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CHANGES IN THE YEAST *SACCHAROMYCES CEREVISIAE* CELL WALL ASSOCIATED WITH AUTOLYSIS IN A SIMULATED INDUSTRIAL PROCESS AND STUDY OF THE ACTIVITY OF RELATED ENZYMES

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DECLARATION

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DEDICATION

To my parents, wife and son.

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SUMMARY

Although production of yeast autolysates is of considerable commercial importance, to date little has appeared in the published literature on the autolysis process. Yeast autolysates are widely used in the food industry because of their flavouring and taste-enhancing potential and for their nutritional benefit. Compounds arising from breakdown of proteins and nucleic acids and their derivitive nucleotides and amino acids, and lipid degradation are of primary importance as flavourings. In developing countries vitamins, and proteins, are of significance in enhancing nutritional value of foodstuffs. However, the dissolution of the yeast cell wall is central to the properties of the final autolysate and, to date, little information on this process has been reported.

In this study the changes in the yeast cell wall that take place during autolysis were examined. A model for industrial autolysis of yeast, simulating a thermal induction that is in widespread commercial usage, was studied in detail using chemical and enzymic analyses and fluorescence light microscopy. Analysis of yeast cells at different stages of autolysis was carried out using scanning and transmission electron microscopy. It was found that general erosion of the cell wall took place during autolysis with the chitinous bud scars becoming increasingly prominent as the process proceeded. Microscopic study of ruptured cells indicated that there were no preferred points of cell

wall fracture. Compositional analysis showed that glucan and mannan components of the cell wall were depleted as autolysis progressed whereas depletion of chitin was not significant.

Analysis of the enzymes in autolysing yeast cells showed that thermal induction of protease, glucanase, mannanase and chitinase took place in the model process. Two major classes of glucanases could be identified: β-1,3 glucanases had the highest specific activity of enzymes in autolysing cells. Study of the glucanases using iso-electric focusing followed by overlaying of gels with yeast glucan showed that multiple individual enzyme species were induced during autolysis.

It was concluded that during the commercial yeast autolysis process, general erosion of the cell wall takes place with depletion of glucan and mannan that can be observed in microscopic studies as a reduction in intensity of staining. Rupture appeared to take place non-specifically at points where erosion had weakened the integrity of the cell wall. It was noted that intact chitin-rich bud scars were prominent in fragmented cell walls.

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INTRODUCTION

1. INTRODUCTION

1.1 History

Although man has been subject to the ravages of pathogenic microorganisms throughout his evolution, yeast appears to be the first microbe to be employed to his benefit. Yeasts can, with justification, be viewed as one of many tools developed by early man since the first records of their usage relate to production of a type of acid beer called "boozah" in Egypt in 6000 B.C., depicted in wall paintings of the period. This beer appears to have been produced by fermentation of an infusion prepared from crushed, germinated barley.

The processes for production of beer and wine and for preparation of leavened bread probably developed in parallel over the next five thousand years. In Egypt by 1200 B.C., the distinction between leavened and unleavened bread was well established and the use of a portion of the previous day's fermentation to inoculate each bakery dough or batch of grape must for wine productions was well established. From Egypt, the technology of brewing and baking passed to successively Greece and Rome and subsequently was spread throughout the Roman Empire (Berry, 1982).

There is a shortage of records on brewing in the period following the fall of the Roman Empire. It is clear, however, that by the thirteenth and fourteenth centuries, brewing was long established in the monasteries of northern Europe. Between 400 and 500 monasteries are known to have

been active in producing beer in Germany during this period. As early as 1188, Henry II levied the first recorded tax on beer in Britain (Berry, 1982).

The origins of distilled beverages are, however, lost in the mists of time. There are reports of distilled beverages in China in 1000 B.C. and it is clear that whiskey distillation was well established in Ireland by 1200 A.D.. It is currently believed that the process of distillation came to Europe from the Middle East and this view is supported by the fact that the word "alcohol" is derived from Arabic. The production of distilled beverages appears also to have been associated with the religious establishments and one of the earliest references to whisky in Scotland refers to its production by a Friar John Cor in 1494 (Berry, 1982).

1.1.1 Early Scientific knowledge of yeast

The elucidation of yeast structure was dependent upon the discovery and development of the light microscope and thus the first description of yeast cells is attributed to the Dutch microscopist Antonie van Leeuwenhoek in 1680. At this time, however, there was no suggestion that the structures described as yeast were living organisms. It is difficult to identify the first scientists to suggest that yeasts, which caused the alcoholic fermentations observed in wines and beer, could grow and multiply. Vitalistic theories proposed in the late eighteenth century suggested that fermentations were spontaneous events and it was only in 1818 that Erxleben suggested that yeasts were responsible

for alcoholic fermentations. However, it is generally agreed that it was the work of Pasteur, published in his "Etudes sur Vin" in 1866, that established beyond doubt the role of yeasts in the fermentation of sugars to alcohol. This work thus represents a milestone in the development of the science of microbiology. Another important event was the establishment of pure cultures of yeasts from single cell isolates, achieved by Hansen in Denmark in 1881. The use of such monocultures has been fundamental to the development of the taxonomy and physiology of yeasts and other microorganisms. In 1897, Buchner obtained the first cell-free extracts by grinding yeasts capable of fermenting sugars to alcohol, and by doing so assisted in the establishment of modern biochemistry. Subsequent similar work in this area made a significant contribution to the elucidation of the Embden-Meyerhof-Parnas (EMP) pathway. Since this time yeast has been a favoured organism for a wide range of physiological and biochemical studies (Berry, 1982).

Early developments in the field of microbial genetics also arose out of studies on yeast. The alternation of haploid and diploid phases in the life cycle of yeast was established by Winge (1935) who subsequently went to demonstrate the Mendelian segregation of genes during sexual reproduction in yeast. These studies opened the way for extensive research into yeast genetics which have made a major contribution to our understanding of the nature of the genetic material and the mechanism of inheritance in

eukaryotic microorganisms.

1.2 Development of classification

Although the characteristic budding form of yeast has been known since the published report by Antonie van Leeuwenhoek in 1680, more precise descriptions and taxonomic classification of yeasts has always presented a problem. Since the vegetative forms of most yeasts do not have distinctive morphological characteristics, they are not readily identifiable by direct gross features. Initially, the name *Saccharomyces* was given to all yeasts isolated from alcoholic beverages and three species were recognized by Meyen (1837) on the basis of the beverage from which they had been isolated: *S. vini* from wine, *S. cerevisiae* from beer and *S. pomorum* from cider. Yeast sexual spores were recognized by Schwann in 1837 but only in 1870 was the genus *Saccharomyces* restricted to those yeasts that produce spores.

1.2.1 Classification of yeasts

Yeasts, defined as unicellular fungi reproducing by budding or fission, are taxonomically diverse and include both ascomycetes and basidiomycetes. Lodder *et al.* (1985) have reviewed in detail the different systems of classification. The most recent classification, reviewed by Kreger van Rij (1984) is given below.

Classification of yeasts: Eumycota

Ascomycotina	
Hemiascomycetes	Sphermopthoracee
Endomycetales	Saccaharomycetaceae
Basidiomycotina	Filobasidiaceae
Ustilaginales	Telio-spore formimg yeasts
Tremallales	Sirobasidiaceae
	Tremallaceae
Deuteromycotina	Crytococcaceae
Blastomycetes	Sporobolomycetaceae

Classification of yeasts: Ascosporogenous yeasts

Spermopthoraceae	Coccidiascus	
	Metschnikowia	
	Nematospora	
Saccharomycetaceae		
Schizosaccharomycetoideae	Schizosaccharomyces	
Nadsonioideae	Hanseniospora	
	Nadsonia	
	Saccharomycodes	
	Wickerhamia	
Lipomycetoideae	Lipomyces	
Saccharomycetoideae		Ambrosiozyme
		Pachysolen
	Arthroascus	Zygosaccharomyces
		Pachytiochospora
	Citeromyces	Pichia
	Clavispora	Saccharomyces
	Cyniclomyces	Saccharomycossis
	Debaryomyces	Schwaniomyces
	Dekkera	
		Sporopachydermia
	Guilliermondella	Stephanoascus
	Hanseula	Torulaspora
	Issatchenkia	Wickerhamilla
	Kluveromyces	Wingea
	Lodderomyces	

1.3 Characteristics of the genus Saccharomyces

The yeasts in this genus multiply asexually by budding and have multilateral budding sites. Occassionally they form pseudomycelia. Asci are unconjugated, ascospores spherical or oval, smooth, seldom warty and are not liberated spontaneously from asci. No pellicle is formed during growth in liquid media. These yeasts ferment sugars vigorously but are unable to assimilate nitrate (Kreger van Rij, 1984). Strains of S. cerevisiae are the industrial yeasts used by most food, feed and beverage companies and by bakers, brewers, and distillers. The vegetative cells of S.cerevisiae may be either haploid or diploid with both phases being equally common. The cells of both genotypes reproduce asexually by budding (Dube, 1983).

1.4 The life cycle of Saccharomyces cerevisiae

S. cerevisie exhibits a haplo-diplobiontic life cycle (Guilliermond, 1970) in which both haploid and diploid phases are equally extensive and important. During growth, cells alternate between genotypes, although the stimulus or trigger for this transformation is not known in detail. Haploid cells differ in mating types and cells of opposite mating types are required for fusions (plasmogamy and karyogamy) that will yield diploid cells. At the end of diploid phases, nuclei undergo meiosis, each forming four haploid nuclei around which ascospores develop. In the laboratory, diploid cells can be induced into formation of ascospores by addition of 0.5% sodium acetate to growth media. Following lysis of the ascus wall, ascospores develop

into vegetative cells, establishing a new haploid growth phase.

Sexual compatibility in *S. cerevisiae* is controlled by a single, 2-allele (a and α) gene that segregates during meiosis. Conjugation may take place either between vegetative cells or between ascospores immediately after their release. Agglutination (sexual aggregation) takes place under favourable conditions which greatly increases the chances of zygote formation.

1.5 The architecture of yeast cell

1.5.1 Gross morphology

The cells of *S. cerevisiae* are round, ovoid or ellipsoidal in shape and vary between 2.5 and 10 µm in width and 4.5 and 21 µm in length. Unstained cells exhibit little detail under the phase-contrast light microscope and it is difficult to differentiate between cytoplasmic vacuoles, granules and nuclei. Although more information can be obtained by using specific stains, it is only since the advent of the electron microscope that cell morphology has been elucidated. Characteristic features of a typical yeast cell (Fig. 1.1) are the thick cell wall within which are such structures as the plasmalemma, nucleus, mitochondria, endoplasmic reticulum, vacuoles, vesicles and granules (Webster, 1980). The distinguishing feature of multiplying yeast cells is the presence of the buds of asexual cell division. In such vegetative growth, daughter cells are initiated as small buds, which enlarge throughout a cell cycle until they reach the same size as the mother cell,

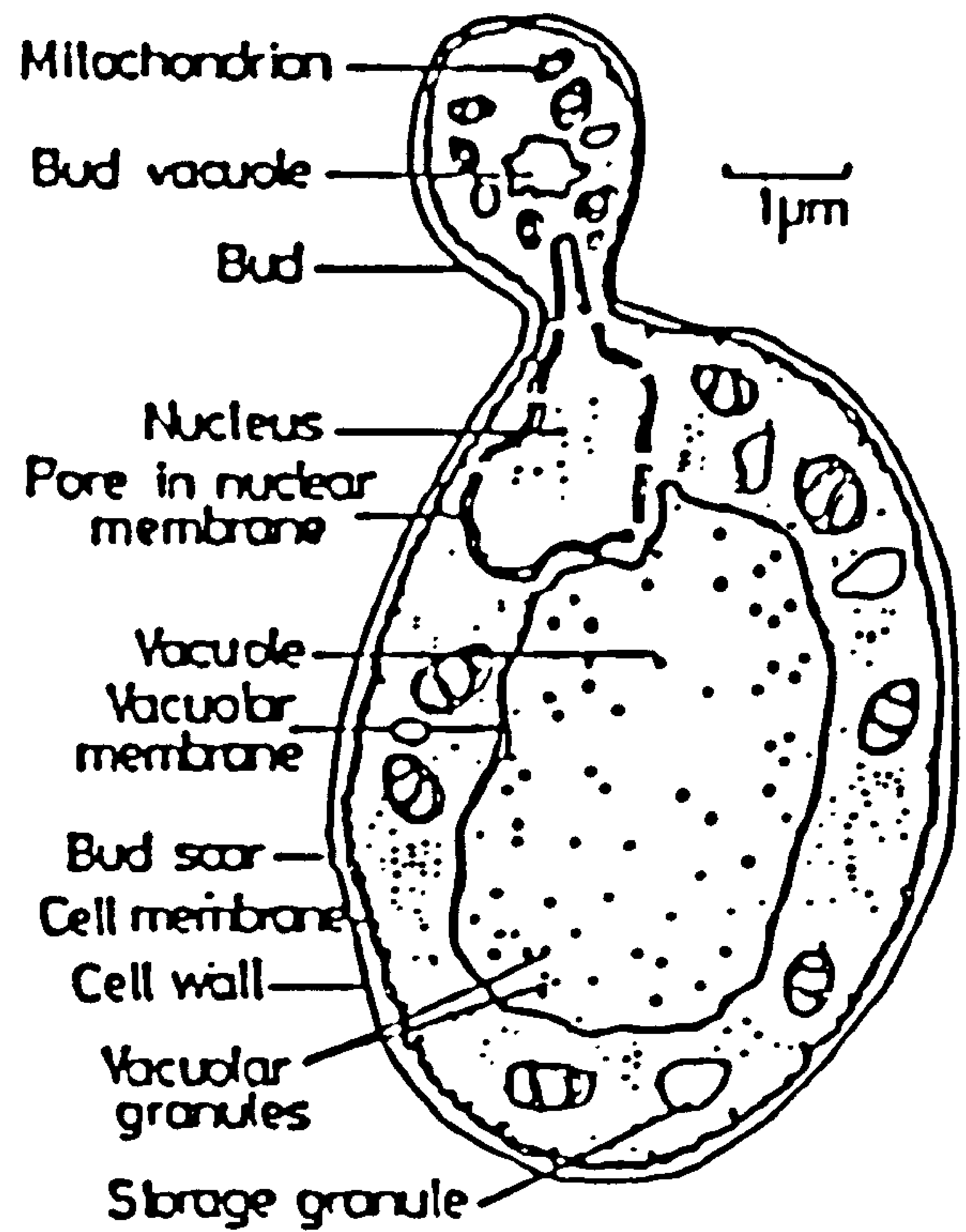


Fig. 1.1 Section through typical yeast cell showing the main features of the cell and their distribution. (Reproduced from Webster, 1980, *Introduction to Fungi*, p. 273. Cambridge University Press.)

when separation takes place. Such fission takes place after mitosis, although, often new rounds of mitosis take place before cell fission has occurred, so that clusters of daughter cells are produced. The site of cell separation can be discerned on mother cells as a structure referred to as a bud scar and on the daughter cell as the birth scar (Matile et al., 1969). Such structures can be seen using fluorescence microscopy with such stains as calcofluor. Being anionic calcofluor is precipitated on the cationic surface of N-acetylglucosamine and is strongly fluorescent when excited by ultraviolet radiation (Paton and Jones, 1971). Bud and birth scars are also very distinct in scanning electron micrographs. In S.cerevisiae, each bud produces a new scar in the mother cell wall. Thus by counting the number of bud scars, it is possible to establish the number of progeny produced by an individual cell, which can be used as a measure of its age. In any yeast population, 50% of cells were produced by the last generation of cell divisions so possess a birth but no bud scar. Of the remaining 50%, 25% will have one bud scar, 12.5% two bud scars and 12.5% more than two bud scars (Al-Shahwani et al., 1978). In flocculent yeasts, cells growing in liquid culture adhere to form clumps that settle to the bottom of the growth vessel. This phenomenon is of considerable importance in the brewing industry, since it promotes beverage clarification following the fermentation.

1.5.2 The cell wall

The yeast cell wall is a rigid structure,

approximately 25 nm thick, that constitutes approximately 25% of cell dry weight. Chemical analysis of cell walls indicates that the major components are the polysaccharides glucan and mannan; chitin and protein being important minor constituents. Glucan is an amorphous polymer of glucose units linked by ß-1,3-glycosidic bonds with ß-1,6 branch points. This carbohydrate, located in the layer adjacent to the plasmalemma, appears to be the major structural component since its removal results in total disruption of the cell wall. Mannan, an amorphous polymer of mannose linked by α-1,2-bonds, occurs mainly in the outer layers of the cell wall (Lampen, 1968). Since it is possible to remove the mannan without altering the shape of cells, it appears that this polymer is not essential for the integrity of the cell wall. The third most abundant carbohydrate component, chitin, is a linear polymer of N-acetylglucosamine that forms less than 1% of the wall composition (Table 1.1).

Table 1.1 Chemical composition of isolated cell walls of *Aspergillus nidulans* and *S. cerevisiae*

Component	*A. nidulans* (% wall dry wt.)	*S. cerevisiae* (% wall dry wt.)
Glucose	39.0	35.2
Mannose	4.0	20.0
Galactose	9.5	-
Galactosamine	2.3	-
Glucosamine	13.5	2.1
Protein	3.5	15.6
Lipid	10.2	2.7
Unspecified residue*	18.0	24.4

a. *A. nidulans* (Bainbridge *et al.*, 1979)
b. *S. cerevisiae* (Farkas, 1985)
* Residual % not specified by authors.

This polymer is only found in association with bud scars in *S.cerevisiae* cell walls. In isolated bud scars, produced by treating the cell wall with appropriate lytic enzymes, chitin is found in a ring around the periphery. Protein constitutes 10% of the dry weight of cell walls: at least some of these proteins being wall-bound enzymes. Enzymic activities described as associated with yeast cell walls include glucanase and mannanase probably involved in the softening of the cell wall to initiate bud formation. Invertase is associated with both plasmalemma cell walls, whereas alkaline phosphatase and lipases are primarily associated with the cell membrane: all of these are mannoproteins. Invertases, for example, can contain up to 50% mannan by weight in polypeptide surface glycosylations. Much of the remaining cell wall protein is also associated with mannan, and it is possible that these proteins and mannan form a structural association. The detailed organization of the yeast cell wall is not yet fully understood, but current theories favour a three-layered structure in which an inner glucan layer is separated from an outer mannan coating by a mannan-protein matrix (Fig. 1.2).

