles, from samples obtained during mashing can be explained by amylase activities. β-Amylase was responsible for the high levels of maltose in the wort, however, the activity of this enzyme is limited to the initial stages of mashing-in (see Section 3.4.1.2.1.). It was therefore unlikely that maltose levels would increase substantially during the later stages of mashing, as shown in Figure 96. Residual β-amylase activity may account for part of this increase. The remainder coming from α-amylase activity on medium sized oligosaccharides (MacGregor and MacGregor 1985), *i.e.*, maltooctaose to maltodecanaose and on the larger dextrin molecules.

The concentration of glucose, through-out the first water during mashing varied only slightly. The differences between samples were not significant, in that no major pattern was observed. Since α-glucosidase was active, but only at very low levels, the production of glucose was limited from this enzyme. Glucose may be produced through the action of α-amylase, on maltoheptaose, and by any limit dextrinase activity present. This type of α-amylase hydrolysis would only be expected after the main liquification on the starch molecules was completed. Activity of this type would increase the levels of

maltohexaose. This effect was present during the period 65 min to 108 min into the first water. However, an increase in maltoheptaose was detected at 85 min, this was probably caused by further α-amylase activity on larger oligosaccharides not resolved by the HPLC analysis.

Increases in the concentrations of maltotriose and maltopentaose were most likely caused by α-amylase activity on the maltooctaose to starch fractions. Whereas, the decrease in concentration of maltotetraose would be caused by the limited β-amylase hydrolysis. Through-out the first water period the total carbohydrate, based on the summation of the glucose to maltoheptaose plus the starch fractions did not vary greatly. However, a slight increase was detected at 108 min, due to an increase in fermentable sugars.

Carbohydrate profiles during fermentation all followed a similar pattern. In each carbohydrate component, an initial increase in concentration took place, after this a decrease was observed. Glucose gave the most dramatic decrease, with most of the glucose used within the first 12 hr. This component was followed by maltose, which was used preferentially to maltotriose, but both attained their minimum levels at approximately 26 hr. The non-fermentable carbohydrate fractions, including starch, were also observed to decrease during the period of fermentation. However, the hydrolysis of these components can be attributed to the action of amylases and carbohydrases that survive through-out the fermentation period.

4.7 Summary and Suggestions for Further Work.

Detailed studies of the enzymology from the Scotch Malt Whisky process have not been reported to our knowledge. The work presented here has concentrated on the carbohydrate hydrolytic enzymes of malt, although the modified and developed assays for these enzymes were suitable for the qualitative determination of yeast and bacterial enzyme systems. More detailed work would have to be undertaken,

especially with regard to the fermentation process to obtain more quantitative data on enzyme activities from the other enzyme systems identified.

As, and when, new substrates are developed for carbohydrate hydrolyzing enzymes they should be used to elucidate other enzyme activities, or to enhance the information already gathered. This is especially so for the complex mixture found in malted barley and the fermentation process. Other enzymes associated with the Scotch Malt Whisky process should be identified and their activities and fate followed throughout the process, *e.g.*, limit dextrinase and those enzymes associated with β-glucan and arabinoxylan hydrolysis.

The study of micromalted and production malted barley varieties, Section 3.3, was carried out from a fixed weight of malt (5.0 g). If comparisons between varieties are to be made, it may be best to compare a fixed number of grains, *i.e.*, 100. Enzyme activity could then be expressed per grain and thus give a more accurate insight into the enzymology of the varieties. Similarly, the same variety from different geographical areas, or from different harvests could be studied in this way.

The assays developed, or modified, in this study may be applicable during the malting processes, to follow enzyme development during steeping, germination and kilning. Enzyme activities were only determined on kilned malted barley. However, the use of relatively unsophisticated assays may aid the development of barley varieties, since present day varieties are superseded much more quickly.

Statistical analysis of production malted barley suggests that continual study of enzyme levels within the malted barleys could lead to a better understanding of how the malt may perform within the process. Each season's malted barley can vary. The Distiller should

be able to judge the quality of the malt using as many variables as possible, this should include basic enzymology. Enzyme levels, when used in conjunction with basic malt parameters, may also aid the development of new barley varieties for the Malting industry. However, the balance between the amount of enzymes and their substrates must be met. Distillers' malted barley with high enzyme levels, but with lower starch content have little use in practice.

The use of statistical analysis may also be useful in determining the performance of malting processes. However, this would entail a much larger study than carried out here. Similarly, different varieties may be more suited to some malting processes than others.

The synthetic substrates used in the assays for amylases and minor carbohydrases, could be further developed for use as specific stains for protein electrophoretic separations. Such techniques would establish if the enzymes under study contained a specific activity associated with one protein molecule, or if an enzyme was associated with more than one substrate.

