THE β-1,3-GLUCANASE OF *BASIDIOMYCETE* QM 806

Studies on it's production and application in yeast cell wall hydrolysis

THESIS

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by

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1. INTRODUCTION

1.1. SUMMARY OF THE PROJECT

In this project the growth and production of β-1,3-glucanase by Basidiomycete sp QM 806 was investigated, with a view to studying its application in β-1,3-glucan degradation and in yeast extract production. The effect of various parameters on β-1,3-glucanase production was examined. The optimal conditions for enzyme production in submerged shake-flask culture were chosen.

Two β-1,3-glucans (laminarin and yeast cell walls) were degraded using the β-1,3-glucanase produced. The degradation products were identified using a high performance liquid chromatographic method developed during this project. The β-1,3-glucanase produced by Basidiomycete sp QM 806 was compared with other commercially available enzyme preparations, namely Kitalase and Novozym-234 which were also used in β-1,3-glucan degradation experiments.

Yeast extract production experiments were conducted using the Basidiomycete sp QM 806 culture supernatant, Kitalase and Novozym-234. These experiments involved treating whole baker's yeast cells (obtained commercially) with the various enzyme preparations and calculating by dry weight the percentage yeast extract produced. A comparison was drawn between the enzyme preparations.

1.2 INTRODUCTION TO BASIDIOMYCETES

Fungi are a diverse group of organisms. They are heterotrophs and may live as saphrophytes or parasites or less frequently as symbionts in commensal association with other organisms. (Smith, 1975; Alexopoulos and Mims, 1979).

They are eukaryotic, having a discrete membrane-bound nucleus and a range of membrane-bound organelles, and their ribosomes are of the 80S type (Deacon, 1984).

Fungi display a wide variety of morphological form ranging from the minute unicellular yeasts to the multicellular macroscopic mushrooms (Smith, 1975).

Basidiomycetes, the most evolved class of fungi are characterised by the formation of basidiospores on the outside of spore-bearing structures known as basidia. Such organisms as rusts, smuts and jelly fungi, as well as mushrooms, puffballs, shelf and coral fungi, stinkhorns, earthstars and bird's nest fungi are Basidiomycetes. They are an important group of fungi including harmful species as well as useful ones. The rusts and smuts cause plant diseases such as stinking smut and black stem rust of wheat. Among the economically important products of the Basidiomycetes are such enzymes as cellulases and ligninases, such toxins as the amanitins, phalloidin, ibotenic acid, and atropine, and hallucinogens such as psilocybine (Lechevalier, 1978). Mushrooms are also economically important Basidiomycetes, particularly members of the genus Agaricus especially Agaricus bisporus which is cultivated extensively. (Hayes and Nair, 1975; Alexopoulos and Mims, 1979).

Basidiomycetes are usually uninucleate and haploid and are usually borne on sterigmata. The mycelium is uninucleate to begin with, then becomes binucleate through plasmogamy, with karyogamy and meiosis occurring only in the basidium. Clamp connections characteristic of the mycelium of many Basidiomycetes play a role in the production of the binucleate condition. The vast majority of basidiomycetes are heterothallic. The sexual compatibility of such organisms depends not on differentiation of distinct sexual

forms, but on "incompatibility factors". These factors regulate both the recognition of a potential mate by a given strain as well as subsequent morphogenesis. (Koltin et al., 1972). The fruiting bodies (basidiocarps), except for those of most rusts and smuts, are quite complex; they may be open or closed, varying in shape and size, from exceedingly tiny to approximately 100mm. (Smith, 1966). The cell walls of the Basidiomycetes that have been examined are of the chitin-glucan type. L-fucose is also found in the wall. (Bartnicki-Garcia, 1968).

Basidiomycetes may be classified as Heterobasidiomycetidae or Homobasidiomycetidae depending on whether the basidia are septate or non-septate.

1.3. MICROBIAL GLUCANASES

β-1,3-Glucanases are ubiquitous enzymes having been found in fungi, bacteria, higher plants, algae and lower forms of sea animals (Reese and Mandels, 1959; Totani et al, 1983).

They are enzymes hydrolysing the β-1,3-linked polymers of glucose (glucans). β-Glucans occur in micro-organisms and higher plants as structural components of cell walls, as reserve materials and as extracellular constituents of uncertain significance. (Reese and Mandels, 1959; Chesters and Bull, 1963).

β-1,3-Glucanases are of significance in the hydrolysis of cell walls of yeast and other fungi. Purification of such enzymes is of interest because of their use in structural analysis of polysaccharides. (Jeffries et al., 1977; Totani et al., 1983)

Ꞵ-1,3-Glucanases are of two types:

a) the endo or random spitting type. Hydrolysis of Ꞵ-1,3-glucans yields laminaribiose (3-O-Ꞵ-D-glucopyranosyl -D-glucopyranose) and higher oligosaccharides. It is found in wheat, barley, rye, marine algae and in Rhizopus arrhizus.

b) the exo or endwise spitting type. Hydrolysis of Ꞵ-1,3-glucans produces glucose as the major and initial product. It is found in almond elmusini and in fungi (Basidiomycete sp QM 806 and Sporotrichum pruinosum) (Manners, 1955; Duncan et al., 1956; Reese and Mandels, 1959; Chesters and Bull, 1963).

1.3.1. Fungal Ꞵ-1,3-glucanases

Ꞵ-1,3-Glucanases are present in most fungi. They are secreted into the medium where they function as digestive enzymes hydrolysing glucans produced by other organisms. Extracellular digestive enzymes (cellulase, chitinase, xylanase) are usually adaptive in fungi. The Ꞵ-1,3-glucanases are constitutive. They may also function in another capacity, one common to all fungi, the intracellular hydrolysis (and synthesis) of a reserve material containing Ꞵ-1,3-glucosidic linkages. As such, these enzymes resemble amylase which is also constitutive in many fungi, which may function intra-and extra -cellularly, and which appears later in the growth cycle than cellulase and other adaptive enzymes.

Although nearly all fungi produce Ꞵ-1,3-glucanase, there are great differences in the amounts produced by the various organisms. Myrothecium verrucaria and Aspergillus niger, organisms which have been used as sources of B-1,3-glucanase

in the works of others. (Aitkin et al., 1956; Stone, 1957). Reese and Mandels (1959) reported that Basidiomycete sp QM 806, Sporotrichum pruinosum and Rhizopus arrhizus were good producers of β-1,3-glucanase with yields of 10-100 times higher than those previously known from other sources. The Basidiomycete enzyme was the best producer of β-1,3-glucanase.

1.3.2. β-1,3-Glucanase from Basidiomycete sp QM 806

Reese and Mandels (1959) in a survey of the occurrence of β-1,3-glucanases in fungi, observed that an unidentified species of Basidiomycete produced a very high level of β-1,3-Glucanase when grown on a starch medium. The β-1,3-Glucanase levels increased rapidly once the starch was consumed. The organism was designated Basidiomycete sp QM 806.

The enzyme has been purified by ammonium sulphate fractionation, D.E.A.E.-cellulose chromatography, and preparative acrylamide gel electrophoresis, yielding a preparation homogeneous as judged by disc electrophoresis on acrylamide gel, its sedimentation characteristics in the ultracentrifuge and the absence of contaminating enzymes. Two isoenzymes of the enzyme have been separated. (Huotari et al., 1968).

The β-1,3-Glucanase removes single glucose residues from a β-1,3-linked glucan chain commencing at the non-reducing terminal. It can bypass β-1,6-linkages yielding gentiobiose (6-O-β-D-glucopyranosyl-D-glucopyranose) quantitatively from this structural feature, and it can cleave β-1,3-linkages adjacent to other types of linkage in the main chain. The enzyme, although typically exo in its mode of attack, can initiate an endo cleavage in that it attacks endo-β-1,3-

bonds adjacent to β-1,6-linkages. The enzyme does not catalyze transglycosylation reactions (Nelson et al., 1969).

The enzyme does not hydrolyse laminarin (a β-1,3-linked glucan with some β-1,6 linkages) which has been modified at both non-reducing and reducing ends by periodate oxidation followed by borohydride reduction. (Nelson et al., 1963). However, following mild acid hydrolysis which removes the modified residues at the non-reducing ends of the laminarin molecules, the glucan is hydrolysed by the enzyme. These findings indicate that the enzyme is a typical exo-enzyme with glucose released in the α-configuration (Parrish and Reese, 1967).

Friebe and Holldorf (1975) studied the control of β-1,3-Glucanase activity from Basidiomycete sp QM 806. They found that the enzyme once synthesised was secreted immediately into the culture medium. The intracellular level of enzyme activity was found to be low compared to the extracellular activity, at all stages of growth. The onset of enzyme synthesis is determined by the concentration of carbon source in the medium. Addition of glucose or other carbon sources to a culture after consumption of the initial carbon source led to an inactivation of the extracellular β-1,3-glucanase by an inactivating system, which was separated from the cells.

Basidiomycete aphyllophoroles has been reported to produce both endo and exo-β-1,3-glucanases. (Totani et al., 1983).

1.4. PRODUCTION OF INDUSTRIAL ENZYMES BY FUNGI

Microbial enzymes have found many applications in industry (Beckhorn et al., 1965; Blain, 1975; Godfrey and Reichelt, 1983). Most of the enzymes used on an industrial scale are

extracellular enzymes. Thus, the fermentation broth from the cultivation of certain micro-organisms e.g. bacteria, yeasts and filamentous fungi becomes a major source of proteases, amylases and to a lesser extent cellulases and lipases. Most industrial enzymes are hydrolases and are capable of acting without complex co-factors and are readily separated from micro-organisms without rupturing the cell walls and are water soluble. (Smith, 1981; Blain, 1975).

Some intracellular enzymes are now being produced industrially such as penicillin acylase, glucose oxidase, and asparaginase. (Smith 1981).

Industrial scale culture of micro-organisms is generally referred to as fermentation. Fermentations can be divided into two categories depending on the growth vessel used.

In earlier traditional methods micro-organisms were grown as surface cultures on solid or semi-solid media in trays. Although the process is still widely used in Japan for fungal enzymes, and may often yield higher initial enzyme concentrations, it is avoided in Western Countries because it occupies too much space, contamination is difficult to avoid and product yield is often low. (Priest, 1984; Blain 1975).

Surface culture techniques are used in the production of amyloglucosidase from Rhizopus species, amylase from Aspergillus oryzae and proteases from Aspergillus niger. (Arima, 1964; Underkofler, 1969; Fogarty, 1983).

Submerged culture methods dominate the industry today because modern methods of process control can be easily adapted to the plant. The method permits greater control of factors such as temperature and hydrogen ion concentration, yields are generally higher and risk of contamination low. (Priest, 1984; Blain 1975).

1.5. FERMENTER CULTIVATION OF FUNGI

Fungal enzymes have been produced increasingly by submerged fermentation cultivation methods. (Blain, 1975; Gaden, 1981), which permit greater control of environmental factors than do surface culture methods.

The design of a fermenter must take into account the following technical considerations: biological kinetics, piping and equipment design to maintain sterility, fluid hydraulics, mass transfer of substrate materials into the micro-organism, mass transfer of atmospheric oxygen through the bulk liquid and into the micro-organism, mass transfer of product material out of the micro-organism into the bulk liquid, heat transfer for removal of metabolic heat and control philosophy. The equipment usually consists of a baffled sterilisable tank, fitted with rotary impellers for agitation and piping for forced aeration. (Gaden, 1981; Rhodes and Fletcher, 1966; Solomons, 1969). Some fermenter designs have no impellers, but use aeration alone to provide mixing and oxygenation of broth. (Barker and Worgan, 1981; Greenshields and Smith, 1971).

Fungi present a number of problems when grown in submerged culture in fermenters, mainly due to the form or morphology of fungal growth. This morphology may be pelleted or filamentous. (Metz and Kossen, 1977; Whitaker and Long, 1973). The type obtained depends on the fungal strain, inoculum, medium and conditions of growth used.

Problems arising from the use of fungi include provision of a suitable inoculum, prevention of fouling of baffles and tubing with growth, and the dispersion of growth in the medium. (Solomons, 1975; Rowley and Bull, 1973).

1.6. STRUCTURE OF YEAST CELL WALLS

Most of the information on yeast cell wall studies are with cell walls from baker's yeast, Saccharomyces cerevisiae and closely related species.

The principal components of Saccharomyces cerevisiae cell walls are glucan and mannan type polysaccharides. A low concentration of chitin (1%) may be present depending on the number of times a cell has produced buds. (Phaff, 1977).

The glucose polymer β-glucan is the most abundant polysaccharide occurring in the cell walls of yeast and comprises approximately 12-14% of the dry cell weight. Glucans provide the structural rigidity of yeast cell walls, hence maintaining the specific morphology and integrity of the cells. (Phaff, 1963; Phaff, 1977; Jamas et al., 1986). The cell wall formed by glucans is insoluble and encapsulates the cell contents. Yeast cell wall glucan is a homopolymer of glucose linked through either β-1,3 or β-1,6-D glucosidic bonds. (Manners et al., 1973 a; Manners et al., 1973 b). Glucans are grouped into fractions on the basis of their solubility in alkali. (Phaff, 1971; Sentandreu et al., 1975).

The alkali-soluble glucan was first described by Eddy and Woodhouse (1968) and subsequently by Fleet and Manners (1976). The alkali-soluble fractions are a minor component (15-20% W/W of total glucan) that is of little structural importance to the cell wall. (Jamas et al., 1986). Structural analyses have revealed the presence of 80-85% β-1,3-linkages and 3-4% β-1,6-linkages and 3-4% branched residues linked through C-1, C-3, and C-6. The molecular weight of the alkali-soluble glucan was estimated to be about 250,000. (Fleet and Manners, 1976). Fleet and Manners (1977) have found that the alkali-soluble glucan is

closely associated with some parts of the mannan component of the cell wall, through the β-1,6-linked glucose residues. This type of glucan may thus represent the bonding material which links the outer mannan layer to the innermost alkali-insoluble glucan layer (Phaff, 1977).

The major glucan component is insoluble in alkali. This fraction is responsible for the structure and integrity of cell walls. The alkali-insoluble fraction consists of a β-1,3-linked backbone of high molecular weight (240,000) containing 3% β-1,6-glucosidic interchain linkages which accounts for 85% of the alkali-insoluble glucan fraction. Manners and co-workers (1973 a) presented possible structures for alkali-insoluble β-glucan, a tree-type structure with multiple branching or a comb-type structure. However, there is no conclusive evidence to support these structures (Phaff, 1977). A water soluble β-1,6-glucan component accounts for the remaining 15% of the alkali-insoluble glucan fraction (Manners et al., 1973 b) This β-1,6-glucan contains about 19% β-1,3-glucosidic linkages which may serve both as inter-residue and interchain linkages. In alkali-insoluble glucan preparations from several other species of yeast, the proportion of the β-1,6-glucan component is significantly greater than in baker's yeast. (Manners et al., 1974; Phaff, 1977).

The structures described do not agree with those originally proposed by (i) Bell and Northcote (1950) who suggested that yeast glucan was a branched β-1,3-glucan with β-1,2-glucosidic interchain linkages, (ii) Peat et al (1958 b) who considered the glucan to be linear and to contain certain sequences of β-1,3 and β-1,6-glucosidic linages, (iii) Misaki et al. (1968) who postulated a branched structure based on a "backbone" of β-1,6-linked glucose residues, to which were attached linear chains containing about 8 β-1,3-linked glucose residues. These suggestions were

before the heterogeneity of baker's yeast glucan was known. Nevertheless, the various structures are one consequence of the difficulties involved in examining an insoluble polymeric carbohydrate whose composition cannot be readily determined and whose physical nature makes the standard techniques of carbohydrate chemistry less effective.

Yeast mannan constitutes one of the main soluble polysaccharides of the yeast wall. Phaff (1963) and Ballou (1974) have reviewed the development of our knowledge of the chemistry of yeast mannan. Mannan is usually extracted with dilute NaOH, followed by precipitation of the mannan with Fehling's solution. The precipitate is dissolved in acid and a mannan-protein complex is recovered by alcohol precipitation after dialysis of the copper salts. The mannan molecule consists of an outer chain (an α-1,6-linked backbone with oligomannoside side chains) and an inner core near the point of attachment to the protein. This linkage fragment (to the protein) of 12 mannose units consists of an α-1,6-linked backbone with di, tri, and tetrosaccharide side chains. The fragment is attached by the last mannose unit of the backbone through a β-1,4-bond to N-acetylchitobiose which in turn is linked to asparagine to the peptide bond (Phaff, 1977; Nakajima and Ballou, 1974).

In summary, the cell wall of baker's yeast represents appromiately 15% of the dry weight of the cell and it is made up of 20-40% mannan, 5-10% protein, 1% chitin and 30-60% glucan. The medium and growth conditions may influence the mannan/glucan ratio of the cell wall (Mc Murrough and Rose, 1967). The protein content of cell wall preparations is likely to be influenced by the extent of purification to which the walls were subjected. (Phaff, 1971). The glucan content is more likely to be in the 50-60% range than in the 30-35% range, often reported in earlier literature. These low values are most likely due to

the elimination of about 20-25% alkali-soluble glucan during cell wall fractionation. Figures by authors, who based glucan and mannan contents on the direct determination of glucose and mannose in wall hydrolysates, can be expected to be more reliable than data based on cell wall fractionation. (Mill, 1966).

In some species of yeast an additional structural wall component occurs, which has been identified as a linear α-1,3-glucan. (Phaff, 1977; Horisberger et al., 1972.

1.7. ENZYMATIC LYSIS OF YEAST CELL WALLS

Since three of the major wall components are potentially water soluble, the effective removal or weakening of the cell wall must involve enzymatic hydrolysis of the long chains of β-1,3-linked glucose residues in the micro fibrillar, alkali-insoluble glucan layer.

Yeast cell walls may be hydrolysed by the yeasts own endogenous β-glucanases or by β-glucanases from microbial and other sources. Autolysis by endogenous β-glucanases of Saccharomyces cerevisiae (Hien and Fleet, 1983 a; Hien and Fleet, 1983 b), Candida utilis (Notario et-al, 1976) and Schizosaccharomyces pombe (Reichelt and Fleet, 1981) has been studied. In Saccharomyces cerevisiae, two exo β-1,3-glucanases and four endo β-1,3-glucanases have been detected (del Rey at al., 1979; Sanchez et al., 1982; Hien and Fleet, 1983 a), which may act in vivo in wall structuring during vegetative growth and sporulation (del Rey et al., 1979; Hien and Fleet, 1983 b). These glucanases might also be responsible for the degradation of walls during in vitro autolysis. The critical enzymes required for yeast autolysis are endo-β-1,3-glucanases, together with specific or non-specific exo-β-glucanases which appear to be

located in the periplasmic space between the cytoplasmic membrane and the innermost glucan layer. (Phaff, 1977).

Other enzyme activities such as periplasmic chitinase (Elango et al., 1982) or mannanase (Maddox and Hough, 1971) may also play a role in yeast wall autolysis, as well as hypothetical envelope associated proteases (Sanz et al., 1985).

Research on the enzymatic digestion of yeast cell walls by exogenous enzymes has been stimulated for three main reasons: (a) the preparation of protoplasts or spheroplasts for physiological studies (b) studies on the biosynthesis and regeneration of cell walls from protoplasts and (c) analysis of native and isolated cell walls. (Phaff, 1971). Wall analysis using selected and specific enzymes avoids the often used harsh treatments of cells or cell wall material with strong acids and alkali. Such enzymes can cleave specific linkages or selectively remove certain groups from macromolecules with minimal modification.

Giaja in the early part of this century observed that the digestive juice of the snail Helix pomatia, which is found in a small vesicle of the alimentary canal, possesses the ability to digest yeast cells. Many subsequent workers have used such preparations, mainly to prepare protoplasts of yeast. It contains 30 or more investigated enzymes, including mannanase, glucanase, cellulase, chitinase, lipase and polygalacturonase, although it is very low in proteolytic activity (Holden and Tracey, 1950). Susceptibility of the yeast cell walls to selected enzymes was first tested by Eddy and Williamson (1957) who found that the complex snail enzyme caused complete dissolution of the cell wall. Anderson and Millbank (1966) used the digestive juice of Helix pomatia in the study of the

degradation of isolated cell-wall preparations from a strain of Saccharomyces carlsbergensis. Brown (1971) studied the susceptibility of yeast cell walls to the digestive juice of Helix pomatia and found that older (stationary-phase) cells were not susceptible, but that cells in exponential phase were susceptible.

Salton (1955) was one of the first to isolate a number of actinomycetes and myxobacteria which showed lytic activity towards cell walls of a yeast, Candida pulcherrima. Strains of Streptomyces showed lytic activity towards walls of gram-positive bacteria and the yeast, but two myxobacteria, Myxococcus fulvus and Cytophaga johnsonii were lytic only on C pulcherrima walls.

Bacon et al (1970 a, 1970 b) have investigated the use of β-glucanases produced by Cytophaga johnsonii and their role in the lysis of yeast cell walls. Chromatographic fractionation of lytic culture fluids showed the presence of two types of endo-β-1,3-glucanase and several β-1,6-glucanases. Extensive solubilization of yeast cell walls was obtained only with preparations of one of these glucanases, an endo-β-1,3-glucanase producing as end products mainly oligosaccharides have five or more residues. Lysis by the other endo-β-1,3-glucanase was incomplete. The β-1,6-glucanases produced a uniform thinning of the cell walls, and mannan-peptide was found in the solution.

Sugimoto (1967) studied the lysis of yeast cell walls with enzymes from Streptomyces species. He found that the Streptomycete β-1,3-glucanases were poorly lytic against heat-treated baker's yeast, but in combination with an alkaline protease from St satsumaensis nov. sp, a large increase in lytic activity was demonstrated. The intact untreated baker's yeast was dissolved very easily by action of β-1,3-glucanase or the protease alone.

Tanaka and Phaff (1965) have described a number of micro-organisms from soil, Bacillus circulans and Streptomyces species which produce β-1,3 and β-1,6-glucanases, mannanases and proteases. Although some alterations were caused by the action of the purified enzyme on the yeast cells, no protoplasts were formed. Nagasaki et al. (1966) obtained yeast protoplasts by means of the combined action of two agents isolated from B circulans. Later Rombouts and Phaff (1976) used the β-1,6-glucanase from Bacillus circulans WL-12 in studies on yeast cell wall lysis.

Monreal et al (1967) have used the purified β-1,3-glucanase from Micromonospora chalcea to attack various yeast cells harvested in the early logarithmic phase, which resulted in the liberation of protoplasts.

Oerskovia species have also been shown to have lytic activity against yeast cells. (Obata, 1977a; Obata, 1977 b; Scott and Schekman, 1980; Mann et al., 1972).

Arthrobacter sp have been shown to produce lytic B-glucanases. The glucanase attacked Saccharomyces cerevisiae glucan, Candida albicans glucan, Saccharomyces fragilis glucan, pachyman, curdlan (linear β-1,3-glucans with some β-1,6-linkages) and laminarin (a linear or branched β-1,3-glucan with some β-1,6-linkages). (Doi et al., 1973 a/b/c). Susceptibilities of Saccharomyces and Candida yeasts, to the yeast cell wall lytic enzyme from Arthrobacter luteus have been extensively studied. (Kaneko et al., 1973; Kitamura et al., 1972; Kitamura et al., 1974). Crude lytic enzymes (predominantly β-1,3-glucanases) from Oerskovia xanthineolytica, Basidiomycete sp QM 806 and Rhizopus arrhizus QM 1032 have been tested against the walls of log-phase yeast cells and germinated fungal spores (Jeffries et al., 1977). The enzyme preparation from

Oerskovia was more active than the other two enzymes for formation of spheroplasts and release of reducing sugars. The Basidiomycete enzyme was also able to attack these viable cells and form spheroplasts, but the rate of release of reducing sugars and the extent of spheroplast formation was lower than for the Oerskovia enzyme. The enzyme from R arrhizus did not attack viable cells extensively or release spheroplasts from the organisms tested.

The exo β-1,3-glucanase from Basidiomycete sp QM 806 was found to cause almost complete solubilization of the yeast cell wall (Reese and Mandels, 1959). The enzyme has also been used in the analysis of cell wall carbohydrates of Saccharomyces cerevisiae and Wickerhamia fluorescens (Bauer et al., 1972).

Other extensive work has been done with the Flavobacterium sp, Alternaria sp and Trichoderma sp β-glucanase enzymes. (Phaff, 1977).

There is considerable difference in the mechanism by which the enzymes, from the various micro-organisms described hydrolyse cell wall glucan. These differences are manifested, in part, by the products of the enzymatic hydrolysis. The degradation of yeast cell walls and laminarin and the products produced by enzymatic hydrolysis is discussed in the following section.

1.8. THE USE OF ENZYMES IN STRUCTURAL STUDIES ON POLYSACCHARIDES

In this section, the enzymes produced by the various micro-organisms mentioned in the previous section will be discussed on the basis of the products they produce. The following groups of enzymes will be discussed (a) enzymes

which cause random hydrolysis of β-1,3-glucans to disaccharides and glucose from polysaccharides; (b) enzymes which produce oligosaccharides of the laminarin series with a degree of polymerization of five or larger. These enzymes may have an exo or endo action pattern or can be debranching enzymes; (c) yeast glucan debranching enzymes; (d) enzymes which produce glucose and gentiobiose from laminarin or from yeast glucan by an exo mechanism; (e) enzymes with action patterns which have not been clearly defined.

1.8.1. Endo-enzymes producing disaccharides and glucose from polysaccharides

The most thoroughly studied enzymes of this group are produced by strains of Bacillus circulans. Horikoshi and Sakaguchi (1958) isolated a strain of this bacterium from soil, which showed lysis of Aspergillus oryzae and Saccharomyces sake cell walls. Later, Horikoshi et al (1963) separated a β-1,3-glucanase from the culture fluid of B circulans grown on Aspergillus oryzae mycelium. This enzyme, which hydrolysed laminarin to glucose and laminaribiose by a random mechanism, also hydrolysed cell walls of a Fusarium species and of Aspergillus oryzae.

Tanaka and Phaff (1965) isolated another strain from soil, which was designated B circulans WL-12. They separated the enzyme complex into an endo β-1,3-glucanase and an endo β-1,6-glucanase. Fleet and Phaff (1974 a) grew B circulans on baker's yeast cell walls and subjected the β-1,3-glucanase (tested on laminarin) and the β-1,6-glucanase (tested on pustulan - a linear β-1, 6-glucan) to more thorough purification. The two highly purified glucanases caused only a very limited hydrolysis of baker's yeast cell walls. This result was unexpected since β-1,3-bonds are the main linkages in yeast cell wall glucan. It seemed likely that some additional enzymes of

the crude culture fluid, which were lytic to yeast cell walls, had been removed during the more extensive purification of the two β-glucanases. Rombouts and Phaff (1976 a/b) tested this possibility. They isolated one lytic β-1,6-glucanase and two lytic β-1,3-glucanases, in addition to the non-lytic β-1,6-glucanase and β-1,3-glucanase. The lytic β-1,6-glucanase hydrolysed pustulan through a series of oligosaccharides, leading to a mixture of gentiotriose, gentiobiose and glucose. The enzyme also produced small amounts of gentiobiose from laminarin and pachyman and on this basis its lytic activity on yeast cell walls was attributed to a debranching of the alkaki-insoluble β-1,3-glucan in the wall. Low molecular weight products from yeast cell walls included gentiotriose, gentiobiose and glucose but β-1,3-linked oligosaccharides were not detected. The lytic β-1,6-glucanase differes from the non-lytic β-1,6-glucanase by it's positive action on yeast cell walls and yeast glucan and its much lower specific activity on soluble pustulan. (Rombouts and Phaff, 1976 a; Rombouts et al., 1978).

The lytic β-1,3-glucanase I (Rombouts and Phaff, 1976 b) caused complete lysis of cell walls in cup plates, while the lytic β-1,3-glucanase II only moderately lysed yeast cell walls. The lytic β-1,3-glucanase I hydrolysed laminarin randomly to laminaribiose and glucose. It's action pattern on laminarin is similar to that of the non-lytic β-1,3-glucanase. (Fleet and Phaff, 1974 a), but it differs from the non-lytic enzyme due to its powerful lytic activity on insoluble yeast glucan.

Kobayashi et al (1974) and Tanaka et al. (1974) have also explored the production of multiple β-glucanases by B circulans WL-12. This organism has also been shown to produce high levels of endo -α-1,3-glucanase when grown on α-1,3-glucan (pseudonigeran) as substrate (Meyer, 1975).

1.8.2. Enzymes producing oligosaccharides of DP 5. and larger from β-1,3-linked glucans

Doi et al (1971) isolated two types of β-1,3-glucanase from the culture fluid from an Arthrobacter species. Yeast glucan was readily solubilized by the glucanase type I of their preparations and the reaction was accompanied by the accumulation of laminaripentaose. Type II glucanase partially solubilized yeast glucan liberating laminaribiose and glucose. Later, Doi et al. (1973 a/b/c) reported further information on the lytic ability of the Arthrobacter species. They suggested that the laminaripentaose is not merely a product that accumulates as a residue of endo-hydrolysis but that the glucanase I cuts out this oligosaccharide from the interior of the long β-1,3-linked portions of insoluble yeast glucan. Further studies showed that the glucanase I component is heterogenous. The enzyme fractions could be grouped into those which exhibited relatively high lytic activity on yeast cell walls and those which showed much lower activity, but the action pattern of the various fractions was apparently the same.