1.5.3 The cell membrane or plasmalemma

Cell membranes (the plasmalemma) can be seen in transmission electron micrographs as three-layered structures closely associated with the inner surface of the cell wall. Whilst normally having a smooth appearance, at certain stages of cell growth, invaginations can be

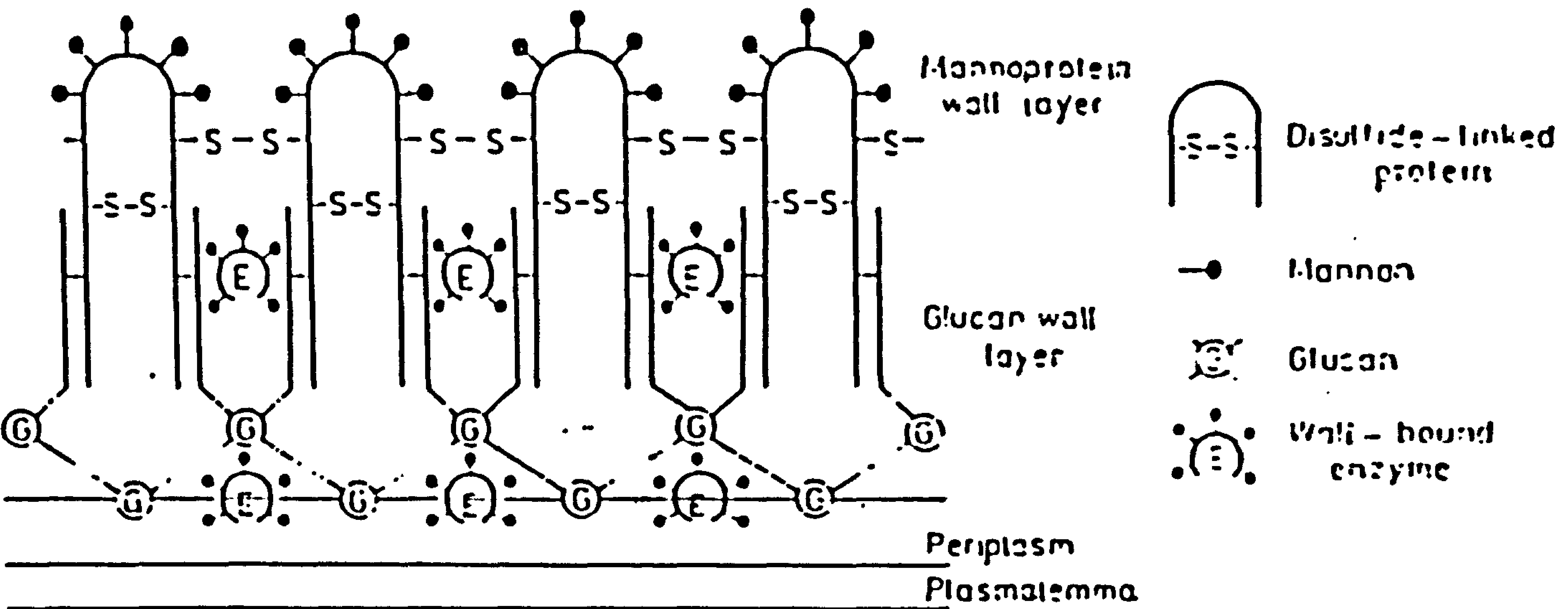


Figure 1.2 Hypothetical model of the cell wall structure of the yeast *Saccharomyces cerevisiae*.

distinguished. Determination of the chemical composition of plasmalemma requires isolation techniques eliminating other cellular components, including membranes. One standard technique involves removal of the cell wall by treatment with suitable lytic enzymes, such as those obtained from the gut of the snail Helix pomata. Such protoplasts remain intact in an isotonic solution of sugars but disintegrate in hypotonic suspension media. Fractions enriched for individual organelles, including the plasmalemma, can be obtained by differential centrifugations of lysed protoplasts. An alternative technique is to mechanically disrupt cells and wash cytoplasmic materials from the insoluble wall preparation. The plasmalemma may be either attached to or associated to cell wall material and liberated on enzymic dissolution of walls.

The plasmalemma is composed of lipids and proteins in approximately equal amounts, with a minor proportion of carbohydrate. The major lipid class are the neutral, principally, mono-, di- and triglycerides, and sterols such as ergosterol and zymosterol with phospholipids representing a minor portion of the total (Berry, 1982). The actual proportions of lipids will vary as a response to growth conditions. Weete (1980) has quoted S.cerevisiae plasmalemma lipid composition as 93.5% neutral lipid, of which ergosterol represents 26.2%, and 6.4% phospholipid, dominated by phosphatidylcholine (34%), phosphatidylethanolamine (20%), phosphatidylserine and phosphatidylinositol (together 28%), and phosphatidic acid (15%).

The nature of proteins in the plasmalemma is less well understood but these probably include enzymes involved in the uptake of sugars and amino acids. Phospholipids are amphipathic and are believed to be arranged in such a manner that hydrophilic parts of the molecule lie on the aqueous interfaces of the membrane and hydrophobic portions internal to the membrane. Protein molecules may be arranged either on the membrane surface or may transverse it.

The plasmalemma is a major organelle, acting as a permeability barrier encircling the cytoplasm controls transport of solutes into and out of the cell. Strong evidence has also been presented that the plasmalemma is involved in the control of cell wall biosynthesis in yeast.

Saccharomyces cerevisiae is unusual amongst yeasts in that it is unable to synthesize certain unsaturated fatty acids and sterols when grown under strictly anaerobic conditions (Berry, 1982). Such essential lipid components must be supplied in media if biomass production is required. Since these lipids are incorporated into cell membranes, it is possible to influence the composition of the plasmalemma. Using such techniques, it has been shown that changes in membrane lipid composition affects, osmotic properties, temperature sensitivity, alcohol tolerance, and solute uptake characteristics of the cell.

1.5.4 The nucleus

The nucleus can be distinguished in phase contrast microscopy and is usually situated between the vacuole and site of daughter buds. Chromatic bodies can be differentiated in the nucleus using specific stains such as acid fuchsin or giemsa. However, until recently limited data was available on yeast chromosomes since they are relatively small, similar in size to the E.coli genome. Moreover, they are not recognizable as discrete structures either by light or electron microscopy. The nuclear membrane, which remains intact throughout the cell cycle, is visible in electron micrographs as a double membrane, perforated at intervals with nuclear pores. Associated with this nuclear membrane is a structure referred to as a plaque. This appears to function in a similar manner to the centrioles of animal cells and appear characteristically as a multilayered disc from which microtubules extend into both nucleus and cytoplasm. Such plaques are considered to form a part of the spindle apparatus of the yeast cytoplasm and they have been shown to play a role in different stages of nuclear division.

1.5.5 Mitochondria

Mitochondria are readily recognizable in electron micrographs of aerobically-grown yeast as spherical or rod-shaped structures surrounded by double membranes. These important organelles consist largely of cristae, structures formed by the folding of the inner membrane with association of mitochondrial-specific enzymes and electron-transport

systems, that play a key role in oxidative metabolism.

Considerable research has taken place into the structure of the mitochondrion and distribution of the large number of mitochondrial enzymes. The enzymes of the tricarboxylic acid cycle are predominantly present in the matrix of the mitochondrion, whereas enzymes involved in electron transport and oxidative phosphorylation are largely associated with the inner membrane, including the cristae.

Until recently, it was considered that mitochondria were absent from anaerobically grown or catabolite repressed yeast since they could not be seen in electron micrographs and because such cells lacked many of the enzymes associated with mitochondria. The development of freeze-etching techniques for preparation of yeasts for microscopy has led to the discovery that the apparent absence of mitochondria was due to inadequate fixation in earlier protocols. Cells grown anaerobically in the absence of exogenous lipids in growth media appear to have very simple mitochondria, consisting of an outer double membrane which appears as lacking cristae. The addition of lipids such as oleic acid and ergosterol, to media results in the formation of mitochondrial cristae. The development of these structures is reported to be influenced by concentrations of oxygen, lipids and glucose in media. Thus *de novo* generation of mitochondria does not take place with shifts from anaerobic to aerobic metabolism.

1.5.6 Other cytoplasmic structures

As with other eukaryotic microorganisms, yeast cytoplasms contain membrane structures known collectively as the endoplasmic reticulum (E.R.). Some of these membranes are associated with ribosomes, the so-called rough E.R., and appear to be the site of translation of mRNA into polypeptides that will either pass into the cytoplasm or the lumen of the E.R. for translocation to other cell compartments. However, the endoplasmic reticulum appears to be involved in many other cellular activities and the relationship between this and other membranous organelles is not clear. It is clear that there is a continuity between the E.R., the outer membrane of the mitochondrion and the plasmalemma. The E.R. is also involved in the formation of vesicles which are present in the cell and in this it behaves in a manner akin to the Golgi apparatus of other organisms. Consequently it is not clear whether a true Golgi apparatus is present in yeast; membranous discs have been observed but appear to be few in number and are not clearly recognizable as a Golgi apparatus. Similarly cytoplasmic lipid granules also appear to be derived from the E.R..

Mature yeast cells contain a single large vacuole that appears to fragment at the point in the cell cycle when bud formation is initiated into smaller vacuoles which become distributed between mother cell and bud. Later on in the cell cycle, these small vacuoles reform a single vacuole in both mother and daughter cells. The function of this organelle vacuole is not clearly established but it has been

reported to contain hydrolytic enzymes, polyphosphates, lipids, low-molecular weight intermediates, and metal ions (Berry, 1982). It may act as a storage reservoir both for nutrients and for hydrolytic enzymes.

Since the technical problems of isolating and characterizing the different membrane components of yeast are considerable, it is perhaps not surprising that our understanding of the functional relationships between several of these structures is limited. Vesicles, vacuoles and other organelles are fragile and easily disrupted and fragments of membrane from individual classes of organelles are almost impossible to separate.

1.6 Yeast cell wall components

1.6.1 Cell wall structure

Structure and biosynthesis of the components of the *S. cerevisiae* cell wall have recently been reviewed in some depth (Farkas, 1979; Wessels and Siestma, 1981; Cabib *et al.*, 1982 and Sentandreu *et al.*, 1984). Briefly, yeast cell wall glucan is predominantly linked by (1,3)-ß-D-glycosidic bonds, although minor amounts of (1,6)-ß-D-glucan are also present. This glucan forms a partially fibrillar core in the internal layers of the wall. This network is embedded in an amorphous matrix of mannoproteins that extends to the more external layers. These external mannoproteins may protect the glucan skeleton from the lytic action of exogenous glucanases. The third component, chitin, a true crystalline polymer, is mainly restricted in location to the bud scar. This polymer has been the subject of structural studies since the 1920s, and is thought to exist as fibres similar to cellulose. It is known that a range of polymorphic forms exists but that ordered arrays of chitin fibrils can be laid down rapidly, often in association with proteins, as during formation of the insect cuticle (Blackwell, 1988). Synthesis of yeast wall mannoproteins is known to take place at the rough endoplasmic reticulum with molecules being subsequently translocated to the cell wall. The secretory route to the extracellular location has been determined using a combination of genetic and physiological approaches, using thermosensitive *sec* mutants (Novick *et al.*, 1980; Novick and Schekman, 1983) . Glycosylation of

polypeptides is carried out by transferases present at the inner surface of the rough E.R. (Ruiz-Herrera and Sentandreu, 1975) and probably also in secretory vesicles (Welten-Verstegen _et al._ , 1980; Santos and Sentandreu, 1981). In contrast, the polysaccharides glucan and chitin appear to be polymerized by synthases localized in the plasma membrane (Duran _et al._, 1975; Shematek _et al._, 1980). Chitin synthetase, according to the proposals of Cabib _et al._ (1982), is secreted as an enzyme precursor that is activated by proteases present at the plasmalemma. However, this enzyme may alternatively be transported in specific organelles, chitosomes, from site of synthesis to its final location at the bud scar (Bracker _et al._, 1976).

Several enzyme activities have been shown to be associated with the yeast cell surface. However, most, if not all, seem to be located in the periplasm. It is uncertain precisely which enzymes are restricted to this compartment since experimentally periplasmic proteins are defined as those released into media when cell walls aredigested under osmotically-stabilized conditions. In addition, such enzymes do not co-sediment with cell wall fractions obtained by mechanical breakage. Herrero _et al._ (1984) reported release of small number of supposedly, together with a larger number of true periplasmic proteins during protoplasting of _S. cerevisiae_ cells.

Mannoproteins have been extracted and solubilized from _S.cerevisiae_ using either SDS or urea and subsequently analysed by SDS polyacrylamide gel electrophoresis (Herrero

et al., 1984). About forty different polypeptide species could be distinguished and glycosylation of polypeptides was confirmed by showing that they were retained as binding to Concanavalin A-Sepharose beads. However, such solubilization treatments did not release high molecular weight mannans studied previously by Ballou *et al.* (1973), Raschke *et al.* (1975) and Nakajima and Ballou (1974, 1975) after disruption of yeast cell walls following extensive autoclaving.

A heterogenous population of mannoproteins was also shown to be liberated by boiling isolated *Candida albicans* cell walls in SDS. No qualitative differences could be observed between yeast and mycelial forms (Chaffin and Stocco, 1983), suggesting that factors other than differences in mannoprotein composition may be central to determination of the cell wall morphogenetic character. Subtle interactions of mannoproteins with other wall components may be involved in these structural changes.

No enzymic role has been assigned to mannoprotein molecules localized in the wall network. High-molecular weight mannan complexes are thought to be located in the external wall layers, since these are responsible for the immunogenic properties of yeast cells (Ballou, 1976). This does not preclude other roles besides the purely structural one for these proteins. In *Saccharomyces kluyveri*, carbohydrate-protein complexes have been released by zymolyase digestion of walls of differing mating types and characterized as sexual agglutinition factors (Pierce and Ballou, 1983). However, similar results have not been

reported for S.cerevisiae. In any case, it seems clear that the wide diversity of mannoprotein components in the wall must reflect a complexity of roles in a structure that has otherwise been considered as almost inert.

1.7 Glucans

1.7.1 Yeast Glucan

The generic term "glucan" covers a large group of homopolymers of D-glucose differing both in type and relative proportions of glycosidic bonds. Yeasts cell wall contain predominantly ß-linked glucans, in which the glycosyl units are interlinked by ß-(1,3) and ß-(1,6) glucosidic bonds (Farkas, 1985). Different classes of glucans can be distinguished on the basis of their solubility in various solvents and these polymers can be separated by virtue of the relative proportions of individual glucosidic linkages (Phaff, 1977: Fleet and Phaff, 1981; Bacon, 1981; Duffus et al., 1982). Some yeasts, such as certain species of Schizosaccharomyces, also contain α-glucans, linked exclusively by α-(1-3) glucosidic bonds, in cell walls (Bacon et al., 1968; Phaff, 1977; Bush et al., 1974; Manners and Meyer, 1977).

In yeasts, but also in other fungi, insoluble ß-(1,3);ß-(1,6) glucans form a microfibrillar network at the inner layer of the wall (Kopecka et al., 1974). The insolubility and crystalline nature of this glucan is thought to be caused by hydrogen bonding between linear portions of ß-(1,3) glucan chains. Increases in the number of ß-(1,6) branch points would diminish the interactions

between individual molecules increasing the solubility of glucans in alkali (Phaff, 1977). However, another possible reason for the insolubility of, at least a portion of, wall glucan may be its covalent attachment to chitin (Sietsma and Wessels, 1981; Wessels and Sietsma, 1981).

Formation of microfibrillar ß-(1,3) glucan can be readily visualised in regenerating yeast protoplasts (Necas, 1971; Kreger and Kopecka, 1973, 1976 a,b and 1978). Until relatively recently, however, attempts at synthesis in a cell free system were unsuccessful. Sentandreu et al. (1975) reported the incorporation of radiolabelled glucose, as exogenous UDP-[U-^{14}C] glucose, into toluene-permeabilized yeast cells, about 60% of the insoluble product formed being ß-(1,3) glucan. Balint et al. (1976) isolated a membrane fraction from disrupted yeast that catalysed the transfer of minute amounts of radioisotopically-labelled glucose from UDP-[U-^{14}C] glucose and GDP-[U-^{14}C] glucose into a product insoluble in 66% ethanol containing both ß-(1,3) and ß-(1,6) glucosidic linkages.

Optimization of reaction conditions enabled Lopez-Romero and Ruiz-Herrera (1977, 1978) to improve the efficiency of glucosyl transfer and to show that UDP-glucose is the principal glucosyl donor in the biosynthesis of yeast glucan. Glucono-s-lactone was a potent non-competitive inhibitor of the reaction (Lopez-Romero and Ruiz-Herrera, 1978). Interestingly, glucan synthesized by a mixed membrane fraction contained only 0.6% ß-(1,6) linked glucosyl residues, whereas material synthesised by a cell wall

fraction contained 2.5% glucosyl units linked by ß-(1,6) glucosidic bonds (Lopez-Romero and Ruiz-Herrera, 1977). These findings indicate that probably two distinct glucan synthases exist, preferentially catalysing the formation of different glucosidic bonds. Alternatively, a " branching enzyme", possibly associated with cell walls, may hydrolyse ß-(1,3) bonds and attach the displaced glucosyl units or laminaridextrins by ß-(1,6) glucosidic bonds to other sites on the ß-(1,3) glucan chains, thus creating branches (Parodi, 1981a; Kreger and Kopecka, 1976a).

A dramatic improvement in the efficiency of conversion of UDP-glucose into ß-(1,3) glucan _in vitro_ was achieved by Shematek _et al._ (1980). Purified yeast plasma membrane (in the presence of UDP-[U-^{14}C] glucose, glycerol, bovine serum albumin and GTP or ATP) transferred within 20 min at 30°C, 20%-50% of the glucosyl moiety from the nucleotide into linear ß-(1,3) glucan chains with an average length of 60 - 80 glucosyl units. The active site of the enzyme was shown to be at the cytoplasmic side of the plasmalemma. Reduction of product glucan with sodium borohydride, followed by acid hydrolysis, led to liberation of ^{14}C-labelled sorbitol, indicating that at least a part of the ß-(1,3) linked polymer was formed _de novo_.

Particulate preparations of ß-(1,3) glucan synthase (UDP-glucose: (1,3)-ß-D-glucosyltransferase, EC 2.4.1.34) have also been prepared from _C.albicans_ (Orlean, 1982; Gopal _et al._, 1982) and from the yeast form of the pathogenic fungus _Paracoccidoides brasiliensis_ (San-Blas, 1979; San-

Blas and San-Blas, 1982). Although *P. brasiliensis* yeast cells walls contain mainly α-(1,3) glucan, only ß-(1,3) glucan synthesis was observed *in vitro* using preparations from both yeast and mycelial cells (San-Blas, 1979). Partial solubilization of yeast ß-(1,3) glucan synthase could be achieved when the cells were disrupted in the presence of 1M sucrose. This enzyme, located in the 54000 x g supernatant, catalysed the formation of ß-(1,3) glucan microfibrils, each fibril consisting of about 80 polysaccharide chains, each containing about 700 glucosyl units (Larriba *et al.*, 1981).

1.7.2 Glucans of filamentous fungi

Certain filamentous fungi also produce glucans as extracellular products often loosely associated with the cell wall and as cytoplasmic reserve materials. Glucans have been reported in many fungal species and it is now recognised that the presence and structure of these glucans may vary with conditions of growth, stage of fungal life cycle and species (Bartnicki-Garcia, 1973).