The HPLC technique for carbohydrate separation has been developed for use within the Research and Technical Services Department of Chivas Bros. Ltd. It is used for the determination of residual carbohydrate concentrations in fully fermented washes. In addition to the profiles being used as an indicator of carbohydrate utilization by the various yeasts available, it has also become important in process trouble-shooting (Sim 1990).

Although the production of Scotch Malt Whisky uses the same basic raw materials as the Brewing industry, *i.e.*, malt, yeast and water, intrinsic differences are found between the two processes. No additions, either chemical or enzymic, are tolerated in the production of the raw materials used in the Scotch Malt Whisky process. Some

are allowed in brewing, namely in water treatment, barley growth promoters and inhibitors, adjuncts and possibly enzyme cocktails. Fundamental areas in processing are different between the two processes. In distilling there is no wort boiling stage, mashing equipment and design may be different, the mashing programmes are invariably different, sanitation is much more strictly adhered to in the Brewing process with no bacterial contamination allowed. Bacteria may enhance the product from a distillery fermentation. Malted barley enzymes are active throughout the Scotch Malt Whisky process, from mashing to fermentation, allowing secondary conversion of substrates.

Much of the published work on malted barley enzymology focuses on brewing practice and purified enzymes. With so many different basic differences between distilling and brewing the published material in this field is not wholly applicable to the distilling industry. The aims of this study were to determine suitable assays for the enzymes to be studied, ascertain their suitability within the heterogeneous mix of a malted barley extract and establish the enzyme activity within the production processes of the Scotch Malt Whisky industry. These aims have been realized and indicate that many of the results and observations are novel.

APPENDICES.

Appendix I. HPLC Injection Programme.

This programme was written for the Gilson 231/401 auto-injector and controls all aspects of the automated HPLC system.

```
 1  RACK CODE 9
 2  INPUT A1/1
 3  IF A1>120
 4  GO TO 2
 5  INPUT A2/23
 6  INPUT A3/5
 7  A4=3
 8  A5=A3+A4
 9  RINSE
10  DISP 0/A3/9
11  TUBE 0/0
12  DISP 0/A3/4
13  B1=0
14  FOR A=1/20
15  FOR B=1/6
16  B1=B1+1
17  IF B1>A1
18  HOME
19  PRINT B1/1
20  HEIGHT
21  ASPIR 0/A4/0
22  TUBE A/B
23  FOR C=1/2
24  ASPIR 0/A2/4
25  DISP 0/A2/4
```

```
26 NEXT C
27 ASPIR 0/A2/1
28 TUBE 0/0
29 DISP 0/A2/1
30 INJECT 1
31 WAIT
32 AUXIL 7/1
33 WAIT 2
34 AUXIL 7/0
35 DISP 0/A5/9
36 RINSE
37 DISP 0/A3/9
38 HEIGHT
39 WAIT 2900
40 INJECT 0
41 NEXT B
42 NEXT A
```

Lines 1 - 8 set up the sample numbers, volumes and parameters. Lines 9 - 12 ensure that the injection port is rinsed before sample injection. Lines 13 - 31 are involved with sample mixing in the vial, and the loading and injection of the sample. The remainder of the programme ensures the data acquisition is triggered (lines 32 - 34), injection port rinsing takes place (lines 35 - 38), the run time is correct (line 39) and finally the next sample is initiated correctly (lines 40 - 42).

Appendix II.

Results of the time course analysis through mashing, wort draining and fermentation.

Table 18. Results of time course analysis of mashing variables studied throughout a typical distillery mashing cycle.

		Temperature (°C)	pH	FAN (mg/l)	Protein (g/l)	Phosphate (mM)
Mashing-in	0 min	65.0	5.54	333.6	1.415	7.93
	22 min	64.5	5.48	276.1	0.832	6.03
After Raking	45 min	63.0	5.39	312.2	0.997	7.10
	65 min	62.0	5.37	323.8	1.051	7.48
	85 min	62.0	5.36	321.6	1.030	7.70
	108 min	61.5	5.32	312.2	0.990	7.73
	125 min	61.0	5.32	324.7	0.967	7.63
	145 min	60.5	5.28	332.3	0.950	7.30
Second Water						
After Raking	195 min	64.0	5.07	168.6	0.282	2.53
	220 min	62.0	4.81	160.6	0.218	2.05
	240 min	61.5	4.70	155.5	0.197	2.03
	260 min	59.0	4.66	148.7	0.166	2.28
Sparge Waters						
After Raking	287 min	73.0	4.63	53.0	0.077	1.23
	307 min	73.0	4.43	33.3	0.058	0.75
After Raking	353 min	81.0	4.71	10.1	0.022	0.18

Table 19. Time course analysis of enzyme activities studied throughout a typical distillery mashing cycle under mashing conditions.