An enzyme preparation from Arthrobacter luteus has been shown to produce laminaripentaose from insoluble laminarin and from heat-treated pachyman, when grown on yeast cells or β-1,3-glucan. The enzyme was named Zymolyase. (Kitamura et al., 1972 a/b) Kitamura et al., 1974). Zymolyase specifically hydrolysed linear glucose polymers with some β-1,3-linkages releasing laminaripentaose, and leaving some higher oligosaccharides (DP≥8) with laminarin as substrate. Pachyman was resistant to hydrolysis unless it was first treated.

Kaneko et al (1973) studied the susceptibilities of yeasts belonging mainly to Saccharomyces and Candida genera, to a yeast cell wall lytic enzyme produced by Arthrobacter

luteus. The yeasts were tested during the logarithmic and stationery phases of growth. The effects of various treatments such as heating, or treatment with 2-mercaptoethanol or sodium dodecylsulphate on their susceptibility were also examined. Most strains of Candida tested were less susceptible than Saccharomyces yeasts, but became as susceptible after treatment with 2-mercaptoethanol. Schizosaccharomyces pombe showed low susceptibility while Rhodotorula and Sporobolomyces species were not susceptible at all., However this may be related to the observation that the cell walls of the later two yeast genera, are composed of chitin and mannan and do not contain glucan (Bartnicki-Garcia, 1968).

Another lytic β-1,3-glucanase which produces mainly oligosaccharides with five or more glucose residues, from yeast glucan, laminarin, and pachyman, has been isolated from the culture fluid of Cytophaga johnsonii (Bacon et al., 1970 a/b; Bacon et al., 1965). This enzyme acts on only long β-1,3-glucan chains, including alkali-treated cell walls, but it has little effect on living yeast cells. Autoclaved whole cells, were more susceptible to its action.

Oerskovia xanthineolytica has been extensively studied as a producer of yeast lytic enzymes. Mann et al (1972) and Jeffries (1975) have reported that the soil actinomycete produces a mixture of several β-1,3-glucanases capable of lysing various viable yeasts. Scott and Schekman (1980) purified the yeast lytic activity from Oerskovia xanthineolytica culture fluid. The lytic activity was found to consist of two synergistic enzyme activities. The first component was a β-1,3-glucanase with a molecular weight of 55,000. The second lytic component was an alkaline protease. Hydrolysis of β-1,3-glucans was endolytic yielding a mixture of products ranging from glucose to oligomers of 10 or more, in a pH dependent fashion. The

glucanase was unable to lyse yeast cells without the second lytic component. The alkaline protease had no effect on the glucanase activity on polysaccharide substrates.

The Oerskovia enzyme has been shown to attack the cell walls of Saccharomyces cerevisiae, Aureobasidium pullulans, Myrothecium verrucaria and Trichoderma viride. Hydrolysis products from the live yeast cells indicated primarily laminaribiose, as well as glucose and laminaritriose. Laminarin hydrolysis yielded large amounts of trimer and tetramer and relatively small amounts of glucose early in the hydrolysis. (Jeffries et al., 1977).

Flavobacterium dormitator var. glucanolyticae FA-5 has been shown to produce five endo-β-1,3-glucanases (Mori et al., 1977). The endo β-1,3-glucanase II has been characterized as a lower oligosaccharide producing type of endo- β-1,3-glucanase, and has been found to be responsible for lysis of living yeast cells and hydrolysis of yeast glucan (Nagasaki et al., 1976). The endo β-1,3-glucanase IV hydrolyses laminarin and yeast glucan to predominantly laminaripentaose, with a small amount of glucose observed after exhaustive hydrolysis, (Yamamoto et al., 1981)

1.8.3. Yeast glucan debranching enzymes

Rombouts and Phaff (1976 b) postulated that the lytic β-1,6-glucanase which they purified from the culture fluid of Bacillus circulans grown on alkali-insoluble yeast glucan might have a debranching action on such glucan. Their conclusion was based on the partial clearing of baker's yeast cell walls in the absence of laminarinase activity and on the excision of gentiobiose from laminarin and pachyman. The latter activity was thought to be caused by the ability of the lytic β-1,6-glucanase to cleave β-1,3-bonds next to a glucose residue substituted in the 6-position. (Parrish et

al., 1960; Marshall, 1974). The partial hydrolysis of yeast glucan was thought to be caused by the cleavage of one or more β-1,3-bonds adjacent to the β-1,6-linkage involved in branching of the alkali-insoluble glucan. The remaining long chains of β-1,3-linked glucose residues are considered responsible for the residual opacity in cell wall plates treated with this enzyme.

Yamamoto et al (1974) have described the B-glucanase from the culture fluid of Rhizopus chinensis. These authors suggested that the enzyme may be an endo-B-glucanase of the debranching type producing oligosaccharides containing 5-7 glucose residues from alkali-insoluble yeast glucan and walls of living yeast. However if the products of this enzyme are indeed laminarin oligosaccharides of D.P. 5-7, it is difficult to see how this enzyme could act as a debranching enzyme, since the β-1,3-linked linear portions of the alkali-insoluble glucan are much longer than seven glucose residues (Manners et al., 1973 a). Some laminaribiose was found to be produced from yeast glucan by action of the Rhizopus chinensis lytic β-1,6-glucanase. (Yamamoto et al., 1974).

1.8.4. Cell-wall lytic enzymes acting in an exo-hydrolytic manner

Exo-enzymes are well known as endogenous enzymes from yeasts (Abd-El-Al and Phaff, 1968; Fleet and Phaff, 1975). However, these enzymes are unable to cause lysis either of cell walls or of alkali-insoluble glucan to a significant extent. The exo-β-1,3-glucanase from Basidiomycete sp QM 806 has the ability to produce protoplasts from several yeasts. (Bauer et al., 1972). The purification (Huotari et al., 1968), action pattern and specificity (Nelson et al., 1969) and the hydrolytic mechanism (Nelson, 1970) have been

thoroughly investigated. The glucanase removes single glucose resides from a β-1,3-linked glucan chain commencing at the non-reducing terminal. It can bypass B-1,6-linkages, yielding gentiobiose quantitatively from this structural feature, and it can cleave β-1,3-linkages adjacent to other types of linkage in the main chain. The enzyme, although typically exo in its mode of attack, can initiate an endo cleavage in that it attacks endo β-1,3-bonds adjacent to β-1,6-linkages. The enzyme does not catalyse transglycosylation reactions (Nelson et al., 1969). Its ability to bypass β-1,6-linked side chains or branch points probably enhances its lytic potential. (Phaff, 1977).

Nelson et al (1963) in structural studies of enzymes on polysaccharides, used the Basidiomycete sp QM 806 enzyme to hydrolyse laminarin. The products yielded were glucose, as the major product, with laminaribiose, gentiobiose, laminaritriose, and several higher oligosaccharides. As the course of the hydrolysis proceeded, laminaritriose and most of the higher oligosaccharides gradually disappeared while gentiobiose remained constant and laminaribiose increased. Action of the glucanase on simple oligosaccharides was also studied. The enzyme was found to attack laminaritriose but not laminaribiose or gentiobiose. However, later work (Nelson et al., 1969) has shown that laminaribiose was attacked by the enzyme at higher concentrations.

The enzyme has been shown to hydrolyse cell walls of Fusarium and of Aspergillus oryzae (Horikoshi, 1973), of Saccharomyces cerevisiae and Wickerhamia fluorescens (Bauer et al., 1972) and of Aureobasidium pullulans, Myrothecium verrucaria, Saccharomyces cerevisiae and Trichoderma viride. (Jeffries et al., 1977).

1.8.5. Lytic enzymes with unclearly defined action patterns

Yamamoto et al (1974 a) have purified and crystallised on enzyme from a Deuteromycete which degrades yeast glucan and log-phase yeast cells. Log-phase cells of S. cerevisiae were disrupted by the enzyme preparation, although addition of 2-mercaptoethanol or phosphomannanase enhanced its effectiveness as a lytic agent. Nagasaki et al (1974) have studied the enzymic and structural properties of the crystallised enzyme.

The action of the Deuteromycete enzyme on yeast glucan was characterised by a rapid reduction in optical density of a glucan suspension. The reaction products from laminarin, consisted of a mixture of oligosaccharides ranging from laminaritriose to laminaridecaose. Since there was no evidence that laminarin oligosaccharides with a degree of polymerisation of less than ten could be hydrolysed further, the smaller oligosaccharides found as products probably arose as remnants of the polymeric β-1,3-glucan. (Yamamoto et al., 1974 a).

Chesters and Bull (1963) studied extensively the enzymatic degradation of laminarin by various fungal species, and reported on the multicomponent nature of these enzymes. The various types of β-1,3-glucanases were separated by a combination of chromatographic procedures. One of their fungal species, Myrothecium verrucaria produced at least six β-1,3-glucanase components, containing both endo and exo-hydrolytic activities. Evidence was obtained for a synergistic effect of these enzyme components, and a working scheme for laminarin hydrolysis was proposed (Bull and Chesters, 1966).

Other lytic enzymes whose action patterns have not been clearly defined have been studied by Sugimori et al (1972).

They screened a number of moulds including Aspergillus, Fusarium, Penicillum, Corticum, Rhizopus and Mucor as well as Streptomyces, for their abilities to release reducing sugars from heat treated baker's yeast.

In summary, there is no universal process by which the alkali-insoluble glucan of Saccharomyces cerevisiae or other species can be hydrolysed enzymatically. There exists a range of lytic β-1,3-glucanases which act in an exo or endo or combined exo and endo hydrolytic manner.

1.9. YEAST EXTRACT PRODUCTION

Yeast extract is a concentrate of soluble material obtained from yeast following treatment. It is widely produced by plasmolysis, autolysis and to a certain extent by mechanical disruption.

Plasmolysis, a simple method for initiating cell disruption can be achieved by raising the temperature to 55-60°C for 40-48 hours at pH 5.5 and by the addition of salt or organic solvents such as ethyl acetate or isopropanol (Kelly, 1983). However, addition of solvents and high levels of salt are undesirable when yeast autolysate is intended for food products. (Knorr et al., 1979 a).

Autolysis or self-digestion of the yeast cell contents involves the use of the yeasts own enzymes acting alone or supplemented with industrial enzymes. (Kelly, 1983).

Methods of mechanical disruption, such as sonication, freeze-thaw, and homogenization are restricted due to apparatus inefficiency, protein denaturation and high cost as well as restriction to laboratory scale (Knorr et al., 1979 b; Mogren et al., 1974).

Yeast extract finds major application as a flavouring agent in the food industry where it contributes a meaty flavour to a wide range of products. Other minor applications include its use in animal feeds, yeast extract tablets and in malt whiskey production. (Kelly, 1983; Hough and Maddox, 1970; Cogman, 1977).

Estimates for total world yeast extract production suggest a figure of 25,000 tonnes yeast extract per annum (Kelly 1983).

Yeast have a wide variety of endogenous enzymes that include lipases, nucleases, mannanases, glucanases and proteases. The most studied are the proteases, Hough and Maddox (1970) have described four yeast proteases which degrade protein to peptide and amino acids. About 50% of the yeast cell wall is made up of glucan. The alkali-insoluble glucan, which is the structural component of the cell wall responsible for its rigidity and tensile strength is predominantly made up of β-1,3-linked glucans. (Manners et al., 1973 b). Phaff (1977) concluded that effective removal or weakening of the cell wall must involve hydrolysis of these β-1,3-linked glucose residues. Saccharomyces cerevisiae contains both exo and endo - β-1,3-glucanases. (Abd-El-Al and Phaff, 1969; Bacon et al., 1970; Arnold, 1972; Cortat et al., 1972; Sanz et al., 1985), which contribute to the yeast autolysis process.

The autolysis procedure may involve supplementing the yeasts own enzymes with other enzymes such as proteases (most frequently papain) which causes an increase in the rate of solubilization and the final yeast extract yield (Kelly, 1983).

Enzyme mixtures have been obtained from various micro-organisms that attack the intact yeast and could be used in

autolysis. These contain β-glucanase and protease activities. Cell walls could be totally solubilized by such enzyme mixtures and an even higher percentage of soluble compounds could be obtained although the flavour could be altered (Kelly, 1983).

2. MATERIALS AND METHODS

2.1. CHEMICALS

Soya-flour (full-fat) and glucose (food-grade) were obtained from Biocon Ltd., Ireland. Fresh and dried bakers yeast were purchased from Irish Yeast Co., Dublin, Ireland. Malt Extract Agar, Nutrient Agar, Skimmed Milk Powder and Technical Agar No. 3 were obtained from Oxoid Ltd., London, England. B.D.H. Ltd., England or Reidel-de-Haen AG, Germany supplied laboratory chemicals. Biochemicals were obtained from Sigma London Ltd., England. Chemicals and biochemicals used were of analytical quality unless otherwise stated. Protease enzymes L.P. conc and Papain were obtained from Biocon Ltd., Ireland. Carbopol-934 was supplied by B.F. Goodrich Ltd., U.S.A. and Tween-80 by Merck Ltd., Germany. Kitalase was obtained from the Kumiai Chemical Co., Japan and Novozym-234 from Novo Biolabs, Denmark. H.P.L.C. grade methanol was purchased from Labscan Ltd., Ireland. The glucose GOD-PAP kit was obtained from Boehringer Mannheim GmbH, Germany.

2.2. FUNGAL CULTURE

2.2.1. Source of Strain:

Basidiomycete sp was obtained from the Commonwealth Mycological Institute, Surrey, England, Number 155711, (also known as Basidiomycete sp QM 806).

2.2.2. Culture Maintenance:

Stock cultures were maintained on Malt Extract Agar (Oxoid) slopes in universal bottles, subcultured monthly and stored at 4°C. Cultures were incubated at 30°C and sporulated after 6-7 days.

2.2.3. Detection of contamination:

Fungal/Yeast contamination of stock cultures, spore suspensions or fermentation samples were detected by plating samples onto Malt Extract Agar (Oxoid) and incubating at 30°C for 3-4 days.

Bacterial contamination was detected by plating samples on Nutrient Agar (Oxoid) containing 0.1mg/l cycloheximide and incubating overnight at 30°C.

2.3. SHAKE-FLASK CULTIVATION

Media were dispensed 90ml or 100ml where stated in 250ml conical flasks. Incubation was conducted at 30°C on an L.H. Engineering 2-tier orbital shaker (model MK II/III) at 150 r.p.m. with a displacement of 50mm for 13-14 days.

2.4. FERMENTER CULTIVATION

The fermenter used on laboratory scale was of 16 litre gross capacity, (Microferm, New Brunswick Scientific). Working volume was 10 litres. Inoculation and sampling of the fermenter were carried out under sterile conditions. The Microferm fermenter was run at 5 p.s.i. back-pressure to control foaming and to reduce the risk of contamination. The levels of aeration and agitation were 0.5 volume air/volume media/minute (v/v/m) and 200 r.p.m. respectively. Incubation was conducted at 30°C.

The dimensions and specifications of the Microferm fermenter are shown in Table 2.1.

TABLE 2.1. DIMENSIONS OF MICROFERM FERMENTER:

(Volumes measured in litres, lengths in cm).

Total volume	16.0
Medium volume	10.0
Vessel diameter	22.0
Impeller number	3
Impeller diameter (Di)	7.0
Impeller spacing	9.5
Impeller speed r.p.m. (N)	200
Impeller-tip speed (πN Di) (a) cms^{-1}	73.3
Impeller shear (N^2 Di^2) (b) x 10^3	0.54
Number of baffles	4
Baffle width	2.0
Distance baffle-wall	1.5
Distance impeller tip-wall	4.0
Aeration maximum level (v/v/m)	2.0

References: (a) Wang et al, 1979

(b) Wang and Fewkes, 1977

2.5. FERMENTATION MEDIA

2.5.1. Medium A (1/2) Basal Medium:

	Components	Concentration
1.	Carbon source Medium A(1) glucose Medium A(2) starch	10g/l
2.	$KH_2\ PO_4$	2g/l
3.	$(NH_4)_2\ SO_4$	1.4g/l
4.	Urea	0.3g/l
5.	$MgSO_4\ 7H_2O$	0.3g/l
6.	$CaCl_2$	0.3g/l
7.	Yeast extract	0.1g/l
8.	Trace elements	
	Fe	1.0µg/l
	Mn	0.5µg/l
	Zn	0.8µg/l

The components were sterilized separately and recombined to the concentrations shown.

2.5.2. Medium B Soya inoculum medium

Soya-flour (full-fat), 10g/l; glucose, 40g/l; at pH 6.0 (Materials used were of food-grade quality).

2.5.3. Medium C (a/b/c) β-1,3-glucanase production medium:

Soya-flour (full-fat), 10g/l; glucose 10g/l; in 0.2M Na_2HPO_4 - 0.1M citric acid buffer at a - pH 3.0, b - pH 4.0, c - pH 5.0.

2.5.4. Medium D (a/b/c) β-1,3-glucanase production medium:

Soya-flour (full-fat), 10g/l; glucose 40g/l; in 0.2M Na_2 HPO_4 - 0.1M citric acid buffer at a - pH 3.0, b - pH 4.0, c - pH 5.0.

2.5.5. Medium E (a/b/c) β-1,3-glucanase production medium:

Soya-flour (full-fat), 10g/l; glucose 10g/l; fresh bakers yeast, 20g dry wt/l; in 0.2 Na_2 HPO_4 - 0.1M citric acid buffer at a - pH 3.0, b - pH 4.0, c - pH 5.0.

2.5.6. Medium F (a/b/c) β-1,3-glucanase production medium:

Glucose, 10g/l; fresh bakers yeast, 20g dry wt/l; in 0.2M Na_2 HPO_4 - 0.1M citric acid buffer at a - pH 3.0, b - pH 4.0, c - pH 5.0.

2.5.7. Source of media formulations:

Medium A was based on that of Reese and Mandels (1959). Medium B was developed by Byrne (1985). Media C, D, E and F were developed during the course of this work.

The method for preparing the 0.2M Na_2 HPO_4 - 0.1M citric acid buffer is outlined in (Appendix I). All shake-flask

media were prepared using distilled water. The medium used in the Microferm fermenter was prepared using tap water.

2.5.8. Sterilization procedures:

Shake-flask media were sterilized in conical flasks at 121°C and 15 p.s.i. for 15 - 20 minutes. The Microferm fermentor was sterilized by steam-injection.

2.6. ANALYTICAL PROCEDURES

2.6.1. Estimation of reducing sugars:

Reducing-sugars were estimated using the Dinitrosalicylic Acid (D.N.S.) method. (Bernfeld, 1955; Miller, 1959). Results were expressed as reducing equivalents g/l using glucose as standards.

D.N.S. Reagent:

3.5-dinitrosalicylic acid, 10g/l; potassium sodium tartarate, 300g/l; sodium hydroxide, 16g/l were dissolved in 600 ml distilled water by heating, without boiling. The solution was cooled and diluted to 1 litre.

Procedure:

A standard curve was prepared using a range of glucose solutions 0.1 - 1.5 g/l. Unknown glucose solutions were determined within this concentration range.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent blank
Glucose sample	1.0ml	-
Distilled water	1.0ml	2.0ml
D.N.S. reagent	2.0ml	2.0ml

2. Tubes were placed in a boiling water bath for 10 minutes, then cooled.

3. 10ml distilled water were added to each tube and the contents mixed.

4. The optical density at 540nm was read using the reagent to zero the spectrophotometer.

5. The optical density at 540nm vs glucose concentration g/l was plotted.

6. Reducing sugar concentrations of unknown solutions were determined as glucose reducing equivalents from the glucose standard curve. All determinations were carried out in duplicate.

2.6.2. Estimation of β-1,3-glucanase activity:

β-1,3-glucanase activity was determined by production of glucose reducing equivalents from laminarin at 50°C. 1 unit β-1,3-glucanase liberates 1mg reducing equivalents per minute. Reducing sugars were determined by the D.N.S. method.

Enzyme assay:

Eyzyme:

Cell-free supernatants (CFS) were diluted with 0.1M citric acid- sodium citrate buffer, pH 5.0. (Appendix I).

Substrate:

2% w/v laminarin in 0.1M citric acid-sodium citrate buffer pH 5.0.

Method:

1. Tubes were prepared as follows:-

Additions	Enzyme Analytical (A)	Enzyme Control (B)	Reagent Blank
Substrate	0.2ml	0.2ml	0.2ml
D.N.S. reagent	-	0.4ml	0.4ml
Diluted enzyme	-	0.2ml	-
0.1M citrate buffer	-	-	0.2ml
Equilibrate tubes 50°C			
Diluted enzyme	0.2ml	-	-
Incubate 50°C 10 mins			
D.N.S. reagent (stops reaction)	0.4ml	-	-

2. Tubes were placed in a boiling water bath for 10 minutes, then cooled.

3. 2ml distilled water were added to each tube and the contents mixed.

Reducing sugars were then determined as glucose using the method described in section 2.6.1. but scaled down to 20% volume. The spectrophotometer was zeroed using the reagent blank. Δ optical density (optical density A - optical density B) was calculated and reducing sugars produced, determined using a glucose standard curve. All determinations were carried out in duplicate.

The β-1,3-glucanase activity was calculated as follows:-

Glucose produced was obtained from standard curve.

$$\text{Activity} = \text{units/ml CFS} \qquad \frac{\text{mg glucose/ml x enzyme dilution (ml) x 5}}{\text{reaction time (mins)}}$$

1 unit of enzyme activity produces 1mg of glucose reducing equivalents per minute under the assay conditions.

2.6.3. Estimation of protease activity:

Two methods were used, one using haemoglobin as substrate based on Anson (1939) and another using skimmed milk powder as substrate which was developed during the course of this work as an indicator method for the presence of protease activity.

2.6.3.1. Haemoglobin protease assay
(Biocon Ltd., Ireland information Sheet No. MA-080-80A)

The method was based on a method developed by Anson (1939). The test is based on the 30 minute enzymatic hydrolysis of a haemoglobin substrate at pH 4.6 and 40°C. Unhydrolysed substrate is precipitated with trichloroacetic acid and

removed by filtration. The quantity of solubilised haemoglobin in the filtrate is determined spectrophotometrically. 1 unit of protease activity is the amount of enzyme that produces in 1 minute a hydrolysate whose absorbance at 275nm is the same as a solution containing 1.10µg/ml tyrosine in 0.006N HCI.

Tyrosine standard curve:

100 mg L-tyrosine (chromatographic grade) was dissolved in 60ml 0.1N HCl. The solution was then diluted to 1 litre. This solution contained 100µg/ml L-tyrosine. Solutions containing 75,50 and 25 µg L-tyrosine /ml were prepared from this stock solution. The absorbance of each at 275nm was determined using 0.006N HCl to zero the spectrophotometer. A standard curve of optical density 275nm vs µg L-tyrosine /ml was plotted.

Enzyme assay:

Enzyme:

Cell-free supernatants (CFS) or commercial preparations were diluted with 1.0M acetic acid - sodium acetate buffer pH 4.6 (Appendix I).

Substrate:

5g Haemoglobin (Sigma) in 100ml distilled water was stirred for 10 minutes to dissolve. The pH was then adjusted to pH 1.7 by the addition of 0.3N HCl. After 10 minutes the pH was readjusted to pH 4.6 with 1.0M sodium acetate. The solution was then diluted with distilled water to 250ml. Trichloroacetic acid solution (T.C.A.). A 7% w/v solution was prepared in distilled water.

Trichloroacetic acid solution (T.C.A):

A 7% w/v solution was prepared in distilled water.

Method:

Tubes were prepared as follows:-

Additions	Enzyme Analytical (A)	Enzyme Control (B)	Reagent Blank
Haemoglobin substrate	5.0ml	5.0ml	5.0ml
Equilibrate tubes at 40°C			
Diluted enzyme	1.0ml	-	-
1.0M acetate buffer	-	-	1.0ml
Incubate tubes 40°C, 30 mins.	10.0ml	10.0ml	10.0ml
TCA solution (stops reaction)			
Diluted enzyme	-	1.0ml	-

The tubes were mixed and allowed to cool for 1 hour with frequent shaking. The tube contents were then filtered through Whatman No. 1 filter paper. The spectrophotometer was zeroed using filtrate C at 275nm and the optical density (A-B) was determined.

One H.U.T. unit is defined as that amount of enzyme which will, under standard conditions, give a hydrolysate in one minute, whose absorbance at 275nm is equivalent to that of 1.10µg/ml tyrosine in 0.006N HCl. The absorbance of a solution containing 1.1µg/ml tyrosine is obtained by dividing the optical density value for the 75µg/ml solution by 68.2 (75/1.1 = 68.2). This absorbance value is required to calculate the protease activity as defined above.

Calculation:

$$\text{H.U.T. (units /ml or g)} = \frac{\text{OD (A-B)}}{\text{OD (1.1µg tyr/ml)}} \times \frac{\text{reaction volume (mls)} \times \text{enzyme dilution}}{\text{reaction time (mins)}}$$

OD = optical density.

2.6.3.2. Skimmed milk method:

25ml of a 2% w/v skimmed milk powder (Oxoid) and 2% w/v technical agar No. 3 (Oxoid) mixture which had been boiled and cooled to hand-hot temperature was dispensed into petri dishes. 0.1ml cell-free supernatant was applied to an 8mm diameter well. The dishes were incubated for 20 hours at 30°C after which time zones of hydrolysis were obtained.

2.6.4. Estimation of glucose:

Glucose was measured using an enzymatic colourimetric method based on Trinder (1969). The test is based on the following principle:-

$$\text{glucose} + O_2 + H_2O \xrightarrow{GOD} \text{gluconate} + H_2O_2$$

$$^{2}H_2O_2 + 4 - \text{aminophenazone} + \text{phenol} \xrightarrow{POD}$$

$$4 - (p - \text{benzoquinone} - \text{mono-imino}) \text{ phenazone} + 4\ H_2O$$

NOTE: GOD = glucose oxidase
POD = peroxidase

A diagnostic kit (Boehringer Mannheim GmbH, Germany) was used to estimate glucose. Two reagents were supplied. These contained the following:-

reagent 1: phosphate buffer, 100 mmol/l; pH7.0; GOD$\geq$ 18 u/ml; POD$\geq$ 1.1 u/ml; 4-aminophenazone, 0.77 mmol/l.

reagent 2: phenol 11mmol/l.

The contents of one bottle containing reagent 1 were dissolved in 200ml redistilled water and the contents of one bottle containing reagent 2 added. The reagent mixture was stored in a dark bottle and stored at 4°C.

Procedure:

A standard curve was prepared using a range of glucose solutions 10-100 mg glucose/100 ml. Glucose, in unknown solutions, was determined within this concentration range.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
redistilled water	0.1ml	0.2ml
glucose sample	0.1ml	-
reagent mixture	2.0ml	2.0ml

2. The tubes were mixed and incubated at 20 - 25°C in the dark for 35-60 minutes.

3. The optical density at 510nm was determined using the reagent blank to zero the spectrophotometer.

4. Optical density 510nm vs glucose concentration mg/100ml was plotted.

5. Glucose concentration in unknown solutions was determined from the standard curve. All determinations were carried out in duplicate.

2.7. AUTOLYSIS PROCEDURE

The autolysis method was developed during the course of this work. Autolysis was carried out in 250 ml conical flasks with 100g yeast slurry per flask. Each 250 ml conical flask contained fresh, pressed or dried bakers yeast (Saccharomyces cerevisiae); 10g dry wt; lytic enzymes (Papain, Kitalase, Novozym-234, Basidiomycete sp QM 806 culture supernatant); distilled water added to bring weight of contents to 100g. β-1,3-glucanase activity in Kitalase, Novozym-234 and the Basidiomycete culture supernatant was assayed according to section 2.6.2. The pH of the slurry was generally pH 5.0. Incubation was conducted at 55°C on an incubator shaker. Samples of 10g yeast slurry were

centrifuged at 7,500g for 15 minutes. The supernatant was recovered and the pellet was resuspended in 10ml distilled water and recentrifuged as before. The supernatants were combined and dried. The pellet was also dried.

Calculation:

Yeast extract produced (g)/10g slurry (1g dry wt yeast)

$$= \frac{\text{dry wt supernatants}}{\text{dry wt supernatants + dry wt pellet}}$$

% yeast extract = yeast extract (g)/10g slurry x 100

2.8. CHROMATOGRAPHIC PROCEDURES

2.8.1. Instrumentation:

The Waters Associates (Milford MA U.S.A.) instruments as detailed in Section 2.10 were used. The column was a specialist Waters "Dextropak" plastic cartridge 10 x 1 cm, which for use was pressurized in the radial compression Z - module. It was packed with a C_{18} - bonded silica specially made and optimised for carbohydrate oligomer separations. The "Dextropak" was stored in methanol, methanol-water (40 : 60), and finally distilled- deionised water. All solvents were filtered (0.45µm filter, Sartorius, England) and degassed ultrasonically. Laminarin samples were filtered through a 0.45 µm filter and glucan samples were centrifuged at 12,000g in an eppendorf centrifuge (Heraeus Christ). 20µl samples were injected.

2.8.2. Working parameters for High Performance Liquid Chromatography (H.P.L.C.):

The following parameters were used:-

1. Column: Waters "Dextropak" plastic cartridge 10 x 1 cm.
2. Flow-rate: 1ml/min.
3. Mobile phase: distilled - deionised water (Milli-Q System).
4. Detector: Refractive Index detector at 25°C.
5. Chart speed: 1cm/min.
6. Injection volume: 20µl.