1.8 Types of glucans

A review of the historical development of our knowledge of yeast cell walls has been given by Phaff (Phaff, 1963; 1971). Most data has come from *S.cerevisiae* cell walls, although more recently other species have been studied. The polysaccarides glucan and mannan together account for approximately 80% to 90% of the dry weight of cell walls. However, the glucan component has been shown to vary greatly in relation to culture conditions (McMurrough and Rose, 1967; Ramsey and Douglas, 1979), protocol for cell wall

preparation, and extraction and fractionation procedure (Bacon *et al*., 1969; Fleet and Manners, 1976).

The wall glucan of *S.cerevisiae* can be fractioned into at least three classes: (i) an alkali-soluble glucan mainly (1,3)-ß-linked with a small number of ß-1,6-linked branches (Fleet and Manners, 1976); (ii) an alkali-insoluble but acetic acid soluble ß-1,6 glucan; (iii) both alkali and acetic acid insoluble ß-1,3 glucan (Misaki *et al*, 1968 and Manners *et al*, 1973b). These glucans are responsible for the tensile strength, rigidity and shape of the cell. Manners and Patterson (1966) amongst others, demonstrated that yeast glucan has a branched structure, based upon a ß-(1,3) glucan backbone with ß-(1,6) branch points. This (1,3)-ß-glucan is thought to form an insoluble capsule, responsible for the rigidity of the cell wall. Alkali-extraction disrupts the amorphous matrix of ß-(1-3) glucan that supports the insoluble outer mannan layer, and also inner rigid, fibrillar (1,3)-ß-glucan (Bacon *et al*., 1969; Kopecka *et al*., 1974; Fleet and Manners, 1976, 1977)

1.8.1 ß-linked glucan

1.8.1.1 ß-1,4-glucan

Cellulose is a crystalline polysaccharide composed of ß-(1,4)-glucans chains of lengths up to 400 residues, interlinked in parallel or antiparallel by hydrogen bonding (Aronson, 1965). X-ray diffraction, solubility in aqueous cupric-ammonium hydroxide (Schweizer reagent) and susceptibility to hydrolysis by cellulase have been used to identify cellulose as a cell wall component in numerous

fungi including *Phytophthora*, *Pythium*, *Saprolegnia*, *Sapromyces* and *Dictyostelium* (Aronson, 1965; Bartnicki-Garcia, 1968; and Gorin and Spencer, 1968), but not in yeasts such as *S. cerevisiae*. More recently, methylation studies have confirmed the presence of (1,4)-ß-linked glucose residues in the walls of Basidiomycete QM 806 (Bush and Horisberger, 1973) and *Phythium acanthium* (Sietsma *et al*., 1975).

1.8.1.2 ß-1,3-glucan

The chemistry of ß-(1,3) linked glucans has been reviewed (Clarke and Stone, 1960; Bull and Chesters, 1966 and Barras *et al*., 1969). Although the (1,3)-ß-glucosidic linkage occurs widely in fungi, it is seldom present in linear homopolymer glucans and molecules are generally branched by (1,6)-ß-glucosidic bonds.

Porio-cocos, a tree-rot fungus, has sclerotia composed of pachyman, an alkali-soluble and water insoluble (1,3)-ß-glucan, an approximately 250-700 glucose units in chain length and with three to six branch points per molecule (Saito *et al*., 1968). Cytoplasmic reserves of a water-soluble (1,3)-ß-linked glucan, mycolaminarin, found in *Phytophthora cinnamoni* and *P. palmiroro*, contain only 30 to 40 glucose residues with one or two branch points per molecule (Wang and Bartnicki-Garcia, 1974). Another type of (1,3)-ß-glucan is found in growth media of *Sclerotium rolfisii* (Johnson *et al.*, 1963), *Pullulvinia pullulans* (Bouveng *et al*., 1963), *Claviceps fusiformis* (Buck *et al*., 1968), *Schizophylum commune* (Wessels *et al*.,1973) and

Monilinia fructigena (Santamaria *et al.*, 1978). In each case the glucan consisted of a main (1,3)-ß-linked chain to which single glucose residues were attached by (1,6)-ß-bonds. Depending upon fungal species and stage of life cycle, side groups are attached regularly to either every second, third or fourth glucose residue and glucans can range from DP 10 to 400. In species of *Claviceps*, *Schizophyllum* and *Monilinia*, glucan production decreases with culture age and it has been suggested that such glucans may serve as an extracellular reserve, being remetabolised upon depletion of other nutrients in growth media.

1.8.1.3 ß-1,6-glucan

Predominantly (1,6)-ß-linked glucans, such as those in yeast cell walls (Manners *et al.*, 1973) have not been described in higher fungi. However, no systematic studies on the presence of these glucans have been reported in the literature. Lutein acid and islandic acid, water-soluble, extracellular polysaccharides secreted by *Penicillium luteum* and *P. islandicum* respectively, are linear molecules consisting of 80 to ß-(1,6) linked glucose residues. Pustulan, a linear water-soluble (1,6)-ß-D-glucan (DP 120), can be extracted from the lichen *Umbilicaria pustulata* and related species (Hellerqvist *et al.*, 1968).

1.8.1.4 Mixed (1,3)-ß- and (1,6)-ß-linked glucans

Glucans containing blocks of unsubstituted (1,3)-ß- and (1,6)-ß-linked glucose residues with interspersed branch points, either ß-(1,3) or ß-(1,6), are found in the cell walls of numerous fungi (Bartnicki-Garcia, 1968; Gorin and

Spencer, 1968). Both alkali-insoluble and alkali-soluble (1,3;1,6)-ß-glucans have been isolated and in a few cases structural features have been determined. The alkali-insoluble glucan of *Phytophthora cinnamomi* is predominantly (1,3)-ß-linked, but is highly branched at carbon 6 (Zevenhuizen and Bartnicki-Garcia, 1969). The alkali-insoluble glucan (termed R-glucan) from *Schizophyllum commune* forms about 55% to 60% of the cell wall and is closely associated with chitin. This glucan is also highly branched containing approximately equal proportions of (1,3)-ß- and (1,6)-ß-linked residues (Wessels *et al.*, 1972; Sietsma and Wessels, 1977).

1.8.2 α-linked glucans

1.8.2.1 Starch and glycogen

Starch and glycogen are both α-linked glucan of considerable commercial importance. Iodine staining suggests that glycogen is found in all fungi, including yeasts, and starch in certain higher fungi, acting as energy reserves. Gorin and Spencer (1970) have summarised early observations on starches in *Aspergillus* and *Penicillum* species.

1.8.2.2 Pullulan and Elsinan

During growth on certain carbon sources *Aureobasidium pullulans*, a single-celled fungus, produces an abundance of a viscous, water-soluble extracellular polysaccharide, pullulan. This homopolysaccharide is composed of maltotriose (α-1,4) units linked by ß-(1,6)-bonds. A similar glucan, elsinan, has been isolated from culture brothes of *Elsinoe leucosplia*, a pathogenic fungi of tea plants. This polymer

is water-soluble with an essentially linear structure consisting of blocks of maltotriose units connected by (1,3)-α-bonds.

1.8.2.3 Mycodextran (or Nigeran) and Pseudonigeran

This is linear glucan has alternating (1,3)-α and (1,4)-α-linked D-glucopyranosyl residues. Mycodextran production has been associated with a number of Aspergillus and Penicillium species.

Pseudonigeran is water- and alkali-soluble glucan consisting of a linear arrangement of (1,3)-α-linked glucose units (Zonneveld, 1971).

1.9 Yeast mannans

1.9.1 Nature and Distribution.

Mannan is a generic name for a number of different polysaccharides, containing a high proportion of mannose residues. Unlike glucan or chitin, mannans are readily soluble in water once extracted from cells and do not appear to contribute to the shape of the organisms. They are usually covalently bound to protein, and apparently their role in yeasts is to bond together different components of the yeast cell wall. These polymers polymers can be divided into three groups on the basis of function: (i) integral components of the cell walls; (ii) surface antigens and agglutinins and (iii) enzymes located in both intra- and extra-cellular compartments.

The structure of yeast mannan appears to be well established and this understanding of polymer structure greatly facilitated studies aimed at elucidating the

molecular mechanism of its biosynthesis. Phaff (1963) showed that yeast cell walls contain 40% mannan, 40% glucan and varying amounts of lipids and protein. Ballou (1976) suggested, as a working hypothesis, that cell wall mannan is differentiated in structure by its specific location.

Mannan is also bound to the surface of enzymes such as invertase and acid phosphatase (Ballou, 1976). Lampen (1968) found that mannan is always associated with protein in the cell wall, occurring mainly near the exterior. Mannan was found to be located on the outer surface of S.cerevisiae, Candida mycoderma and Sporoblomyces roseus but seems to be overlayed by other components in certain other yeasts.

Yeast mannoproteins have covalently-linked carbohydrate of up to 150 mannosyl monomers, linked via N,N'-diacetylchitobiose bridges to asparagine as N-glycosylations. Carbohydrate can be removed polypeptides by treatment with endo-N-acetyl-ß-D-glucosaminidase H (Endo H) which cleaves glycosidic bonds between the two N-acetyl-D-glucosamine residues, or hydrazinolysis, destruction of N-glycosidic bonds to protein.

The "inner core" of N-glycosylations, previously thought to consist of 12 to 17 mannosyl units (Nakajima and Ballou, 1975), was subsequently shown to contain, on average, only 11 mannose residues. The structure of the yeast mannan inner core resembles those of "high-mannose" animal glycoproteins (Kornfeld and Kornfeld, 1976). A second carbohydrate moiety of yeast mannan, typically short manno-oligosaccharides, is linked glycosidically to serine

and/or threonine residues in polypeptides. These O-glycosylations can be removed from peptides by ß-elimination in weak alkali (Sentandreu and Northcote, 1969; Nakajima and Ballou, 1974). In certain yeasts α-(1,6)-linked mannoses can be interspersed with α-(1,3) and α-(1,2) bonds, or alternate monosaccharides can be present in side chains (Ballou, 1976).

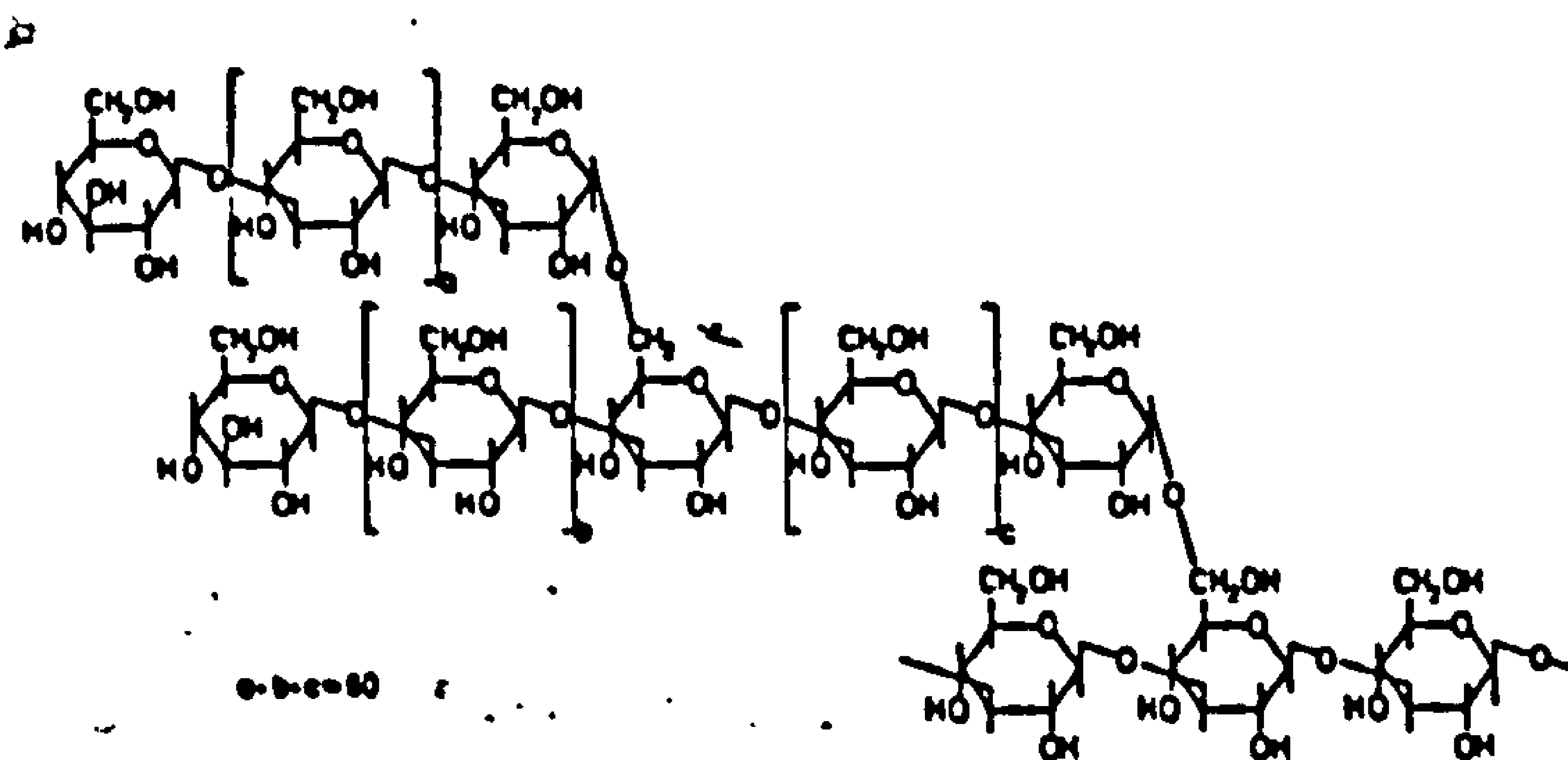
1.10 Yeast chitin

1.10.1 Nature and distribution of chitin

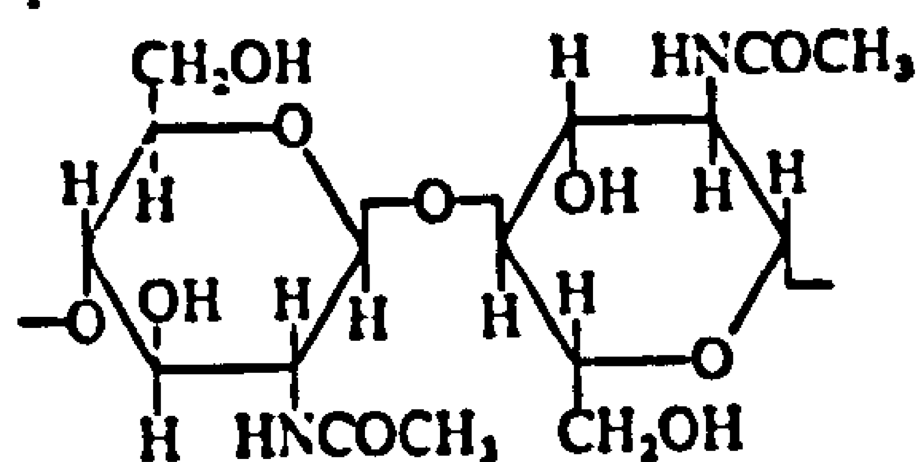
Chitin is an important microfibrillar component of fungal cell walls although relatively little (in *S. cerevisiae* <1% by weight) is present in yeasts and its structural significance is less evident: it is reportedly absent from *Schizosaccharomyces*. In crustacean shells, chitin fibrils, linked to protein are embedded in a matrix of calcium carbonate, and phosphate (Muzzarelli, 1970).

The polymer was first identified in *S. cerevisiae* cell walls by its characteristics X-ray diffraction pattern (Houwink and Kreger, 1953) and Bacon *et al.* (1969) reported it was restricted to in and around the bud scars in *S. cerevisiae*. In higher fungi, chitin is the major component of cell walls and septa and quantifications of this and other polysaccharides, has been an key character in fungal taxonomy (Bartnicki-Garcia, 1968). Chitin fibrils vary in length depending on species and cellular location (Gow and Gooday, 1983; Hunsley and Burnett, 1970). However, in *S. cerevisiae*, chitin is exclusively located in the primary septum forming between mother and daughter cells (Cabib *et*

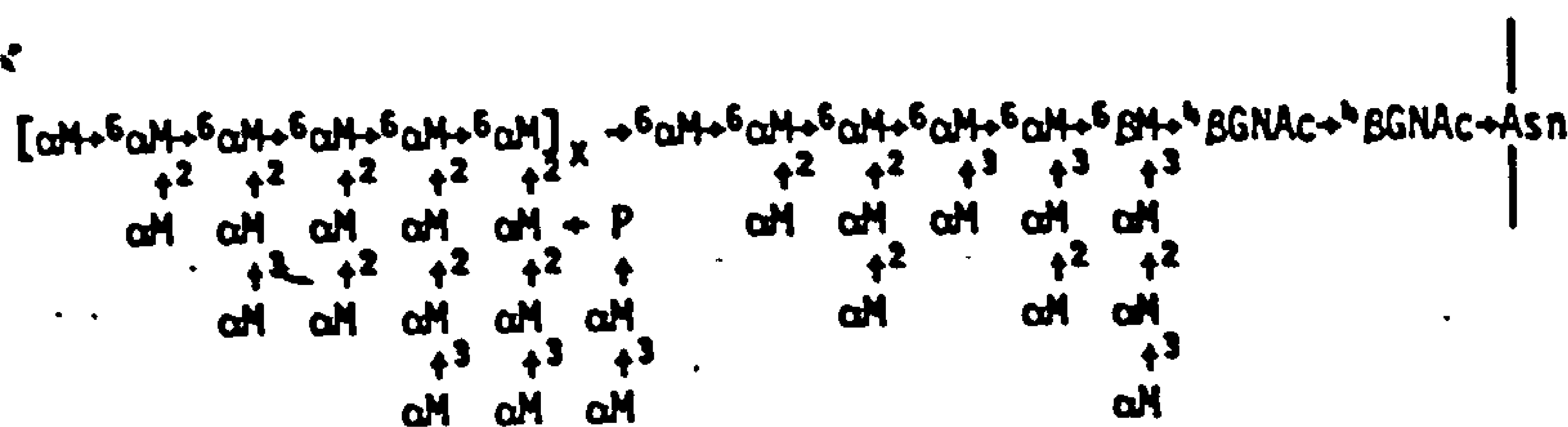
al., 1979). Thus because primary septum formation has been extensively studied as a model for morphogenesis (Cabib, 1981; Cabib et al., 1974, 1982 ; Cabib and Shematek, 1981). Chitin and cell wall ß- glucan are intimately associated by covalent linkage (Sietsma and Wessels, 1979; Molano et al., 1980) but in M. rouxii, a part of the chitin is reported to be deacetylated, yielding chitosan (Bartnicki-Garcia, 1968).



A. Yeast glucan



B. Repeating unit of chitin



C. Structure of yeast mannan

1.11 Enzymes involved in the degradation of yeast cell wall components

1.11.1 Yeast ß-glucanases

Synthesis of yeast ß-glucanases is thought to be coupled to the morphogenetic events in which these enzymes are involved (Farkas *et al.*, 1973). Glucanases have been studied extensively in both bacteria and fungi (Tanaka and Phaff, 1965; Bull and Chester, 1966) but their occurrence and function in yeast has received relatively little attention. In yeasts, the presence of an enzyme system capable of hydrolysing the ß-1,3-glucosidic linkage was inferred from the observation that certain yeast species were able to grow on laminarin (Arnold, 1981b). Brock (1965) first reported the presence of an intracellular ß-glucanase in baker's yeast and subsequently a number of ß-glucanases have been isolated from a range of yeast species.