		α-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylglucos-aminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
Mashing-in	0 min	4595	1.360	14.393	28.546	28.066	33.265	42.522	27.673	11.973	5.045	29.096
	22 min	4761	1.422	2.086	18.750	3.531	24.254	9.613	12.850	10.278	2.261	15.058
After Raking	45 min	3787	1.179	1.118	17.781	1.905	22.349	4.076	8.736	9.099	2.171	11.247
	65 min	3337	0.936	1.028	16.600	1.481	17.508	1.716	7.223	9.674	2.171	6.134
	85 min	2860	0.545	0.604	13.606	1.330	14.938	1.342	4.833	10.007	2.352	4.863
	108 min	2489	0.604	0.755	14.393	1.573	14.090	0.866	5.681	11.579	2.291	5.075
	125 min	2219	0.332	0.816	14.302	1.028	13.243	0.893	5.438	11.854	2.654	4.863
	145 min	2149	0.302	0.665	13.727	0.736	12.607	0.961	5.136	12.185	2.897	4.561
Second Water												
After Raking	195 min	414	0.332	1.118	4.229	0.489	3.684	1.353	1.928	26.039	0.900	2.480
	220 min	184	0.241	0.453	4.150	0.422	2.503	0.264	0.749	25.254	0.900	1.997
	240 min	12	0.271	0.422	3.350	0.422	2.291	0.120	0.688	23.045	0.870	1.844
	260 min	12	0.210	0.241	3.562	0.392	2.140	0.054	0.325	21.411	0.859	1.634
Sparge Waters												
After Raking	287 min	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	307 min	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
After Raking	353 min	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 20. Optimal enzyme activities from the time course analysis of amylases, minor carbohydrases and acid phosphatase a typical distillery mashing cycle.

		α-Amylase	β-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylgluc-osaminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
Mashing-in	0 min	17296	42824	1.785	14.57	38.23	35.51	32.48	111.90	71.32	135.8	8.281	378.0
	22 min	12700	3503	1.360	2.99	29.40	8.25	23.89	27.01	14.06	120.0	3.048	183.9
After Raking	45 min	15444	3817	1.785	2.44	28.19	4.74	22.98	5.65	10.22	118.2	2.202	134.6
	65 min	12300	2255	1.481	1.99	25.37	3.32	18.69	2.84	8.10	112.8	3.531	130.3
	85 min	11795	979	1.481	2.08	23.38	2.41	15.12	2.69	5.72	112.2	3.291	109.4
	108 min	11812	976	1.330	1.81	26.37	2.53	14.62	2.63	6.92	117.9	3.986	103.1
	125 min	9867	891	1.028	1.75	27.28	2.11	13.58	1.27	5.44	125.2	3.168	88.0
	145 min	9103	823	0.997	1.49	26.28	1.63	12.34	1.18	4.98	125.2	3.017	76.2
Second Water													
After Raking	195 min	2039	18	0.967	1.23	7.80	1.37	3.65	0.88	1.41	33.0	2.232	67.8
	220 min	808	4	0.755	0.84	7.04	1.17	2.44	0.55	1.26	27.6	1.082	47.7
	240 min	329	5	0.604	0.66	6.77	0.36	1.26	0.55	1.20	24.0	0.900	46.2
	260 min	168	2	0.241	0.26	4.14	0.24	1.08	0.15	0.51	21.0	0.876	41.1
Sparge Waters													
After Raking	287 min	0	0	0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.000	0.0
	307 min	0	0	0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.000	0.0
After Raking	353 min	0	0	0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.000	0.0

Table 21. Optimal enzyme activities from the time course analysis for phytase, general proteinase and selected proteases, during a typical distillery mashing cycle.

		Phytase	General Proteinase Type	Serine Protease Type	Leucine Aminopeptidase Type	Carboxy-peptidase A Type
Mashing-in	0 min	137.553	59.845	24.905	20.998	22.018
	22 min	81.881	57.178	23.532	20.331	21.006
After Raking	45 min	24.525	59.179	21.179	23.998	21.000
	65 min	32.962	58.678	15.492	22.998	20.266
	85 min	49.829	52.677	13.923	21.998	19.002
	108 min	31.272	52.677	7.844	20.665	21.000
	125 min	21.151	51.344	10.982	21.331	21.000
	145 min	14.398	47.176	13.923	23.998	22.518
Second Water						
After Raking	195 min	16.088	25.172	12.158	20.998	8.352
	220 min	16.088	22.505	8.236	19.998	0.000
	240 min	14.398	21.004	8.040	19.331	0.000
	260 min	14.398	19.004	7.844	18.331	0.000
Sparge Waters						
After Raking	287 min	0.000	0.000	0.000	0.000	0.000
	307 min	0.000	0.000	0.000	0.000	0.000
After Raking	353 min	0.000	0.000	0.000	0.000	0.000

Table 22. Time course analysis of wort draining process variables studied throughout a typical distillery wort draining cycle.