2.8.3. Glucose estimation using H.P.L.C.:

Glucose solutions were prepared in distilled water in the range 2 - 10 mg/ml. 20µl glucose solution was injected onto the column. The determinations were carried out in duplicate. A standard curve of glucose peak height (cm) vs glucose concentration was plotted. The retention time for glucose was found to be 3.70 minutes. A chromatogram for glucose is enclosed in Appendix III.

2.8.4. β-gentiobiose estimation using H.P.L.C.

β-gentiobiose (Sigma) solutions were prepared in distilled water in the range 1.25 - 10mg/ml. 20µl β-gentiobiose solution was injected onto the column. Determinations were carried out in duplicate. A main peak with a shoulder peak was obtained. The retention time for the main peak was 5.00 minues and 5.20 minutes for the shoulder peak. The peak height (cm) for the main peak vs β-gentiobiose concentration mg/ml was plotted to obtain a standard curve. The presence

of the main and shoulder peak indicated that β-gentiobiose was present in anomeric form. A β-gentiobiose chromatogram is enclosed in Appendix III.

2.8.5. Laminaribiose estimation using H.P.L.C.

Laminaribiose (Sigma) solutions were prepared in distilled water in the range 2.5 - 10 mg/ml. 20µl laminaribiose solution was injected onto the column. Two peaks were obtained, one at a retention time of 5.90 minutes and the second at 6.45 minutes indicating that laminaribiose was also present in anomeric form. A standard curve of peak height (cm) for the main peak at 5.90 minutes was plotted against laminaribiose concentration mg/ml. Determinations were carried out in duplicate. A laminaribiose chromatogram is enclosed in Appendix III.

2.8.6. Yeast glucan degradation:

Yeast glucan was prepared by a method based on that of Bacon et al (1969). The method used is described in Appendix II.

Procedure:

Yeast glucan was treated with (a) Basidiomycete sp QM 806 culture supernatant (b) Kitalase and (c) Novozym - 234 for 3½ hours at 50°C and pH 5.0. Degradation products were assayed using H.P.L.C.

Substrate:

20 mg/ml glucan in 0.1m citric acid-sodium citrate buffer pH 5.0 (Appendix I).

Enzyme:

Kitalase, Novozym - 234 and Basidiomycete sp QM 806 culture supernatant preparations were diluted to 5U β-1,3-glucanase/ml (Section 2.6.2.) in 0.1m citric acid - sodium citrate buffer pH 5.0.

Method:

1. 10mg glucan were treated with 2.5U β-1,3-glucanase by taking equivolumes of the substrate and enzyme solutions in test tubes. Incubation was conducted at 50°C. The time at which the glucan and enzyme solutions were mixed equalled to.

2. Samples were taken at To, 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, 180 and 210 minutes. Reaction was stopped by boiling for 10 minutes.

3. Samples were cooled and centrifuged at 12,000g in an eppendorf centrifuge for 5 minutes.

4. 20µl samples were injected (in duplicate) onto the column. Peak heights (cm) were measured and degradation products identified from their retention times.

5. Amounts of glucose, β-gentiobiose and laminaribiose produced were estimated using the standard curves already prepared.

2.8.7. Laminarin degradation:

Procedure:

Laminarin was treated with (a) Basidiomycete sp QM 806 culture supernatant (b) Kitalase and (c) Novozym - 234 for

3½ hours at 50°C and pH 5.0. Degradation products were assayed using H.P.L.C.

Substrate:

20 mg/ml laminarin in 0.1M citric acid - sodium citrate buffer pH5.0 (Appendix I).

Enzyme:

Kitalase, Novozym - 234 and Basidiomycete sp QM 806 culture supernatant preparations were diluted to 5U β-1,3-glucanase/ml (Section 2.6.2.) in 0.1M citric acid sodium citrate buffer pH 5.0.

Method:

1. 10mg laminarin were treated with 2.5U β-1,3-glucanase by taking equivolumes of the substrate and enzyme solutions in test tubes. Incubation was conducted at 50°C. The time at which the laminarin and enzyme solutions were mixed equalled To

2. Samples were taken at To, 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, 180 and 210 minutes. Reaction was stopped by boiling for 10 minutes.

3. Samples were cooled and filtered through a 0.45 µm filter (Sartorious).

4. 20µl samples were injected (in duplicate) onto the column. Peak heights (cm) were measured and degradation products identified from their retention times.

5. Amounts of glucose, B-gentiobiose and laminaribiose were estimated using the standard curves already prepared.

2.8.8. Laminaribiose degradation:

Procedure:

Laminarin (Sigma) was treated with Kitalase, Novozym - 234 and Basidiomycete sp QM 806 culture supernatant preparations for 1 hour at 50°C and pH 5.0.

Substrate:

2 mg/ml laminaribiose in 0.1M citric acid - sodium citrate buffer pH5.0 (Appendix I).

Enzyme:

Enzyme preparations were diluted with 0.1M citric acid sodium citrate buffer pH to 5U B-1,3-glucanase /ml (Section 2.6.2.).

Method:

1. 1mg laminaribiose was treated with 2.5U B-1,3-glucanase by taking equivolumes of the substrate and enzyme solutions in test tubes. Incubation was conducted at 50°C.

2. The reaction was stopped after 1 hour by boiling for 10 minutes.

3. Glucose produced from laminaribiose was assayed using the GOD-PAP method (Section 2.6.4.).

2.9. GROWTH ASSESSMENT

Biomass determinations (by dry weight) were not carried out routinely due to the insoluble nature of soya-flour which was used extensively in fermentation media. However, an attempt was made to estimate growth using dry weight measurement (Appendix IV). As a rough guide, good growth where stated, would be equivalent to approximately 400mg growth/100mls culture medium.

Growth was assessed visually throughout this work. Pelleted growth with pellet sizes of the order 0.1mm to 0.5mm were obtained in all shake-flask media. Mycelial growth was obtained in the Microferm fermenter.

2.10 ROUTINE MEASUREMENTS AND INSTRUMENTATION

pH was measured using a Philips PW 9420 pH meter. Spectrophotometric measurements were conducted on a Pye-Unicam SP6-550 u.v./vis spectrophotometer or on a LKB Ultraspec II 4050 u.v./vis spectrophotometer with a 1cm light path.

Balances used routinely included a Sartorius 1219MP electronic balance (600g ± 0.01g) and Precisa 80A electronic balance (30g ± 0.0001g).

Centrifugation was carried out using a bench-top Heraeus Christ model 600, an eppendorf centrifuge Heraeus Christ Biofuge at 12,000g (set speed) and a floor centrifuge DuPont Instruments Sorvall RC-5B Refrigerated Superspeed Centrifuge.

High performance liquid chromatography was carried out using the following Waters Assoc. Instruments: M600 pump; U6K injector; R401 refractive index detector; and a radial

compression Z-module. The refractive index detector was maintained at 25°C using a circulating water bath (Haake DI). A Linseis L6512 single channel recorder was used. H.P.L.C. samples and solvents were filtered through Sartorius 0.45 µm filters.

The incubater shaker used during autolysis was a LH Fermentation MK X Incubator Shaker.

For microphotography, samples of growth were wet-mounted onto slides and photographed under a Nikon Optiphot phase-contrast microscope. An Olympus OM 10 camera was used.

Microbiological techniques were generally carried out according to Collins and Lyne (1979).

3. RESULTS

3.1. GROWTH AND PRODUCTION OF β-1,3 GLUCANASE BY BASIDIOMYCETE SP QM 806 IN SUBMERGED CULTURE

Basidiomycete sp QM 806 produces β-1,3-glucanase in submerged culture. This enzyme is of significance in the hydrolysis of cell walls of yeast and other fungi. β-1,3-glucanases are also of interest due to their use in structural analysis of polysaccharides (Totani et al., 1983; Jeffries et al., 1977). The β-1,3-glucanases are of two types a) exo or end-wise splitting type yielding glucose from β-1-3 glucans, and b) endo or random splitting type yielding laminaribiose and higher oligosaccharides from β-1,3-glucans (Reese and Mandels 1959). Studies on the growth of Basidiomycete sp QM 806 and on its production of β-1,3-glucanase were conducted. The effect of different parameters on β-1,3-glucanase production were investigated. These investigations included testing the effect of pH, glucose feeding, and medium supplements in submerged culture in shake-flasks. (Reducing sugar and β-glucanase determinations were conducted using procedures described (Sections 2.6.1 and 2.6.2.).

The Basidiomycete β-1,3-glucanase was used in subsequent experiments on laminarin and yeast glucan degradation and on yeast autolysis.

3.1.1. β-1,3-Glucanase production in basal media

The medium used was the basal medium described by Reese and Mandels (1959), (Medium A Section 2.5.1). Basidiomycete sp QM 806 was grown on a carbon source (10g/l) in this medium. 250ml Conical flasks containing 100ml of the medium were inoculated with the culture contents of one malt extract

agar slope. The flasks were incubated for 14 days under standard conditions (Section 2.3) Duplicate flasks were used. Starch (medium A.2 Section 2.5.1) and glucose (medium A.1, Section 2.5.1) were used as carbon sources. Cell-free supernatants were prepared by vacuum filtration through Whatman No 1 filter paper.

β-1,3-glucanase production and pH were monitored. The results are presented in Figures 3.1 and 3.2.

Growth in both the starch and glucose containing basal media was monitored visually with maximum growth occurring at 5-6 days. Pelleted growth was obtained (Section 2.9).

A trend was observed from the pH values and β-1,3-glucanase level profiles. β-Glucanase production was not initiated until the pH had reached a minimum value pH 3.5. This data was in good agreement with observations of Reese and Mandels (1959) and Friebe and Holldorf (1975). The decrease in pH was found by these to be associated with the consumption of the carbon source and the subsequent increase in pH with the production of β-1,3-glucanase.

FIGURE 3.1.

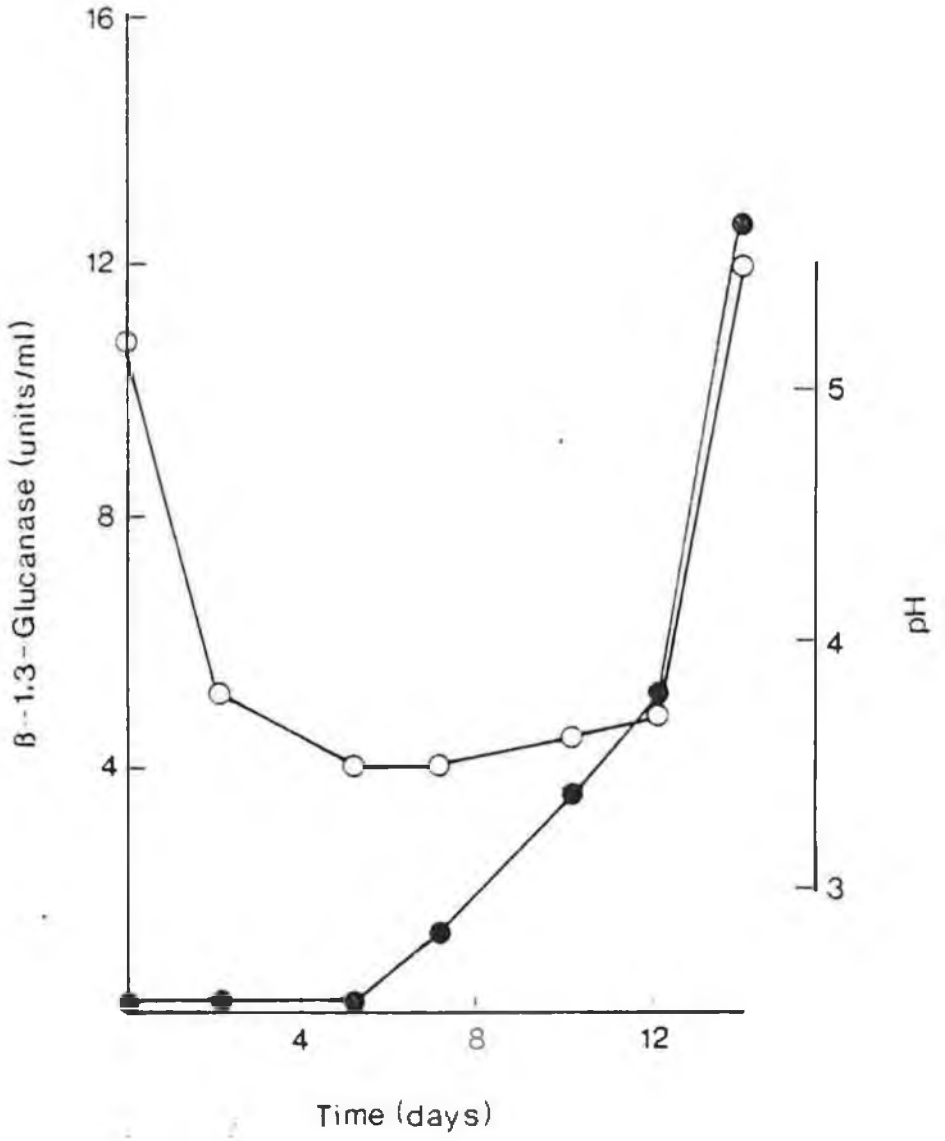

Figure 3.1. Production of β-1,3-glucanase by Basidiomycete sp QM 806 in basal medium, containing glucose. (Medium A.1), 100 ml in 250 ml conical flasks, inoculated with growth contents of one malt extract agar slope and incubated at 30°C and 150 r.p.m. for 14 days. β-1,3-Glucanase levels (●) and pH (○) were monitored.

FIGURE 3.2.

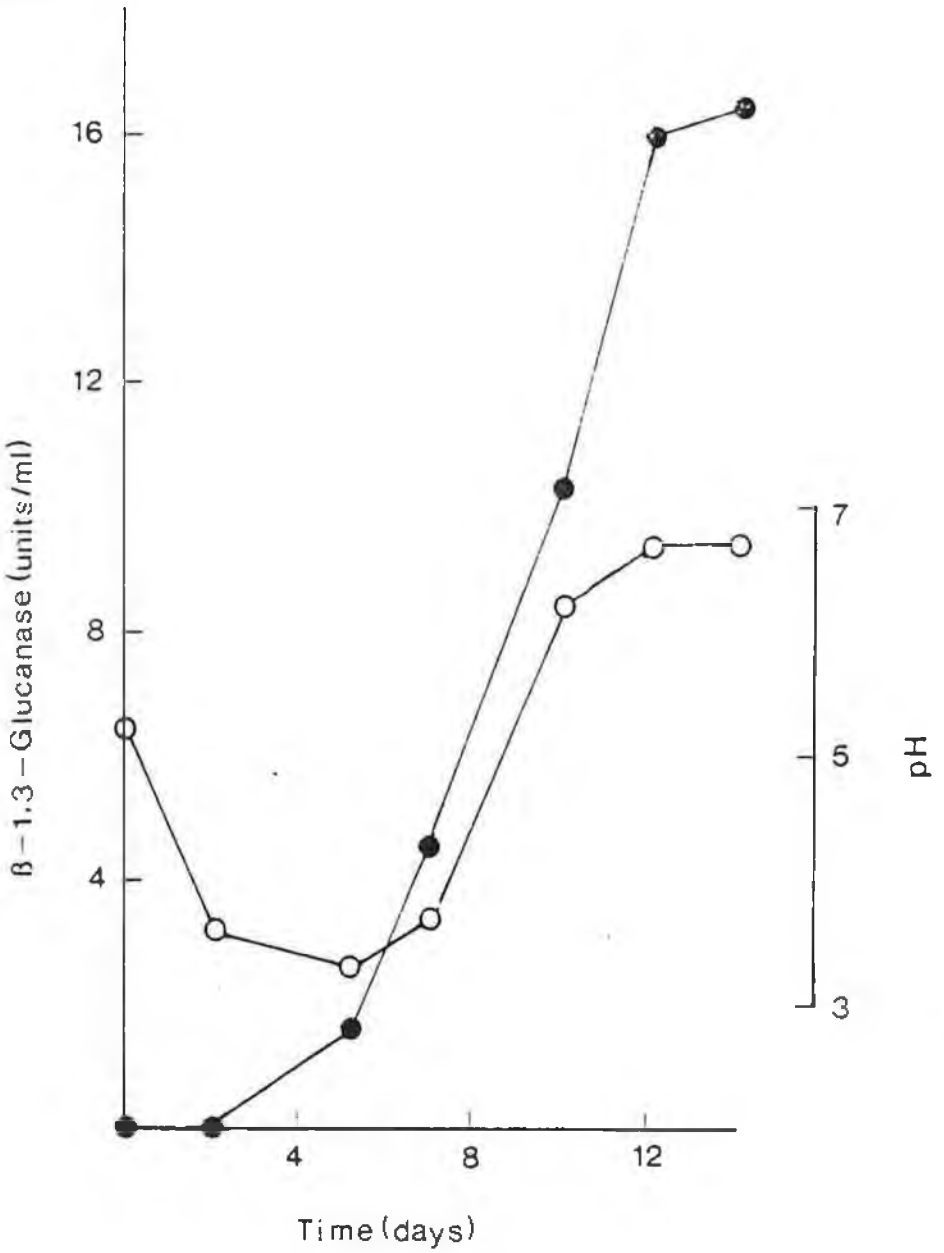

Figure 3.2. Production of β-1,3-glucanase by Basidiomycete sp QM 806 in basal medium, containing starch. (Medium A.2), 100 ml in 250 ml conical flasks, inoculated with growth contents of one malt extract agar slope and incubated at 30° C and 150 r.p.m. for 14 days. β-1,3-Glucanase levels (●) and pH (○) were monitored.

3.1.2. β-1,3-Glucanase production in complex medium

The medium contained soya-flour (full-fat), 10g/l; glucose (food-grade), 40g/l; pH 6.0 (medium B, Section 2.5.2). Inoculation, incubation and harvesting procedures were the same as those used in the previous section (Section 3.1.1).

β-glucanase production and pH were monitored. The results are presented in Figure 3.3.

Growth was monitored visually with maximum growth at 5-6 days. Pelleted growth was obtained. (Section 2.9). The trend which occurred using the basal medium was also observed in the complex medium, with β-1,3-glucanase production being initiated when the pH had reached a minimum due to glucose consumption.

FIGURE 3.3.

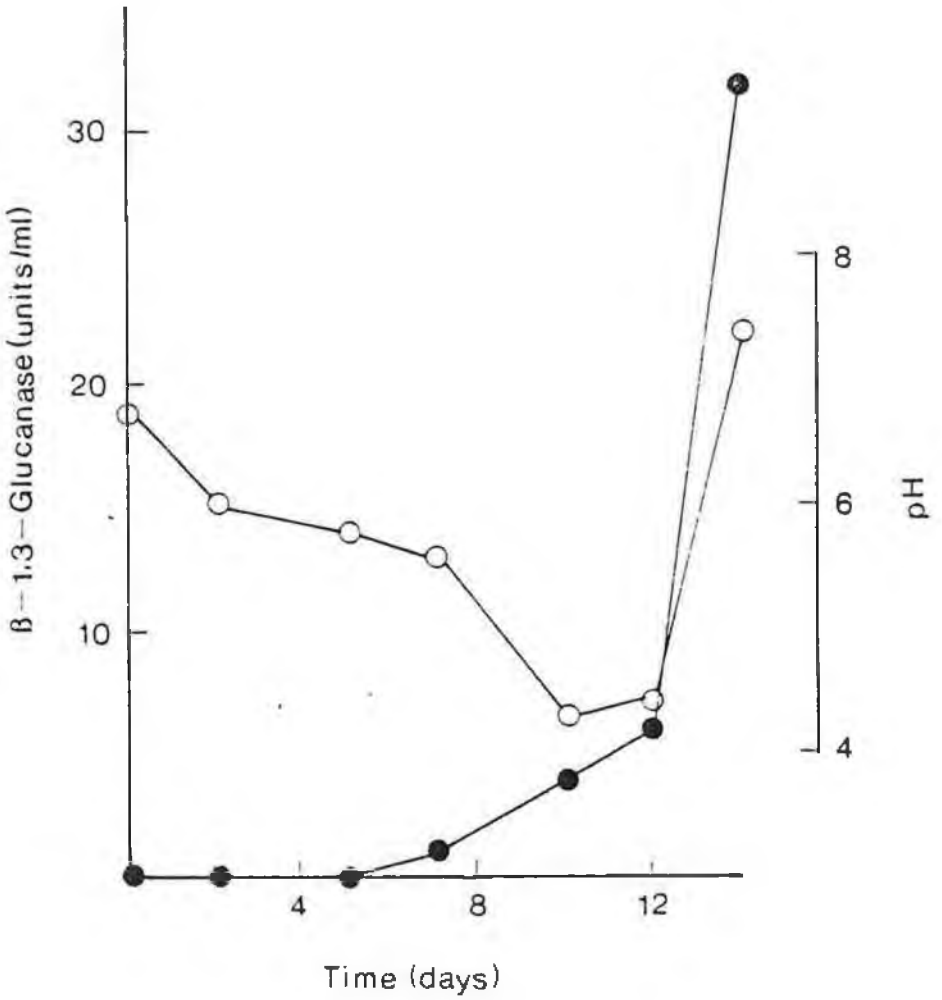

Figure 3.3. Production of β-1,3-glucanase by Basidiomycete sp QM 806 in complex medium (Medium B.), 100 ml in 250 ml conical flasks, inoculated with growth contents of one malt extract agar slope, and incubated at 30°C and 150 r.p.m. for 14 days. β-1,3-Glucanase levels (●) and pH (○) were monitored.

3.1.3. Effect of pH and medium constituents on β-1,3-glucanase production

The effect of pH on β-1,3-glucanase production was investigated. Various media (Table 3.1) were used and buffered with 0.2M Na_2HPO_4 - 0.1M citric acid buffer at pH 3.0, pH 4.0 and pH 5.0. The method for preparing these is given in Sections 2.5.3 -2.5.6.

A vegetative inoculum was used instead of a spore inoculum. Using a spore inoculum, maximum growth was obtained after 5-6 days. (Sections 3.1.1 and 3.1.2). A vegetative inoculum would provide active biomass for transfer into other media with the minimum of lag in subsequent media, and thus earlier enzyme production.

Table 3.1

Composition of media used to investigate β-1,3-glucanase production

Medium	Soya-flour (full-fat) g/l	Glucose (food-grade) g/l	Fresh baker's Yeast dry wt. g/l
C	10	10	-
D	10	40	-
E	10	10	20
F	-	10	20

Fresh baker's yeast was chosen as a media component to induce lytic enzyme production against this yeast. Baker's yeast was used in subsequent experiments on yeast autolysis. Phaff (1977) reported that due to considerable variation in cell wall composition between yeast species, the most effective lytic enzyme complex is obtained using the same strain as an inducer.

Media were dispensed 90ml per 250ml conical flask. Each enzyme production flask was inoculated with 19ml vegetative inoculum from a 3-4 day old shake-flask culture of Basidiomycete sp QM 806 grown in medium B, which had been inoculated with growth from a malt extract agar slope. Flasks were incubated under standard conditions (Section 2.3) for 13 days. Duplicate flasks were used.

Growth was assessed visually and by the rate of reducing sugar consumption.

Residual reducing sugar levels, β-1,3-glucanase levels and pH were monitored. The results are presented in Figures 3.4 - 3.7.

The buffering capacity maintained the pH of each medium. In some cases the pH rose by 1-2 pH units.

Visually maximum growth was obtained after 3-4 days. Good biomass occurred in media buffered at pH 3.0, with moderate biomass at pH 4.0 and poor-moderate biomass at pH 5.0. Pelleted growth was obtained in all media (Section 2.9). Accordingly higher enzyme yields were obtained at the pH at which most growth occurred. The highest enzyme levels were obtained in media buffered at pH 3.0, with 56.5U in medium D, 47.0U in medium C, 38.0U in medium E and 46.0U in medium F. β-1,3-Glucanase production was initiated at residual reducing sugar levels of 5-6 g/l. At levels above this no β-1,3-glucanase activity was found. In media C,E, and F β-1,3-glucanase activity was found after 7-9 days when the reducing sugar levels had decreased to 5-6 g/l. In medium D, enzyme production was not initiated until later due to the high initial reducing sugar level (40 g/l).

No appreciable difference in enzyme levels were found when using baker's yeast as a medium component. Enzyme was produced earlier but this was probably due to consumption of the sugar supply.

Employing a vegetative inoculum resulted in shorter lag periods with earlier and higher enzyme levels being achieved. Production of β-1,3-glucanase in media inoculated with spores was initiated later with lower enzyme yields attained (Sections 3.1.1 and 3.1.2)

FIGURE 3.4

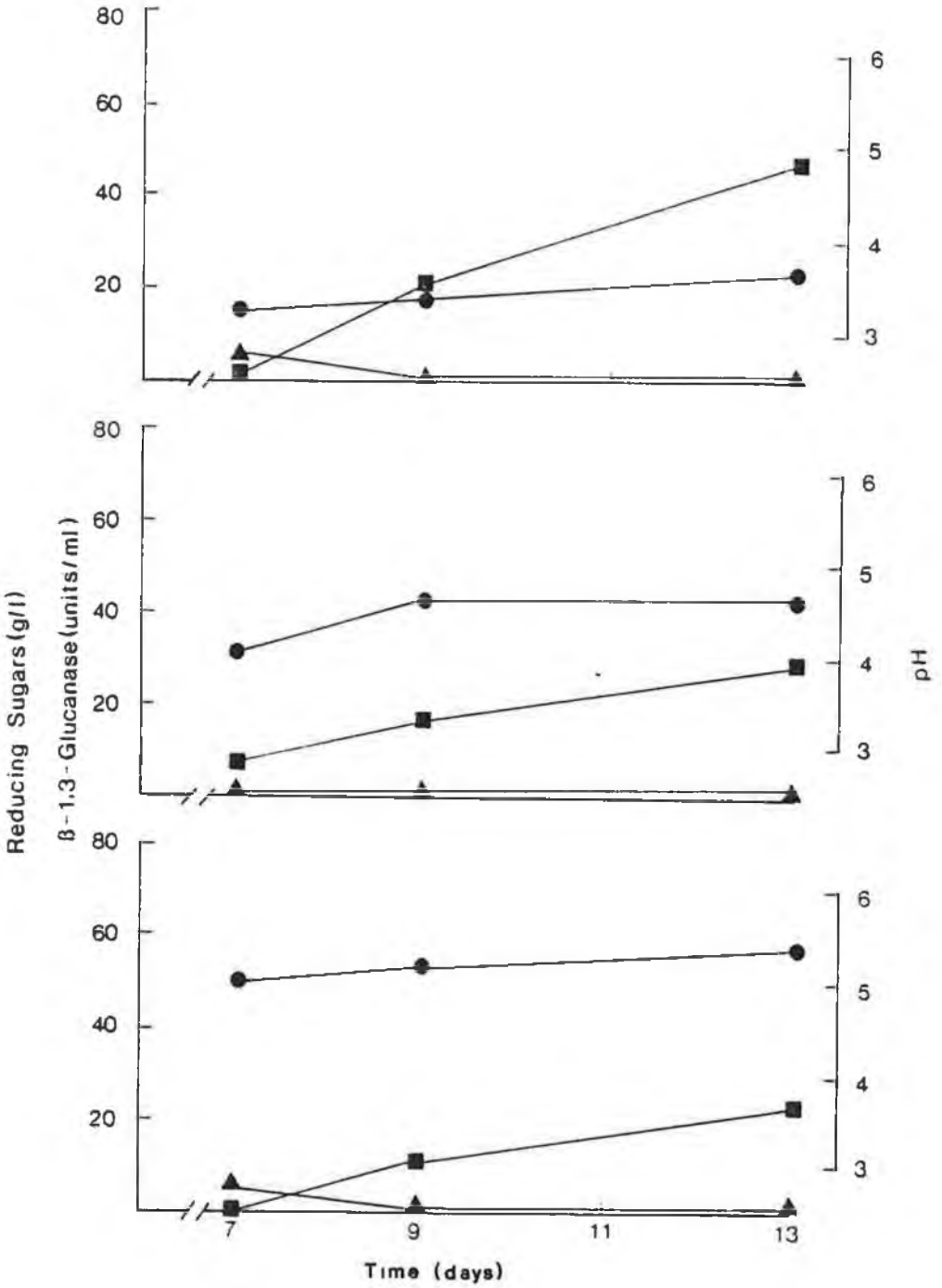

Figure 3.4. Production of β-1,3-glucanase in Medium C buffered at pH 3.0, pH 4.0 and pH 5.0, 90ml in 250ml conical flasks, inoculated 10% from a 3-4 day old culture grown in Medium B, and incubated at 30°C and 150 r.p.m. for 13 days. β-1,3-Glucanase production (■), reducing sugar levels (▲) and pH (●) were monitored.