1.11.1.1 Degradation of glucan

Yeast wall glucans are currently thought to undergo little turnover during vegetative growth (Budd, 1974; Kratky *et al.*, 1975; Villa *et al.*, 1980). Limited site-specific hydrolysis of rigid skeletal wall ß-glucans, carried out by endogenous ß-glucanases, probably takes place during such morphogenetic processes as budding, wall growth, conjugation and ascus formation. Indirect evidence for participation of ß-glucanases in wall growth was reported by Notario *et al.* (1982a) who observed that there is a good correlation between ß-glucanase activity and rate of $[^{14}C]$ glucose incorporation into ß-glucan in *C.albicans* cell walls.

Andaluz *et al.* (1986) have proposed a model for ß-glucan biosynthesis in which glucan molecules are synthesized on a non-glucan primer, presumably a protein. Thus, wall-associated ß-glucanases would carry out release of newly-formed glucan chains from their primers, enabling polysaccharides to be incorporated into the wall. The action of cell wall hydrolases must be finely attuned to the rate of synthesis and incorporation of new polymers into the wall (Johnson, 1968; Bartnicki-Garcia, 1973). During autolysis, the equilibrium will be shifted so that the action of lytic enzymes prevails, resulting in the breakdown of walls and lysis of cells (Arnold, 1981b).

A characteristic property of yeast ß-glucanases is that they are primarily located external to the plasmalemma in association with their substrates. Cortat *et al.* (1972) reported that approximately 80% of ß-(1,3) glucanase activity is released when yeast cells are converted into protoplasts, indicating that enzymes must be located in the periplasm or associated with the wall. Barras (1972) estimated that 80% of total cell endo-ß-(1,3) glucanase was associated with the wall in *Schiz. pombe*. When isolated cell walls were incubated in buffer for prolonged periods, glucose, laminarioligosaccharides and high molecular-weight soluble wall polymers were released. During this hydrolysis previously wall-bound endo-ß-(1,3) glucanase was also liberated. Similar results were obtained by Fleet and Phaff (1973, 1974) with *Schiz. versatilis* var. *japonicus* and a number of other yeasts. The same authors reported the

isolation of an endo-ß-(1,3) glucanase and an exo-ß-glucanase from supernatants of autolysed walls of *Schiz. versatilis*. This exoglucanase split both ß-(1,3) and ß-(1,6) glucosidic bonds but was found to be unable to hydrolyse isolated cell walls (Fleet and Phaff, 1975).

The nature of the association between ß-glucanases and cell walls is not entirely clear. Various solubilizing agents, such as salts, organic solvents, thiol reagents, proteases and detergents were all ineffective in dissociation of ß-glucanases from purified cell walls (Arnold, 1972; Fleet and Phaff, 1974; Reichelt and Fleet, 1981; Notario, 1982). Similarly sonication did not enhance soluble glucanase activity. Release of ß-glucanases during autolysis of cell walls could, however, be suppressed by the proteinase inhibitor phenylmethylsulphonyl fluoride, indicating that ß-glucanases may be covalently bonded attached to walls so that polypeptide hydrolysis is required to generate free enzyme (Reichelt and Fleet, 1981).

Some ß-glucanase activity has been shown to be secreted into culture media during yeast growth (Abd-El-Al and Phaff, 1968; Biely *et al.*, 1972; Villa *et al.*, 1975). Vegetative cells and protoplasts of *S. cerevisiae* secrete at least three different ß-glucanases (Farkas *et al.*, 1973; Sanchez *et al.*, 1982a): an endo-ß-(1,3)-glucanase and at least one exo-ß-glucanase hydrolysing yeast glucan and pustulan (ß-(1,3) and ß-(1,6) glucosidic bonds). Similarly, Villa *et al.* (1975) observed the secretion of three different ß-glucanases by intact cells and protoplasts of *Pichia*

polymorpha. One enzyme was shown to be an endo-ß-(1,3) glucanase, a second a non-specific exo-ß-(1,3); ß-(1,6) glucanase and the third a specific exo-(1-3) glucanase. Four endo- and two exo-ß-(1-3) glucanases were identified in cell extracts and wall autolysates from *S. cerevisiae* by Hien and Fleet (1983).

1.11.1.2 Exo-ß-glucanases

As discussed previously, it is clear that differing isoenzymes of ß-1,3-glucanases are present in cell extracts, cell walls and culture fluids of various yeasts. Abd-el-al and Phaff (1968) first demonstrated the presence of exo-ß-glucanases in culture fluids and cell extracts of *Fabospora fragilis*, *Hansenula anomala* and *S. cerevisiae*. These authors also showed that the catalytic properties of glucanases varied between yeast species and yeast exo-ß-glucanases were distinctly different from those of the fungi studied.

1.11.1.3 Endo-ß-glucanases

The presence of endo-ß-glucanase activity was reported first by Abd-el-al and Phaff (1969) in studies of bipolarly budding yeasts such as *Hanseniospora valbyensis* and *H. uvarum*. Later Fleet and Phaff(1974) characterized an endo-glucanase from *Schizosaccharomyces* and reported the occurrence of similar enzymes in cell walls of *Saccharomyces*, *Kluyveromyces*, *Hansenula* and *Pichia* species. Endo-ß-1,3-glucanases have, in particular, been studied in *S. cerevisiae* (Arnold, 1972) and Villa *et al.* (1978) isolated ß-glucanases from *Pichia*, *Candida* and

Kluyveromyces. In certain yeasts ß-1,6 and α-1,3 glucanases have been reported (Arnold, 1972) but very little is known about these enzymes. Interestingly, α-1,3-glucanases have not only been found in yeasts where α-1,3-glucan is a component of the cell wall (e.g.*Cryptococcus albicans*) but also in yeasts where this glucan is absent from cell walls.

1.11.1.4 Function and regulation of ß-glucanase activities

Since endogenous glucanases have the potential to hydrolyse the rigid component of yeast cell walls and are thought to be autolysins (enzymes responsible for autolysis of yeast cell walls), much speculation has been given to the function and control of these enzymes. Possible functions include cell wall expansion during budding, cell separation (bud abscission), formation of conjugating tubes and cellular fission during mating (Crandall *et al.*, 1977), and release of ascospores from asci (Phaff, 1977). Brock (1965) reported that the level of ß-1,3-glucanase in cell extracts of conjugating cells of *Hansenula wingii* was much higher than in extracts of vegetatively growing cells. These authors proposed that this glucanase may be responsible for softening and degradation of cell walls during conjugation and fission. Similarly, sharp increases in the ß-1-3-glucanase activity of *Schiz. versatalis* during early stages of cell agglutination were reported by Fleet and Phaff (1975).

In *Kluyveromyces*, *Hansenula* and *Schizosaccharomyces*, where spores are rapidly liberated from asci on spore maturation, exhibit higher levels of endogenous ß-1,3

glucanases than species where spores are liberated more slowly by swelling processes, implying the enzymes are involved in spore release (Fleet and Phaff, 1975).

In budding yeasts such as *S. cerevisiae*, cell division involves early modification of properties of the wall at the location of the new bud. Rapid extrusion of buds seems to depend on local softening of the existing cell wall. Cytological studies have shown that budding is initiated by localized vesiculation in the endoplasmic reticulum. Small vesicles fuse with the cell wall at the site of the nascent cell; it has been reported that ß-1,3 glucanase activity is associated with such vesicles (Cortat *et al.*, 1972). Endogenous glucanase activity in multiplying budding cells is significantly higher than that in static non-budding cells, supporting the concept that glucanases are involved in plasticizing and degradation of cell walls during the budding (Maddox and Hough, 1971).

Yeast autolysis is effected with cell walls undergoing self degradation or autolysis during incubation in buffer (Kroning and Egel, 1974). In a *Kluyveromyces* species, which had two exo- and two endo-ß-glucanases, it was found that both endo-glucanases exhibited lytic attack on yeast cell walls. In addition, a typical exo-glucanase, with activity against periodate-oxidized laminarin, also degraded wall material. It has been concluded that these three enzymes act synergistically on the cell wall (Kroning and Egel, 1974)

Vesiculation, as described by Cortat *et al.* (1972), is one means of compartmentalizing and localizing endogenous

glucanase activity during transport to the presumptive bud site. Final glucanase activity is external to the cytoplasmic membrane and is located in the periplasmic space and further may be tightly associated with its cell wall substrates (Villa *et al.*, 1975). It has been reported that low-molecular weight carbohydrates like laminaribiose may influence glucanase activity by feedback inhibition (Fleet and Phaff, 1974). It has also been suggested that endogenous glucanases may be secreted in inactive or zymogen forms, requiring controlled activation by protease action as for chitin synthetase (Cabib *et al.*, 1974).

However, Reichelt and Fleet (1981) reported that protease treatment activated wall-associated endo-ß-1,3 glucanase in *Schizosaccharomyces pombe*. Molina *et al.* (1987) found that protoplast lysates or extracts from cells treated with acid (0.1M HCl) were devoid of glucanase activity, only regaining traces of activity following prolonged incubations. Furthermore, glucanase activity in supernatants of cell free extracts following high speed centrifugation was reported to be very low when assayed immediately after their preparation. However, increased enzyme activities were obtained by maintaining such preparations at 0°C for several days suggesting active exo-1,3-ß-glucanases are generated from inactive precursors by proteolysis after secretion.

A number of recent studies have shown that certain yeasts may contain a number of exo- and endo-ß-glucanase isozymes with functions in different phases of the cell cycle. Although total glucanase activity is known to vary

during the cell cycle, the contributions of individual isozymes may differ with control of activity operating at either transcription or translation (Rey *et al.*, 1979).

Purified endo-1,3-ß-glucanases from *Schizosaccharomyces versatilis* are very active on isolated cell walls of yeast, suggesting an *in vivo* wall degradative function (Fleet and Phaff, 1974), whereas the non-specific exo-1,3-ß-glucanase from this yeast shows negligible hydrolytic activity. This isoenzyme may only act on the products of endo-glucanase depolymerisation of glucan (Fleet and Phaff, 1975).

Santos *et al.* (1979a) *S. cerevisiae* mutants defective in secretion of the non-specific exo-ß-glucanase, which grew normally implying that suggests that this enzyme is not essential for cell wall modification during morphogenesis and growth. The fact that no exo-ß-glucanase activity could be detected in *Schiz. pombe* supports this hypothesis (Reichelt and Fleet, 1981). This yeast, however, secretes two endo-ß-glucanase isozymes, only one of which was found to be lytic against isolated cell walls. A role for ß-glucanases in dimorphism, transition from yeast to mycelial form, has been proposed for *Paracoccidioides brasiliensis* by Flores-Carreon *et al.* (1979). These authors demonstrated that transition from yeast to mycelium was accompanied by an increase in ß-1,3-glucanase activity. However, the generality of this hypothesis was not supported by similar studies on *C. albicans*, reported by Molina *et al.* (1987), since in this organism glucanase activities decreased substantially with the transition.

Hien and Fleet (1983) demonstrated the presence of six ß-glucanases in wall autolysates and cell extracts of *S. cerevisiae*, although it cannot be concluded that these are encoded by as many genes. Two isozymes were exo-acting and four were endo-glucanases. Each protein species differed in molecular weight, kinetic properties and activities against cell walls. Glucanases were designated as I, II, IIIA, IIIB, IV and V with II and IIIA being exo-glucanases and isozymes I, IIIB, IV and V endo-glucanases. Individual glucanases varied in the extent of hydrolysis of isolated cell walls; since these are composed of at least three different populations of glucans, a complex of glucanases will be required for significant degradation or modification.

Molina *et al.* (1987) reported the presence of single exo- and endo-acting hydrolases in *S. cerevisiae*. Cell-free extracts of a *exb1* mutant (diploids containing two differing defective alleles of the EXB1 gene) were totally deficient in exo-glucanase activity whereas endo-glucanase activity appeared as multiple heterogenous forms, probably due to incomplete glycosylation of polypeptides. However, protoplast lysates of wild type and *exb1* strains appeared identical in their complement of glucanases, each consisting of two enzymes clearly distinguishable from periplasmic glucanases. These authors further showed that both wild type and *exb1* protoplasts secreted small amounts of glucanases when cultured in osmotically-stabilised regeneration medium. However, wild types also secreted periplasmic exo-ß-glucanase absent from the mutant strain. It was concluded

that these results confirmed that the classical glucanases of *S. cerevisiae*, located in the periplasmic space must be activated following secretion as inactive precursors.

Although it is known that there are two ß-glucanases in *S. cerevisiae*, several reports have made it difficult to maintain the simple concept and role of two glucanases in the vegetative cells. Since defective exo-ß-glucanases mutants grow, mate and sporulate normally, Santos *et al.*, (1982) suggested that the exo-ß-glucanases are non-essential or their function can be complemented by other enzymes although these although reported the presence of a second glucanase, serologically related.

To summarise, it is clear that a number of glucanase isozymes may be present in each yeast species and different enzymes may function at different stages of the yeast cell cycle. The regulation of the ß-glucanase system of *S. cerevisiae* seems to be very complex with individual enzymic activities varying independently through out the life cycle. Such variations are possibly due to *de novo* synthesis or enzyme activation or both. Neither is the precise function of each enzyme known. Direct association of individual glucanase activities with particular morphogenetic events will require detailed knowledge of all of the glucanases in an individual strain of yeast with estimation of activities of each enzyme at different stages during the cell cycle.

It can be concluded that our knowledge of the role of glucanases in yeast physiology is at best fragmentary, often conflicting and would merit further investigation.

1.11.2 Proteolytic enzymes

A considerable amount of data on yeast proteases has been built up through the work of Vines (1901, 1904 and 1909); Dernby (1918 a,b); Hecht and Civin (1936); Johnson and Berger (1940) and Johnson (1941,1948). It was also known that proteinases and peptidases were present in yeast autolysate. Johnson (1941), in particular, studied the characteristics of yeast exopeptidases in great detail.

Lenney (1956) reported the presence of proteases A and B in chloroform autolysate of S. cerevisiae. These proteases were found to differ in properties. Isozyme A was found to have a pH optima of 3.7 with acid-denatured haemoglobin as a substrate and was found to be extremely labile in urea solutions; protease B showed a neutral pH optima of 6.2 on urea-denatured proteins and was stable in urea solution but was inhibited by sulphydryl reagents.

Hata et al. (1967) reported the presence of three proteases in yeast, designated as proteases A B and C. Later, Hayashi et al. (1968) provided evidence of the presence of inactive precursors of proteases B and C in baker's yeast, suggesting their existence in vivo predominantly as inactive precursors whereas protease A was present only as the active enzyme.

Maddox and Hough (1969) characterized four proteolytic enzymes, designated as A,B,C and D, and found that isozyme A was the most abundant protease in the strain of S. cerevisiae studied. Protease A appeared to be less thermostable at temperatures higher than 30°C and to have a

more alkaline pH optimum activity than other isoenzymes. The products of protease A activity were found to be either amino acids or low-molecular weight peptides whereas proteases B,C and D gave products with molecular weights >500 Daltons. All four isozymes were characterised as glycoproteins containing both glucose and mannose residues in varying proportions.

1.11.2.1 Proteolysis during autolysis process

Vosti and Joslyn (1954) studied conditions that influence autolysis in S. cerevisiae. They showed that in aerobic growth autolysis of cells is relatively frequent, whilst in the absence of aeration, autolysis was less frequent. The optimum pH for autolysis in aerobic growth was 5.0 as compared with 4.6 for anaerobic cultures. Further experiments showed that yeast grown under favourable conditions autolyses less rapidly than that grown under less favourable conditions. Proteolytic activity was induced when protein was used as sole nitrogen source for growth whereas the activity was absent when either amino acids or ammonium salts and calcium and magnesium ions enhances activity.

Calleja et al. (1977) observed that approximately 15% of conjugating cells of a Schizosaccharomyces sp. lysed during the process, with cell disruption occurring at the site of union. It has been suggested that such conjugation-induced lysis is either due to faulty fusion or badly-controlled glucanase activity (hyperactivity) during cross-wall removal.

1.11.2.2 Regulation of protease activity

Regulation of extracellular protease production in *Candida lipolytica* was extensively studied by Ogrydziak *et al.* (1977). These authors showed that extracellular protease production is regulated in response to at least three different metabolic signals: reflecting the carbon, nitrogen, and sulphur status of the cell. They also reported that proteases were induced by the presence of proteins in media and that anabolic glutamate dehydrogenase did not appear to be involved in this regulation.

Klar and Halvorson (1975) obtained data showing increases in the specific activities of proteases A,B and C during sporulation of *S. cerevisiae*. This increase in activity appeared to be through *de novo* synthesis of enzymes, although no new sporulation-specific proteases were observed.

Yeast vacuoles have been studied morphologically as well as with regard to the localisation of proteases and other autolytic enzymes (Matile and Wiemken, 1967). Results obtained suggest that the vacuoles may serve the function of lysosomes in the yeast cell.

1.11.3 Yeast mannanases

Although mannan is a major wall component, known to be subject to metabolic turnover (Pastor *et al.*, 1982; Kratky *et al.*, 1975), little research has been reported on mannanases in yeasts. Both exo- and endo-mannanases are presumed to participate in breaking down cell wall mannan and modifying mannoprotein glycosylations. Although yeast

mannanases have been studied extensively by Fleet (1984), Kratky *et al.* (1975), Gale *et al.*(1980) and Villa *et al.* (1978), none of these authors were able to detect mannanase activity in cell-free extracts of *S. cerevisiae*, *C.albicans* and *P.polymorpha*. These results were consistent with findings that polymeric mannan, rather than low-molecular weight mannose or oligosaccharides, is solubilised during autolysis of the *S. cerevisiae* cell walls.

However, Maddox and Hough (1971) and Lyons and Hough (1970) have reported the presence of mannanase activity in culture fluids and autolysates of *S. cerevisiae* and *S. carlsbergensis* and noted cyclical variation of this activity during budding and a major increase in activity during the stationary phase. Augustin *et al.* (1980) reported the production of an extracellular mannanase by *S. cerevisiae* and 47 other yeast species during growth on mannan as sole carbon substrate. Mannanase has also been detected in extracts of *C. albicans* by Barrett-Bee *et al.* (1982). Unfortunately none of these studies reported in detail on the specificity of the mannanase enzymes.

1.11.4 Degradation of chitin

Although often neglected, chitin breakdown is a significant part of the carbon cycle. Chitin, a homopolymer of N-acetylglucosamine linked by ß-1,4 bonds, has an important structural role in the exo-skeletons of arthropods and crustaceans. In breakdown processes, the insoluble crystalline material is depolymerised by chitinases to yield low-molecular weight oligosaccharides, notably the

dimer diacetylchitobiose. These oligosaccharides are converted into the monomer by ß-N-acetyl-hexosaminidases or chitobiases. Chitinases also have important physiological functions in animals with chitinous exoskeletons where hydrolysis of the cuticle or shell forms an initial step in the moulting process.