	Gravity	Temperature (°C)	pH	FAN (mg/l)	Protein (g/l)	Phosphate (mM)
1st Water 0 min	68.0	20.0	5.14	394.2	1.057	11.78
10 min	69.5	20.0	5.25	437.9	1.042	12.41
20 min	69.0	20.0	5.29	420.9	1.048	12.68
30 min	68.0	20.0	5.30	387.4	1.027	12.08
40 min	65.5	20.0	5.30	364.6	1.007	10.72
50 min	62.5	20.0	5.32	328.6	0.991	10.05
60 min	60.0	20.0	5.32	305.3	0.963	9.49
70 min	58.0	20.0	5.32	315.5	0.970	9.42
80 min	56.0	20.0	5.31	296.6	0.950	9.35
90 min	54.0	20.0	5.31	293.0	0.983	10.05
1st Water in WB.	67.0	20.0	5.11	342.8	0.995	9.95
2nd Water 0 min	52.0	20.0	5.25	296.3	0.243	9.25
10 min	28.0	20.0	5.13	158.1	0.185	5.23
20 min	25.0	20.0	5.13	145.9	0.179	4.74
30 min	24.0	20.0	5.08	131.3	0.167	3.71
40 min	21.0	20.0	5.02	130.4	0.165	3.21
50 min	22.0	20.0	4.92	134.8	0.167	2.64
2nd Water in WB.	52.0	20.0	5.02	274.1	0.959	7.86

Table 23. Time course analysis of enzyme activities studied throughout a typical distillery wort draining cycle under wort draining conditions.

	α-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylglucos-aminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
1st Water 0 min	1075	0.990	0.656	20.200	1.747	4.682	2.746	8.069	8.644	4.076	40.738
10 min	1101	0.568	0.627	18.079	1.475	3.411	0.658	6.376	7.041	3.593	32.363
20 min	1008	0.476	0.539	12.939	0.485	3.321	0.556	4.833	6.951	3.593	31.878
30 min	940	0.476	0.539	13.533	0.476	1.565	0.536	2.866	5.165	3.426	24.195
40 min	793	0.507	0.390	10.819	0.386	1.565	0.527	2.020	4.833	3.442	18.870
50 min	628	0.174	0.113	6.883	0.323	1.324	0.518	2.143	3.925	3.472	12.578
60 min	348	0.027	0.045	7.189	0.205	1.353	0.444	1.839	4.257	3.291	10.037
70 min	303	0.075	0.000	7.345	0.113	1.777	0.417	1.294	4.894	2.958	8.373
80 min	265	0.235	0.009	8.087	0.113	1.082	0.264	1.082	4.439	3.442	7.162
90 min	236	0.293	0.205	9.309	0.041	1.202	0.449	0.919	5.136	2.897	6.890
1st Water in WB	652	0.386	0.586	9.039	0.568	1.898	0.544	3.684	8.281	3.894	25.737
2nd Water 0 min	177	0.144	0.417	6.272	0.054	0.537	0.113	0.144	4.198	0.545	5.106
10 min	63	0.018	0.205	1.457	0.054	0.325	0.054	0.295	2.414	0.536	1.172
20 min	38	0.027	0.045	0.827	0.018	0.032	0.018	0.032	2.140	0.514	0.809
30 min	31	0.027	0.027	0.413	0.018	0.018	0.009	0.023	1.869	0.509	0.749
40 min	23	0.018	0.000	0.090	0.027	0.018	0.005	0.000	1.684	0.523	0.537
50 min	31	0.027	0.000	0.413	0.000	0.023	0.000	0.018	1.989	0.523	0.476
2nd Water in WB	369	0.235	0.235	6.272	0.476	1.414	0.313	2.805	6.678	0.917	19.264

Table 24. Optimal enzyme activities from the time course analysis of amylases, minor carbohydrases and acid phosphatase during a typical distillery wort draining cycle.