FIGURE 3.5

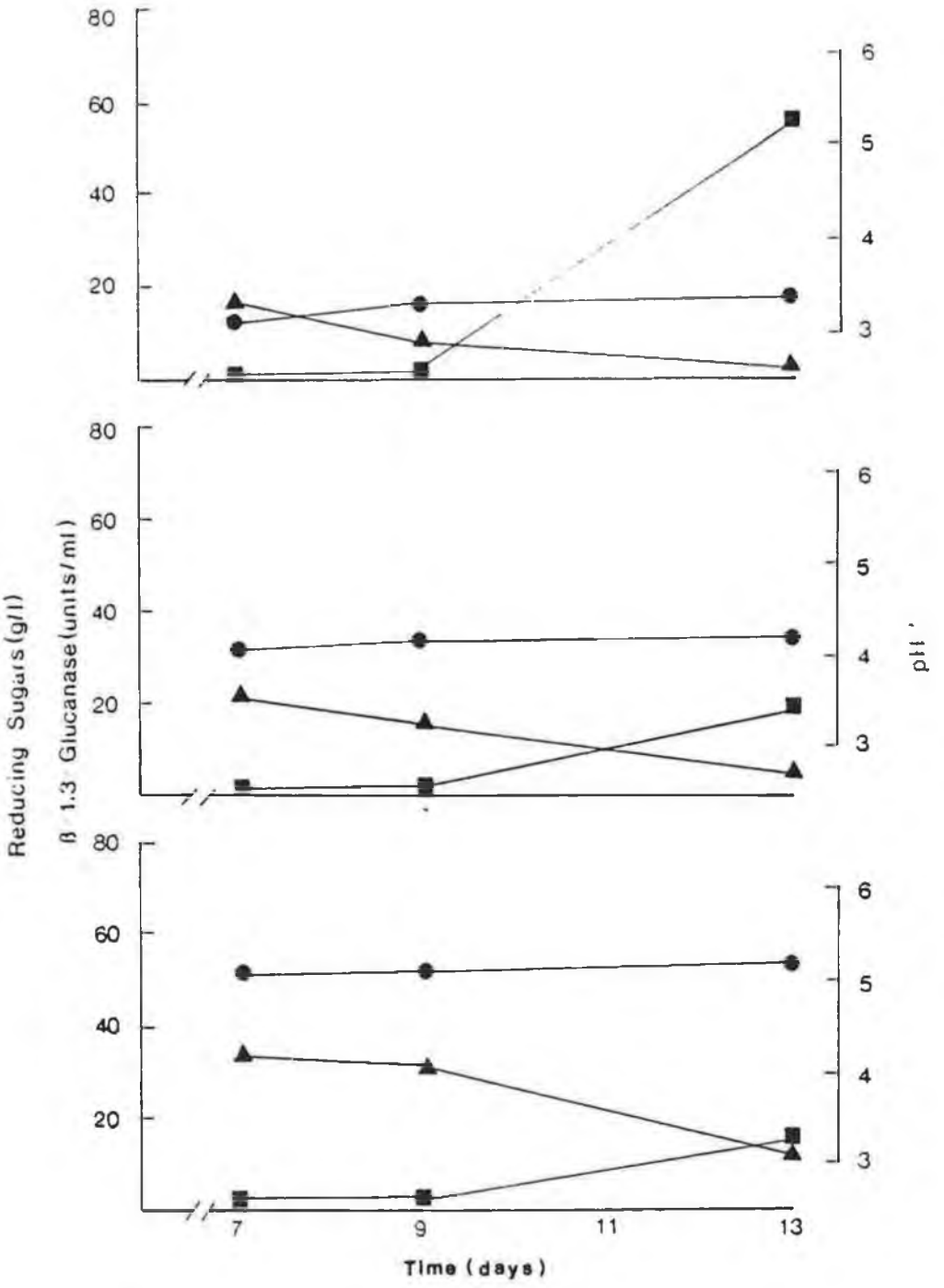

Figure 3.5. Production of β-1,3-glucanase in Medium D buffered at pH 3.0, pH 4.0 and pH 5.0, 90ml in 250ml conical flasks, inoculated 10% from a 3-4 day old culture grown in Medium B, and incubated at 30°C and 150 r.p.m. for 13 days. β-1,3-Glucanase production (■), reducing sugar levels (▲) and pH (●) were monitored.

FIGURE 3.6

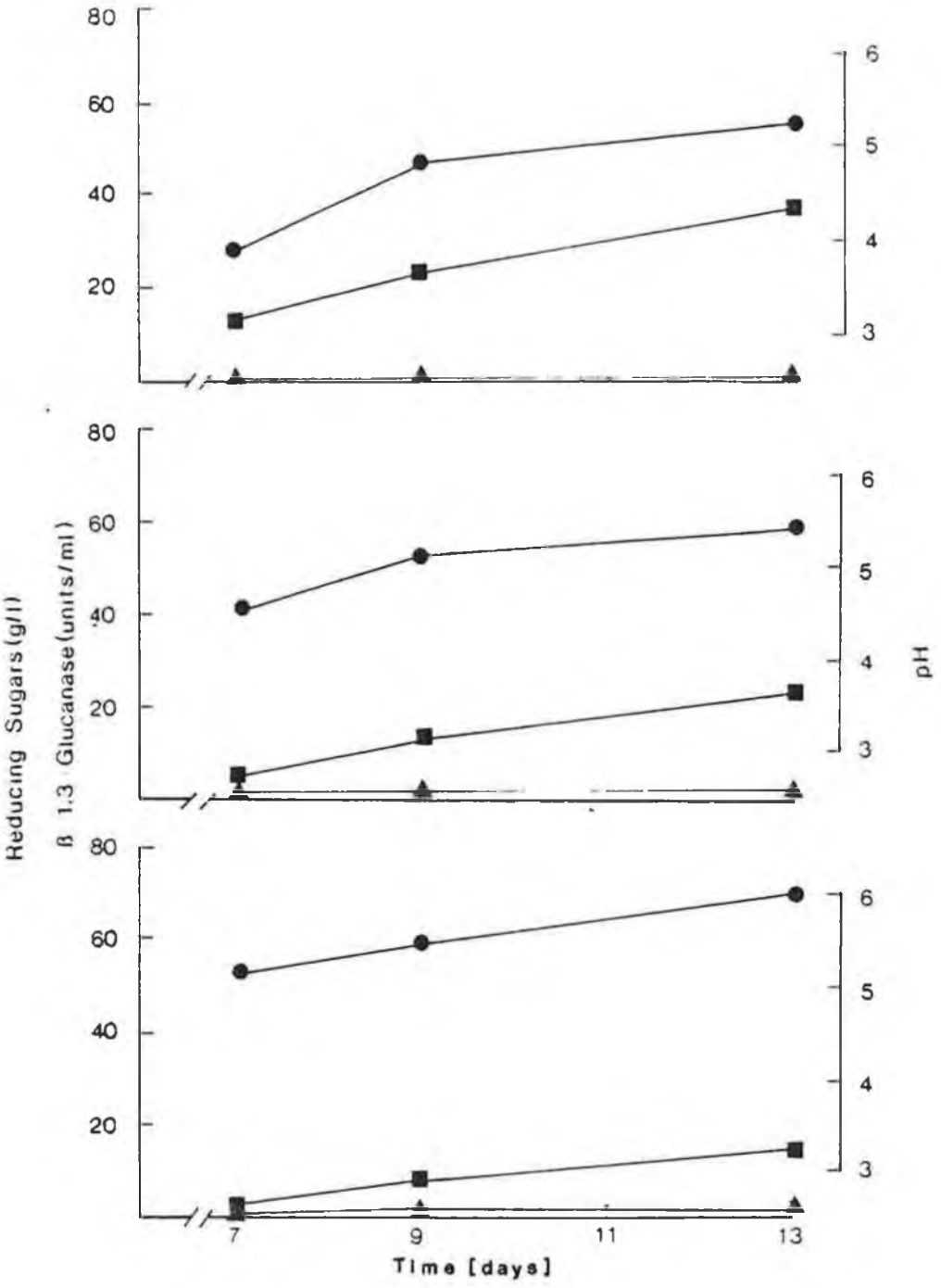

Figure 3.6. Production of β-1,3-glucanase in Medium E buffered at pH 3.0, pH 4.0 and pH 5.0, 90ml in 250ml cronical flasks, inoculated 10% from a 3-4 day old culture grown in Medium B, and incubated at 30°C and 150 r.p.m. for 13 days. β-1,3-Glucanase production (■), reducing sugar levels (▲) and pH (●) were monitored.

FIGURE 3.7

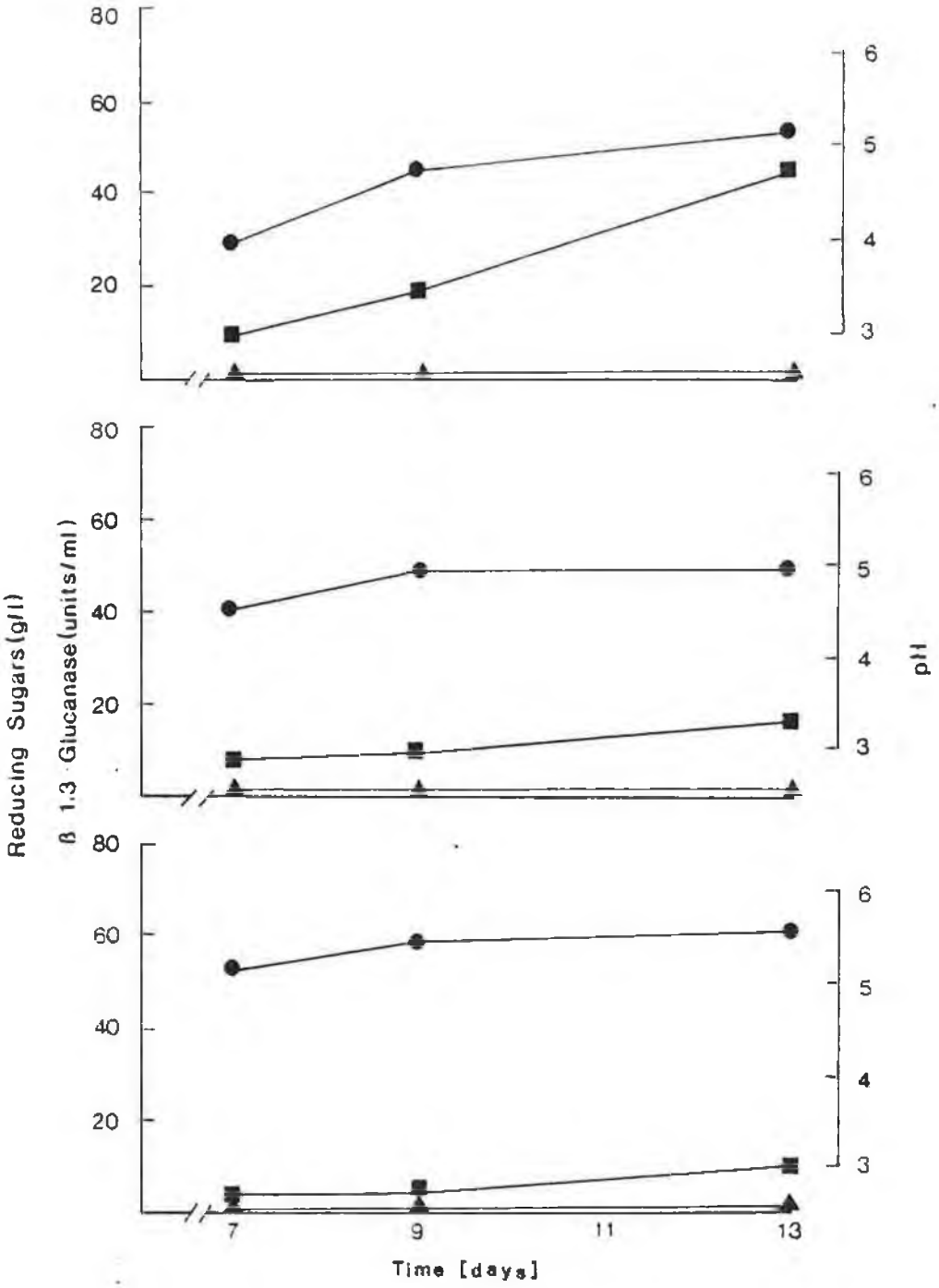

Figure 3.7. Production of β-1,3-glucanase in Medium F buffered at pH 3.0, pH 4.0, and pH5.0, 90ml in 250ml conical flasks, inoculated 10% from a 3-4 day old culture grown in Medium B, and incubated at 30°C and 150 r.p.m. for 13 days. β-1,3-Glucanase production (■), reducing sugar levels (▲) and pH (●) were monitored.

3.1.4. Effect of varying soya-flour and glucose levels on β-1,3-glucanase yields

The effect of varying soya-flour and glucose levels in enzyme production media was investigated. 100ml media in 250ml shake flasks were inoculated with 10% v/v of a vegetative inoculum prepared as described in Section 3.1.3. Flasks were incubated under standard conditions (Section 2.3. for 14 days. Media were prepared in 0.2M Na_2HPO_4 -0.1M citric acid buffer pH 3.0. pH, growth and enzyme levels were monitored. Medium constituents and the data obtained are presented in Table 3.2.

Good growth was obtained in all media except in the medium containing the lowest glucose and soya-flour levels used.

The buffering capacity of the citrate-phosphate buffer maintained a low pH but as enzyme was produced, the pH increased by 1 pH unit.

In media containing 10g/l and 20g/l soya-flour, the enzyme yield was higher in media containing the highest glucose concentration.

Good yields of enzyme were achieved in media containing 30 g/l soya-flour, with 55.5U β-1,3-glucanase being produced in the medium without glucose. In this case the soya-flour was the sole carbon source.

Generally better enzyme yields were obtained in media with the higher glucose or soya-flour content.

TABLE 3.2

EFFECT OF VARYING INITIAL SOYA-FLOUR AND GLUCOSE CONCENTRATIONS ON β-1,3-GLUCANASE PRODUCTION BY *BASIDIOMYCETE* sp QM 806

Medium Constituents g/l		Fermentation Time (days)									
		0		3		7		10		14	
Soya-flour (full-fat)	Glucose (food-grade)	pH	Enzyme activity U/ml	pH	Enzyme activity U/ml	pH	Enzyme activity U/ml	pH	Enzyme activity U/ml	pH	Enzyme activity U/ml
10	0	2.97	0.0	3.02	0.0	3.26	14.5	3.53	22.0	3.59	32.0
10	5	3.03	0.0	3.09	0.0	3.36	19.0	3.61	29.0	3.73	31.0
10	10	3.16	0.0	3.21	0.0	3.27	9.0	3.53	3.0	3.82	52.0
20	0	2.97	0.0	3.05	0.0	3.53	17.0	3.89	24.5	4.40	31.0
20	5	2.97	0.0	3.06	0.0	3.33	18.5	3.96	29.5	4.15	40.0
20	10	2.96	0.0	3.05	0.0	3.16	16.0	3.67	29.0	4.10	43.0
30	0	3.02	0.0	3.11	0.0	3.63	13.5	4.15	27.5	4.56	55.5
30	5	3.00	0.0	3.11	0.0	3.49	14.5	4.03	24.5	4.49	45.0
30	10	2.03	0.0	3.03	0.0	3.13	11.0	3.75	17.0	4.21	45.0

Basidiomycete sp QM 806 was grown in media buffered at pH3.0 in 0.2M Na_2HPO_4-0.1M citric acid buffer, 90ml in 250ml conical flasks, inoculated 10% v/v vegetative inoculum (Section 3.1.3) and incubated at 30°C and 150 r.p.m for 14 days.

3.1.5. Glucose feeding during fermentation

The effect of glucose feeding during the fermentation was investigated.

Enzyme production flasks, containing 90ml medium C.A. (section 2.5.3) were inoculated with a 10% v/v vegetative inoculum prepared as described in Section 3.1.3. Flasks were incubated under standard conditions (Section 2.3) for 13 days.

Glucose consumption was monitored from the third to the sixth day of the fermentation and adjusted as required. When the level of reducing sugars had decreased to less than 5g/l, the level was adjusted to 5g reducing sugar /l. A control was used in which the levels of reducing sugar were not monitored.

Pelleted growth was obtained. (Section 2.9).

The results are presented in Table 3.3.

The β-1,3-glucanase level produced after six days in the adjusted medium was much lower than that obtained in the non-adjusted medium. When the level was no longer adjusted and the glucose consumed β-1,3-glucanase production was initiated. In the non-adjusted control medium, enzyme was produced earlier due to the non-adjustment of the glucose level once consumed.

Friebe and Holldorf (1975) also conducted experiments on glucose feeding during fermentation. They found that addition of low concentrations of glucose caused a limited temporary decrease in activity and high concentrations of glucose resulted in a complete loss of β-1,3-glucanase activity.

Table 3.3

Effect of glucose feeding on β-1,3-glucanase production

Time (days)	Non-adjusted medium pH	β-1,3-glucanase activity U/mlCFS	Adjusted medium pH	β-1,3-glucanase activity U/mlCFS
7	3.20	26.5	3.09	8.5
9	3.46	50.0	3.34	35.0
13	3.56	70.0	3.56	51.0

Basidiomycete sp QM 806 was grown in medium C.A. (Section 2.5.3), 90ml in 250ml conical flasks, inoculated with a 10% v/v vegetative inoculum (Section 3.1.3) and incubated at 30°C and 150 r.p.m. for 13 days.

Glucose was fed when the reducing sugar level was less than 5g/l. Feeding was discontinued after the sixth day of fermentation.

3.1.6. Effect of culture medium supplements on growth and enzyme production by Basidiomycete sp QM 806

The effect of two supplements was investigated. Carbopol-934 and Tween-80 were added to media. Carbopol-934 an anionic, carboxypolymethylene polymer, has been shown to be effective in dispersing growth of Aspergillus niger (Elmayergi et al, 1973) and Rhizopus arrhizus (Byrne, 1985).

Tween-80 a surfactant has been shown to stimulate enzyme production in a range of fungi, including Basidiomycete sp QM 806 (Reese and Maguire, 1969) and to disperse growth (Takahashi et al, 1960).

Basidiomycete sp QM 806 was grown in medium C-A (Section 2.5.3) with or without supplements, 90ml in 250ml conical flasks, inoculated with a 10% v/v vegetative inoculum prepared as described in Section 3.1.3 and incubated under standard conditions. (Section 2.3) for 14 days. β-1,3-Glucanase production growth and pH were monitored. The media constituents and data obtained are presented in Table 3.4.

Dispersed growth was not obtained in the media supplemented with Carbopol-934 or Tween-80. The usual form of pelleted growth was found. Tween-80 did not stimulate enzyme production, rather it appeared to inhibit production. The conditions used in this investigation were different to those used by Reese and Maguire (1969), who used a medium containing 0.3% lactose and 0.2% cellobiose. Under more favourable conditions enzyme stimulation may have occurred.

Table 3.4

Effect of supplementing media with Carbopol-934 or Tween-80

Time (days)	Medium C.A. No supplement		Medium C.A. 0.1%v/v Tween-80		Medium C.A. 0.3% v/v Carbopol-934	
	pH	β-1,3-glucanase U/ml	pH	β-1,3-glucanase U/ml	pH	β-1,3-glucanase U/ml
0	2.98	0.0	2.95	0.0	2.95	0.0
3	3.03	0.0	2.99	0.0	2.99	0.0
7	3.10	11.5	3.05	3.0	3.03	11.5
10	3.57	25.0	3.42	3.0	3.43	25.5
14	3.55	39.0	3.47	3.0	3.60	45.0

Basidiomycete sp QM 806 was grown in medium C.A (Section 2.5.3), with or without Tween-80 or Carbopol-934, 90ml in 250 ml conical flasks, inoculated with a 10% v/v vegetative inoculum (Section 3.1.3) and incubated at 30°C and 150 r.p.m. for 14 days.

3.1.7. Measurement of protease side activity

The lytic effect of the Basidiomycete sp. QM 806 culture supernatant in addition to the effect of other commercial enzymes (Kitalase, Novozym-234) on yeast autolysis was investigated in other experiments. The lytic effect is mainly due to the presence of β-glucanases and proteases. It was decided to assay the protease activity present in the Basidiomycete culture supernatant.

A simple method of applying culture supernatant to wells in skimmed milk agar was developed (Section 2.6.3.2). After incubation, zones of hydrolysis were found, where the protease present in the supernatant had acted upon the skimmed milk powder present in the agar. Zones of 1.2cm diameter on average were obtained, measuring from the centre of the well to the edge of the zone of hydrolysis. The 0.1ml enzyme supernatant applied to the well contained 5U β-1,3-glucanase activity as determined by the method described in Section 2.6.2. This method of measuring protease activity was used qualitatively to determine the presence or absence of protease activity.

A quantitative method was also used to determine protease activity. The method was based on Anson's method (1939) and involved using haemoglobin as a substrate for the protease. Other proteases were assayed in conjunction with the Basidiomycete sp QM 806 protease. Protease activity in the Basidiomycete supernatant was assayed under standard conditions. (Section 2.3). The data obtained are presented in Table 3.5. Protease levels in the other preparations appear much higher than those present in the Basidiomycete supernatant, however levels were expressed as activity /g whereas activity /ml was used to define the Basidiomycete protease activity.

Table 3.5
Levels of protease activity in various preparations

Enzyme	Protease activity HUT/g or ml
L.P. conc (protease)	600,000 /g
Papain (protease)	200,000 /g
Kitalase (yeast lytic enzyme)	40,000 /g
Basidiomycete protease	400 /ml

The protease: β-1,3-glucanase ratio for the Basidiomycete sp QM 806 enzyme is 8:1 and for Kitalase 267:1. (Kitalase when assayed for β-1,3-glucanase (Section 2.6.2) was found to have 150 U/g)

3.1.8. Appraisal of results

This work showed that β-1,3-glucanase production was initiated once the carbon source was consumed to levels <5g/l.

It was found that the best growth and enzyme yields occurred at pH 3.0 in a medium consisting of soya-flour and glucose. A standard medium containing: soya-flour (full-fat), 10g/l; glucose (food-grade), 40g/l; adjusted to pH 6.0 (used as the vegetative inoculum) and a medium containing : soya-flour (full-fat), 10g/l, and glucose (food-grade), 10g/l; in citrate-phosphate buffer at pH 3.0 (used as the production medium) were developed. Vegetative inocula were found to reduce the lag when transferred into production medium when

compared to spore inocula. Levels of about 50 U/ml Β-1,3-glucanase were produced after 13-14 days under these conditions.

400 units /ml protease were produced by the Basidiomycete organism. The protease: Β-1,3-glucanase ratio was 8:1 for the Basidiomycete enzyme supernatant. Glucose feeding was found to inhibit Β-1,3-glucanase production but once the feeding was discontinued and glucose consumption was resumed, enzyme was produced. Tween-80 and Carbopol-934 were found to have no effect on growth or enzyme production, in fact Tween-80 appeared to inhibit production.

3.2. SCALE UP OF Β-1,3-GLUCANASE PRODUCTION PROCESS

Having established that Basidiomycete sp QM 806 grew well and produced reproducible levels of Β-1,3-glucanase in soya-flour/glucose medium, an attempt was made to grow the organism in this medium in a Microferm fermenter. (Section 2.4).

3.2.1. Growth in a Microferm Fermenter

A 10% v/v inoculum was developed in Medium B, under standard conditions. (Section 3.1.3). 10 inoculum flasks were used to develop the 1 litre inoculum. The production medium used was medium C.A. with the pH held at pH 3.0 with HCl rather than with citrate-phosphate buffer at pH 3.0. The fermenter maintained the pH at pH 3.0 and fed in HCl as required. The geometry of the fermenter and the conditions used are described in Section 2.4.

The results obtained are presented in Figure 3.8.

FIGURE 3.8

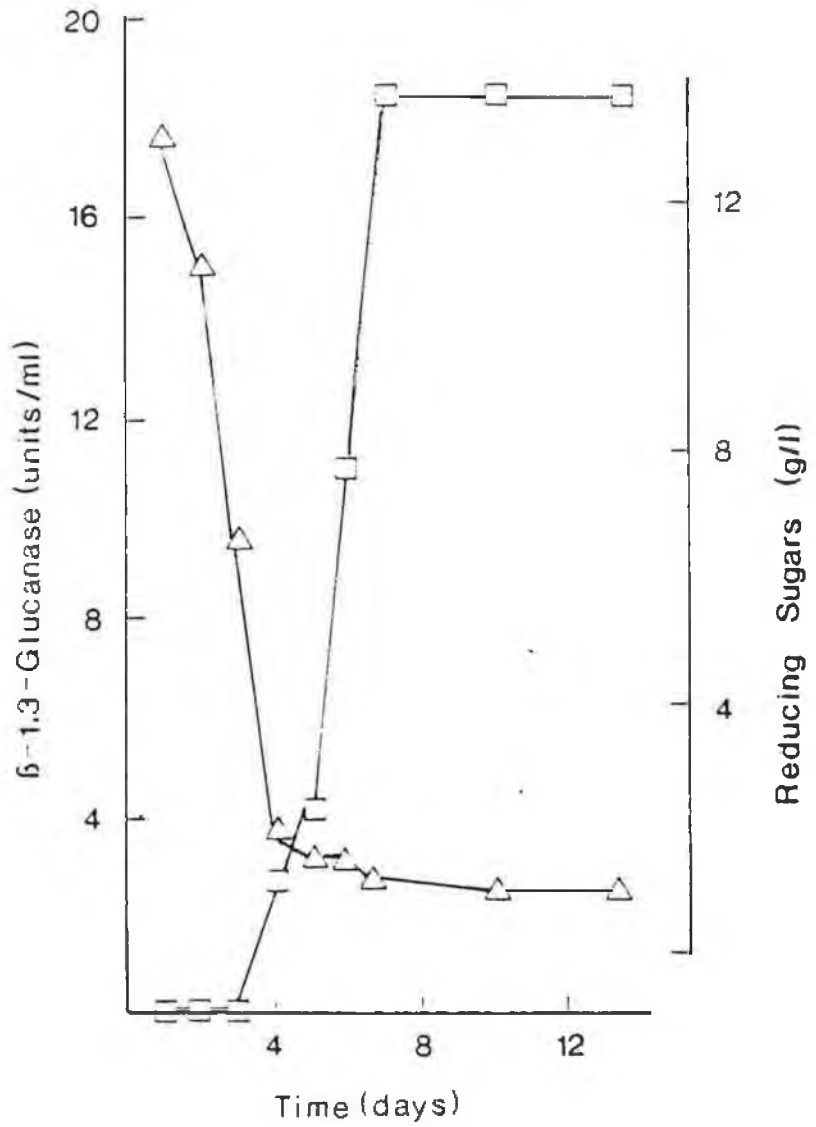

Figure 3.8. Basidiomycete sp QM 806 was grown in a Microferm fermenter, containing 9 litres Medium C, (Section 2.5.3) with pH adjusted to pH 3.0 with HCl, inoculated 10% v/v vegetative inoculum from a 3-4 day old culture in Medium B (Section 2.5.2) and incubated at 30°C, 200 r.p.m, and 0.5 v/v/m (Section 2.4) for 13 days. Reducing sugar levels(△) and β-1,3-glucanase production(□) were monitored.

The level of β-1,3-glucanase activity found in the fermenter was 18.5 U/ml cell free supernatant (CFS) after 13 days compared to about 50U/mlCFS in shake-flasks.

Mycelial growth was obtained after 3-4 days incubation and was maintained throughout the fermentation. In shake-flasks growth was always of the pelleted type. Photographs of both morphologies are presented in Figures 3.9 and 3.10.

The change in morphology from shake-flask to fermenter could be due to a number of reasons. The dimensions of the fermenter were different to those of a shake-flask as were the aeration and agitation levels.

Further experiments are required to elucidate the optimal conditions for β-1,3-glucanase production in fermenters.

FIGURE 3.9

Figure 3.9. Basidiomycete sp QM 806 was grown in a Microferm fermenter, containing 9 litres Medium C, with pH adjusted to pH 3.0 with HCl, inoculated 10% v/v vegetative inoculum from a 3-4 day old culture in Medium B, and incubated at 30°C, 200 r.p.m. and 0.5 v/v/m for 13 days. The photograph shows mycelial growth, taken at X400 magnification, obtained after3-4 days incubation.

Figure 3.10

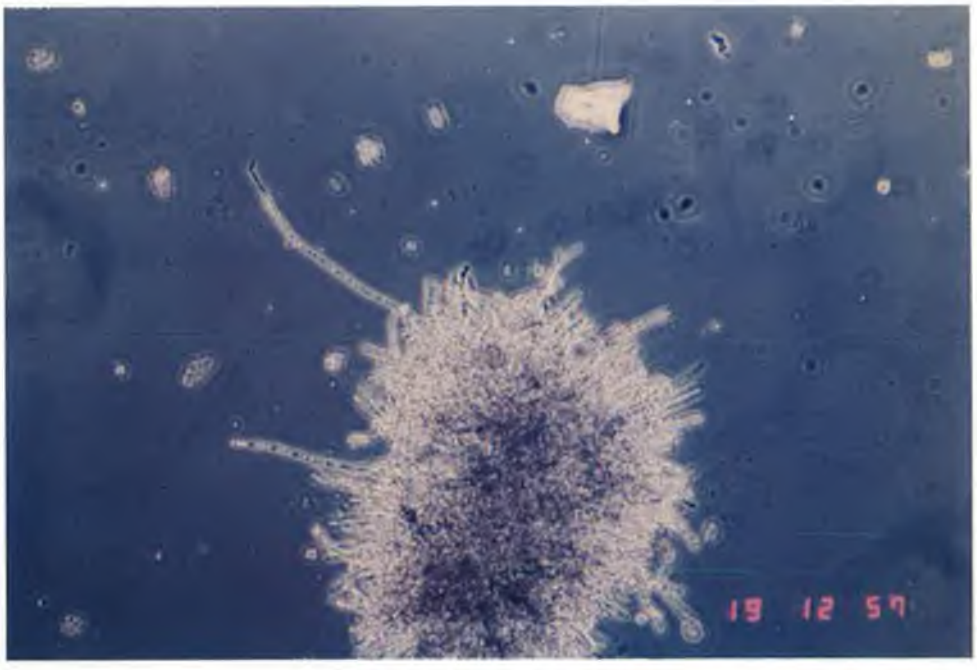

Figure 3.10. Basidiomycete sp QM 806 was grown in a 250ml conical flask, containing 90mls Medium C, buffered at pH3.0, inoculated 10% v/v vetetative inoculum from a 3-4 day old culture in Medium B, and incubated at 30°C and 150 r.p.m. for 13 days. The photograph shows pelleted growth, taken at X400 magnification, obtained after 3-4 days incubation.