1.11.4.1 Yeast chitinases

Benecke (1905) was the first worker to reported the isolation and study of a chitin-decomposing microbe but concluded that although "Bacillus chitinovorus" (a non spore former) must have possessed a chitinase, he could find no evidence of the products of chitinase activity. Most of the published reports of microbial chitin decomposition are concerned with taxonomic and physiological studies of microorganisms, rather than with the enzymic aspects of the degradative process (Mommsen, 1978). Enzymic hydrolysis of chitin to N-acetyl-D-glucosamine is thought to be performed by two enzymes working in synergy. The first poly 1,4-ß-(2-acetamido-2-deoxy-D-glycoside) glycanohydrolase or chitinase, removes chitobiose units; and the second chitobiose acetaminodeoxyglycohydrolase or chitobiases, degrading diacetylchitobiose to N-acetyl-D-glucosamine.

Chitin forms up to up to 16% of the dry weight in Basidiomycetes (Gooday, 1979). Biosynthesis of chitin takes place by addition of the precursor uridine di-phosphate N-acetyl-D-glucosamine to growing chains of acetyl-D-glucosamine catalysed by the enzyme chitin synthase, a process extensively described in S. cerevisiae (Cabib, 1981;

Cabib *et al.*, 1982). In contrast, little has been published on its degradation and modification during budding, growth and conjugation. Cabib *et al.* (1982) and Elango *et al.* (1982) have described the isolation and purification of an endo-chitinase from *S. cerevisiae* that was a mannoprotein found in both the vesicules and in the periplasm. Chitinase activity was found to increase during exponential growth, implying a role in the budding process (Phaff, 1984). Chitin production and deposition increase during cell conjugation (Schekman and Brawley, 1979) and yeast-mycelial conversion (Sullivan *et al.*, 1983). It would seem likely that modification of the polymer during such events will require chitinase activity.

2. Manufacture of autolysed yeast products

2.1 The process of autolysis

Autolysis is a general term used to describe the dissolution of cell components that follows cell death. The action of endogenous cell enzymes leads to an active autofermentation, detected in light microscopy as rapid staining of the cytoplasm followed by characteristic granulation. Proteolysis and breakdown of nucleic acids are major autolytic activities leading to the accumulation of amino acids, nucleotides and their derivitives as well as low molecular-weight carbohydrates (Vosti and Joslyn, 1954).

The action of intracellular enzymes, particularly proteases, produces amino acids that react with sugars on heating to give Maillard (non-enzymic browning) reaction products with a pronounced meat-like flavour (Prescott and Dunn, 1982). Proteolysis is thus, regarded as one of the most critical components in determining autolysate flavour but choice growth media also has a significant impact. Although, according to Hough and Maddox (1970) 'autolysis' means 'self-destruction' of the yeast, the term is now expanded to include processes in which a whole range of artificial methods by which yeasts and their cellular contents are converted to solubles for use in the food industries.

2.2 The biochemistry and enzymology of autolysis

Initially, in autolysis, autofermentations of reserve carbohydrates (mainly glycogen) deplete the cell of storage polymers. Subsequently endogenous protease and nuclease activities increase and the cell membrane adjacent to the cell wall loses its selectivity, through modifications of protein functionality, leaking soluble products into culture media followed by an increase in cell wall porosity increases through ß-glucanase action (Arnold, 1981).

Extracellular proteolysis also occurs as autolysis proceeds (Peppler, 1982) and the release of protein and its breakdown into amino acids is generally considered to be the most important aspect of yeast autolysis for development of flavoured yeast products (Hough and Maddox, 1970). It has also been suggested that yeast proteases that play important regulatory roles in living cells are involved in the process of cell wall dissolution (Arnold, 1981).

During autolysis, carbohydrate is also released from the cell in the form of glycoprotein which appears to be the result of the action of at least two ß-glucanase isozymes and possibly amino peptidases during dissolution of the cell wall (Hough and Maddox, 1970; Arnold, 1981).

2.3 Effect of temperature and other factors on autolysis

Autolysis is normally induced during industrial manufacture of yeast products by heating cells to a temperature at which killing is achieved but enzyme activities preserved, typically between 40 and 60 °C (Peppler, 1967; Hough and Maddox, 1970; Reed and Peppler, 1973; Prescott and Dunn, 1982; Hill, 1981). Autolysis has been reported as induced by 10 h incubation at 47 °C or 2 h at 90 °C. Different processes will yield commercial products differing in functional properties and flavour profiles although Maillard products, with important effects on yeast product aroma, will vary (Darrington, 1986). Degradative enzyme action during autolysis forms pools of low-molecular weight Maillard precursors for flavour-forming reactions. Thus although elevated temperatures initiate autolysis, heating of yeast cells in boiling water inactivates enzymes and limits the extent of autolysis (Arnold, 1981).

A number of organic compounds have been reported at one time or another to induce autolysis in yeasts (Arnolds, 1981). For example, ethyl acetate, amyl acetate, toluene, ethanol, thymol, phenol, ether, formaldehyde, and chlorinated hydrocarbons such as chloroform (not permitted in USA) have been utilised to kill cells and to serve as antiseptics, suppressing growth of thermophiles and altering cell permeabilities. Other plasmolysing agents, e.g. sodium chloride, sucrose, potassium chloride and combinations of salt and ethanol are used for labilization of cell membranes

during drying of the yeast before autolysis (Hill, 1981; Peppler, 1982; Prescott and Dunn, 1982).

Autolysis is carried out until the desired degree of polymer breakdown, measured as level of soluble α-amino nitrogen is achieved (Reed and Peppler, 1973; Prescott and Dunn, 1982). However, the degree of solubilisation of the yeast biomass will depend upon autolysis temperature and pH, incubation time and the strain of yeast and its propagation. Vosti and Joslyn (1954) and Hough and Maddox (1970) have suggested that pH and temperature are the primary factors determining not only the rate and degree of autolysis and solubilisation but also the quality and flavour profile of the resulting product.

Autolysates of primary yeasts (grown specifically for biomass production) have been reported as having higher protein contents at 60 - 75% than secondary (e.g. spent brewers') yeasts at 45 to 55% (Przybyla, 1986). Sarwar *et al.* (1985) showed that growth of *C. utilis* and *S. cerevisiae* on different substrates affected the levels of nucleic acid breakdown products and other nutrients in derived autolysates. Levels of both nucleic acid and protein-derived compounds were higher in *C. utilis* autolysates than similar products obtained from *S. cerevisiae*. Lee *et al.* (1981) showed that the period of autolysis had a major effect on the extract although complete solubilisation was not achieved. In any autolysis a significant proportion by weight of biomass (cell wall material, other carbohydrates such as glycogen and mannan, and proteinaceous material)

remains insoluble (International Hydrolysed Protein Council, 1977). Thus, in industry, research has been targeted towards increasing extraction yields in the new yeast product manufacturing processes including the initiation of cell lysis by addition of exogenous enzymes such as lysozymes, glucanases and proteases from various organisms. In addition, mechanical rupture and comminution of cells by grinding and cell wall fracture often destroys cell compartmentalisation releasing endogenous autolytic enzymes. Although suitable equipment for such processing is commercially available, the additional energy costs have limited their economic attraction (Hill, 1981; Peppler, 1982).

Other chemical, biochemical and physical treatments have been reported to accelerate yeast autolysis, e.g. stirring has a positive effect on extraction yield (Lee *et al.*, 1982). Addition of early autolysates to cells in the later stages of solubilisation, microwave treatments, dilute alkali treatment and high-pressure extrusion of yeast cells have all been described as autolysis accelerators (Choi and Shim, 1984).

2.4 Production of yeast extracts

Although "Yeast extract" is a general descriptor for inactive yeast products the term is used as a synonynm for yeast autolysates (Reed and Peppler, 1973; Prescott and Dun, 1982). It may also be used to designate hot water extracts of autolysed yeasts (Reed and Peppler, 1973). Thus, some clarification of the terminology is helpful.

2.4.1 Definition

The International Hydrolysed Protein Council (1977) has reported that autolysed yeast extracts are currently used as food ingredients and natural food flavours throughout the world. Such products are composed primarily of:

a. Breakdown products of proteins and nucleic acids (including amino acids, peptides and nucleotides) that have arisen through enzymic hydrolysis of the microbial polymers and reactions promoted by thermomechanical processing of breakdown intermediates (pyrroles, pyrazoles and other Maillard products).

b. Breakdown products, such as sugar and lipid derivitives, arising from thermomechanical processing of solubles.

c. Food-grade salts added in processing (Prescott, 1982).

Thus, autolysed yeasts are marketed, with insolubles and cell wall material removed, as vacuum-evaporated concentrates in the form of translucent or opaque soluble pastes or spray-dried as "autolysed yeast" or "yeast extracts" (Peppler, 1982; Przybyla, 1986). If cell walls and

insolubles are not removed and the yeast slurry is roller dried the product is referred to as dried autolysed yeast; if dewatered to yield a paste, yeast extract (Peppler, 1967; Prescott and Dun, 1982; Przybyla, 1986; Dziezak, 1987). Inactivated (heat-killed, non-autolysed) yeasts are generally designated as "dried yeast" and are obtained either by recovery and processing of residual brewer's yeast or yeast produced specifically as a human or animal dietary supplement. Such products are utilised as sources of proteins, flavour,and vitamins of the B complex (Peppler, 1979). However, autolysed yeast extract and yeast autolysate will be the two main products considered in this thesis.

2.4.2 Applications of yeast autolysates

Yeast autolysates are approved for use as food flavourings and flavour enhancers by the U.S. Food and Drug Administration and are widely regarded as "natural" products for such applications. They are included in formulations, as condiments for such foods as meat products, meat pie fillings, hot dogs, sausages and ham, and other savoury foodstuffs. Such additives not only act to enhance flavour profiles but also can be employed to minimise the use of expensive meat extracts (Peppler, 1982; Dziezak, 1987). In powder form, they are regarded as effective bases for preparation of savoury flavours and are widely used to increase the savoury flavour of cheese products, soups, ham product, onion, spices and almost all savoury products (Lyall, 1970).

In the bakery industries, yeast products (especially

whole autolysed baker's yeast) are regarded as a natural component of many kinds of baked goods (Lyall, 1970; Peppler, 1982). When added to the formulation of snack foods notably extruded products with cheese flavourings, they are regarded not only as imparting or enhancing flavour but also conveying "naturalness" and strengthening the image of "wholesomeness" of the product (Hough and Maddox, 1970). Such products are also used in formulations of soups, bouillons, sauces and gravies to develop and enhance savoury characteristics (Dziezak, 1987). Moreover, they serve as ingredients to enhance the nutritional value of such foodstuffs (Binsted and Devey, 1970).

Both, yeast extracts and autolysates produced from brewer's yeasts are regarded as having a characteristic 'bouillon' or "beef stock" flavour which may be appropriate in soups and gravies (Lyall, 1970). Paste concentrates of yeast autolysates are widely used in Australia and by vegetarians as a savoury bread spread (Reed and Peppler, 1973; Prescott and Dun, 1982). Such products are also widely used in pet foods and animal feeds to enhance "meatiness", particularly in aroma.

In enhancing nutritional values of foods and feeds, yeast derivitives act as reliable and economical sources of peptides, amino acids, minerals and B vitamins. Thus extracts and autolysates are frequently included in formulations of health and baby foods as nutritional supplements or for vitamin fortification (Peppler, 1982; Dziezak, 1987). In addition yeast derivitives are used in

formulation of media for growth of microorganisms and other cell culture systems (Peppler, 1982; Prescott and Dun, 1982). Peppler (1982) mentioned that by combining yeast extracts and autolysates with plant protein hydrolysates (HPP), which are also strongly flavoured, some truely unique flavours can be obtained. The flavour-enhancing potential conferred by the presence of sodium salts of glutamic acid (monosodium glutamate or MSG), a central nitrogen pool amino acid in yeast, 2', 3' and 5'-nucleotides has been utilised both in the orient and West. Yeast derivitives are thus often combined with vegetable juices, herbs and spices to provide extra flavour and enhance nutritional value economically in consumer products (Binsted and Devey, 1970).

Other yeast derivatives are used commercially for a range of other food formulation functions aside from flavour enhancement and nutritional fortification such as texturizing, serving as stabilizers and thickeners as well as obviate or reducing the need for additives such as salts or monosodium glutamate (Dziezak, 1987). It can be concluded that many additional applications remain to be explored.

2.4.3 Processing

Preparation of yeast extracts and autolysates begins with the biomass in aqueous suspension or cream, press cake (70% moisture) or active dry yeast granules (8% moisture) (Peppler, 1982). In general, slurries of brewer's yeasts and primary yeast creams are commercially-important starting materials (Peppler, 1979, 1982; Prescott and Dunn, 1982). Fast growing yeasts of the genera *Saccharomyces*, *Candida* and

Torula are cultivated on carbon sources that can be obtained cheaply at the site of production, such as molasses, malt extract, whey, sulphite liquor, ethanol or alkanes (Hill, 1981).

It is regarded that the critical parameters determining uniformity and high flavour quality are: a. selection of yeast strains with high protein contents b. mild conditions of autolysis (<50 °C) c. use of efficient modern stainless-steel equipment d. appropriate, consistent process control and e. propgation to ensure high levels of both proteins and nucleic acids in biomass (Peppler, 1982).

The principal steps in typical autolysate production are summarised in Figure 2.1.

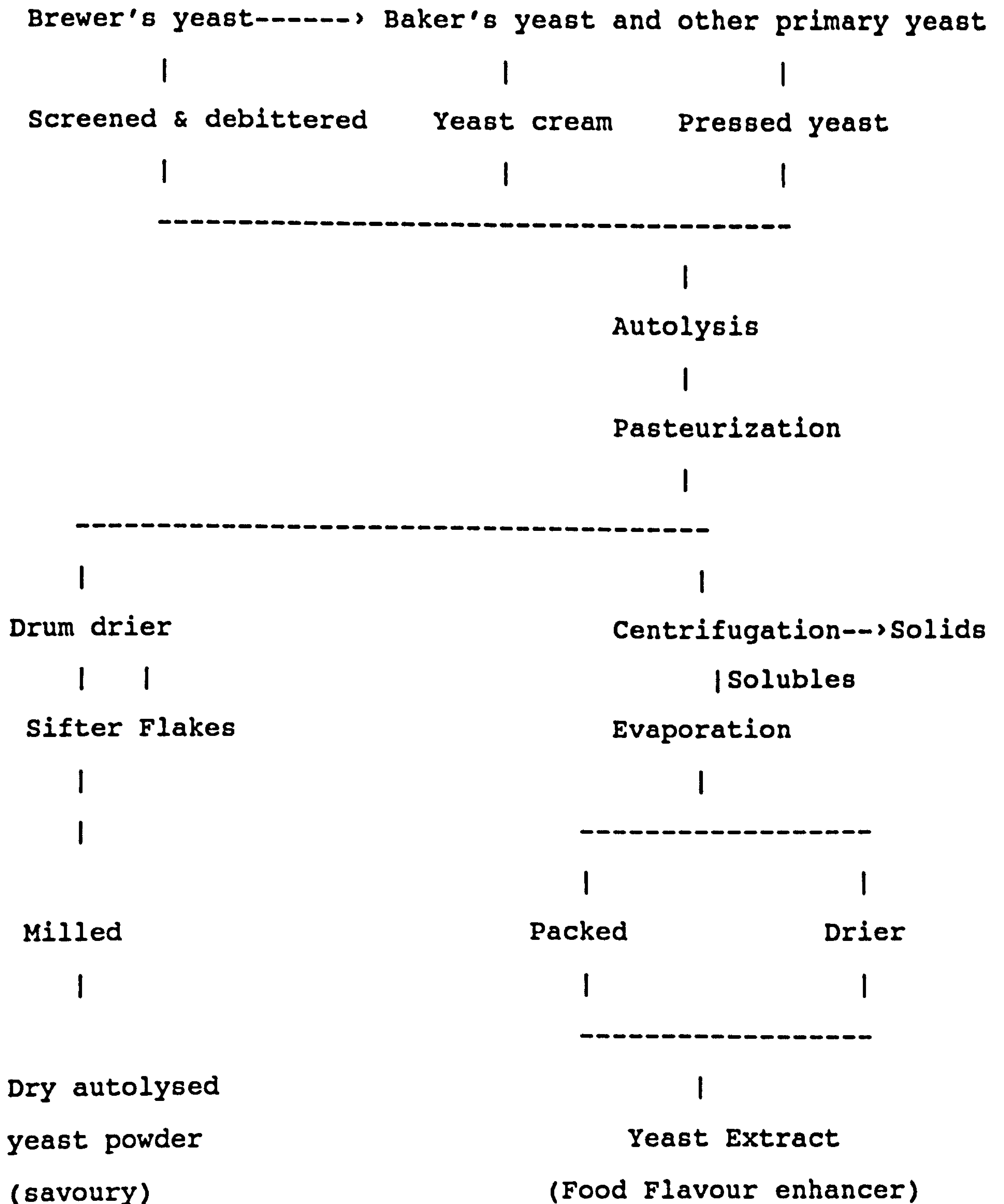


Figure 2.1 Outline of yeast product manufacturing processes

2.4.4 Composition of yeast products

Yeast extract and autolysate (Table 2.1) typically have high contents of proteins, amino acids, vitamins and minerals and are low in both lipids and carbohydrates (Davidek _et al._, 1979; Dziezak, 1987).

Table 2.1 Proximate chemical composition of a standard commercial autolysed yeast extract

Component	Amount (%, w/w)
Moisture	3.3
Carbohydrates	12.0
Protein	57.0
Lipids	0.1
Organic acids	2.0
Ammonium chloride	0.7
Ash*	25.8

From Select Committee on Generally Regarded as Safe Substances (1977).
* including added salts

The composition of yeast autolysates and extracts will vary depending on the degree of variability in the initial yeast biomass and differences in process conditions. Peppler (1982) and Sarwar _et al._ (1985) reported conditions for autolysis and biomass production, including growth substrate, will determine final levels of nucleic acids and flavour congeners in the yeast products. In addition, yeast strains are known to differ in protease pH optima and final degree of proteolysis in autolysates (Vosti and Joslyn, 1954; Hough and Maddox, 1970; Sarwar _et al._, 1985).

In current industrial practice, extract free amino acid or total α-amino nitrogen content is regarded as the most useful index of flavour potential. On completion of

autolysis, approximately 94 - 98 % of biomass nitrogen is recovered as amino acids or nucleic acids and their derivitives. Most current commercial products have high contents of glutamic acid (Lee et al., 1981; Peppler, 1982; Sarwar et al., 1985) known for its flavour-enhancing savoury taste, is a key indicator of flavour potential. However, most amino acids are tasteless, or nearly so, including D- and L-isomers of arginine, aspartic acid, isoleucine, lysine, proline, serine, threonine and valine. L-alanine is sweet whereas L- isomers of tyrosine and leucine are reported to be bitter (Peppler, 1982).