	α-Amylase	β-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylgluc-osaminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
1st Water 0 min	15840	2705	1.446	0.688	24.80	1.959	18.03	10.096	37.95	127.0	5.295	159.4
10 min	16170	2532	0.809	0.749	24.68	1.475	15.60	6.466	35.69	156.0	5.018	158.4
20 min	13780	2166	0.695	0.961	22.77	1.438	13.70	3.562	30.43	140.3	5.018	121.8
30 min	12920	1493	0.507	0.598	21.14	1.414	11.94	1.657	22.71	126.4	5.235	95.8
40 min	10590	715	0.537	0.539	17.84	0.485	10.28	1.202	15.27	105.8	5.174	64.1
50 min	8210	404	0.356	0.539	17.12	0.413	7.83	1.082	10.01	90.7	5.000	39.3
60 min	6915	293	0.144	0.386	15.97	0.325	7.07	0.556	7.28	85.5	5.000	34.1
70 min	6210	219	0.083	0.323	16.21	0.325	6.29	0.568	6.01	84.6	5.050	28.1
80 min	5370	176	0.325	0.270	15.00	0.235	6.10	0.537	5.68	73.4	5.083	22.0
90 min	4850	147	0.356	0.264	12.67	0.144	6.50	0.749	4.77	77.4	5.032	20.8
1st Water in WB	10950	945	0.719	0.839	17.87	0.839	10.19	2.291	17.48	95.8	4.598	80.7
2nd Water 0 min	3430	129	0.174	0.449	12.06	0.144	5.71	0.325	3.65	71.0	3.168	17.2
10 min	990	83	0.054	0.323	3.23	0.063	1.93	0.054	0.96	29.3	2.958	4.8
20 min	740	90	0.041	0.234	5.26	0.027	1.54	0.049	0.69	24.1	2.352	4.2
30 min	560	50	0.027	0.180	5.26	0.023	1.02	0.036	0.60	21.4	2.414	3.3
40 min	112	74	0.023	0.023	5.47	0.018	0.93	0.041	0.60	18.7	2.654	3.6
50 min	224	70	0.009	0.032	5.62	0.018	1.38	0.023	1.11	21.4	2.685	3.3
2nd Water in WB	7400	659	0.480	0.446	12.46	0.627	7.28	1.291	12.46	72.8	3.260	49.2

Table 25. Optimum activities from the time course analysis for phytase, general proteinase and selected protease activities during a typical distillery wort draining cycle.

	Phytase	General Proteinase Type	Serine Protease Type	Leucine Aminopeptidase Type	Carboxy-peptidase A Type
1st Water 0 min	71.706	86.0	27.45	19.332	26.75
10 min	29.579	94.7	29.42	19.332	25.08
20 min	26.534	99.0	31.18	19.998	25.17
30 min	23.515	90.7	37.26	19.998	24.67
40 min	17.246	85.5	39.22	20.664	24.67
50 min	20.979	81.2	39.22	20.664	23.50
60 min	21.912	86.7	35.30	21.332	10.54
70 min	22.377	82.8	33.34	19.998	7.17
80 min	13.981	80.7	30.49	21.332	6.98
90 min	9.357	77.3	37.50	21.332	8.71
1st Water in WB	17.177	86.0	37.26	21.998	11.96
2nd Water 0 min	15.950	68.3	2.16	21.664	6.38
10 min	15.848	53.0	2.94	21.664	5.29
20 min	14.448	49.8	2.13	19.998	2.75
30 min	12.256	50.0	3.53	19.998	2.83
40 min	9.659	47.8	3.33	21.332	2.67
50 min	5.848	42.7	2.94	21.332	1.00
2nd Water in WB	11.237	71.2	35.30	22.664	6.75

Table 26. Time course analysis of fermentation process variables studied throughout a typical distillery fermentation.

Fermentation Time (hr)	Specific Gravity	Temperature (°C)	pH	Viability (%)	Mass (g/l)	FAN (mg/l)	Protein (g/l)	Phosphate (mM)
0	1052	21.0	4.92	99.0	9.75	246.5	0.929	7.54
2	1051	21.0	4.81	99.0	11.50	247.0	0.972	7.05
4	1050	21.0	4.62	99.0	14.50	221.5	0.939	6.73
6	1048	21.0	4.60	99.0	18.25	227.8	0.777	6.34
8	1046	22.5	4.54	99.0	21.25	198.4	0.788	5.75
10	1042	23.0	4.42	99.0	24.75	175.1	0.875	5.23
12	1036	24.0	4.26	99.0	31.00	140.2	0.826	4.62
14	1027	26.0	4.15	98.0	34.75	104.1	0.907	4.52
16	1024	27.5	4.13	98.0	38.00	96.6	0.907	4.58
18	1018	28.0	4.08	98.0	40.00	90.2	1.035	4.52
20	1013	29.0	4.02	98.0	40.75	85.0	1.126	4.42
22	1010	30.0	4.04	97.0	41.75	84.4	1.094	4.65
24	1009	30.0	4.05	95.0	43.25	79.2	1.057	4.62
26	1002	30.5	4.09	94.0	42.50	72.3	0.970	4.78
30	1000	31.0	4.11	92.0	43.00	72.2	0.729	5.20
34	999	31.5	4.16	80.0	42.75	83.8	0.789	5.53
38	998	31.5	4.19	71.0	42.25	87.8	0.779	5.49
42	998	31.5	4.33	50.0	37.00	104.7	0.755	6.01
46	998	32.0	4.27	30.0	32.25	112.7	0.744	6.05

Table 27. Time course analysis of selected fermentation products studied throughout a typical distillery fermentation.