3.3. CHARACTERIZATION OF Β-1,3-GLUCANASE

The following experiments were carried out to establish the pH and temperature optima of the enzyme.

3.3.1. Effect of pH on Β-1,3-glucanase activity

Β-1,3-Glucanase activity in Basidiomycete sp QM 806 shake-flask culture supernatants was assayed in a range of buffers, from pH 2.5 to pH 8.0 at 50°C. The laminarin substrate (2%) was prepared in 0.2M Na_2HPO_4-0.1M citric acid buffers (Appendix 1). The enzyme dilutions were prepared in distilled water. The assay was conducted according to the method described in Section 2.6.2.

The results are presented in Figure 3.11.

The maximum activity was found at pH 4.5 - 5.0.

FIGURE 3.11

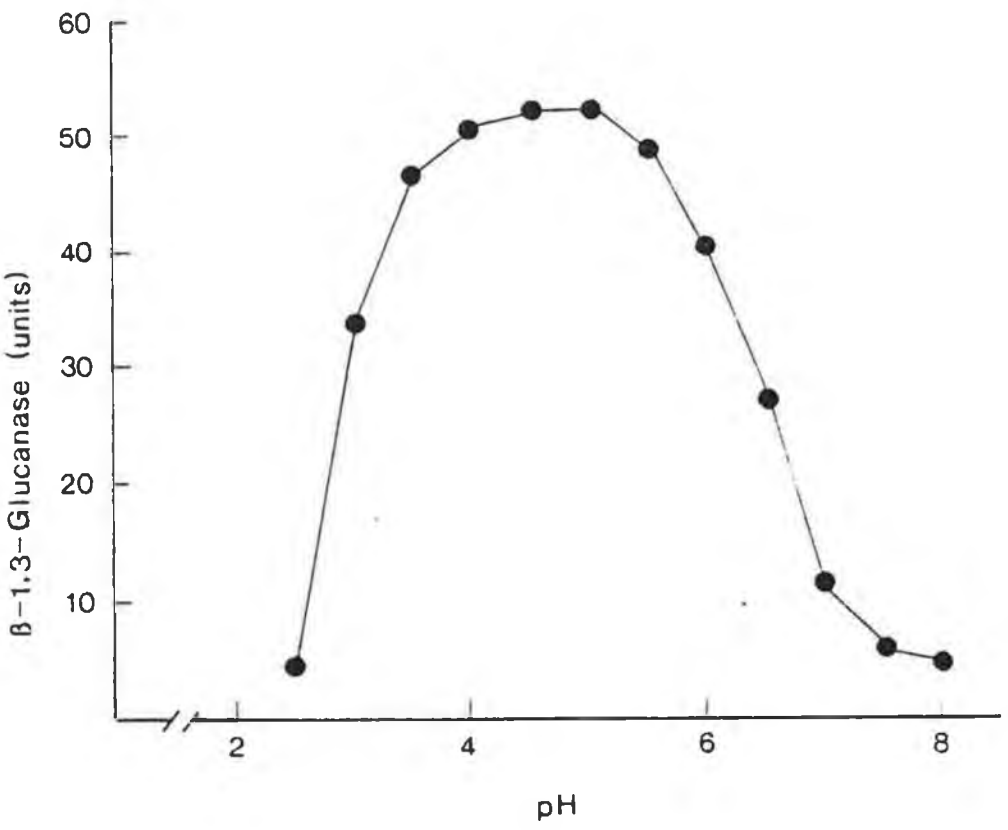

Figure 3.11. Effect of pH on β-1,3-glucanase activity. β-1,3 -Glucanase activity was assayed at 50°C in the pH ranges pH 2.5 - pH 8.0.

3.3.2. Effect of temperature on β-1,3-glucanase activity

Basidiomycete sp QM 806 culture supernatants were assayed at pH 5.0 and at a range of temperatures from 4°C to 70°C (Section 2.6.2).

The results are presented in Figure 3.12.

The temperature at which activity was at maximum was 60°C. Above this inactivation was rapid. 50°C has been used in all β-1,3-glucanase assays as this was considered to be a good operating temperature.

FIGURE 3.12

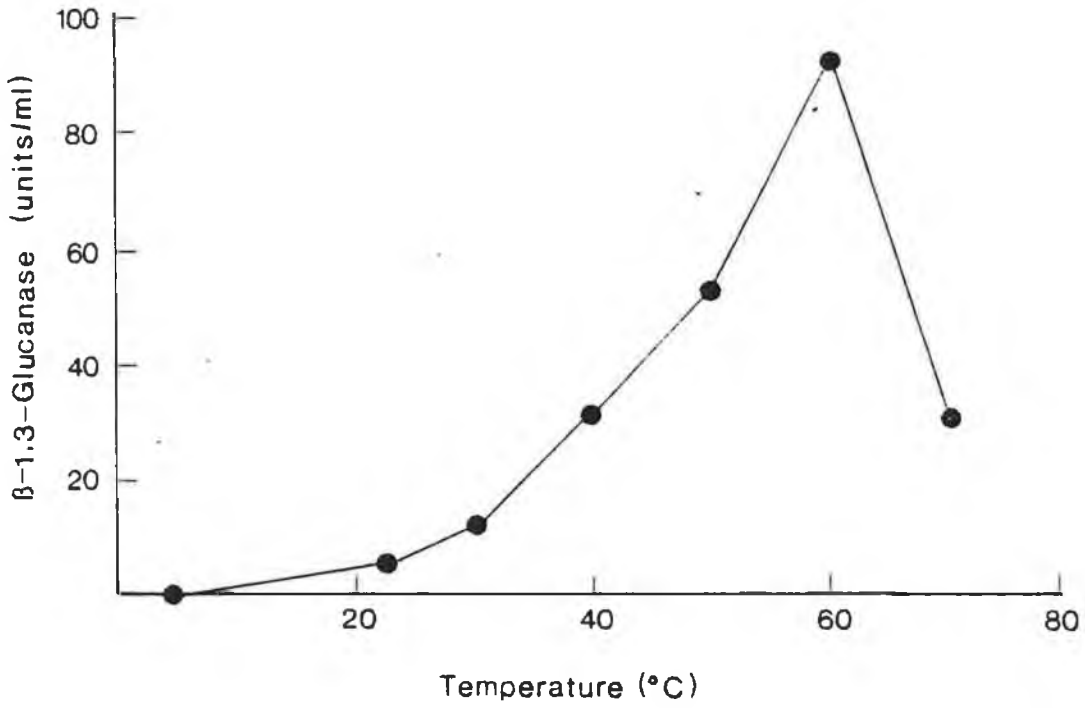

Figure 3.12. Effect of temperature on β-1,3-glucanase activity. β-1,3-Glucanase activity was assayed at pH 5.0 in the temperature range 4° C-70° C.

3.4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (H.P.L.C) ANALYSIS OF YEAST GLUCAN AND LAMINARIN DEGRADATION PRODUCTS

High Performance Liquid Chromatography (HPLC) of unsubstituted sugars using Refractive Index detection is a rapid and convenient method of analysis. The column used in this work was of the reversed-phase type and has been developed especially for the separation of oligomers. Monosaccharides elute first, with the higher oligomers eluted later in order of their degree of polymerization.

Oligosaccharides derived from starch, dextran, cellulose and xylan have been separated using this column (Cheetham and Sirimanne, 1981).

In this work oligosaccharide production from laminarin and yeast glucan by enzyme hydrolysis has been investigated. Laminarin and yeast glucan have a preponderance of β(1-3) links. Kitalase, Novozym-234, and the Basidiomycete sp QM 806 culture supernatant have β-1,3-Glucanase activity which act upon those β(1-3) links to yield various products. The type of product yielded indicates the hydrolytic mechanism of the β-1,3 glucanase. If glucose is the major product, this indicates a major exohydrolytic or endwise-spitting mechanism. Di, tri and higher saccharides indicate a major endohydrolytic or random-spitting mechanism.

3.4.1 Laminarin degradation

Laminarin is known to predominantly contain glucose as the major subunit linked by β-1,3-glycosidic bonds.

Laminarin (Sigma) a crude preparation from Laminaria digitata was treated with Kitalase, Novozym-234 and

Basidiomycete sp QM 806 culture supernatant. The laminarin degradation products were analysed using H.P.L.C., according to the method described in Section 2.8.7. Well defined peaks were obtained and the anomeric forms of β-gentiobiose and laminaribiose were observed.

3.4.1.1. Effect of the Basidiomycete sp QM 806 β-1,3-glucanase on laminarin

Laminarin was enzymatically degraded using the culture supernatant from Basidiomycete sp QM 806 (2.5U β-1,3-glucanase/ 10mg laminarin). Breakdown product production was followed with time, and the products analysed using HPLC (Section 2.8.7). The chromatogram obtained after 210 minutes treatment is contained in Appendix III.

Peaks with retention times of 2.9, 3.75, 4.70, 5.50 and 11.2 minutes were obtained. The first peak at 2.9 minutes was due to citrate buffer. Peaks obtained at 3.75, 4.70 and 5.50 minutes were found to be due to glucose, β-gentiobiose and laminaribiose respectively, when compared to a standard solution containing the three saccharides. Glucose, β-gentiobiose, and laminaribiose production were followed with time by using information from the chromatograms, and amounts of each produced were estimated from the standard curves (Sections 2.8.3, 2.8.4, 2.8.5 and 2.8.8). The results are presented in Figure 3.13.

The peak with the retention time of 11.2 minutes, was probably due to a higher oligomer, with a degree of polymerization (DP) greater than two since disaccharides had been eluted after 5-6 minutes. This peak was first observed after 5 minutes degradation and reached a maximum peak height and therefore concentration, at 120 minutes, and gradually decreased. This decrease was probably due to its degradation.

FIGURE 3.13

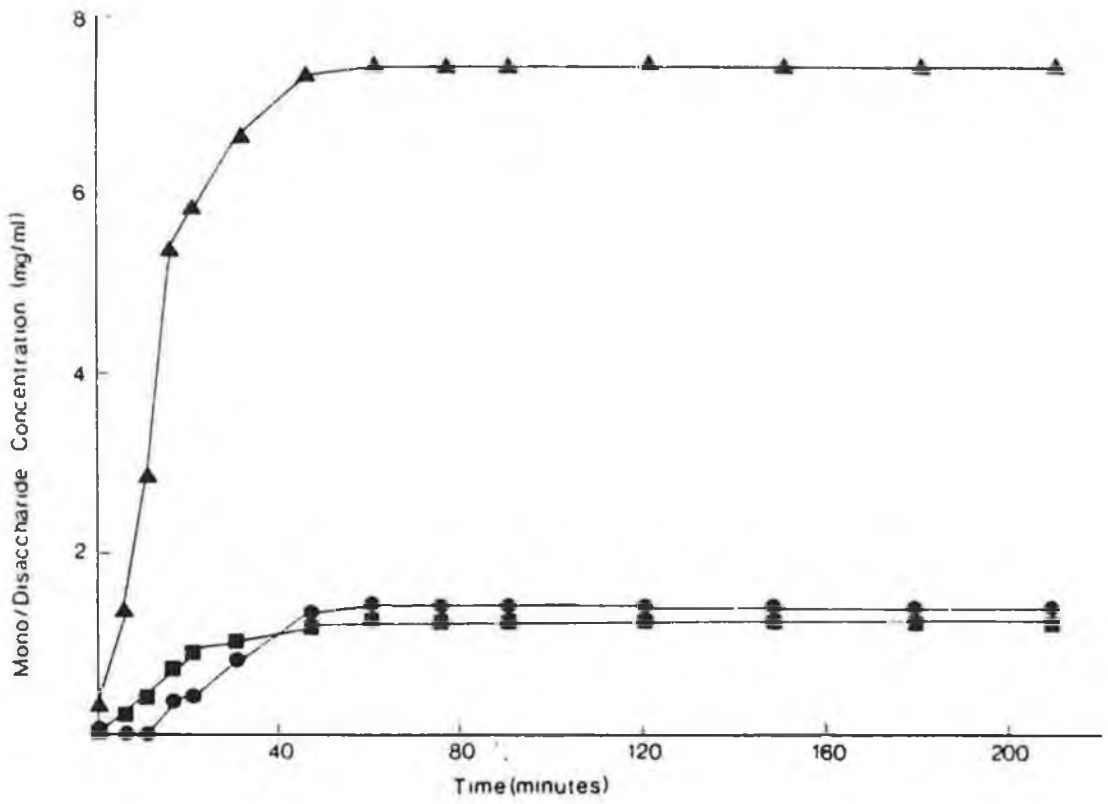

Figure 3.13. Laminarin was treated with the enzyme supernatant from Basidiomycete sp QM 806. (Section 2.8.7), for 210 minutes at 50° C. Degradation products were analysed using H.P.L.C., and glucose (▲), B-Gentiobiose (■) and laminaribiose (●) production followed with time.

Glucose and β-gentiobiose were produced after 5 minutes and laminaribiose after 15 minutes. A background level of 0.25 mg glucose /ml (To) was found, this was probably due to residual sugar left in the culture medium or due to glucose contamination of laminarin.

Glucose was the major product, with 7.30 mg/ml yielded, and 1.35 mg/ml and 1.30 mg/ml β-gentiobiose and laminaribiose yielded respectively after 210 minutes treatment. This indicated a major exohydrolytic β-1,3-glucanase component with a minor endohydrolytic component. Chesters and Bull (1963) in their paper chromatographic analysis of laminarin digests also found a major exohydrolytic component and a minor endohydrolytic component in the Basidiomycete sp QM 806 culture supernatants.

After 60 minutes treatment, no further production of glucose, β-gentiobiose or laminaribiose took place, with levels of each remaining constant.

3.4.1.2. Effect of Kitalase on laminarin

Laminarin was enzymatically degraded using Kitalase (Kumiai Chemical Co), 2.5U β-1,3-Glucanase/10mg laminarin for 210 minutes (Section 2.8.7). This preparation is an enzyme mixture from Basidiomycetes aphyllophoroles. Degradation products were analysed using H.P.L.C. The chromatogram obtained after 210 minutes treatment is contained in Appendix III.

The elution times for citrate buffer, glucose, β-gentiobiose, and laminaribiose were the same as those found in Section 3.4.1. No other peaks were observed. Glucose, β-gentiobiose and laminaribiose production were followed with time using the chromatograms and the amounts of each produced, estimated. The results are presented in Figure 3.14.

FIGURE 3.14

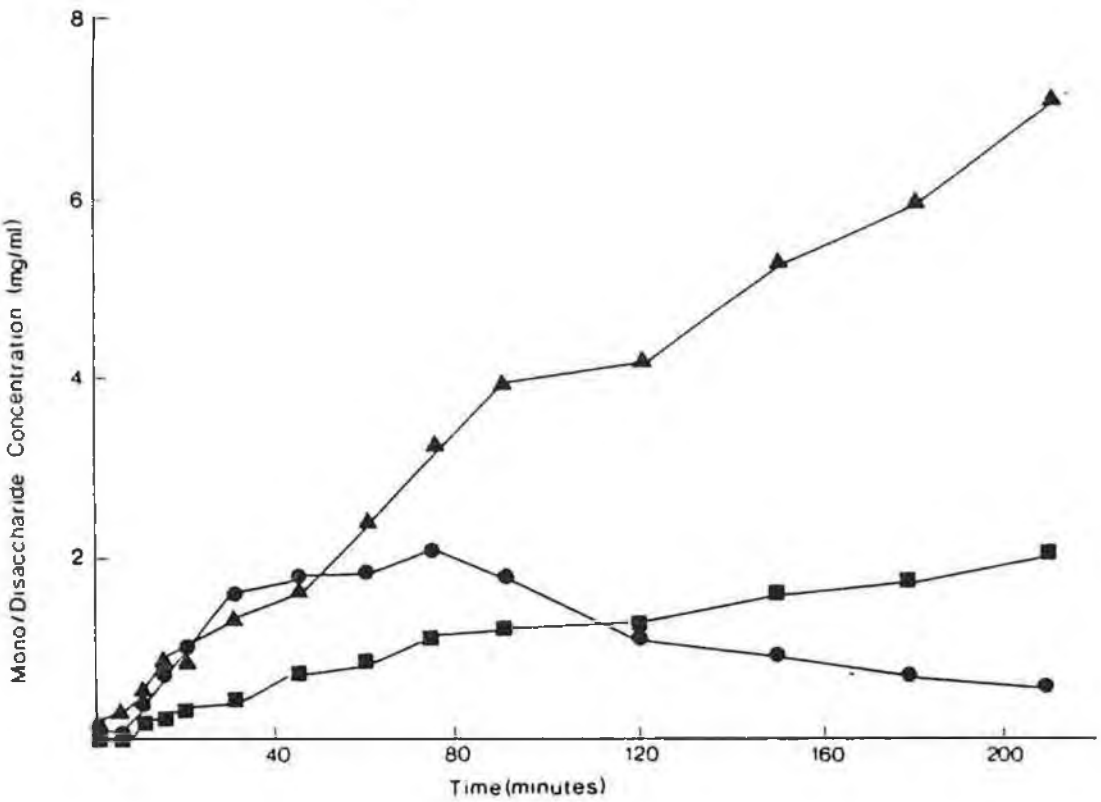

Figure 3.14. Laminarin was treated with Kitalase, (Section 2.8.7) for 210 minutes at 50°C. Degradation products were analysed using H.P.L.C., and glucose (▲), β-Gentiobiose (■) and laminaribiose (●) production followed with time.

Glucose was produced after 5 minutes enzymatic treatment and β-gentiobiose and laminaribiose after 10 minutes. Glucose and β-gentiobiose were still being produced after 210 minutes. Laminaribiose production had reached a maximum of 2.2 mg/ml after 75 minutes but thereafter the levels decreased with time, and after 210 minutes only 0.65mg/ml laminaribiose remained. This decrease suggested that laminaribiose was being degraded, possibly to a smaller saccharide such as glucose.

3.4.1.3 Effect of Novozym-234 on laminarin

Laminarin was degraded using Novozym-234 (Novo Biolabs), 2.5U β-1,3-glucanase/10mg laminarin for 210 minutes at 50°C. Novozym-234 contains α-1,3-glucanase, laminarinase, xylanase, chitanase and protease activities. The method used is described in Section 2.8.7. The laminarin degradation products were analysed using H.P.L.C. The chromatogram obtained after 210 minutes treatment is included in Appendix III.

The elution times for citrate buffer, glucose, β-gentiobiose and laminaribiose were the same as those found in Section 3.4.1.1 No other peaks were observed. Glucose, β-gentiobiose and laminaribiose production was monitored and the results are presented in Figure 3.15.

Glucose production continued for 150 minutes, after which time levels remained constant at 6.3 mg/ml. β-gentiobiose production also continued, for about 60 minutes after which time levels stabilized at 1.35 mg/ml. Laminaribiose production reached a maximum of 2.7 mg/ml after 15 minutes, then the level decreased gradually to 0.20 mg/ml after 210 minutes.

FIGURE 3.15

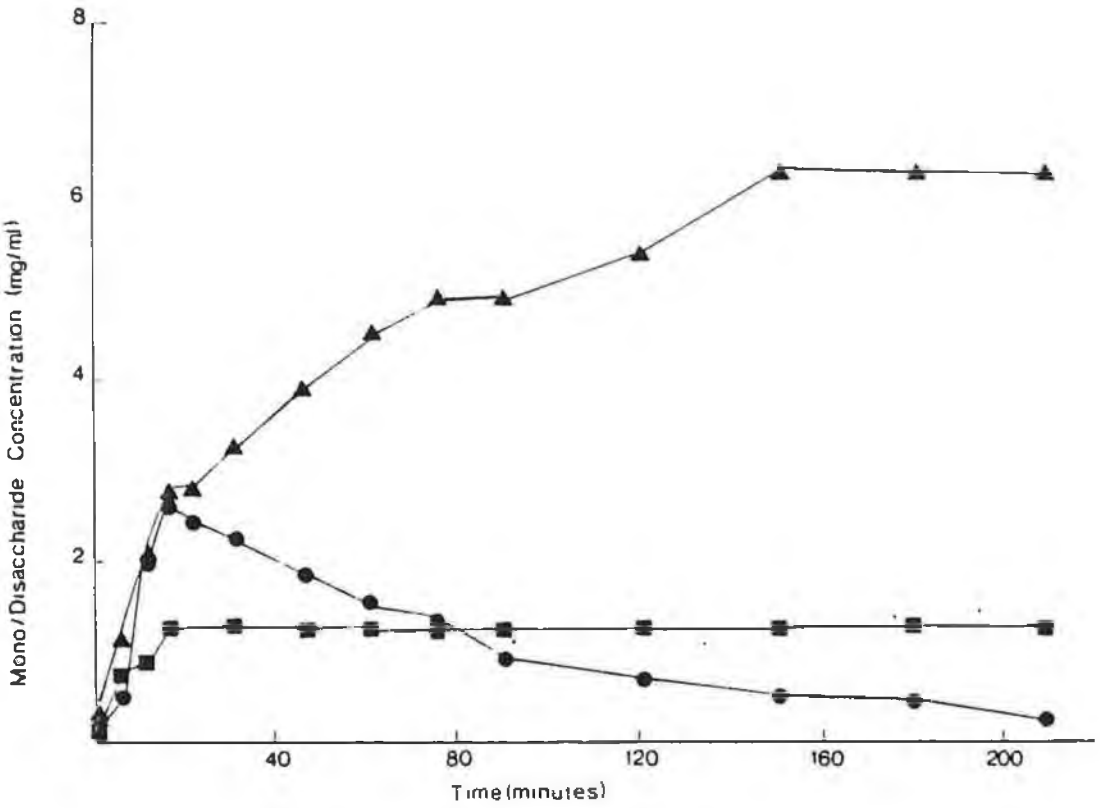

Figure 3.15. Laminarin was treated with Novozym-234,(Section 2.8.7) for 210 minutes at 50° C. Degradation products were analysed using H.P.L.C. and glucose (▲), β-Gentiobiose (■) and laminaribiose (●) production followed with time.

3.4.2 Yeast glucan degradation

Yeast glucan treatment with Kitalase, Novozym-234 and the Basidiomycete sp QM 806 culture supernatant was investigated and the degradation products were analysed using H.P.L.C. (Section 2.8.7). Yeast glucan was prepared by a method based on that of Bacon et al (1969). The procedure is described in Appendix II.

Generally the β-gentiobiose and laminaribiose peaks were not as well defined as the standard peaks or those obtained from laminarin degradations.

3.4.2.1. Effect of Basidiomycete sp QM 806 β-1,3-glucanase on yeast glucan

Degradation products yielded after yeast glucan was treated with the Basidiomycete enzyme were analysed using H.P.L.C. (2.5U β-1,3-glucanase/10mg yeast glucan). The chromatogram obtained after 210 minutes is contained in Appendix III. Section 2.8.6.

Peaks with retention times of 2.1, 3.0, 3.75, 4.7 and 5.4 minutes were obtained. The peaks at 3.0, 3.75, 4.7 and 5.4 minutes were due to citrate buffer, glucose, β-gentiobiose and laminaribiose respectively. The peak at 2.1 minutes was due to an unknown substance. It was first observed after 30 minutes and was still being produced after 210 minutes.

Glucose, β-gentibiose and laminaribiose production were monitored and levels of each estimated from standard curves (Sections 2.8.3, 2.8.4 and 2.8.5). The results are presented in Figure 3.16.

Glucose was the major product with 1.6mg/ml produced after 210 minutes. Lower amounts of β-gentiobiose 0.15mg/ml and laminaribiose 0.55mg/ml were obtained.

FIGURE 3.16

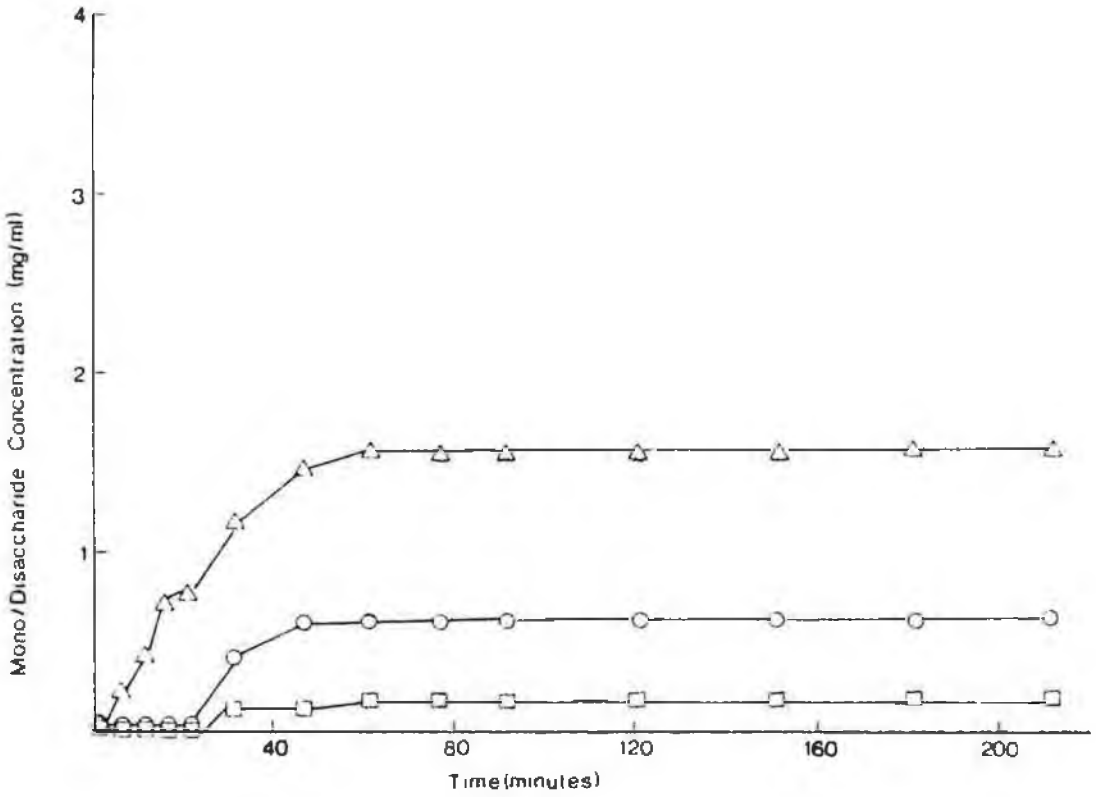

Figure 3.16. Yeast glucan was treated with the enzyme supernatant from Basidiomycete sp QM 806 (Section 2.8.6) for 210 minutes at 50° C. Degradation products were analysed using H.P.L.C. and glucose (△), Б-Gentiobiose (□) and laminaribiose (O) production followed with time.

3.4.2.2. Effect of Kitalase on yeast glucan

Yeast glucan was treated with Kitalase (2.5U β-1,3-glucanase/ 10mg Yeast glucan). Degradation products were analysed using H.P.L.C.. The chromatogram obtained after 210 minutes treatment is included in Appendix III. See Section 2.8.6. The elution times for citrate buffer, glucose and β-gentiobiose and laminaribiose were the same as those found in Section 3.4.2.1. A peak at 2.15 which was also produced from yeast glucan treatment with Basidiomycete sp QM 806 enzyme, appeared after 10 minutes and its concentration increased with time.

Production of the saccharides was followed with time and the data obtained are presented in Figure 3.17.

Glucose was the major product with 3.55 mg/ml being produced after 210 minutes degradation, and was produced steadily for 150 minutes after which time glucose levels remained constant. β-gentiobiose appeared after 45 minutes and after 210 minutes 0.45 mg/ml was yielded. Laminaribiose was produced after 5 minutes and reached maximum concentration after 15 minutes. Laminaribiose levels decreased to zero after 150 minutes.