A number of nucleic acid components reported to be present in commercial autolysates (Solms, 1969) include guanosine (GMP), xanthosine (XMP) and inosine (IMP) 5' monophosphate , a commercially valuable flavour booster derived from RNA autolysis considered to impart a "meaty" flavour to yeast products (Moore, 1977).

2.4.5 Development of Maillard browning products

Maillard or non-enzymatic browning is important because this process yields the majority of cooked or processed flavour congeners in the human diet. Such compounds are of considerable importance because they generate the "roasted" and "grilled" flavours and contribute to heat processed beverage raw materials such as tea, cocoa and chocolate, and coffee. In processing, nonenzymatic browning (NEB) takes place when carbonyls react with amines producing a range of aromas (Table 2.2 and Fig. 2.2). Dominant sources of carbonyls in foodstuffs are the keto-forms of low-molecular

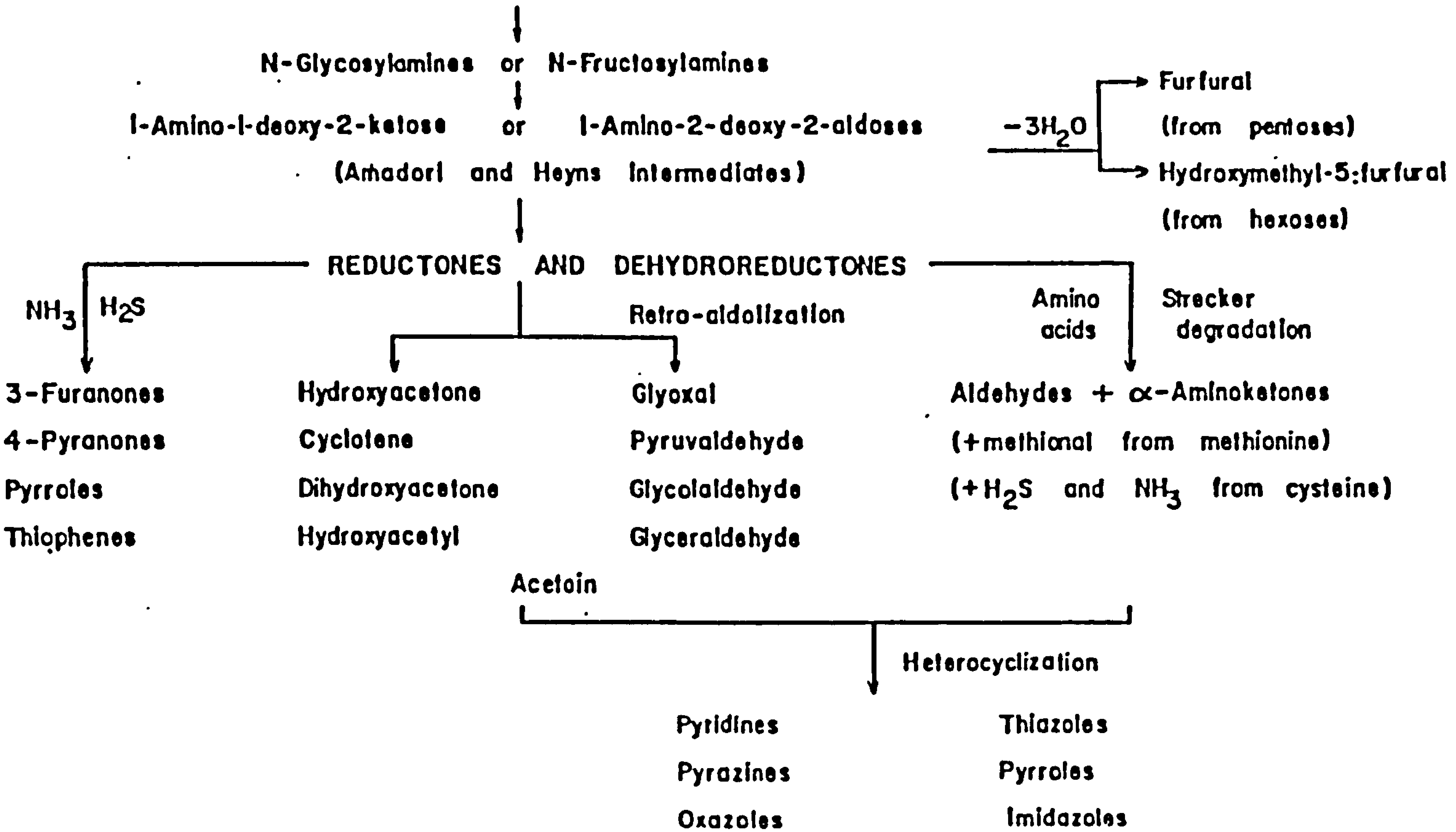


Fig. 2.2 Formation of flavor compounds via nonenzymatic browning.
Vernin and Parkanyi 1982

weight sugars; amino groups are available from amino acids.

Table 2.2 Aromas generated by amino acids with sugars

	Glycine	Glutamate	Lysine	Methionine	Phenylalanine
Glucose	Burnt candy	Chicken tray	Burnt fried potato	Cabbage	Caramel
Fructose	Beef broth	Chicken	Fried potato	Bean soup	Dirty dog
Maltose	Beef broth	Baked ham	Stale potato	Harsh, horseraddish	Sweet
Sucrose	Beef broth	Charred meat	Boiled meat	Overcooked cabbage	Chocolate

From El'Ode _et al._ (1966)

Consequently, in manufacture of yeast products the coupling of sugars and amino acids during heat processing, primarily during dewatering, but also in roller- and spray-drying, is central to flavour development (Fig. 2.3). The type of sugar available has been reported to influence yield and distribution of pyrazines formed during browning (Koehler _et al._, 1969). Other factors that affect yield will include the amino acids available for flavour generation (Leahy, 1985). Maximal reaction rates for browning reactions have been observed at water activities of 0.6 - 0.7 (Labuza, 1980).

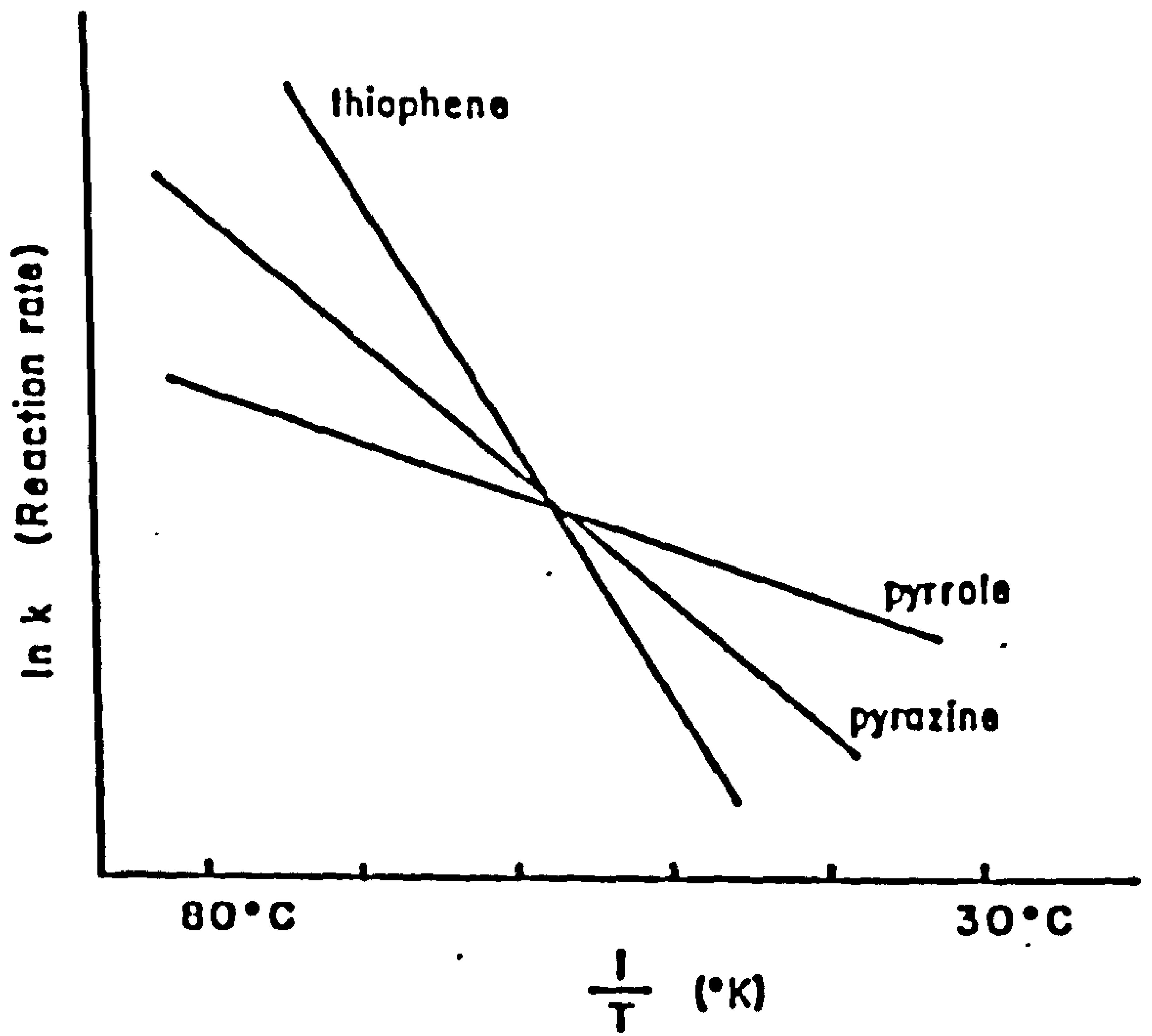


Fig. 2.3 Influence of temperature on rate of formation of various flavor compounds.

2.5 Objective

Although a great deal of data has been published on the occurrence and function of autolytic enzymes, to date, very little is known about the industrial model autolysis process. The main objective of this thesis was therefore, focussed on the following study.

a. An industrial model autolysis process simulating thermal induction of ß-glucanase, mannanase, chitinase and protease.

b. Solubilization of yeast solids during autolysis.

c. Compositional changes of yeast cell wall components during autolysis.

d. Ultrastructural changes of autolysing yeast cells.

e. Autolysis of purified cell walls.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Microorganism and cultural condition

Saccharomyces cerevisiae strain D6, a distilling strain, was obtained from the Department of Bioscience and Biotechnology, University of Strathclyde. The culture was routinely maintained on YEPG medium. Yeast cells were grown in YEPG broth (500 ml) containing 1% yeast extract, 2% peptone and 2% glucose in 1000 ml conical flasks. Batch cultures were incubated at 30 °C on a rotary shaker at 150 rpm. These conditions have been shown previously to result in fully aerobic growth.

3.2 Autolysis of whole yeast cells

After 18 h of growth in YEPG medium, yeast cells were collected by centrifugation at 3000 x g and washed three times with sterile distilled water at 4 °C. After washing 15 g wet weight of cells were suspended in 300 ml distilled water. Cells were then autolysed at different temperatures in 50 ml conical flasks containing 20 ml aliquots of cell suspensions in a shaking water bath for 0-24 h. Samples were collected every 6 h of intervals up to 24 h.

3.3 Enzyme Assays

3.3.1 ß-1,3 glucanase

Substrate: Laminarin (Sigma; from *Laminaria digitata*). Laminarin was routinely used as substrate for the assay of ß-1,3 glucanases. The general properties and structure of this polysaccharide have been reviewed (Bull and Chesters, 1966) and apart from ß-1,3 linkages the molecule also

possesses a small percentage of ß-1,6-glucosidic bonds. Laminarin has been widely used for ß-1,3 glucanase activity assays (Fleet and Phaff, 1974).

Procedure,

Laminarin (0.25 g) was dissolved in 100 ml of 0.05M Na-acetate buffer, pH 5.0. It was poorly soluble in cold water but readily dissolved when heated and remained dissolved for at least a week upon storage at 4 °C.

The substrate (0.5 ml) was added to suitably diluted enzyme solution (0.5 ml) and the mixture was incubated at 30 °C for 30 min. The reaction, which was linear with time, was stopped by boiling the mixture in water bath for 5 min. The amount of reducung sugar produced was subsequently determined by the method of Somogyi (1945) and Nelson (1944). Enzyme activity was expressed as microgram of reducing sugar equivalent as glucose per millilitre of sample under the assay condition.

3.3.2 ß-1,6 glucanase

Substrate: Pustulan (Calbiochem; from Umbilicaria papullosa). This substrate which is a ß-1,6-linked glucan was routinely used for ß-1,6 glucanase assays. It was poorly soluble in cold water but soluble in hot water. After dissolving, pustulan solution was filtered through filter paper to obtain a clear solution. Acid hydrolysis and methylation studies have not revealed the presence of any ß-1,3-linkages in this substrate.

Procedure,

Pustulan (0.25 g) was dissolved in 100 ml of 0.05M Na-

acetate buffer (pH 5), warmed and and allowed to cool. The substrate solution (0.5 ml) was dispensed into labelled test tubes. Suitably diluted enzyme solution (0.5 ml) was added and the mixture incubated at 30 °C for 30 min. A blank containing buffer and enzyme solution and a second blank containing substrate and buffer were included. After 30 min. the reaction was stopped by placing the tubes in the boiling water bath for 5 min. Reducing sugars produced was quantified by Nelson (1944) and Somogyi (1945). Enzyme activity was expressed as described for ß-1,3 glucanase.

3.3.3 Mannanase assays

Substrate: Mannan from *S. cerevisiae* (Sigma).

Procedure,

Mannan (0.25 g) was dissolved in 100 ml of 0.05M Na-acetate buffer (pH 5). The solution was usually stored at 4 °C.

Mannanase Assay

Substrate (0.5 ml) was added to suitably diluted enzyme solution (0.5 ml) in labelled test tubes and incubated at 30 °C for 30 min. The reaction was terminated by boiling the mixture in a boiling water bath for 5 min. The amount of reducing sugars released was determined by Nelson (1944) and Somogyi (1945) method. Enzyme activity was expressed as described for ß-1,3 glucanase.

3.3.4 Chitinase assays

Substrate: Chitin (Purified chitin from crab shells, Sigma). This substrate was prepared by the method of Skujins *et al.* (1965) for the assay of chitinase. Chitin (1 mg ml^{-1})

was suspended in Na-phosphate buffer (pH 6.3) and heated to produce a uniform suspension. Since this substrate was a dispersed chitin, it did not settle and was suitable for chitinase assay. Chitin suspension was shaken thoroughly before being used as substrate.

Glusulase (Snail gut extract) was obtained from DuPont Pharmaceutical and was diluted and filtered through Sephadex G-25 as described by Cabib (1988), which resulted in an overall three fold dilution with respect to the original material. Glusulase which is a good source of ß-N-acetylhexosaminidase, was used to hydrolyse, diacetylchitobiose formed by the action of chitinase. N-acetylglucosamine formed by glusulase was determined according to the method described by Rondle and Morgan (1955).

Procedure,

Chitin suspension (0.5 ml) was added to suitably diluted enzyme solution (0.5 ml) and incubated at 30 °C for 1 h. After the incubation, the solution was placed in a boiling water bath to inhibit further enzyme activity. The solution was then cooled in running tap water for 5 min and further incubated for 1 h at 30 °C with an equal volume of Glusulase and N-acetylglucosamine formed was determined by the method of Rondle and Morgan (1955). The enzyme activity was expressed as µg of N-acetylglucosamine per ml of sample under the experimental condition.

3.3.5 Protease assays

Substrate casein from Sigma (1%) was prepared by dissolving 1.0 g of casein in 1 M Na-phosphate buffer (pH 6) and the volume was made to 100 ml. The solution was kept at 4 °C.

Procedure,

Substrate casein (0.5 ml) was added to suitably diluted enzyme solution (0.5 ml) and incubated at 30 °C for 30 min. After incubation, the mixture was placed in a boiling water bath for 5 min to inhibit further enzyme activity and a-amino acid formed was measured by ninhydrin reagent (Karkalas, 1988, unpublished). The enzyme activity was expressed as µg of α-amino acid per ml of sample under the condition used.

3.4 Determination of reducing sugar (Nelson, 1944; Somogyi, 1945)

Principle,

Reducing sugars react with cupric ions to give a red precipitate of Cu_2O at a rate proportional to the concentration of sugars in the solution. The blue colour developed by the addition of arsenomolybdate reagent was measured spectrophotometrically at 500 nm. This assay is both convenient and reproducible.

Reagents used,

a. Somogyi's reagent containing: Anhydrous disodium phosphate, Sodium hydroxide, Sodium potassium tartrate, Cupric sulphate and Anhydrous sodium sulphate.

b. Nelson's arsenomolybdate reagent: ammonium molybdate,

H_2SO_4, $Na_2HAsO_4,7H_2O$.

Procedure,

Into all test tubes 1 ml of Somogyi's reagent was added and mixed well using a Rotamixer. Tubes were then immersed in a boiling water bath for 20 min and cooled in running tap water for 5 min. After cooling, 1 ml of Nelson's arsenomolybdate reagent was added and mixed immediately in a Rotamixer. After 3 min, 7 ml of distilled water was added to all test tubes and mixed by inverting the tubes several times. Absorbance was then read in the spectrophotometer at 500 nm. A reagent blank and a glucose standard were also included. In all cases, triplicate observations were made.

3.5 Determination of N-acetylglucosamine

N-acetylglucosamine was determined by the method described by Rondle and Morgan (1955) in which N-acetylglucosamine reacts with acetylacetone to form a chromogenic material which gives a red colour with p-dimethylaminobenzaldehyde.

Reagents used:

i) 1M $NaHCO_3$-Na_2CO_3 buffer (pH 9.6).

ii) Acetylacetone reagent: This was prepared by addition of 2.5 ml of acetylacetone to 50 ml buffer. This reagent was prepared freshly before use.

iii) High grade ethanol.

iv) p-Dimethylaminobenzaldehyde (0.8 g) was dissolved in 30 ml of ethanol prior to addition of 30 ml of conc. HCl. This reagent was stored at -10 °C.

Procedure,

Into each test tube containing samples (1 ml), 1 drop of phenolphthalein indicator followed by 1N NaOH was added to give a faint pink colour. Distilled water was added to make the volume 2 ml and 1 ml of acetylacetone was added. After mixing, the tubes were heated in a boiling water bath for 20 min, then placed in cold water for 5 min. After cooling 5 ml of ethanol was added followed by 1 ml of acetylacetone reagent and ethanol to make a volume of 10 ml. The solution was mixed well and placed in a water bath at 65-70 °C for 10 min to accelerate liberation of CO_2 and finally cooled to room temperature. A reagent blank and standards containing known amounts of N-acetylglucosamine were treated in the same way. The absorbance was read at 530 nm.

3.6 Determination of α-amino nitrogen

The determination of protease activity was based on the release of α-amino nitrogen from casein which subsequently reacted with ninhydrin reagent to yield spectrophotometrically detectable colour.

Reagent used,

i) Ninhydrin reagent: 0.5 g ninhydrin, 0.3 g fructose, 10 g Na_2HPO_4 (anhydrous), 6 g KH_2PO_4.