Fermentation Time (hr)	Ethanol (g/l)	Glycerol (g/l)	Succinate (g/l)	Lactate (g/l)	Acetate (g/l)
0	1.13	0.179	0.032	0.266	0.030
2	1.87	0.240	0.029	0.302	0.035
4	3.07	0.315	0.053	0.303	0.042
6	5.08	0.394	0.066	0.307	0.037
8	7.77	0.477	0.071	0.304	0.025
10	11.48	0.637	0.104	0.302	0.013
12	19.22	0.884	0.127	0.310	0.010
14	24.15	1.105	0.147	0.323	0.007
16	30.65	1.265	0.155	0.318	0.007
18	34.07	1.362	0.167	0.332	0.007
20	42.00	1.618	0.158	0.336	0.009
22	43.46	1.713	0.159	0.340	0.018
24	44.94	1.744	0.136	0.370	0.022
26	50.57	1.797	0.132	0.378	0.022
30	50.88	1.824	0.130	0.337	0.024
34	54.24	1.817	0.152	0.426	0.054
38	57.92	1.837	0.139	0.398	0.048
42	56.58	1.868	0.154	0.442	0.108
46	57.38	1.883	0.172	0.484	0.165

Table 28. Time course analysis of enzyme activities studied throughout a typical distillery fermentation under fermentation conditions.

Fermentation Time (hr)	α-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylgluc-osaminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
0	344	0.133	0.288	0.343	0.288	2.606	0.343	3.406	13.36	1.005	28.02
2	331	0.547	0.935	1.700	0.622	3.442	0.726	4.207	15.80	1.109	29.20
4	352	0.586	0.516	1.586	0.552	2.640	0.447	3.337	12.60	0.516	27.26
6	279	0.794	0.794	0.794	0.726	2.466	0.622	3.788	15.11	0.726	24.99
8	282	0.492	0.343	0.726	0.099	2.049	0.288	3.127	16.92	0.203	26.31
10	115	0.388	0.794	1.109	0.412	1.734	0.374	3.023	17.09	0.690	24.02
12	81	0.656	2.049	0.969	0.516	1.457	0.377	3.406	17.30	0.622	21.34
14	13	0.447	3.163	1.945	1.073	1.247	0.900	4.241	20.88	1.396	22.14
16	14	0.748	3.422	2.016	1.377	1.288	1.029	3.406	16.88	1.577	21.06
18	7	1.247	3.233	2.397	1.630	1.387	1.257	4.660	14.10	1.804	17.12
20	7	0.847	1.874	1.909	1.247	1.700	1.457	4.164	13.85	1.351	14.58
22	6	0.482	0.760	1.492	1.247	0.900	1.005	4.660	15.51	1.143	13.92
24	6	0.413	1.770	2.327	1.213	1.005	0.992	4.933	20.60	1.109	13.19
26	5	0.447	0.794	2.013	0.690	0.690	0.516	5.564	23.56	1.143	9.92
30	11	0.377	0.726	1.830	0.547	0.516	0.343	5.217	26.38	0.734	8.73
34	14	0.208	0.203	1.213	0.447	0.465	0.133	4.730	28.79	0.412	6.02
38	13	0.029	0.119	1.005	0.586	0.307	0.029	5.113	29.13	0.482	5.56
42	12	0.099	0.079	1.666	0.794	0.133	0.070	5.043	30.49	0.482	6.51
46	14	0.029	0.029	0.794	0.237	0.173	0.029	4.416	31.33	0.792	5.29

Table 29. Optimal enzyme activities for the time course analysis for amylases, minor carbohydrases and acid phosphatase during a typical distillery fermentation.