FIGURE 3.17

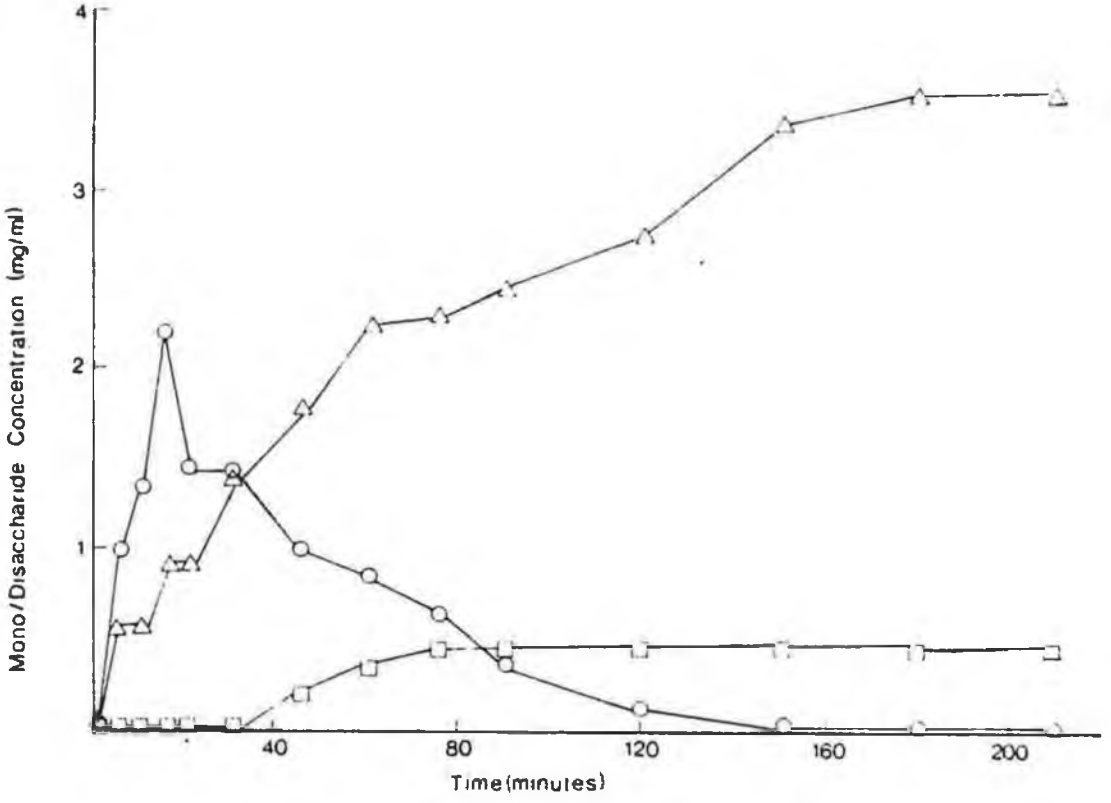

Figure 3.17. Yeast glucan was treated with Kitalase (Section 2.8.6) for 210 minutes at 50°C. Degradation products were analysed using H.P.L.C. and glucose (△), B-Gentiobiose (□) and laminaribiose (O) production followed with time.

3.4.2.3. Effect of Novozym-234 on yeast glucan

Yeast glucan was treated with Novozym-234, 2.5U β-1,3-glucanase /10mg yeast glucan (Section 2.8.6). Degradation products were analysed using H.P.L.C. The chromatogram obtained after 210 minutes treatment is contained in Appendix III.

The elution times for the unknown peak (2.1 minutes), glucose, β-gentiobiose and laminaribiose were the same as those found in Section 3.4.2.1. The unknown peak at 2.10 minutes was not as well defined as those at 2.1 minutes produced by Kitalase and Basidiomycete sp QM 806 culture supernatant treatments. This peak was just a shoulder on the buffer peak, however, its height and therefore concentration increased with time. The shoulder was observed after 45 minutes treatment. Saccharide production was allowed with time and the results are presented in Figure 3.18.

Glucose was again yielded as the major product, with 3.15 mg/ml produced after 210 minutes. β-gentiobiose was produced after 10 minutes and the level remained constant at 2.25 mg/ml. Laminaribiose was produced after 45 minutes with 0.4 mg/ml being produced after 60 minutes, the level then remained constant until 150 minutes, after which time the level decreased to 0.0 mg/ml.

FIGURE 3.18

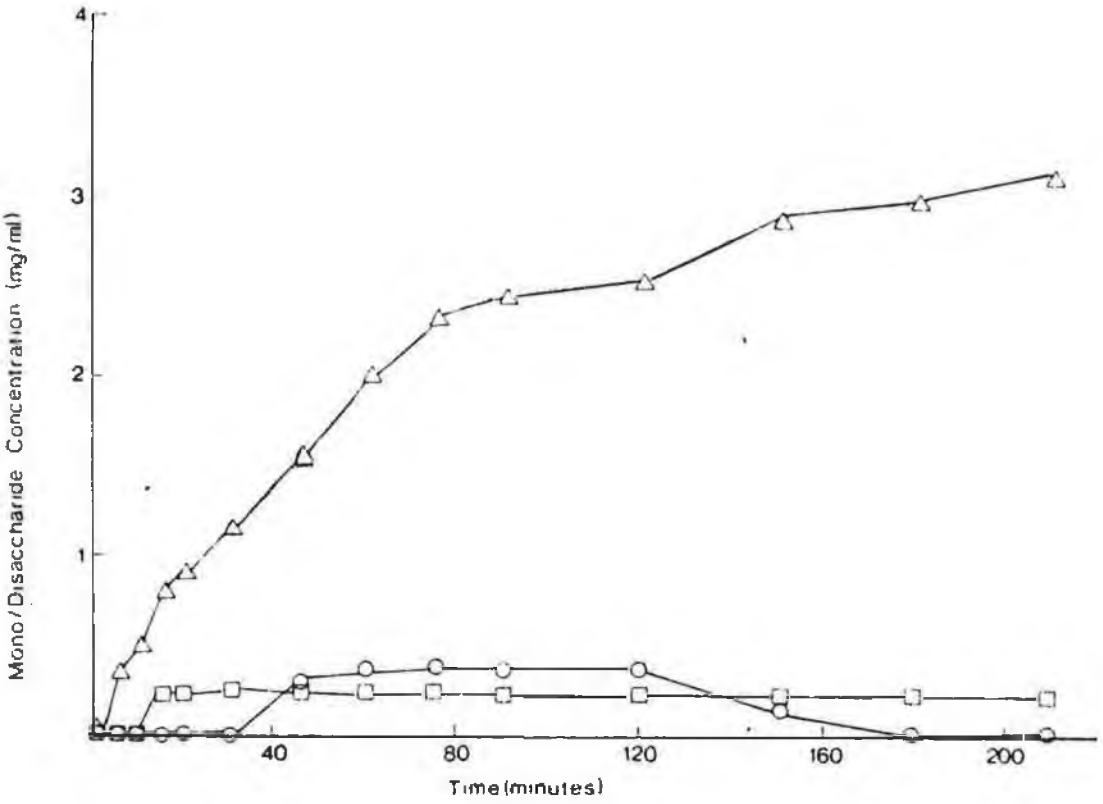

Figure 3.18. Yeast glucan was treated with Novozym-234 (Section 2.8.6) for 210 minutes at 50°C. Degradation products were analysed using H.P.L.C. and glucose (△), β-Gentiobiose (□) and laminaribiose (O) production followed with time.

3.4.2.4. Appraisal of results

Glucose was the major product when laminarin (6-7 mg glucose /ml) and yeast glucan (2-4 mg glucose /ml) were treated with the β-1,3-glucanase from Kitalase, Novozym-234 and the Basidiomycete sp QM 806 culture supernatant, indicating the exohydrolytic mechanism of the enzyme. A minor endohydrolytic component appeared to be present in all preparations, with β-gentiobiose and laminaribiose being produced.

Levels of laminaribiose produced during Kitalase and Novozym-234 treatment of laminarin and yeast glucan decreased after a maximum level was attained. In some cases laminaribiose disappeared altogether. This decrease or disappearance of laminaribiose suggested that the disaccharide itself was being degraded. During laminaribiose degradation, glucose production continued, perhaps laminaribiose was being degraded to glucose. Laminaribiose levels remained constant in Basidiomycete sp QM 806, β-1,3 glucanase treated laminarin and yeast glucan. Interestingly using the Basidiomycete enzyme, the levels of laminaribiose and glucose reached a maximum level at about the same time, and no futher production or reduction of either was observed.

Unlike laminaribiose, β-gentiobiose levels did not decrease after maximum production but remained stable, indicating that it was not being degraded.

Generally other higher polysaccharides were not observed, except for the Basidiomycete sp QM 806 enzyme treatment of laminarin, in which a peak with a retention time of 11.2 minutes was found. Perhaps the level of β-1,3-glucanase used caused their (polysaccharide) instant degradation upon production. Other workers including Phaff (1963) and Nelson

et al (1963) have found higher oligosaccharides, produced during acid and enzyme hydrolysis of yeast glucan and laminarin.

The peak which appeared at 2.1 minutes was present in all chromatograms of enzyme treated yeast glucan but was absent in enzyme treatment of laminarin. This peak was probably due to a monosaccharide or other low molecular weight compound, since glucose eluted after 3.8 minutes.

3.4.3. Laminaribiose degradation

In this investigation, laminaribiose was treated with Novozym-234, Kitalase, and the Basidiomycete enzyme supernatant. (Section 2.8.8), and glucose production after 1 hour treatment estimated using the GOD-PAP method (Section 2.6.4). The results are presented in Table 3.6.

Table 3.6

GLUCOSE PRODUCTION FROM LAMINARIBIOSE

Enzyme	mg Glucose/ml produced
Basidiomycete sp QM 806 culture supernatant	0.09
Novozym-234	0.97
Kitalase	0.98

A solution containing 0.5U β-1,3 glucanase / mg laminaribiose was incubated at 50°C for 1 hour, after which time glucose produced from laminaribiose was estimated.

These results confirm that laminaribiose produced during enzymatic hydrolysis of laminarin and yeast glucan (Sections 2.8.6 and 2.8.7) was being degraded to glucose. During treatment of laminarin and yeast glucan, levels of laminaribiose decreased using Novozym-234 and Kitalase, whilst using the Basidiomycete supernatant, the levels remained stable. A similar result was obtained in this study. Laminaribiose was degraded to glucose using Novozym-234 and Kitalase with 1mg /ml produced, and less than 0.1 mg glucose / ml was produced when laminaribiose was treated with the Basidiomycete preparation. This level may have been due to residual sugars remaining in the culture supernatant.

3.5. STUDIES ON YEAST AUTOLYSIS

Yeast extract is widely produced by plasmolysis and autolysis, although a mechanical cell breakage procedure has been used in the United States.

Autolysis or self-digestion of the yeast cell contents is achieved by the yeasts own enzymes.

Papain is widely used to augment the yeasts endogenous proteases for protein hydrolysis during yeast extract production.

It was decided to evaluate the application of β-1,3-glucanase from Basidiomycete sp QM 806, Kitalase and Novozym-234 in conjunction with Papain in yeast extract production.

Kitalase, a powerful yeast lytic enzyme, is an enzyme mixture from Basidiomycetes aphyllophoroles. The preparation which contains β-1,3-glucanase and protease

activities, finds application in the hydrolysis of yeast cell walls and the extraction of cell components.

Novozym-234 contains α-1,3-glucanase, β-1,3-glucanase laminarinase, xylanase, chitinase and protease activities. The preparation is useful in protoplast production from yeasts and fungi.

Basidiomycete sp QM 806 culture supernatant, Kitalase and Novozym-234 were assayed for β-1,3-glucanase activity using the method described in Section 2.6.2.

3.5.1. The effect of Papain and Kitalase on the autolysis of fresh and dried baker's yeast Saccharomyces cerevisiae

Both fresh and dried baker's yeast were autolysed using Papain/Kitalase combinations. Yeast extract production was monitored from the twelfth to the forty second hour of autolysis and samples taken after 12,18,24,36 and 42 hours for yield of extract determination. The procedure is described in Section 2.7. Results are presented in Figure 3.19.

FIGURE 3.19

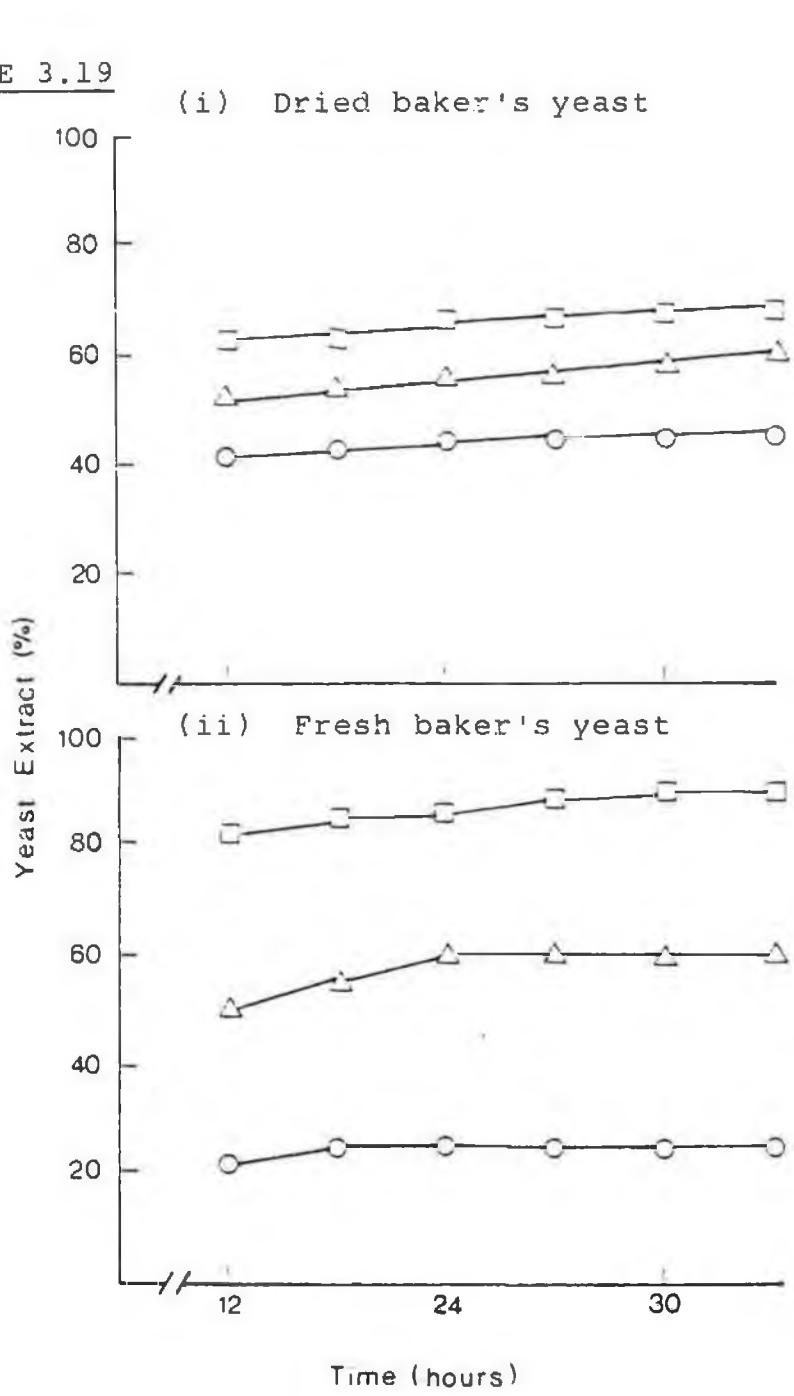

Figure 3.19. Yeast extract production from (i) dried and (ii) fresh baker's yeast using:- No added enzymes (○): Papain, 0.1g/100g slurry (△); Kitalase 15U β-1,3-Glucanase /100g slurry + Papain, 0.1g/100g slurry (□), at 55° C.

Supplementing the yeast's own enzymes with Papain and Papain/Kitalase increased the amount of yeast extract produced compared with the amount obtained when enzymes were not added. This effect was more noticeable when fresh yeast was autolysed, although 45% extract was obtained from dried yeast and 25% from fresh yeast after 42 hours autolysis. Perhaps the drying process leaves the yeast more susceptible to self-digestion once reconstituted. Levels of extract obtained using Papain only, were similar for both fresh and dried yeast. Treatment with Papain and Kitalase augmented the effect of Papain by about 30% for fresh yeast and 10% for dried yeast.

Yields of extract obtained after 42 hours autolysis were similar, (within 10%) to those obtained after 12 hours autolysis. Treatment for 42 hours was not economical in terms of time or in the extra amount of extract obtained.

3.5.2. The effect of Papain and Kitalase on autolysis of fresh baker's yeast over 12 hours

In the previous experiment, yeast extract production was not monitored during the early hours of autolysis. This experiment investigated yeast extract production from fresh baker's yeast, from 0-12 hours autolysis. Fresh yeast was used rather than dried yeast as the autolytic effect was found to be more dramatic using fresh yeast (Section 3.5.1). The same conditions and enzyme levels were used as described in Section 3.5.1. Samples were taken after 1,3,5 and 8 hours autolysis. Levels of yeast extract produced were evaluated and the results are presented in Figure 3.20.

The effect of Papin and Papain/Kitalase treatment was observed after one hour autolysis. The effect of Papain plus Kitalase was even more noticeable after 3 hours, where

FIGURE 3.20

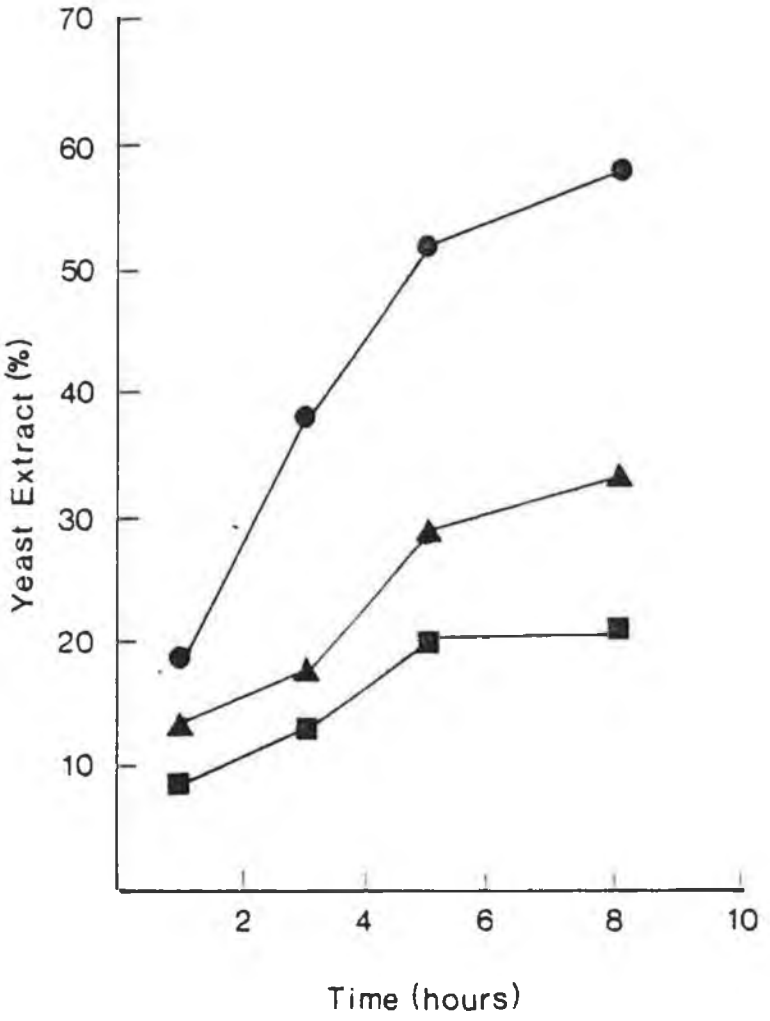

Figure 3.20. Yeast extract production from fresh baker's yeast using:- No added enzymes (■); Papain, 0.1g/100g slurry, (▲); Kitalase, 15U β-1,3-Glucanase /100g slurry + Papain, 0.1g /100g slurry (●), at 55°C.

40% yeast extract was obtained compared to 20% after one hour. After 8 hours, 21%, 33% and 58% extract yields were obtained for flasks with a) no added enzymes b) Papain and c) Papain plus Kitalase respectively compared to 23%, 56% and 83% extract after 12 hours. Thus the four hours between the eight and twelfth hour of autolysis yielded an extra 2% for the flask with no added enzyme, 23% for the flask with Papain and 30% for the flask containing both Papain and Kitalase. This 23% and 30% represent the largest single increase in yeast extract production over the 42 hours investigated. After 12 hours autolysis the rate of extract production decreased (Section 3.5.1).

The results from Section 3.5.1. using fresh yeast are combined with results from this section, to produce the yeast extract production profile from 0-42 hours autolysis which is presented in Figure 3.21.

FIGURE 3.21

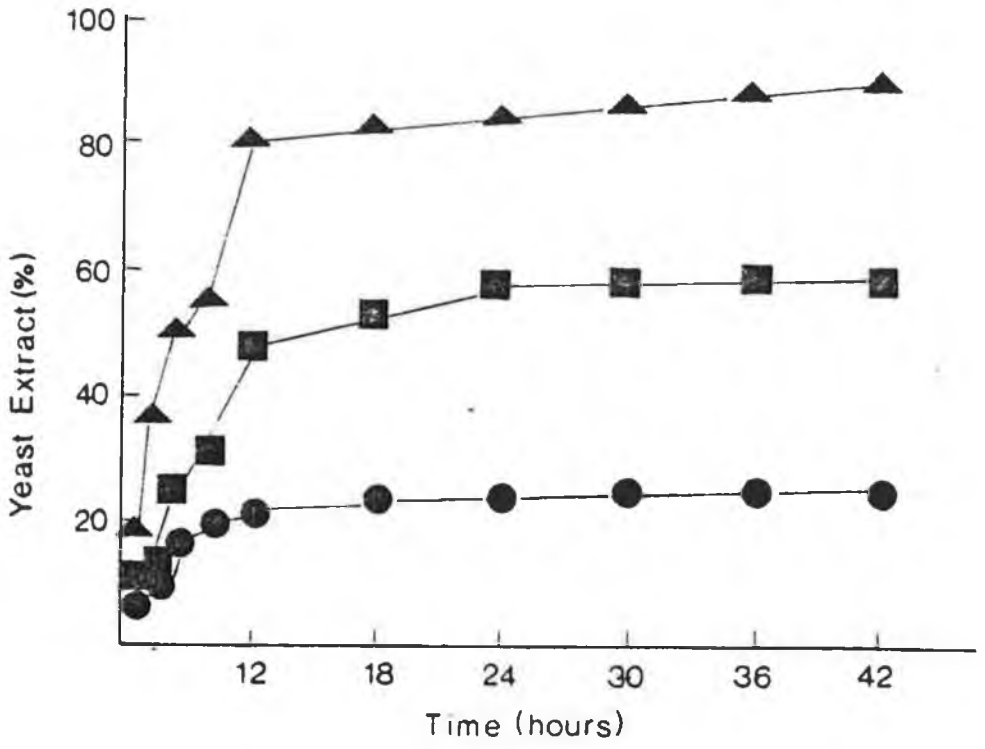

Figure 3.21. Yeast extract production from 0-42 hours of autolysis at 55°C. Enzyme levels used were:- No added enzymes (●); Papain, 0.1g/100g slurry (■); Kitalase 15U β-1,3-Glucanase /100g slurry + Papain, 0.1g/100g slurry (▲).

3.5.3. The effect of Papain and varied Kitalase levels on yeast autolysis

The effect of varied Kitalase levels in conjunction with Papain on fresh yeast autolysis was investigated. Levels of 7.5U, 15.0U and 30.0U β-1,3-Glucanase activity / 10g dry weight yeast were used. Autolysis was conducted for 24 hours, with sampling sfter 4, 8, 12 and 24 hours. Controls using no added enzyme or just Papain were used. The autolysis procedure according to Section 2.7 was followed. The results are presented in Figure 3.22.

25% yeast extract was achieved when no enzymes were added, whilst 67% extract was obtained using 0.1g Papain / 100g slurry, after 24 hours autolysis. Addition of Kitalase greatly enhanced the yields of extract obtained even at the lowest level used. After 24 hours autolysis, more than 90% extract was achieved using 7.5U β-1,3-Glucanase, which was the lowest Kitalase dosage. At all doses, Kitalase substantially accelerated the rate of extract production, achieving for example, yields of extract in 4 hours at the low Kitalase dose similar to those obtained with Papain alone after 24 hours.

FIGURE 3.22

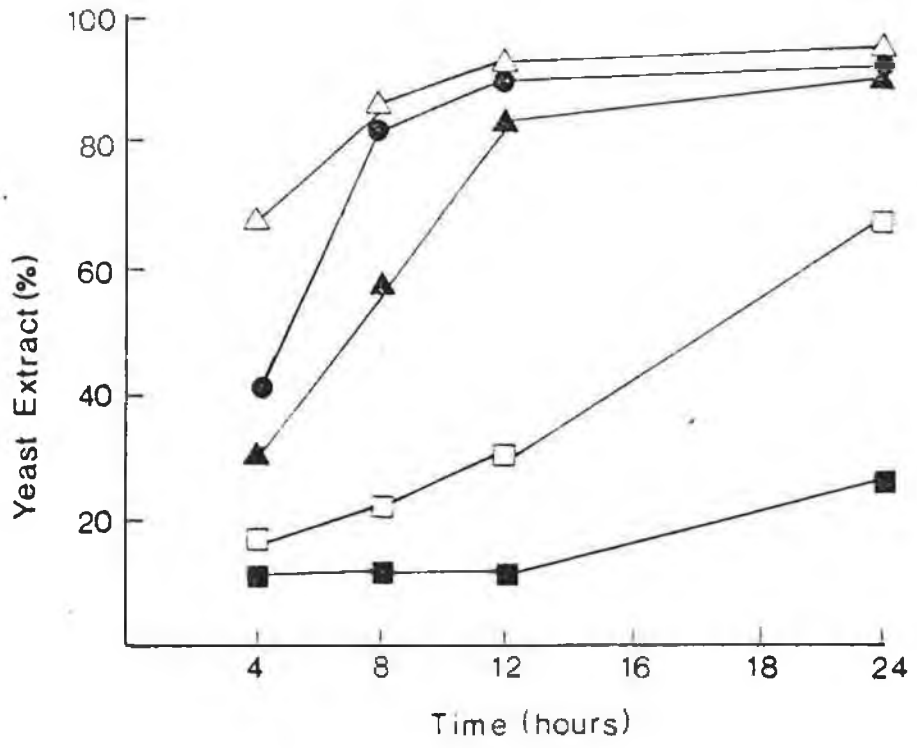

Figure 3.22. Yeast extract production using Papain and varied Kitalase levels at 55°C. Enzyme levels used were:- No added enzymes (■); Papain, 0.1g/100 slurry (□); Papain, 0.1g/100g slurry + Kitalase, 7.5U β-1,3-Glucanase /100g slurry (▲); Papain, 0.1g/100g slurry + Kitalase, 15U β-1,3-Glucanase/ 100g slurry (●); Papain, 0.1g/100g slurry + Kitalase, 30U β-1,3-Glucanase / 100g slurry (△).

3.5.4. Effect of Papain and Basidiomycete sp QM 806 culture supernatant on yeast extract production

The effect of Papain and the β-1,3-glucanase from Basidiomycete sp QM 806 on fresh yeast autolysis was investigated. Controls with no added enzyme and just Papain (0.1g / 100g slurry) were tested also. Levels of 25U, 100U and 225U β-1,3-Glucanase/100g slurry were used. Samples were taken after 4,8,12 and 24 hours. The procedure described in Section 2.7 was followed.

The results are presented in Figure 3.23.

An extract yield of 54% was obtained after 24 hours with Papain alone, compared to 29% for the no enzyme added control. Addition of β-1, 3-Glucanase caused a dramatic increase in yield of extract. Yields obtained after 24 hours were 67%, 85% and 89% for 25U, 100U and 225U β-1,3-glucanase respectively per flask. Results obtained using the Basidiomycete enzyme were similar to those using Kitalase, in that both accelerated the rate of extract production.

FIGURE 3.23

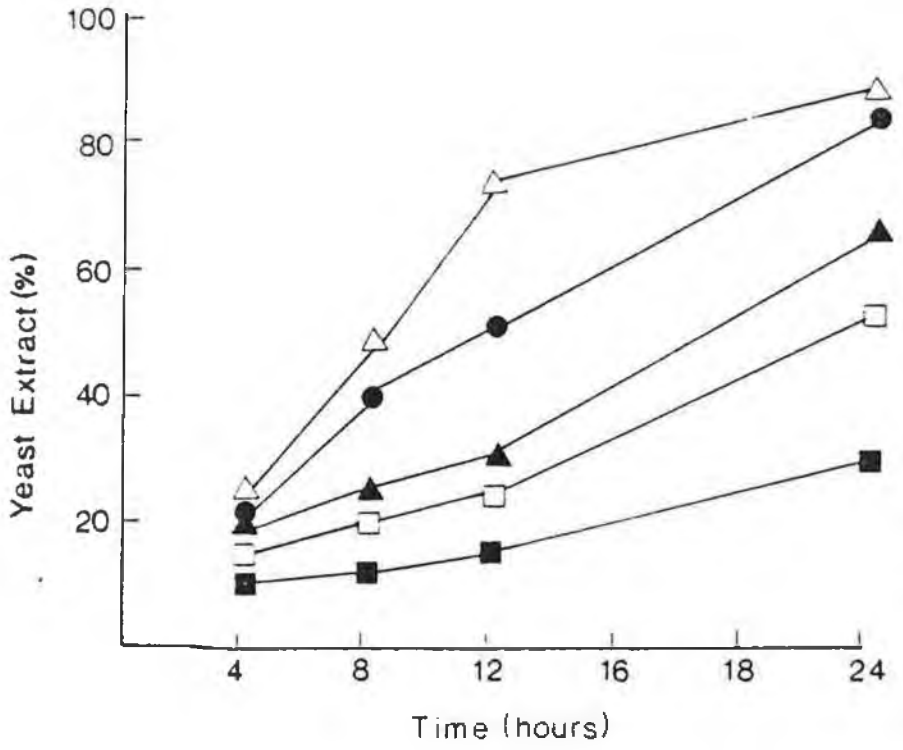

Figure 3.23. Yeast extract production using Papain and varied B-1, 3-glucanase levels from Basidiomycete sp. QM 806 at 55°C. Enzyme levels used were:- No added enzymes (■). Papain, 0.1g / 100g slurry (□); Papain 0.1g /100g slurry + 25U Basidiomycete sp QM 806 B-1,3-Glucanase / 100g slurry (▲); Papain, 0.1g/100g slurry + 100U Basidiomycete sp QM 806 B-1,3-Glucanase /100g slurry (●); Papain, 0.1g/100g slurry + 225U Basidiomycete sp QM 806 B-1,3-Glucanase / 100g slurry (△).