The above reagents were dissolved in distilled water and the volume was made up to 100 ml in a volumetric flask. This solution was stored in a brown bottle at 4°C not more than one week.

ii) Ethanolic potassium iodate: 40 ml of ethanol was measured in a graduated cylinder and the volume was made up

to 100 ml with distilled water. KIO_3 (1 g) was added and stirred with a magnetic stirrer for 2 h to saturate the solution which was then filtered and stored in a stoppered bottle.

iii) Stock solution of leucine: 93.5 g of leucine was dissolved in 100 ml of distilled water in a volumetric flask.

iv) Standard solutions of leucine: 1,2,3,4 and 5ml of stock solution of leucine respectively were transferred in 100 ml volumetric flask and the volume made up to the mark.

Procedure,

To the test tube containing 1 ml of sample or standard solution, 0.5 ml of ninhydrin reagent was added and mixed thoroughly using a Vortex mixer and placed in a boiling water bath for 15 min. The tube was then cooled in running tap water for 5 min and 2.5 ml of ethanolic potassium iodate was added with thorough mixing by repeated inversions of the tubes. The absorbance was then read at 570 nm within 30 min. A standard curve was plotted and a-amino nitrogen calculated.

3.7 Determination of protein

The protein contents of the samples were determined according to the method described by Lowry <u>et al.</u> (1951). Soluble proteins are treated with $NaOH-Na_2CO_3-CuSO_4$ reagent before addition of Folin-Ciocalteau reagent. Insoluble proteins were initially dissolved by boiling in NaOH.

Reagents used

i) 5% Na_2CO_3

ii) 0.5% $CuSO_4$, $5H_2O$ in 1% sodium potassium tartrate

iii) To 50 ml of reagent (i), 2 ml of reagent (ii) were added immediately before use.

iv) Folin Ciocalteau reagent: This regeant was diluted in water in a ratio of 1:1 before use.

v) Standard protein: bovine serum albumin (0-200 $\mu g\ ml^{-1}$).

3.7.1 Procedure for intracellular protein:

To 0.5 ml of sample, 0.5 ml of 1M NaOH was added in a test tube then placed in boiling water bath for 5 min and cooled in running cold tap water for 2 min. To the tube 2.5 ml of reagent (iii) was added and mixed thoroughly in Rota mixer and then was allowed to stand at room temperature for 10 min. Reagent (iv) (0.5 ml) was added rapidly and mixed thoroughly and allowed to stand for 30 min for full colour development. Absorbances were read at 750 nm in a spectrophotometer.

A reagent blank containing 0.5 ml of distilled water instead of the sample and a standard protein sample (50-200 µg protein) were treated in the same way. For extracellular protein, all treatments were as intracellular protein but the protein extraction was excluded.

3.8 Determination of glucose by glucose oxidase method (Trinder, 1969).

Principle,

In the presence of glucose oxidase, glucose is oxidised to gluconic acid and H_2O_2 at pH 7. The H_2O_2 produced is

proportional to the glucose present. Hydrogen peroxide is quantitatively decomposed by peroxidase and a pink colour is produced which is quantified spectrophotometrically.

Reagent used,

$Na_2HPO_4,12H_2O$ (11.5 g), KH_2PO_4 (2.5 g), phenol (500 mg), 4-aminophenazone (75 mg), glucose oxidase (3500 units), peroxidase (3500 purpurogallin units), water (500 ml).

Procedure,

Into each tube containing 1 ml of sample, 5 ml of glucose oxidase reagent was added and mixed on a vortex stirrer. The samples were then incubated at 35 °C for 45 min in a water bath which was protected from direct light. After the incubation, the samples were cooled to room temperature for 10 min in the dark and absorbance was taken at 505 nm. A reagent blank and a standard were also included.

3.9 Dry Weight Determination

Duplicate portions of a well-mixed cell suspension (40 ml) were harvested by filtration through preweighed Whatman GF/C filters (0.45 µm pore size; 4.7 cm dia). The resulting pellets were washed thrice with equal volume of distilled water and dried to constant weight for 12 h in an ordinary oven at 105 °C. Filters containing dried cells were left in a dessicator for 15-30 min before weighing. The difference in weight between preweighed filters and the filters plus pellets gave the dry weight of the cell suspension per sample volume of culture. Results were expressed as grams dry weight per litre.

Dry weights of autolysing yeast cells was determined in preweighed test tubes in duplicate. During autolysis, 10 ml aliquots of cell suspensions were collected in preweighed test tube by using a positive displacement pipette at period between 0 and 24 h. Cells were then collected by centrifugation at 10000 x g and washed thrice with equal volumes of distilled water. The resulting supernatants were placed in preweighed aluminium cups. Dry weights of both pellet and supernatant were determined in an oven at 105 °C for 12 h. Samples were put in the dessicator for 15-30 min before being weighed so that constant weight can be achieved. Percent solubilization of biomass was calculated using the following equation.

$$S\ (\%) = \frac{s}{p + s}$$

where, S = solubilization of biomass,
p = dry weight of pellet
s = dry weight of supernatant

3.10 Microscopy

3.10.1 Yeast viability

Principle,

Viable cells contain enzymes which are able to reduce methylene blue to a colourless compound. The enzymes of living cells, on immersion in methylene blue, decolourize the dye. Dead cells in which enzymes are inactive, or unable to decolourise dye and accordingly stain blue. The percentage of unstained cells is therefore, a measure of viability and although occasionally erroneous is widely used in the published literature.

Methylene blue dye solution

Methylene blue (0.01 g) was dissolved in 10 ml distilled water. 2.0 g of sodium citrate (di-hydrate) was added and the mixture stirred until dissolved. The mixture was filtered through a membrane filter paper (Millipore; 0.45 µm; 2.5 cm dia) and the filtrate was made up to 100 ml in a volumetric flask as described by Pierce (1970).

Procedure,

The dye solution (0.5 ml) was mixed on a Vortex mixer with an equal volume of suitably diluted sample (0.5 ml) in test tubes. After 20 min of incubation at room temperature, 4 ml distilled water was added to each tubes and the suspensions again mixed. Samples from each tube were loaded on to Spencer Bright-Line Hemacytometer. A drop of a well mixed suspension of cells was put onto a haemocytometer counting chamber. Each counting chamber slide has a surface divided into squares of known area. The counting was performed under the microscope at a magnification x 400-600.

Samples were generally diluted after vigorous agitation with a Vortex. The haemocytometer cover slip was pressed firmly on to the two support areas of the chamber until Newton's rings appeared on the edges. A drop of cell suspension was dispensed into the counting chamber. The slide was then placed on to the platform of the microscope and the cells counted. Buds were generally ignored unless their sizes were greater than one half of the parent cells. The counts were carried out in duplicates and the average

count taken as the cell count of the diluted cell suspension. The total count, T, per ml was obtained by using the equation;

$$T = \frac{x \times 4 \times 10^6 \times D}{n}$$

where x = Number of cells counted in "n"squares

D = Dilution factor

n = Number of small squares

Area of the chamber = 1/400 mm^2

Depth of the chamber = 0.1 mm

3.10.2 Fluorescence microscopy

Equal volume of suitably diluted autolysing yeast cells (0.5 ml) was added to the calcofluor dye solution (1%). After 20 min of incubation, 4 ml of distilled water was added and mixed on a Vortex mixer. Samples were loaded on to a Spencer Bright-Line Hemacytometer with the help of a micropipette. The cells were then examined by Leitz epifluorescence microscope (Orthoplan) with an illuminator fixed on the microscope tube which was arranged to make it possible to switch from ultra-violet irradiation to ordinary visible illumination. The incident light source was quartz halogen lamp. The cells were examined under the microscope at a magnification x 500 (N.A. 1.00) with water immersion objective. The cells were then photographed using 200 ASA Kodachrome film (Kodak plc).

3.11 Elecron microscopy

3.11.1 Scanning electron microscopy

Yeast cells before and after autolysis were fixed by 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min followed by three rinses with the same buffer by centrifugation. The post-fixation of the cells was carried out by 1% OsO_4 in buffer for 30 min followed by three rinses with distilled water using centrifugation. The specimen were stained with uranyl acetate in the dark for about 60 min followed by washing of cells once with distilled water. Specimens were dehydrated in different concentrations of acetone in purpose built metal chambers in which cells were trapped in between two filters. Critical Point Drying was carried out using the direct acetone-CO_2 method for 1 h. Finally, specimens were gold coated for 8 min before being examined under the scanning electron microscope.

3.11.2 Transmission electron microscopy

Yeast suspension was placed in microfuge tubes, centrifuged and supernatant was removed. The pellets were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1-2 h. After fixation the materials were rinsed in buffer (3 x 10 min changes). After centrifugation, the pellets were resuspended in bovine fibrinogen and one or two drops of bovine thrombin were then added and tubes were then warmed between the hands until the clot was formed. The clot was removed from the tube and cut into small pieces and

placed in buffer. The materials were dehydrated through an ethanol series (2 x 4 h changes). The pellets were then placed in a 50/50 mixture of absolute ethanol and 1,4-dioxan for 4 h and 2 changes of 1,4-dioxan for 6-8 h in each. Spurr resins without accelerator was then added in the proportion of 3:1 and then 2:1 followed by 1:1 for 4 h each. After 4 h in 1:1, caps were removed from the tubes and 1,4-dioxan was allowed to evaporate overnight. The tubes were placed under vacuum for 6-8 h and then resins were removed and replaced with fresh resins and returned to vacuum for further 8 h. This was repeated twice with accelerator. Samples were then placed in moulds and polymerised for 48 h at 60 °C.

3.12 Gas chromatographic analysis of autolysing yeast cells

Constituent monosaccharides of autolysing yeast cell walls were chromatographed and quantified according to the method of Englyst and Cummings (1988).

Principle,

Aldoses present in sulphuric acid hydrolysate were reduced to corresponding alditols with sodium borohydride in an alkaline aqueous solution which was terminated by addition of glacial acetic acid. Aliquots of acidic alditol were used to prepare alditol acetates by addition of ammonium hydroxide to neutralize hydrolysates and N-methyl imidazole acted as a catalyst for acetylation. Addition of ethanol eliminates excess acetic acid, forming a layer of ethyl acetate containing product alditol acetate esters. Total polysaccharides were calculated as the sum of released neutral sugars as estimated by gas chromatography.

Reagent used,

1) Sulphuric acid (1 M and 12 M),

2) Ethanol (85% v/v)

3) Ammonia solution (3 M and 12 M)

4) Allose solution

5) Sodium hydroxide solution

6) Potassium hydroxide (7.5 M)

7) N-methyl-imidazole

Procedure,

Alditol acetate esters were quantified in a gas chromatograph (PYE Series 104 Chromatograph) fitted with a 1.8 cm x 2 mm ID glass column packed with Supelcoport (100/120) GP 3% of SP2330 using flame ionization detector. Injector and oven temperatures were 225 °C. Carrier nitrogen and FID hydrogen were supplied at flow rates of 30 ml min^{-1}. Air flow rate was 500 ml min^{-1}. Peak areas were estimated with a Pye Unicam DP 88 computing integrator.

For the determination of N-acetylglucosamine 0.9 cm x 2 mm ID glass column packed with Supelcoport (100/120) GP 3% SP 2340 was used. Column temperature was programmed in such a way that it remained at 180 °C for an initial 6 min and was then increased to 240 °C with 2 °C rise per min. N_2 was used as carrier gas with flow rate of 40 ml min^{-1}. Flame ionization detection was used and peak areas were measured using Pye Unicam DP 88 computing integrator.

3.13 Preparation of cell extracts and cell walls

A method described by Fleet and Phaff (1974) was adopted for the preparation of cell extracts and cell walls.

Immediately after harvest, yeast cells were washed six times with 0.05 M sodium succinate buffer (pH 5) and finally resuspended as a thick slurry in the same buffer. Cells were disrupted by mechanical shaking in a Braun homogenizer with an equal volume of glass beads for 1-2 min. Sufficient liquid carbon dioxide was delivered to the shaking chamber to maintain the temperature below 5 °C but without freezing. Generally 99-100% cell breakage was obtained and this was confirmed by phase contrast microscopy. After homogenization, the cell walls were centrifuged at 10000 x g for 30 min. The cell walls were then washed four times with 0.05 M sodium succinate buffer (pH 5). After each washing, cell walls were resuspended in the same buffer and supernatants collected by centrifugation at 10000 x g for 30 min were taken as cell wall washes. Once cell walls had been thoroughly washed, they were suspended in 0.01 M sodium succinate buffer (pH 5) and incubated for autolysis at 30 °C.

3.14 Autolysis of purified yeast cell walls

Autolysis was carried out by the method described by Hien and Fleet (1983). Washed cell walls were suspended in 0.01 M sodium succinate buffer (pH 5) containing 0.001 M -p-methylsulfunyl fluride as protease inhibitor and 0.01% sodium azide as antimicrobial agent. The cell walls suspended in buffer were incubated at 30 °C for 0-24 h. The enzymes solubilized by autolysis were recovered in the supernatants after centrifugation at 10000 x g for 15 min.

3.15 Gel electrophoresis

Proteins in autolysates and in cell extracts were separated by isoelectric focusing method as described in a LKB Application Note. In this technique proteins are separated in a pH gradient on the basis of their respective isoelectric points. Proteins migrate until they align themselves at the pH at which the protein possesses no net overall charge and concentrate at this point as migration ceases. This is therefore, an equilibrium technique in which effects of diffusion are overcome and it is the electrophoretic technique with the highest resolution in which components that differ by 0.001 of a pH unit or less can be resolved. Therefore, IEF was chosen for the separation and analysis of proteins. Proteins were resolved in a gel containing 13.86% acrylamide, 7.5% bisacrylamide and 2.5% ampholine. Samples were run in a wide pH gradient gel (pH 3.5-9.5) for 1500 volt h^{-1} at 25 mA at a constant temperature of 10 °C whereas in narrow pH ranges (pH 4.5-7.0), the samples were focused for 3000 volt h^{-1} at 18 mA at 10 °C. After electrofocusing, the gel was placed in the fixing solution containing trichloroacetic acid and sulphosalicylic acid for 30 min which irreversibly precipitated proteins. The gel was then placed in the destaining solution for 15 min to wash out the residual ampholine. The proteins in the gel were then stained with Coomassie blue for 10 min at 60 °C and then, the gel was placed in destaining solution for at least 30 min with

several changes of solution.

3.16 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel (10%) electrophoresis was used for the determination of molecular weights of proteins. The method used was adapted from that of Laemmli (1970).

Reagents for gel making

1) 3 M Tris-HCl (pH 8.8)

2) 1 M Tris-HCl (pH 6.8)

3) Acrylamide (35% w/v)

4) N-N-Methylene- bis-acrylamide (2% w/v)

5) 10% SDS

6) Ammonium persulphate

7) TEMED

Reagents for electrophoresis

8) Running buffer containing 129 µM Glycine, 25 µM Trizma base and SDS (pH 8.3)

9) Sample buffer containing glycerol, 1 M Tris (pH 6.8), SDS, Pyronin Y and Distilled water.

10) Staining and destaining solution

Procedure,

After polymerization of the separating gel (10%), stacking gel was poured in and allowed to complete polymerization for 1 h. The samples were then put in the sample moulds of the gel and run overnight in a gel tank at 10 mA at ambient temperature. Both high and low molecular weight markers were also resolved in the gel.

3.17 Detection of enzyme activity on agarose gel

Zone of glucanase activity were detected using agarose gels containing substrates, following the method reported by Teather and Wood (1982) and Bartley *et al.* (1984). After the isoelectric focussing, gels were overlaid on an agarose gel containing laminarin or pustulan (0.5%) in Na-succinate buffer (pH 5) and incubated at 50 °C for 10 min. Following incubation, the gels were flooded with Congo red (1mg ml^{-1}) for 15 min to stain carbohydrates. Congo red was then poured off and the gels were flooded with 1 M NaCl for 15 min.

During that time a clear zone of hydrolysis appeared and further enzyme activity was stopped by adding 1 M HCl to the gel.

3.18 Localization of ß-glucanases in SDS-PAGE

A method described by Wolfgang *et al.* (1987) was employed to detect the presence of glucanases in SDS-polyacrylamide gels. Substrate ß-glucan (From Sigma) was incorporated into the gel prior to polymerization. The gel was then run overnight according to the method reported by Laemmli (1970). Upon completion of electrophoresis, the gel was washed five times for at least 30 min with cold 0.1 M sodium succinate buffer (pH 5.8) containing 10 mM DTT. After renaturing the proteins , the gel was incubated in 0.1 M sodium succinate buffer (pH 5.8) for 30 min at 60 °C. Following the incubation, the gel was stained with Congo red (1mg ml^{-1}) for glucanases for 10 min at room temperature and then destained with 1 M NaCl for another 10 min with several changes of destaining solution.

3.19 Thermostability study

Cell extracts prepared by homogenization of yeast cells were used as enzyme solution for thermostability study of mannanase while cell-free extracts were used for ß-glucanase and chitinase. Enzyme activity was normalised against activity at 50 °C since maximal activity was observed at this temperature. After incubating with respective substrates for 0-30 min for different times, tubes containing the mixture were placed in the boiling water bath for 5 min to eliminate further enzyme activity. The activity of ß-glucanase, mannanase and chitinase were determined according to the procedure described elsewhere in this section.

RESULTS

4. RESULTS

4.1 Growth curve for yeast grown at 30 °C

These experiments were carried out to estimate the growth of the yeast strain under the conditions used for biomass production (Fig. 4.1). After 18 h of growth exponential phase of growth was complete and 2.75 g l^{-1} of cells had been produced. Growth continued slowly at this temperature so that after 36 h and 72 h values of 3.25 g l^{-1} and 3.75 g l^{-1} were recorded.

4.2 Uptake of media glucose during yeast growth at 30 °C

Uptake of glucose from YEPG medium during cell growth was analysed to determine if the observed pattern was similar to that of biomass production. Results obtained (Fig. 4.2 and Table 4.1) showed that maximal uptake of glucose occurred in the first 18 h of growth and afterwards the level was very low.