Fermentation Time (hr)	α-Amylase	β-Amylase	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase	N-Acetylgluc-osaminidase	β-Xylosidase	Arabinosidase	Acid Phosphatase
0	5500	459	0.482	0.679	6.993	1.092	7.897	1.065	9.847	93.60	2.119	113.4
2	4800	526	1.283	1.479	6.993	1.230	10.021	1.317	11.622	102.30	1.804	106.8
4	5350	524	1.109	1.757	7.584	1.082	8.913	1.804	11.971	98.47	1.492	111.4
6	4800	544	1.535	1.445	7.340	2.096	7.897	1.172	9.638	88.70	1.666	106.8
8	3600	449	0.674	3.202	8.037	1.474	6.993	1.073	9.742	81.16	1.804	96.1
10	3000	517	0.377	5.100	8.490	1.162	4.939	0.864	9.822	72.93	1.387	86.0
12	750	523	0.690	5.657	8.211	2.120	2.570	0.900	10.857	57.42	1.770	54.9
14	600	583	0.203	6.561	8.540	1.740	1.526	0.997	10.474	60.67	1.213	41.8
16	550	600	0.482	7.850	8.664	3.180	1.377	1.200	8.524	46.54	1.596	39.9
18	350	587	0.726	6.805	8.245	3.320	1.630	1.351	10.752	40.26	1.923	35.5
20	320	531	0.288	3.673	7.410	2.813	1.934	1.656	9.672	43.08	2.340	33.1
22	300	486	0.169	2.036	7.340	1.997	1.492	1.283	10.021	51.19	2.606	25.9
24	300	586	0.302	2.002	8.211	2.240	1.239	1.317	11.309	47.20	2.109	23.9
26	290	594	0.900	1.534	9.325	2.310	1.005	1.143	10.786	49.59	0.969	16.9
30	337	547	0.777	0.851	9.279	1.823	0.969	0.690	10.404	49.01	0.830	13.5
34	305	450	0.726	1.166	9.394	1.818	0.629	0.377	8.490	50.36	0.460	10.8
38	338	367	0.552	0.505	6.436	1.092	0.516	0.203	7.376	48.96	0.387	10.1
42	342	321	0.237	0.364	7.768	1.370	1.213	0.409	9.638	49.87	0.858	9.6
46	337	262	0.146	0.205	6.967	1.158	1.109	0.273	9.430	50.22	0.869	10.7

Table 30. Enzyme activities from the time course analysis of phytase, general proteinase and selected proteases during a typical distillery fermentation.

Fermentation Time (hr)	Phytase	General Proteinase Type	Serine Protease Type	Leucine Aminopeptidase Type	Carboxy-peptidase Type
0	9.115	63.5	3.5	22.0	12.0
2	9.583	66.5	6.1	21.7	15.7
4	9.848	69.8	14.3	23.3	10.8
6	8.183	66.3	16.3	19.3	9.3
8	8.183	60.7	14.5	21.3	7.3
10	10.048	67.0	12.2	25.0	5.0
12	9.583	70.6	7.1	25.0	6.3
14	11.448	71.7	6.1	25.7	8.3
16	15.179	73.5	5.5	26.0	8.7
18	10.510	75.8	4.7	26.3	9.7
20	8.650	75.2	7.1	24.7	11.2
22	9.169	72.5	10.3	24.3	12.3
24	10.048	71.3	9.2	23.3	8.7
26	10.450	61.8	7.3	22.0	6.8
30	11.448	58.3	9.1	20.3	13.0
34	10.717	60.8	10.2	20.0	17.8
38	10.158	63.2	10.2	21.3	17.3
42	9.483	68.5	8.6	22.3	16.2
46	9.115	69.3	6.9	22.7	15.8

Table 31. Analysis of maltosaccharides throughout a typical distillery mashing cycle.

		Starch	Malto-heptaose	Malto-hexaose	Malto-pentaose	Malto-tetraose	Malto-triose	Maltose	Glucose	Total Carbohydrate
Mashing-in	0 min	10.52	2.10	1.94	0.86	4.68	15.51	79.72	14.25	129.58
	22 min	8.15	2.00	1.97	0.98	2.92	20.93	80.59	14.51	132.06
After Raking	45 min	7.80	2.11	1.95	1.28	2.75	20.74	82.39	13.58	132.60
	65 min	7.28	2.06	1.95	1.44	2.75	20.68	82.32	13.42	131.88
	85 min	7.03	2.24	2.09	1.58	1.58	20.63	82.58	13.65	131.37
	108 min	6.84	1.64	1.94	1.86	1.83	21.97	85.31	14.33	135.71
	125 min	6.28	1.60	1.91	1.78	1.71	20.96	84.07	14.08	132.38
	145 min	6.23	1.53	1.94	1.86	1.81	20.94	83.21	14.03	131.54
2nd Water.										
After Raking	195 min	0.82	0.25	0.38	0.49	0.59	5.06	22.35	3.34	33.27
	220 min	1.08	0.27	0.41	0.67	0.84	5.88	25.57	3.79	38.51
	240 min	1.01	0.25	0.39	0.61	0.71	5.17	22.62	3.25	34.01
	260 min	0.95	0.26	0.39	0.61	0.79	5.23	22.87	3.18	34.27
Sparge Waters										
After Raking	287 min	0.81	0.15	0.29	0.37	0.62	2.58	10.89	1.27	16.98
	307 min	0.49	0.10	0.22	0.23	0.35	1.65	7.05	0.82	10.91
After Raking	353 min	0.30	0.07	0.10	0.13	0.13	0.56	2.40	0.29	3.91

Table 32. Analysis of maltosaccharides throughout a typical distillery wort draining cycle.