3.5.5. The effect of Papain and Novozym-234 on yeast extract production

The autolysis of fresh pressed baker's yeast using Papain and Novozym-234 was investigated using 50U, 250U and 500U β-1,3-glucanase per 100g yeast slurry in addition to Papain (0.1g/100g slurry). Controls containing no enzyme and Papain alone were used.

The autolysis procedure described in Section 2.7 was followed with samples taken after 4, 8, 12 and 24 hours. Yields of extract obtained are presented in Figure 3.24.

FIGURE 3.24

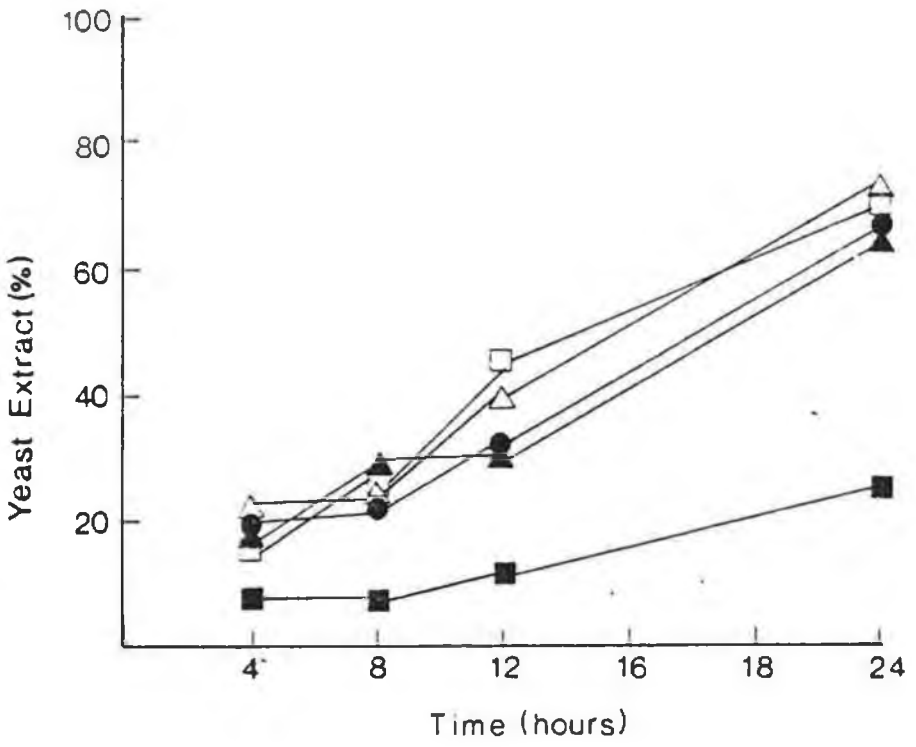

Figure 3.24. Yeast extract production using Papain and varied Novozym-234 levels at 55° C. Enzyme levels used were:- No added enzymes, (■); Papain, 0.1g/100g slurry, (□); Papain, 0.1g/100g slurry + Novozym-234, 50U β-1,3-Glucanase /100g slurry (▲); Papain, 0.1g/100g slurry + Novozym-234, 250U β-1,3-Glucanase / 100g slurry (●); Papain, 0.1g /100 slurry + Novozym-234, 500U β-1,3-Glucanase /100g slurry (△).

3.5.6. The effect of using Kitalase, Novozym-234 and Basidiomycete sp QM 806 culture supernatant on yeast autolysis without Papain additions

In this study, Novozym-234, Kitalase and Basidiomycete sp QM 806 β-1,3-glucanase were used without the Papain addition. Levels of 125U, β-1,3-Glucanase from the Basidiomycete culture supernatent, 15U from Kitalase and 500U from Novozym-234 per 100g yeast slurry were tested. See Table 3.7.

TABLE 3.7

Levels of enzymes used in autolysis of fresh bakers yeast

Flask	Papain g/100g slurry	Novozym-234 U β-1,3 glucanase /100g slurry	Kitalase U β-1,3 glucanase /100g slurry	Basidiomycete sp QM 806 U β-1,3 glucanase /100g slurry
1	0.0	0.0	0.0	0.0
2	0.1	0.0	0.0	0.0
3	0.0	0.0	15.0	0.0
4	0.0	500.0	0.0	0.0
5	0.0	0.0	0.0	125.0

The procedure according to Section 2.7 was followed. Samples were taken after 4, 8, 12 and 24 hours and yields of extract produced determined. Results are presented in Figure 3.25.

Similar yields of extract were obtained after 24 hours using Papain, the Basidiomycete culture supernatant and Novozym-234. Treatment with Kitalase yielded 70% yeast extract after 8 hours autolysis, exceeding levels obtained after 24 hours using the other enzyme preparations. Use of

FIGURE 3.25

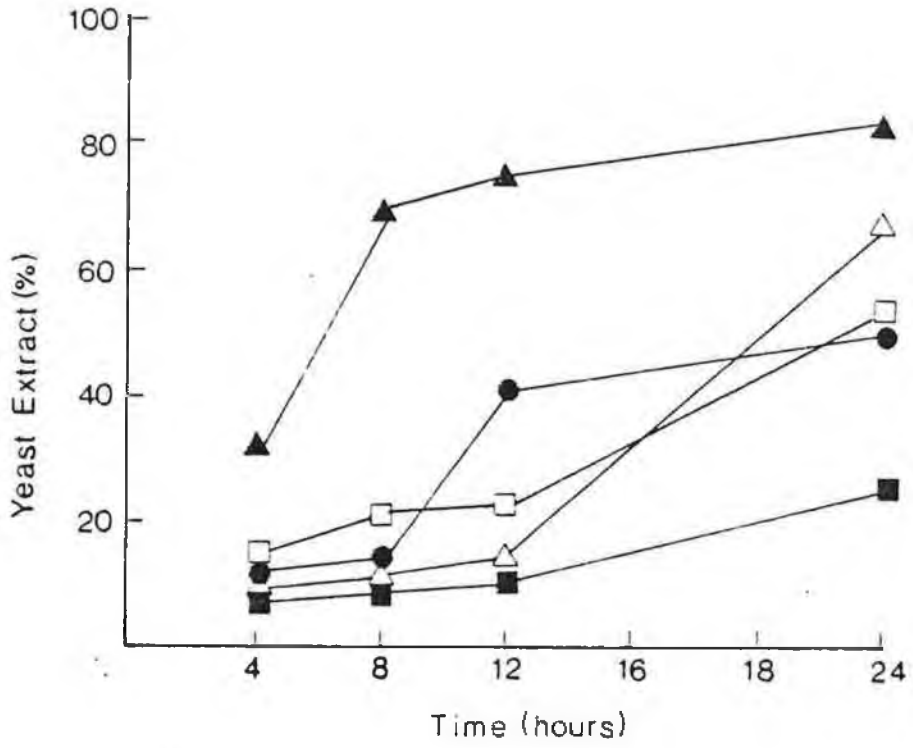

Figure 3.25. The effect of various enzyme preparations on yeast autolysis at 55° C. Enzyme levels used were:- No added enzymes (■); Papain, 0.1g/100g slurry (□); Kitalase, 15U β-1,3-glucanase/100g slurry (▲); Novozym-234, 500U β-1,3-glucanase /100g slurry (△); Basidiomycete sp QM 8 6 culture supernatant, 125U β-1,3-glucanase/100g slurry (●).

the Basidiomycete culture supernatant alone (125U β-1, 3-glucanase) yielded 48% extract, compared to 85.5% extract being produced when Papain plus 100U β-1,3-glucanase were used (Section 3.5.4) after 24 hours. The protease contributed substantially to the lytic effect.

This effect was not found using Novozym-234 plus Papain, with approximately the same extract yield produced using Novozym-234 alone (Section 3.5.5).

Yields of extract obtained after Kitalase only treatment were considerably greater than those achieved after the other enzymic treatments investigated in this study, even after 4 hours autolysis. After 24 hours, 83% yeast extract was produced using Kitalase only, compared to about 50% after treatments with Novozym-234, Papain or the Basidiomycete culture supernatant. The high level of protease activity (40,000 HUT as defined in Section 3.1.7) present in the Kitalase preparation probably contributed to the lytic effect.

3.5.7 Appraisal of results

In these studies on yeast autolysis, the effect of various enzymes on yeast extract production was investigated. Fresh, pressed baker's yeast was chosen for autolysis studies over dried baker's yeast because the fresh yeast was more susceptible to enzymic treatments, with higher yields obtained.

The enzymes used were the Basidiomycete sp QM 806 culture supernatant, Novozym-234, Kitalase and Papain. Levels of Basidiomycete culture supernatant, Kitalase, and Novozym-234 used were defined according to their β-1,3-glucanase component. Papain was used 0.1g/100g yeast slurry throughout the experiments.

The amount of yeast extract produced from fresh, pressed yeast when no enzymes were added was generally about 25%, whilst using Papain alone yields of 55-70% were obtained. Basidiomycete sp QM 806 culture supernatant used separately resulted in 50% extract being produced and when supplemented with Papain, 85.5% extract was achieved after 24 hours. 100-125U β-1,3-glucanase /ml Basidiomycete culture supernatant were used. The protease contributed substantially to the lytic effect. The combined usage of Papain and Basidiomycete culture supernatant would find application in the yeast extract production industry.

Novozym-234 was found not to be particularly suitable for yeast extract production under the conditions used. Approximately the same yields of extract were obtained (within 10%) using Novozym-234 and Novozym-234 plus Papain, in fact autolysis using Papain alone yielded more extract. The presence of Papain or Novozym-234 did not augment the effect of either.

Kitalase was found to be a very powerful yeast lytic enzyme, with higher levels of extract produced and in a shorter time. The high protease level present in the preparations, augmented the effect of the β-1,3-glucanase. β-1,3-glucanase levels used were much lower than those used, using the Basidiomycete culture supernatant or the Novozym-234 preparation, levels of 15U were found to be effective. Kitalase would find major application in yeast extract production.

4. DISCUSSION

Basidiomycete sp QM 806 synthesizes β-1,3-glucanase which is excreted into the culture medium. The organism was grown in both basal and complex media. Higher yields of β-1,3-glucanase were obtained when the basidiomycete was grown in complex media. This was expected since the complex media were nutritionally richer than the basal salts media. Levels of complex media constituents (glucose and soya-flour) were varied in order to obtain the optimal conditions for enzyme production. Higher enzyme yields were found in media with the higher glucose or soya-flour content (Section 3.1.3).

In unbuffered media where the pH was not controlled, the pH decreased dramatically from onset until about the seventh day of the fermentation after which the pH rose (Sections 3.1.1. and 3.1.2). This decrease in pH, has been found to be associated with the digestion of the carbon source and the subsequent increase with the production of β-1,3-glucanase (Reese and Mandels, 1959; Friebe and Holldorf, 1975).

Basidiomycete sp QM 806 grown in complex media, buffered at pH 3.0, produced higher levels of β-1,3-glucanase than in unbuffered complex media (Section 3.1.3). Levels of β-1,3-glucanase produced in media buffered at pH 4.0 and pH 5.0 were about the same as those produced in unbuffered media.

Enzyme synthesis was shown to be initiated when the glucose concentration in the medium had decreased to 5-6 g/l (Section 3.1.3). This process may be considered as a derepression. At glucose levels above this, β-1,3-glucanase was not synthesized. Thus the onset of enzyme synthesis was determined by the concentration of carbon source in the

medium. Ƀ-1,3-Glucanase production was initiated later in the media containing a high glucose supply (40g/l) compared to media with a lower level of glucose (10g/l) because it took longer for the cells to consume the glucose supply. This was found by other workers, Friebe and Holldorf (1975), who showed that at extremely high glucose levels, the cells could not exhaust the glucose supply and therefore Ƀ-1,3-glucanase was not produced.

Addition of glucose after consumption of the initial carbon source inhibited Ƀ-1,3-glucanase production. The glucose addition maintained the carbon source at a level slightly higher than the critical level at which Ƀ-1,3-glucanase synthesis occurs, When glucose feeding was discontinued, and the glucose remaining in the culture medium was consumed to the critical level, Ƀ-1,3-glucanase production proceeded. (Section 3.1.8). Friebe and Holldorf (1975) conducted extensive studies on the control of the Basidiomycete sp QM 806 Ƀ-1,3-glucanase, including investigations on the addition of new carbon sources after consumption of the initial source. Addition of glucose, starch or fructose at any stage of the fermentation caused a decrease in Ƀ-1,3-glucanase activity. The extent of Ƀ-1,3-glucanase inactivation depended on the amount of carbon source added. Low concentrations of carbon source caused a limited temporary decrease of activity and high concentrations, resulted in a complete loss of Ƀ-1,3-glucanase activity.

Basidiomycete sp QM 806 grew in pelleted form in all media, both basal and complex and at all pH's investigated. Various polymers have been shown to disperse growth of Aspergillus (Elmayergi et al, 1973; Takahashi et al., 1960) and Rhizopus (Byrne, 1985). Media were supplemented with Carbopol-934 and Tween-80. Carbopol-934 has been shown to disperse growth of Aspergillus niger (Elmayergi et al., 1973) and Rhizopus arrhizus (Byrne, 1985). The polymer has

been reported to increase mass transfer into fungal mycelia, and to increase respiration and glucose consumption (Elmayergi and Moo-Young, 1973; Moo-Young et al., 1969). Growth in a filamentous form is more efficient than pelleted growth in terms of oxygen and nutrient transfer (Huang and Bungay, 1973; Phillips, 1966). Carbopol-934, in contrast to the previous findings for Aspergillus and Rhizopus species was found not to disperse growth of Basidiomycete sp QM 806. The organism grew in pelleted form, with pellet sizes of the order 1mm to 5mm (Section 3.1.6).

Tween-80 has been shown to stimulate enzyme production from Aspergillus, Basidiomycete, Penicillium and Trichoderma species. (Reese and Maguire, 1969) and to disperse growth (Takahashi et al, 1960). Tween-80 has been reported to cause a 100% increase in β-1,3-glucanase activity from Basidiomycete sp QM 806 (Reese and Maguire, 1969). However, again in contrast, Tween-80 was found to inhibit rather than stimulate enzyme production. Enzyme yields were 10-15 times lower than usually obtained without the supplement (Section 3.1.6). Growth was pelleted rather than dispersed.

The Basidiomycete sp QM 806 culture supernatant contained both β-1,3-glucanase and protease activities. (Section 3.1.7). The presence of both these activities has importance in yeast cell wall lysis (Section 3.5).

Basidiomycete sp QM 806 grew in mycelial form in the Microferm fermenter (Section 3.2) compared to the pelleted form of growth obtained in all shake-flask cultures (Section 3.1) However, β-1,3-glucanase levels were much lower in the fermenter culture medium than in shake-flask culture media. The change in morphology from shake-flask to fermenter could be due to the different agitation and aeration levels used. The dimensions of the fermenter were also much different to those of a 250 ml conical flask.

Laminarin and yeast glucan degradation products have been investigated mainly using paper chromatographic techniques. (Nelson et al., 1963; Chesters and Bull., 1963; Fleet and Manners, 1976; Fleet and Manners, 1977). In this project a High Performance Liquid Chromatographic method was used. The method has previously been used to separate degradation products from acid and enzymic hydrolysates of starch (Cheetham and Sirimanne, 1981).

The effect of three enzyme preparations, Kitalase, Novozym-234 and the Basidiomycete sp QM 806 culture supernatant, on laminarin and yeast glucan was investigated. All three preparations were found to have a major exohydrolytic mechanism with a minor endohydrolytic content (Section 3.4). Kitalase is an enzyme mixture from Basidiomycete aphyllophoroles and Novozym-234, a mixture from Trichoderma harzianum. Glucose was the main product with β-gentibiose and laminaribiose being produced in smaller amounts.

On unidentified compound which eluted before glucose, indicating that this unknown was a low molecular weight compound or some other monosaccharide, was found during yeast glucan degradation but not during laminarin degradation. This unknown compound may be mannose. This monosaccharide has been found in trace amounts from total and partial acid hydrolysis of the alkali-soluble fraction of yeast glucan (Fleet and Manners, 1977; Manners et al., 1974). The glucan preparation used in this project was obtained from baker's yeast by a method of Bacon et al (1969) which involved repeated breaking (using a bead-beater) and washing of yeast cells. Thus, this preparation consisted of cell wall material rather than yeast glucan. Other cell wall constituents such as mannan were not separated from the glucan component. If the unidentified compound was mannose, then this monoaaccharide

may have originated from traces of mannan present in the yeast glucan preparation. (Phaff, 1971; Manners, 1974). This mannose may also have originated from a glucan-mannan-protein complex. (Fleet and Manners, 1976; Fleet and Manners, 1977). These workers found that the alkali-soluble glucan component of baker's yeast cell walls contained an associated mannan fragment.

Fleet and Manners (1976; 1977) have conducted extensive studies on baker's yeast glucan (the alkali-soluble component) degradation products after enzymic and acid hydrolysis. Total acid hydrolysis yielded only glucose while partial acid hydrolysis yielded glucose, laminaribiose, laminaritriose, gentiobiose, laminaritetrose, laminaripentose, gentiotriose, laminarihexose, gentiotetrose, plus higher unidentified oligosaccharides.

Enzymic hydrolysis of yeast cell walls using the endo-β-1,3-glucanase from Bacillus circulans WL-12, yielded glucose, laminarisaccharides, small amounts of gentiobiose and a soluble polysaccharide fraction. This soluble fraction released gentiosaccharides on partial acid hydrolysis and on enzymic digestion by the endo-β-1, 3-glucanase from Bacillus circulans WL-12 and yielded glucose and mannose after total acid hydrolysis.

The Bacilus circulans β-glucanases acted in an endo hydrolytic manner. The enzymes used in this project were exohydrolytic, producing glucose as the major product, with gentiobiose and laminaribiose also released from baker's yeast cell wall preparations. Higher laminarisaccharides or gentiosaccharides were not obtained.

The polysaccharide laminarin is a reserve carbohydrate of the sublittoral brown algae (Phaeophyceae) and occurs principally in the Laminariae. Two types of laminarin are

recognised, the water-insoluble laminarin, which is precipitated spontaneously from an aqueous acid extract of the weed and the soluble form which separates from solution only on the addition of a precipitant, such as ethanol. (Peat et al; 1958 a). No clear cut differences in structure have been established. Insoluble laminarin has been shown to contain mannitol to which are attached chains composed of glucose residues. The glucose chains in laminarin are linked by both β(1-6) and β(1-3) bonds.

Investigations have shown that laminarin is heterogenous and is composed of at least two components. One is a reducing substance termed laminarose, which consists of glucose residues linked by both β-(1-6) and β-(1-3) bonds. The other component is a non-reducing substance, termed laminaritol, which appears to be two chains similar to laminarose, both terminating in the same mannitol residue (Nelson et al, 1963). Soluble laminarin probably has a similar two component structure.

The laminarin used in this project was the soluble type from Laminaria digitata, and hydrolysis of this polysaccharide produced glucose as the major product in addition to laminaribiose and gentiobiose. An unidentified compound which was eluted much later than the disaccharides was probably a higher laminarisaccharide or gentiosaccharide, with a degree of polymerisation of about five. This compound disappeared as hydrolysis proceeded.

The Basidiomycete sp QM 806 has been shown to produce glucose as the major product with smaller levels of laminaribiose, gentiobiose, laminaritriose and several higher oligosaccharides. Some of the higher oligosaccharides were shown to contain mannitol. As the course of hydrolysis proceeded the trisaccharide and higher oligosaccharides disappeared, while gentiobiose levels

remained constant and laminaribiose levels increased. (Nelson et al 1963). Glucose was probably produced from the non-reducing ends of the polysaccharide with laminaribiose being produced from the reducing ends of the laminarose component of laminarin and gentobiose from the β-(1-6) linkages that occur in both the laminarose and laminaritol components.

In this present study, as laminarin and yeast glucan hydrolysis proceeded, glucose, β-gentiobiose and laminaribiose levels remained constant, using the Basidiomycete sp QM 806 enzyme. In the Kitalase and Novozym-234 treated laminarin and yeast glucan preparation, laminaribiose levels were shown to decrease due to the degradation of this disaccharide to glucose. Unlike the Kitalase and Novozym-234 preparations, the Basidiomycete sp QM 806 enzyme was shown to attack laminaribiose only slightly. (Section 3.4).

The Basidiomycete sp QM 806 enzyme has previously been shown to be incapable of attacking laminaribiose. (Nelson et al., 1963). However, later investigations have shown that the disaccharide was attacked by the enzyme at higher concentrations (Nelson et al., 1969).

The reported glucan content of cell walls of Saccharomyces cerevisiae has ranged from 30-60% depending on the study. (Northcote and Horne, 1952; Roelofsen, 1953; Mill, 1966; Bowden and Hodgson, 1970; Fleet and Manners, 1976). This large variation seems related to yeast cultural conditions, (Mc Murrough and Rose, 1967; Ramsay and Douglas, 1979), methods of cell wall preparation, and glucan extraction and fractionation (Bacon et al., 1969; Fleet and Manners, 1976).

The nature of the glucan component of the cell wall of the yeast Saccharomyces cerevisiae has attracted considerable

attention for a number of years. Although it's nature as essentially a β-1,3-glucan has long been realised, the elucidation of it's fine structure did not prove possible by non enzymic methods but was achieved by the application of appropriate enzymes. (Marshall, 1974).

Wall and glucan analysis with selected and specific enzymes avoids the often used harsh, analytical treatments with strong acids and alkalis but great care must be taken of using only highly purified enzyme preparations to avoid false conclusions. (Marshall, 1974; Fleet and Phaff, 1981).

The exo-β-1,3-Glucanase excreted by Basidiomycete sp QM 806 has the ability to produce protoplasts from several yeasts. (Bauer et al., 1972). However, Fleet and Phaff (1975) point out that the enzyme preparation used by Bauer et al (1972) may not have been tested sufficiently for homogeneity and thus the possible presence of traces of a lytic β-1,3-glucanase contaminant working synergistically with the exo-β-glucanase cannot be definitely excluded.

This may also be true of the Basidiomycete sp QM 806 preparation used in this project as the enzyme supernatant was not subjected to purification in any way. The preparation was found to contain protease activity which probably contributed to the lytic effect. The presence of other activities which may also have contributed to the lytic effect cannot be ruled out.

Complete hydrolysis of yeast cell walls and wall glucans by exogenous enzymes has been shown to be greatly facilitated by the synergistic action of a number of different glucanases and proteases. (Rombouts and Phaff, 1976 b; Tanaka et al., 1978; Obata et al., 1977 a; Scott and Schekman, 1980), thereby under scoring the overall

complexity of yeast cell structure. Selective and limited hydrolysis of isolated cell walls and wall glucan components with specific purified, exogenous enzymes can contribute significantly to an understanding of this structure. (Kopecka et al., 1974; Fleet and Manners, 1977).

The β-1,3-glucanases from Arthrobacter have been reported to hydrolyse insoluble but not soluble laminarin. (Matheson and McCleary, 1985; Doi et al., 1973 b), suggesting that long uninterrupted segments of β-1,3 linked glucose units were required for binding. Use of suitable specific and purified enzymes may prove a convenient method of examining in greater detail the differences in structure between soluble and insoluble forms of laminarin which have not been clearly established. (Marshall, 1974).

Autolysed yeast extract which is composed of the protein, low molecular weight polypeptides and other intracellular material has long been used as an additive to food products such as soups, sauces, gravies and meat products. (Hough and Maddox 1970; Cogman, 1977; Kelly, 1983). Yeast extracts are prepared by autolysis, plasmolysis and hydrolysis. (Johnson, 1977) as well as by chemical and mechanical means (Lindblom, 1974; Mogren et al., 1974).

Yeasts contain a full complement of intracellular proteolytic enzymes which have been extensively studied (Rostum et al., 1971; Pringle, 1975; Diezel et al., 1972; Hata et al., 1967). These enzymes are liberated and activated after cells are disintegrated either by autolysis (Hough and Maddox, 1970; Shetty and Kinsella, 1978) or by mechanical disruption (Follows et al., 1971; Lindblom, 1977).

The use of proteolytic enzymes to disrupt yeast cells and aid in proteolytic breakdown during autolysis has been reported. (Corteel, 1972; Reed and Peppler, 1973).

Enzymatie lysis of yeast cell walls using exogenous lytic and protease enzymes has been shown to be effective (Knorr et al., 1979 (a); Knorr et al., 1979 (b)).

In this project, the effect of various enzyme preparations on baker's yeast autolysis was investigated.

Kitalase, an enzyme mixture with β-glucanase and protease activities was found to be a very powerful yeast lytic preparation, producing very high levels of extract as determined by dry weight, and in a reasonably short period of time. (Section 3.5).

The Basidiomycete sp QM 806 culture supernatent enzyme mixture was also found to be effective in yeast autolysis, provided it was used in conjunction with Papain. The supernatant contained much lower levels of protease compared to Kitalase, so for effective autolysis, papain supplementation was required.

Novozym-234 used with or without papain was found not to be suitable for yeast extract production under the experimental conditions used.

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7. APPENDIX 1

The following buffers were used in media preparation or as a β-(1,3) glucanase diluent.

1. SODIUM CITRATE-CITRIC ACID BUFFER, PH 5.0 (0.1M)

A 0.1M solution of citric acid, H_2O is prepared by dissolving 21.01g in water and diluting to 1 litre. Sodium citrate $2H_2O$, 29.4g, is dissolved and diluted to 1 litre to make a 0.1M solution.

These solutions are then mixed in the following proportions:

Buffer pH	Citric acid H_2O	Sodium citrate $2H_2O$
	Solution (ml)	Solution (ml)
5.0	7	13

2. Na_2 HPO_4 - CITRIC ACID BUFFER, (McILVAINE), pH 2.5-8.0.

A 0.2M solution of Na_2HPO_4. $2H_2O$ is prepared by dissolving 35.61g in water and diluting to 1 litre. Citric acid-H_2O, 21.01g, is dissolved and diluted to 1 litre to make a 0.1M solution.

These solutions are then mixed in the following proportions:

Buffer PH	Na_2HPO_4 $2H_2O$	Citric acid H_2O
2.5	1.71	18.29
3.0	4.11	15.89
3.5	6.07	13.93
4.0	7.71	12.29
4.5	9.09	10.91
5.0	10.30	9.70
5.5	11.37	8.63
6.0	12.63	7.37
6.5	14.20	5.80
7.0	16.47	4.53
7.5	18.45	1.55
8.0	19.45	0.55

3. SODIUM ACETATE-ACETIC ACID BUFFER, pH4.6

A 1.0M solution of sodium acetate .$3H_2O$ is prepared by dissolving 136.08g in water and diluting to 1 litre. Acetic acid (17.5M stock) is diluted to 1.0M. To 70mls of 1M Sodium Acetate add sufficient 1M Acetic Acid to give a pH of 4.6 (35mls approx is required for this purpose).

7. APPENDIX II

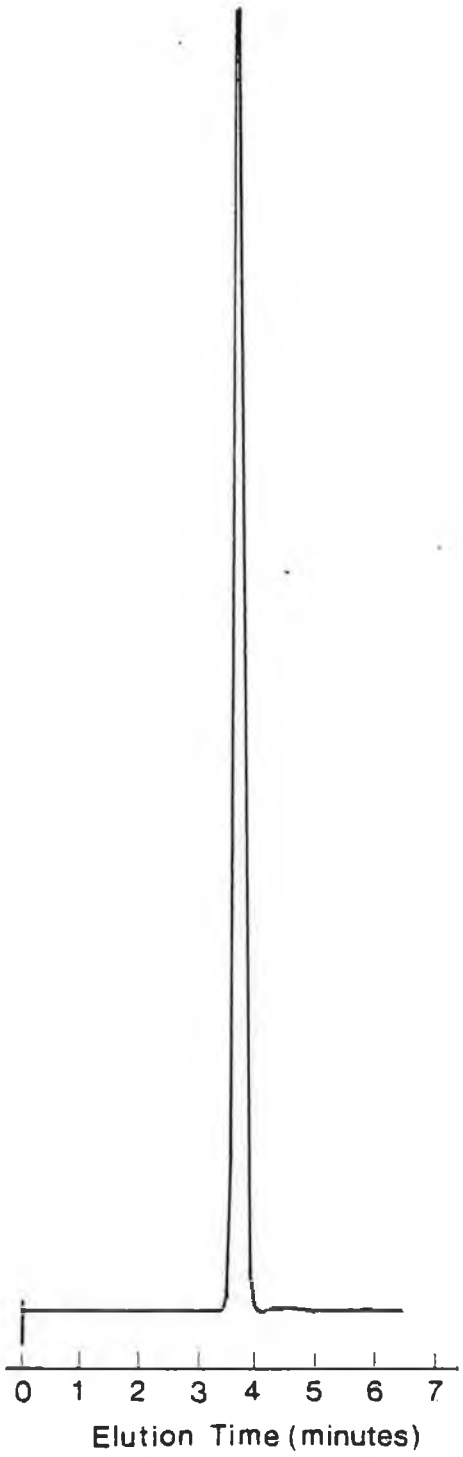
GLUCAN PREPARATION

Pressed yeast (500g) is suspended in 800mls distilled water and centrifuged at 10,000 r.p.m. at 1°C, for 20 minutes. A thin dark layer is scraped off the top of the pellet and the remainder is mixed with glass beads (0.5mm diameter), which half fill the Bead-beater chamber (Biospec Products, Oklahoma). The chamber is cooled prior to use with ice and vibration is applied for three, 3 minute periods. The mixture of beads and broken cells is filtered through three layers of gauze which retain the beads, with iced distilled water. The suspension is then centrifguged at 10,000 r.p.m., at 1°C for 10 minutes, and the supernatant discarded. The yeast walls (the pellet) are then washed four times with distilled water by centrifuging at 10,000 r.p.m., at 1°C for 20 minutes. These treatments break a large proportion of the cells and wash away much of the cell contents. The yeast walls are mixed with glass-beads in the Bead-beater chamber and vibration is applied for 4 minutes. This completes cell breakage. The cell walls are washed three times by centrifuging at 10,000 r.p.m., at 1°C for 20 minutes. After each centrifugion the pellet is cautiously resuspended so that the heaviest part (which includes dark particles of dirt) can be discarded. The walls are finally suspended in 50ml distilled water, heated at 75° for 30 minutes to inactivate endogenous glucanases, and freeze-dried. 500g pressed yeast yields 3-4g yeast cell walls.

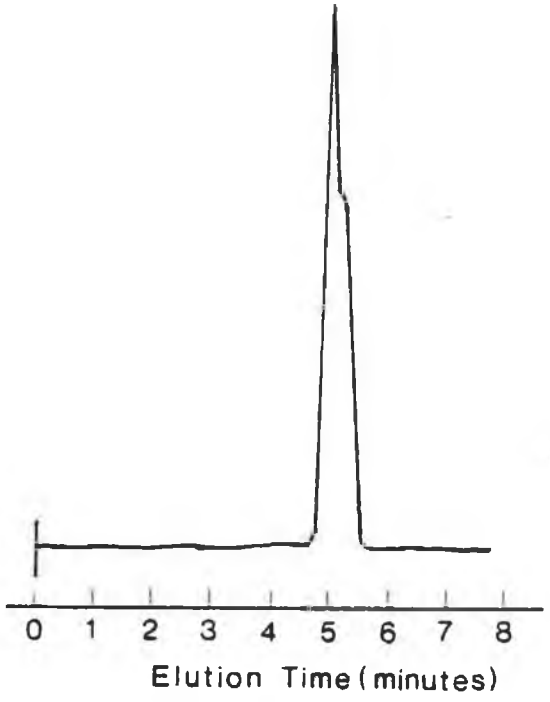
7. APPENDIX III

CHROMATOGRAMS

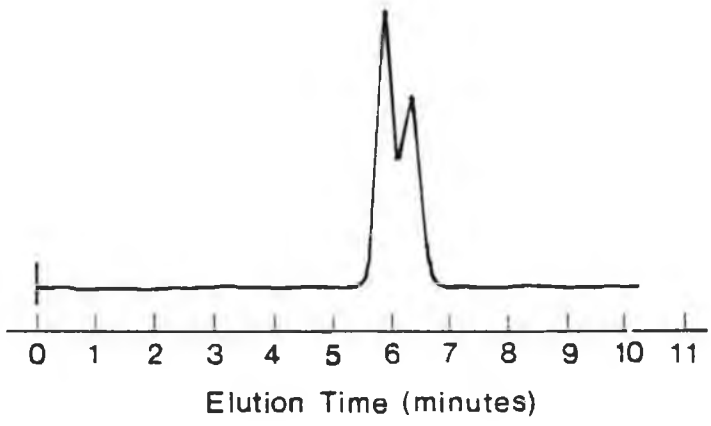
Sample chromatograms are presented. These include the chromatograms of the monosaccharide (glucose) and disaccharides (laminaribiose and β-gentiobiose) used as standards. The remaining chromatograms are those obtained after 210 minutes enzymic hydrolysis of laminarin and yeast glucan, at 50°C. The enzyme preparations used were Kitalase, Novozym-234 and the Basidiomycete sp QM 806 culture supernatant.



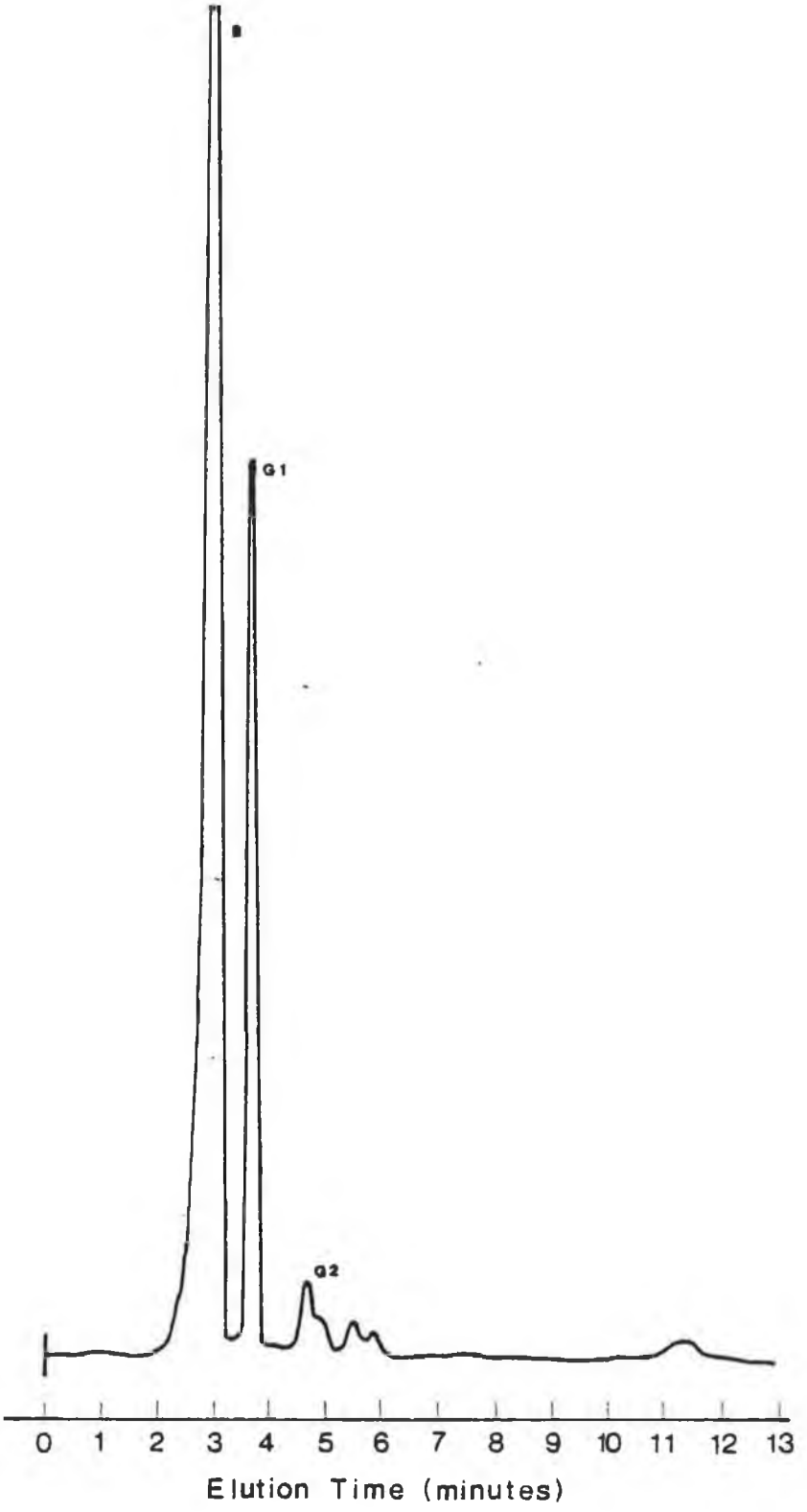
Chromatogram 7.1. 10 mg/ml glucose separated on a Dextropak column. Flow-rate, 1.0 ml/min. Eluent, water.



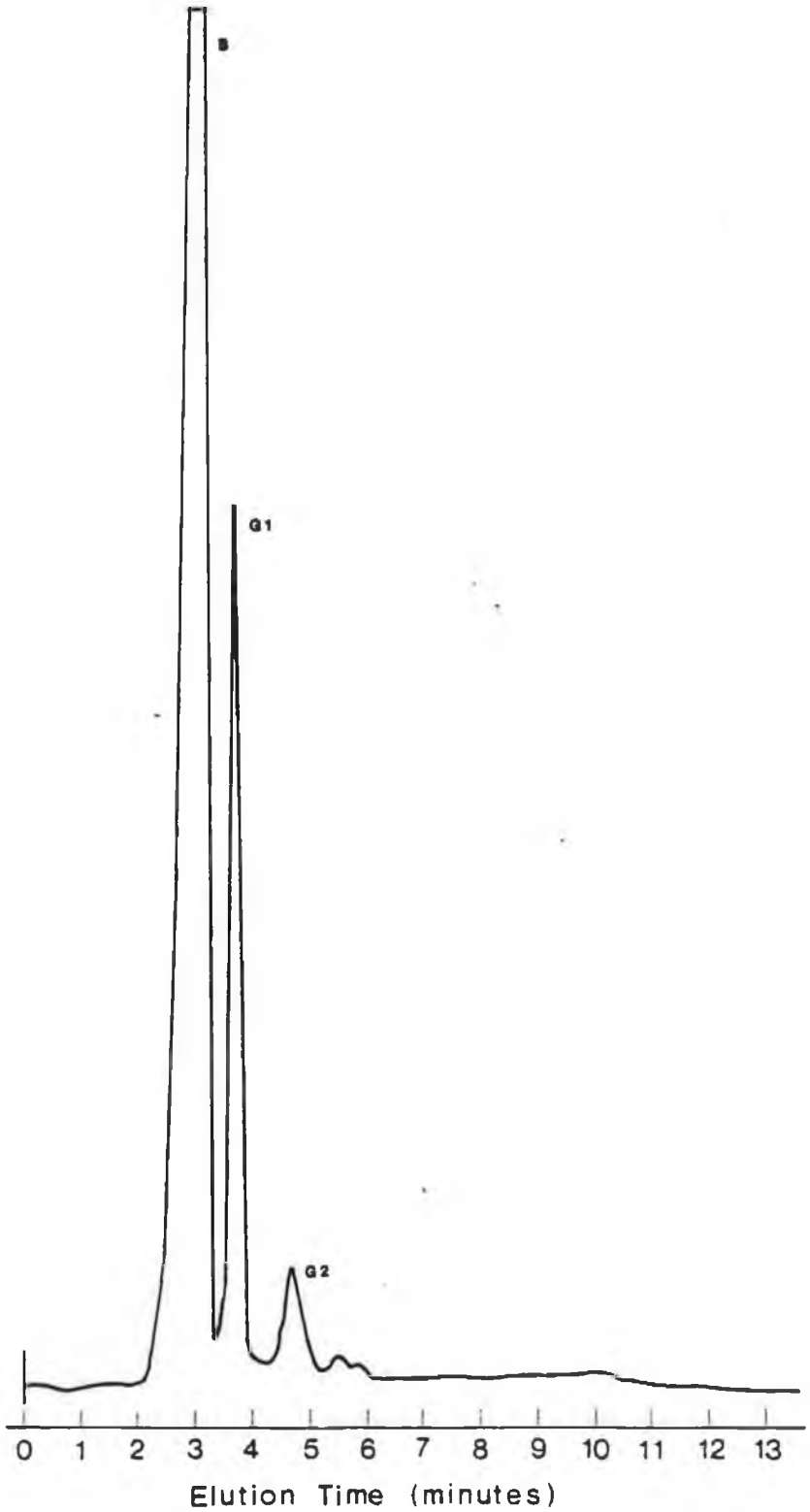
Chromatogram 7.2.10 mg/ml β-gentiobiose separated on a Dextropak column. Flow-rate, 1.0 ml/min. Eluent, water.



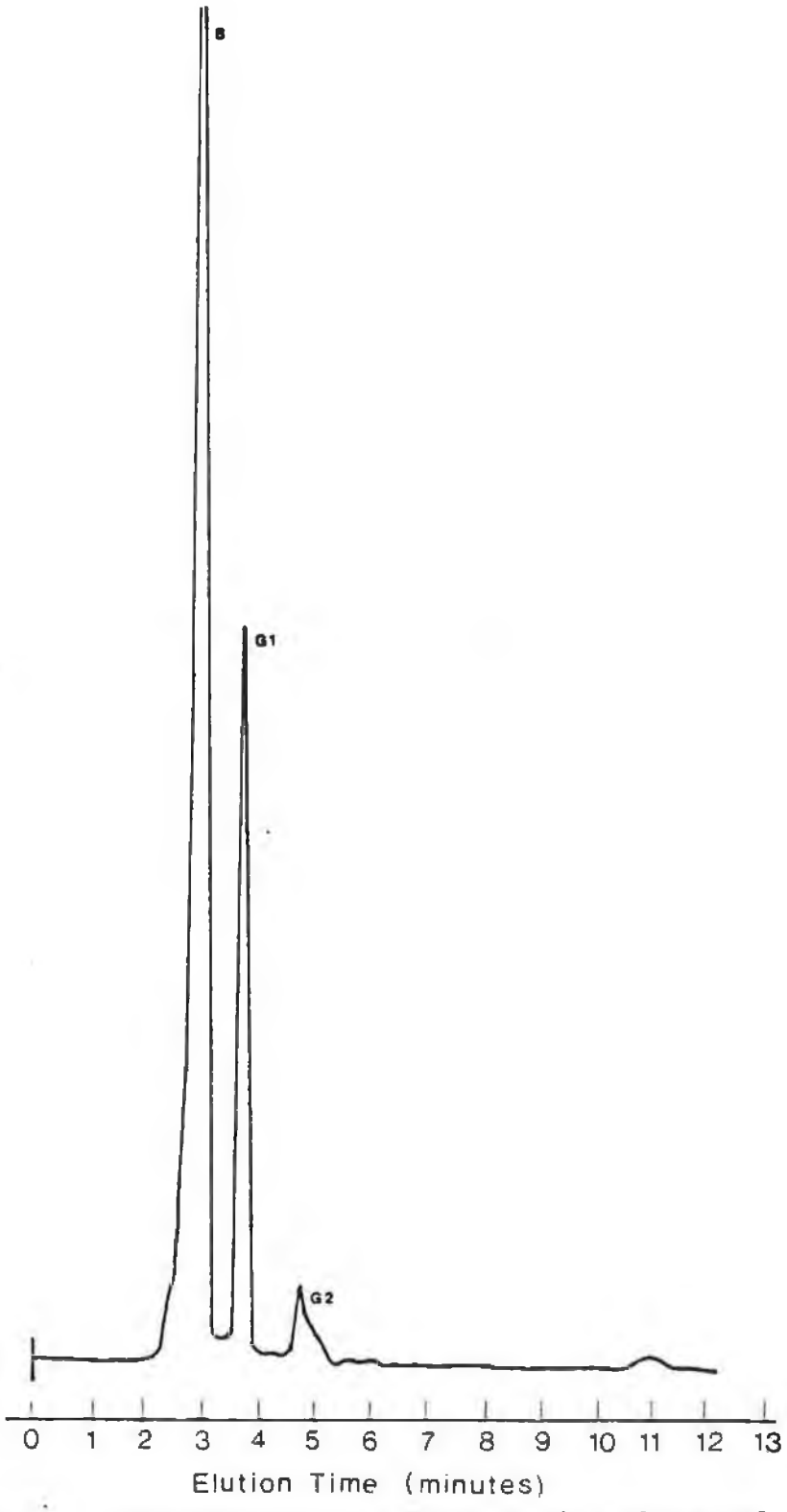
Chromatogram 7.3. 10 mg/ml Laminaribiose separated on a Dextropak column. Flow-rate, 1.0 ml/min. Eluent, water.



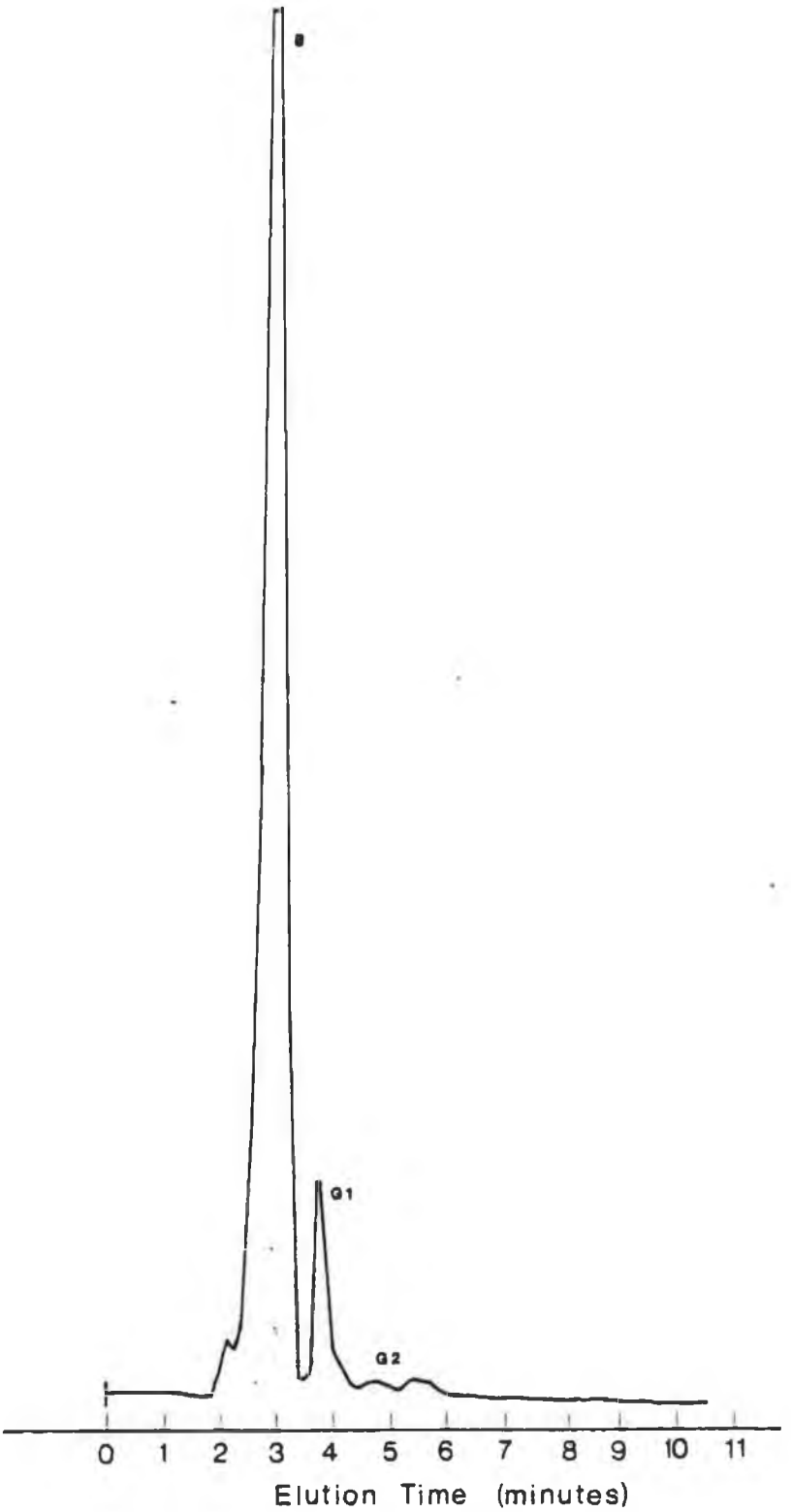
Chromatogram 7.4. Separation on a Dextropak column of oligosaccharides from the enzyme hydrolysis of laminarin. Using the Basidiomycete sp QM 806 culture supernatant (Section 3.4.1.1.). B denotes buffer, G1 denotes glucose and G2 denotes β-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate, 1.0 ml/min. Eluent, water.



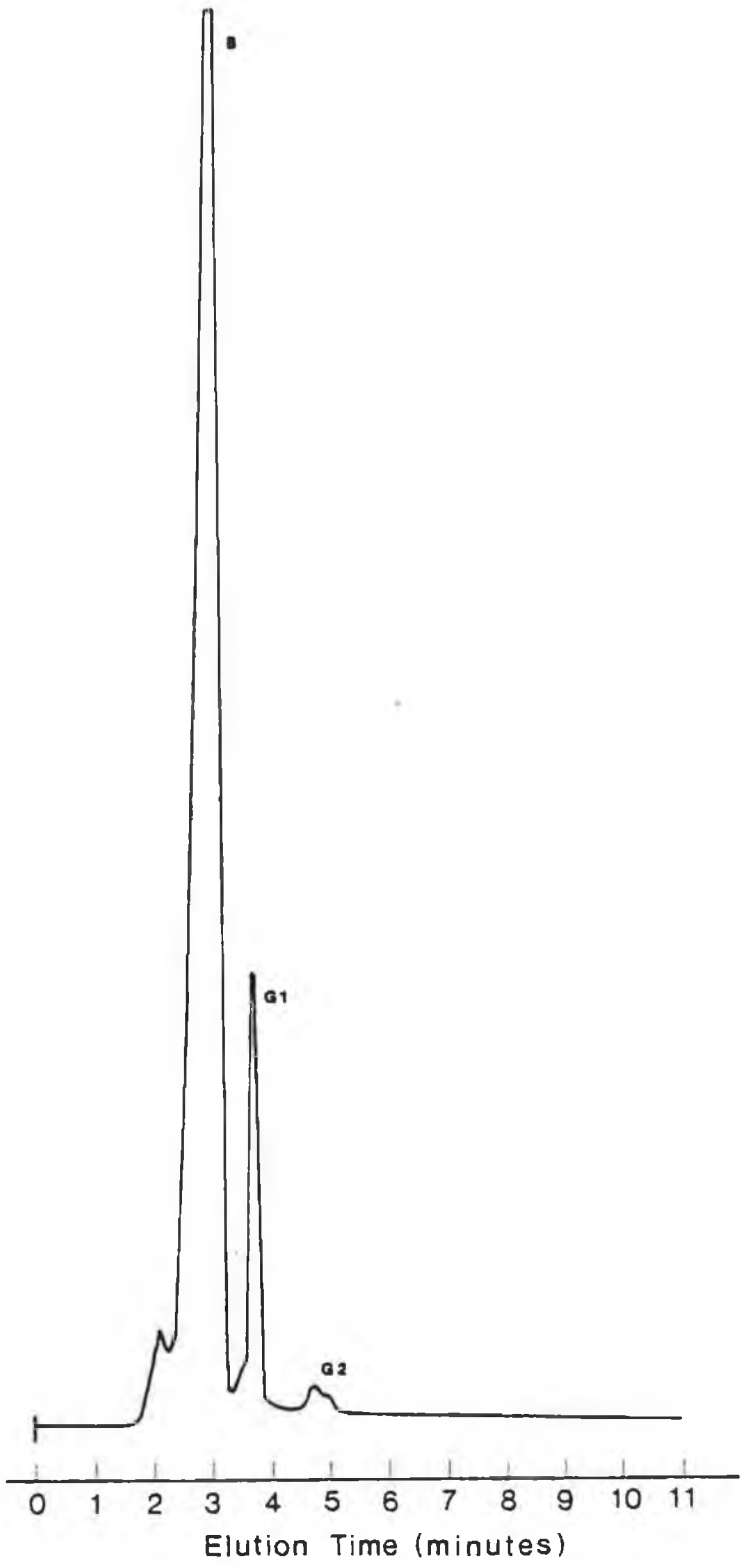
Chromatogram 7.5. Separation on a Dextropak column of oligosaccharides from the enzyme hydrolysis of laminarin using Kitalase, (Section 3.4.1.2.) B. denotes buffer, G1, glucose and G2, β-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate, 1.0 ml/min. Eluent, water.



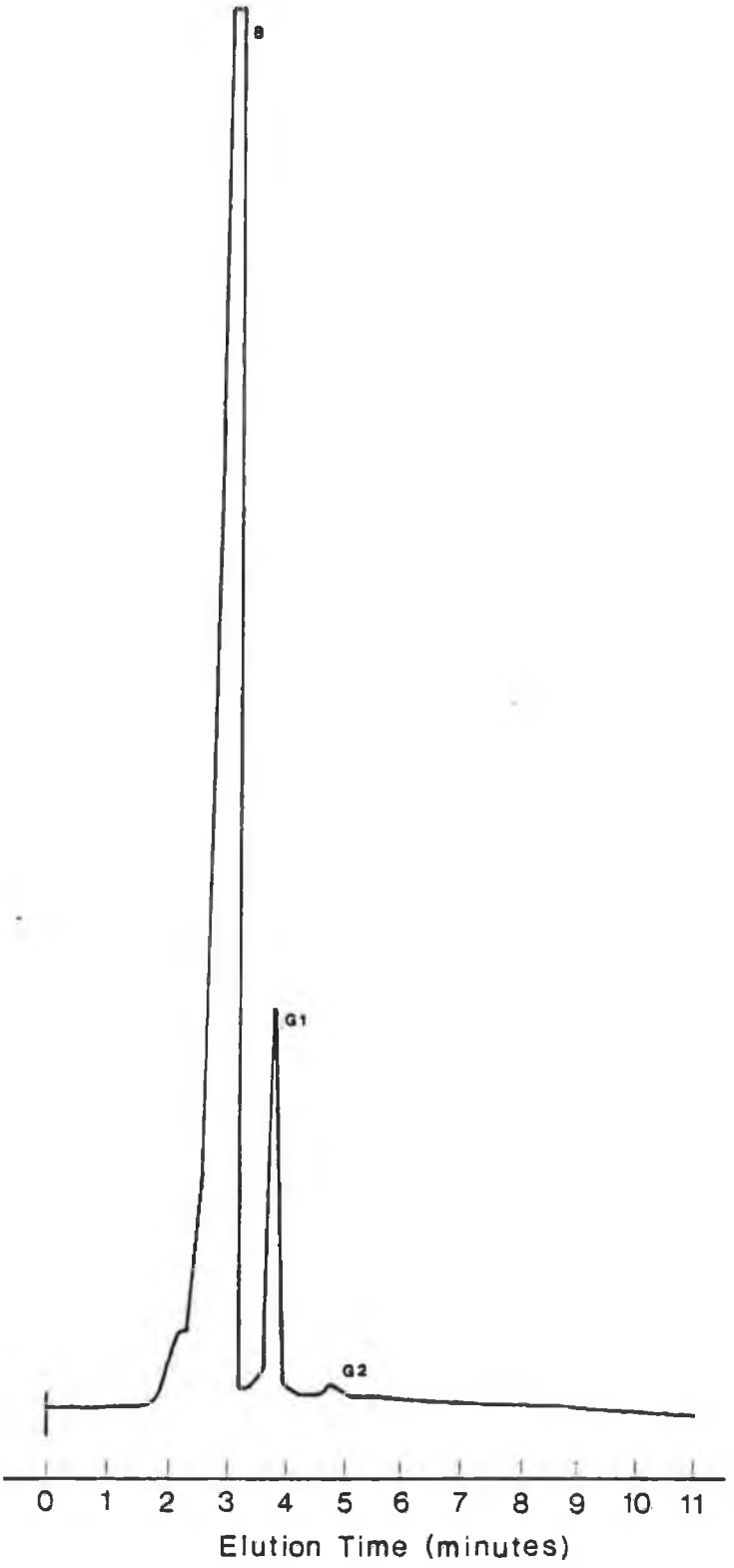
Chromatogram 7.6. Separation on a Dextropak column of oligosaccharides from enzyme hydrolysis of laminarin, using Novozym-234. (Section 3.4.1.3.). B denotes buffer, G1, glucose and G2, β-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate, 1.0 ml/min. Eluent, water.



Chromatogram 7.7. Separation on a Dextropak column of oligosaccharides from enzyme hydrolysis of yeast glucan, using the Basidiomycete sp QM 806 culture supernatant. (Section 3.4.2.1.). B denotes buffer, G1, glucose and G2, β-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate, 1.0 ml/min. Eluent, water.



Chromatogram 7.8. Separation on a Dextropak column of oligosaccharides from enzyme hydrolysis of yeast glucan, using Kitalase (Section 3.4.2.2.). B denotes buffer, G1, glucose, and G2, β-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate, 1.0 ml/min. Eluent, water.



Chromatogram 7.9. Separation on a Dextropak column of oligosaccharides from enzyme hydrolysis of yeast glucan using Novozym-234. (Section 3.4.2.3.). B denotes buffer, Gl, glucose and G2, B-gentiobiose (peak 1) and laminaribiose (peak 2). Flow-rate 1.0 ml/min. Eluent, water.

7. APPENDIX IV

BIOMASS DETERMINATION (CALAM 1969)

The procedure is as follows: Filter papers (Whatman type 1, 9cm diameter) are dried at 100° to constant weight. These are then transferred to a dessicator and cooled under vacuum. The papers are then weighed on a balance weighing grams to 4 decimal places. Biomass is harvested from flask cultures using the filter papers on a Buchner funnel. Growth is washed with three culture volumes of distilled water. The filters are then dried, cooled and weighed as before. Dry weight is determined by difference. Determinations were carried out in triplicate.

7. APPENDIX V

RESEARCH COMMUNICATIONS

MEETINGS

"Effect of β-1,3-glucanase from Basidiomycete QM 806 on yeast extract production".

Poster presented at the Society for General Microbiology meeting, April 1985, at Warwick University, England.

PUBLICATIONS

Ryan, E.M. and Ward, O.P. (1985). Study of the effect of β-1,3-glucanase from Basidiomycete QM 806 on yeast extract production.
Biotechnology Letters 7, 409-412.