	Starch	Malto-heptaose	Malto-hexaose	Malto-pentaose	Malto-tetraose	Malto-triose	Maltose	Glucose	Total Carbohydrate
1st Water 0 min	10.36	4.22	3.74	2.17	3.88	24.83	104.13	22.28	175.62
10 min	11.22	4.23	3.88	2.64	2.33	28.71	108.98	24.61	186.61
20 min	11.03	3.89	3.76	2.84	2.46	29.80	109.83	24.78	188.34
30 min	9.59	3.16	3.13	2.47	2.16	26.42	102.04	20.97	169.93
40 min	8.56	2.66	2.70	2.18	2.01	24.68	95.75	18.46	157.00
50 min	7.78	2.27	2.36	1.96	1.88	22.98	89.73	16.46	145.52
60 min	7.70	2.06	2.21	1.92	3.58	22.52	86.72	15.43	142.13
70 min	7.04	1.90	2.07	1.85	3.80	22.13	83.66	14.48	136.94
80 min	6.75	1.76	1.97	1.78	3.92	22.14	80.79	13.17	132.28
90 min	6.47	1.71	1.90	1.70	3.68	21.03	78.48	13.03	128.00
1st Water in WB.	7.79	2.62	2.75	2.11	4.12	23.58	93.11	17.31	153.38
2nd Water 0 min	5.82	1.51	1.76	1.63	3.64	20.40	75.49	11.25	121.50
10 min	3.35	0.72	0.85	0.95	2.71	11.71	43.20	6.24	69.72
20 min	3.05	0.62	0.75	0.88	2.99	10.32	37.65	5.40	61.65
30 min	2.80	0.55	0.66	0.83	2.95	9.46	34.39	4.86	56.50
40 min	2.67	0.50	0.63	0.78	2.74	8.46	31.83	4.70	52.33
50 min	2.58	0.50	0.62	0.72	2.16	8.61	33.04	4.77	53.00
2nd Water in WB.	5.58	1.65	1.95	1.74	1.55	19.33	78.89	11.89	122.56

Table 33. Analysis of maltosaccharides throughout a typical distillery fermentation.

Fermentation Time (hr)	Starch	Malto-heptaose	Malto-hexaose	Malto-pentaose	Malto-tetraose	Malto-triose	Maltose	Glucose	Total Carbohydrate
0	6.26	1.46	1.76	1.73	2.24	19.07	74.91	12.55	119.98
2	8.12	2.38	2.72	2.71	2.02	21.71	94.68	18.04	152.38
4	6.26	1.42	1.78	1.78	1.99	19.19	76.68	8.79	117.89
6	5.56	1.36	1.86	1.93	1.99	11.22	78.93	9.37	112.22
8	5.56	1.30	1.76	1.86	1.94	18.23	74.11	3.64	108.38
10	5.21	1.18	1.66	1.87	1.89	17.48	66.83	2.43	98.54
12	4.93	1.06	1.54	1.89	1.77	18.18	56.31	1.57	87.24
14	4.13	0.94	1.44	1.95	1.67	13.92	45.46	1.49	70.99
16	4.74	1.02	1.56	2.09	1.77	17.75	37.03	1.54	67.50
18	4.16	0.90	1.34	1.98	1.68	14.85	24.52	1.50	50.94
20	3.88	0.85	1.19	1.98	1.67	12.10	15.71	1.48	38.85
22	3.24	0.77	1.05	1.80	1.67	6.22	10.76	2.60	28.11
24	2.97	0.70	0.84	1.62	1.63	7.28	10.19	1.27	26.51
26	2.78	0.68	1.82	1.42	1.55	3.71	4.17	1.03	16.15
30	2.76	0.70	0.90	1.22	1.58	2.27	2.01	1.10	12.53
34	2.71	0.74	0.91	1.18	1.47	2.08	1.51	1.03	11.62
38	2.77	0.71	0.97	1.24	1.53	2.08	1.12	0.89	11.31
42	2.53	0.62	0.87	1.10	1.35	1.85	0.77	0.66	9.75
46	2.57	0.61	0.87	1.11	1.36	1.64	0.76	0.64	9.56

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