Table 4.1 Uptake of media glucose during yeast growth at 30 °C

Time of incubation (h)	Conc. of glucose in the medium (mg ml^{-1}) (Mean/standard deviation)
0	19.43/0.5700
18	0.011/0.0030
36	0.004/0.0001
72	0.002/0.0001

4.3 ß-glucanase activity during yeast autolysis

Autolysis of yeast biomass may be induced by increases in temperature, addition of plasmolysers to cultures, mechanical disruption of cells and other factors disrupting

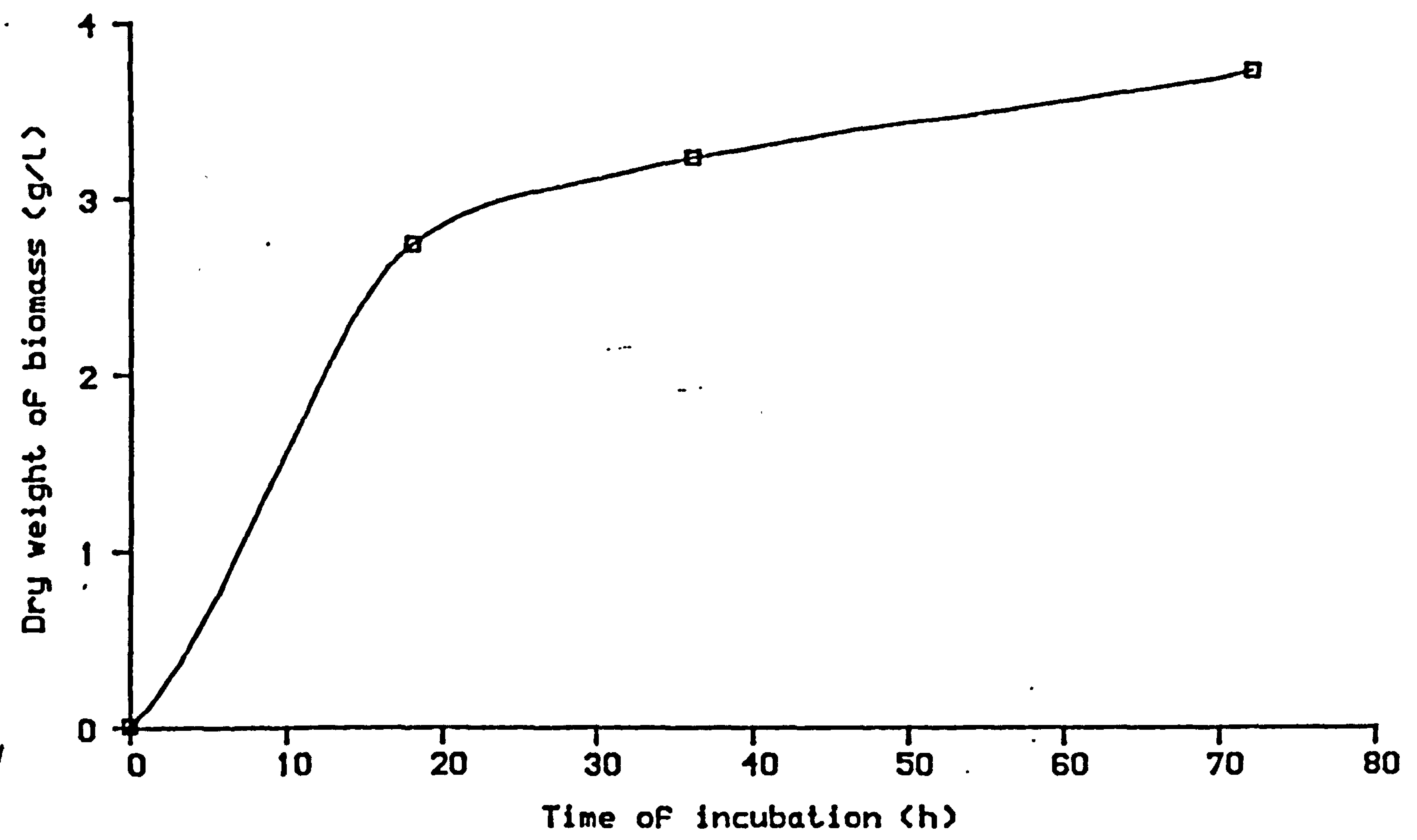


Fig. 4.1 Growth curve for yeast grown on YEPG medium at 30 °C

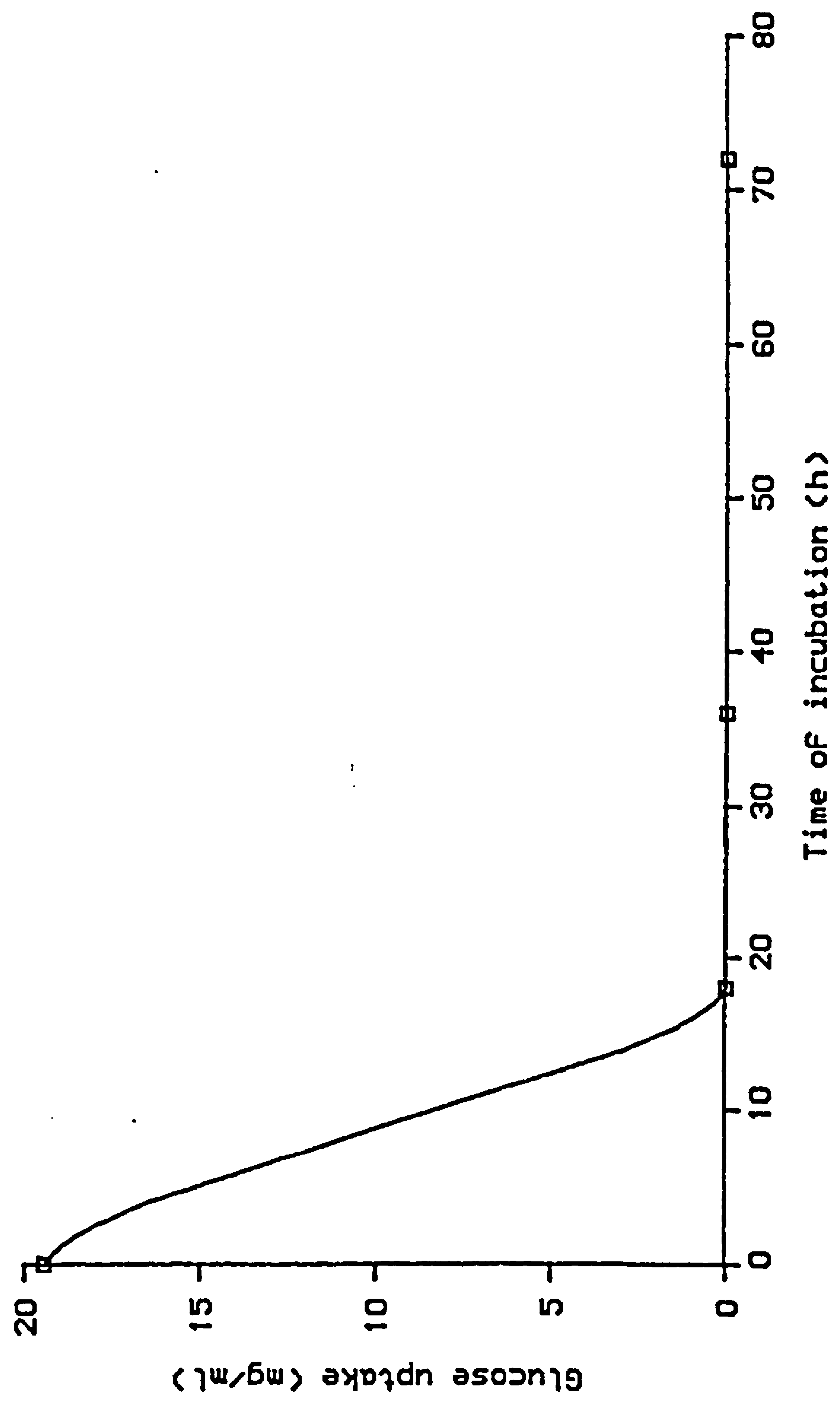


Fig. 4.2 Uptake of media glucose during yeast growth at 30 oC

the cytoplasmic membranes to allow diffusion of lytic enzymes from cytoplasmic compartments (Babayan *et al.*, 1981). The polysaccharide network of the yeast cell wall, predominantly ß-1,3 glucan in *S. cerevisiae*, is attacked during autolysis to yield low molecular weight sugars (Barras, 1974). Thus, an initial objective was to study the ß-glucanase activity during autolysis at a range of temperatures for differing periods.

The ß-glucanase assay used was based upon estimation of reducing sugar released from laminarin (ß-1,3 glucan) and pustulan (ß-1,6 glucan) by crude autolysate, cell-free supernatants or extracts, and cell pellets (predominantly cell wall material but including other insolubles). In each case, changes in total reducing sugar concentration were determined by the method of Nelson (1944) and Somogyi (1945).

Using a glucose solution, a standard calibration curve for reducing sugar concentration against absorbance at 500 nm was obtained (Fig. 4.3). It was found that the range of linearity was between 5 and 200 µg of glucose.

Yeast autolysates were prepared by producing biomass through aerobic growth on YEPG for 18 h at 30 °C at 150 rpm in an orbital incubator. Cells were harvested by centrifugation at 5000 x g for 10 min at 4 °C and washed three times in cold sterile water prior to autolysis. Defined quantities of biomass (15 g wet weight) were resuspended in 300 ml batches of sterile water. Autolysis of these cell suspensions was induced in 20 ml aliquots in

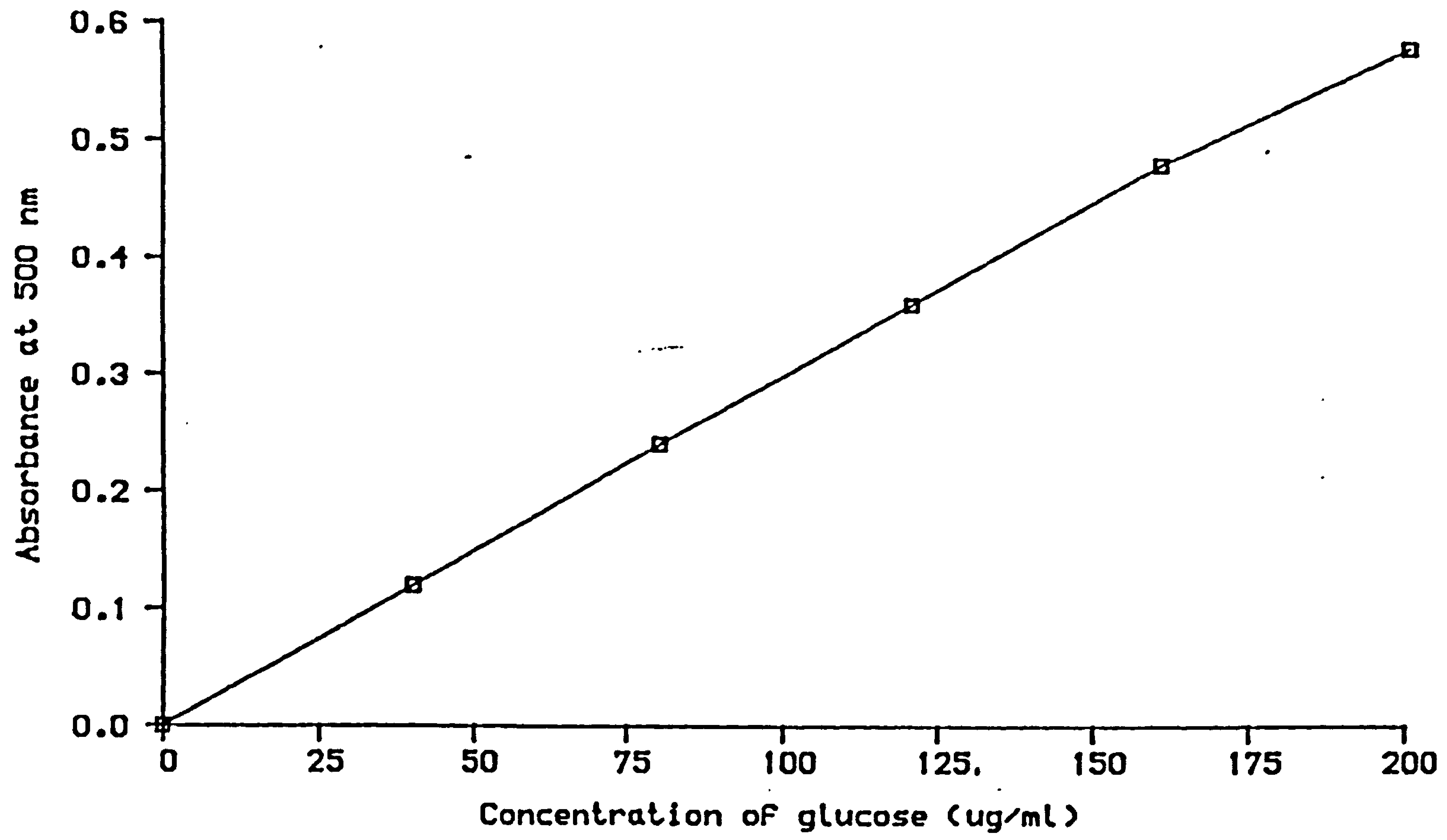


Fig. 4.3 Standard curve for reducing sugar (Nelson, 1944 ; Somogyi, 1952).

conical flasks (50 ml) by incubation at differing temperatures in shaking water bath. Glucanase activities were determined at 6 h intervals up to 24 h of autolysis and expressed as µg product generated per ml of autolysate during a 30 min incubation with the substrate.

4.3.1 ß-1,3 glucanase activity during 30 °C autolysis

Estimation of ß-glucanase activity of autolysing cells incubated at 30 °C (Fig. 4.4) demonstrated the presence of ß-1,3 glucanase activity in total autolysates, cell-free supernatants and cell wall pellet. Total glucanase activity in the pellet (40 µg ml^{-1}) was twice that observed for supernatant (20 µg ml^{-1}) after 6 h of autolysis. Pellet glucanase activity reached a maximum (48 µg ml^{-1}) after 12 h of autolysis and supernatant activity was also maximal at this time (25 µg ml^{-1}). After this time, activities in pellet and in supernatant were found to decrease so that after 24 h of autolysis values of 35.5 and 14.2 µg ml^{-1} were recorded for pellet and supernatant respectively.

Recalculation of data (Table 4.2) obtained in this experiment showed the amount of glucanase released into the soluble fractions during autolysis. It can be observed that 32.9% of ß-1,3 glucanase activity was released into the medium from cell pellets after 6 h of autolysis whereas after 12 h the total activity increased to 75.2 µg ml^{-1} although the balance of activity was similar to the 6 h samples. After 12 h of autolysis the relative proportion of total activity exhibited by the soluble glucanases was maximal (34.3%). No further increases in relative activity

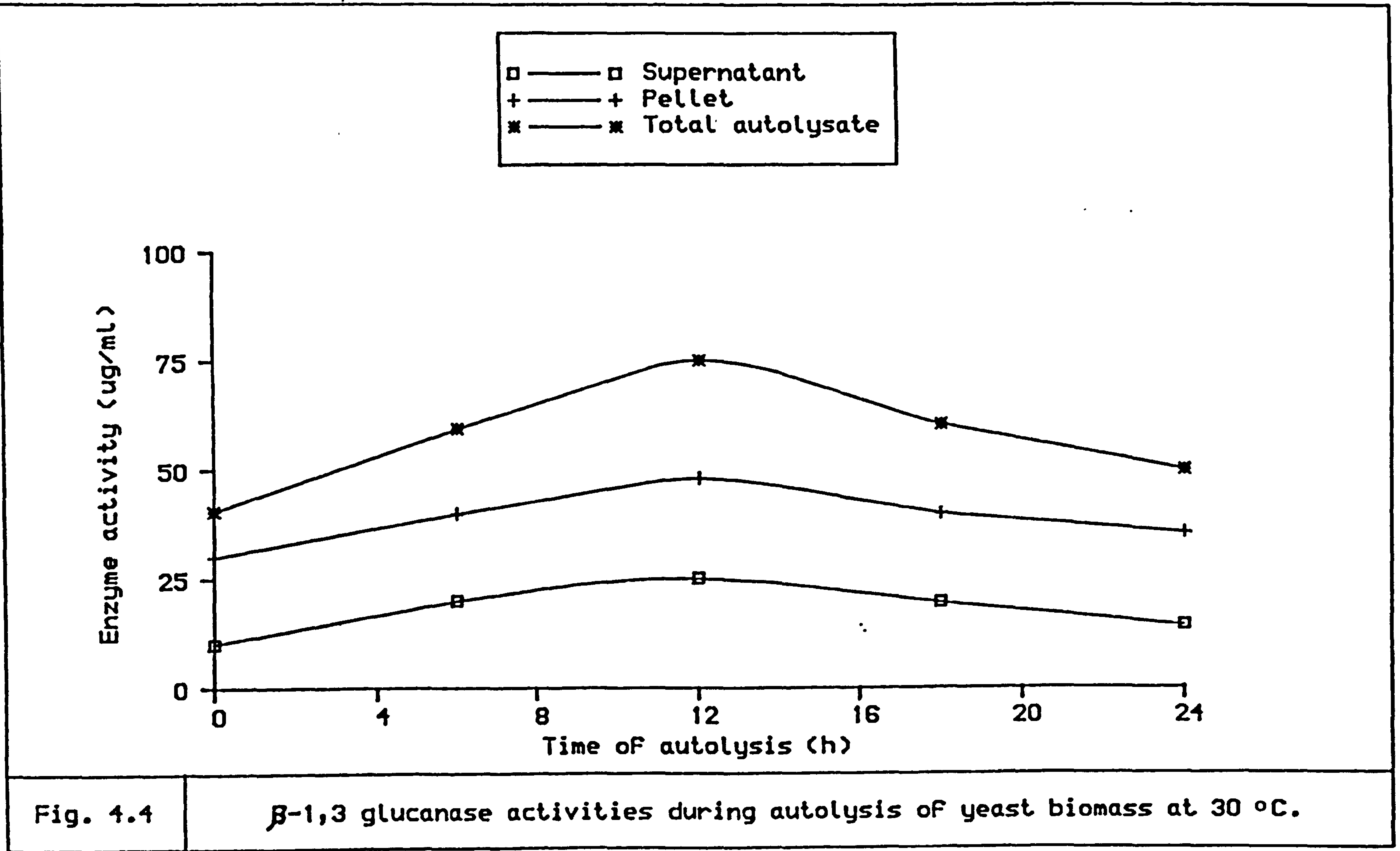


Fig. 4.4 β-1,3 glucanase activities during autolysis of yeast biomass at 30 °C.

was observed following 24 h autolysis.

Table 4.2 Release of ß-1,3 glucanase activity into the medium during autolysis of yeast biomass at 30 °C

	ß-1,3 glucanase activity		
Time (h)	Pellet (Mean/sd.) ($\mu g\ ml^{-1}$)	Supernatant (Mean/sd.) ($\mu g\ ml^{-1}$)	Activity released (%)
0	30.33/3.01	3.58/0.88	10.6
6	40.20/0.26	19.75/0.25	32.9
12	48.03/2.00	25.06/0.12	34.3
18	40.08/1.37	19.50/0.87	32.7
24	35.53/0.68	14.20/0.75	28.6

4.3.2 ß-1,3 glucanase activity during autolysis at 40 °C

The results obtained in these series of experiments (Fig. 4.5) showed that the activity of ß-1,3 glucanase was also maximal after 12 h of autolysis at 40 °C. At this time total glucanase activities were 50 $\mu g\ ml^{-1}$ and 30.2 $\mu g\ ml^{-1}$ for cell pellet and supernatant respectively. However, after 24 h of autolysis activities had decreased to 43.5 $\mu g\ ml^{-1}$ and 21.8 $\mu g\ ml^{-1}$ suggesting that in both fractions glucanases were degraded by proteases.

In these experiments it was found that 34.9 % of activity was solubilised in the inital 6 h of autolysis and this reached a maximal value of 37.6% after 12 h. Following 24 h of autolysis, only 33.4% of the activity was found to be in the soluble fraction (Table 4.3).

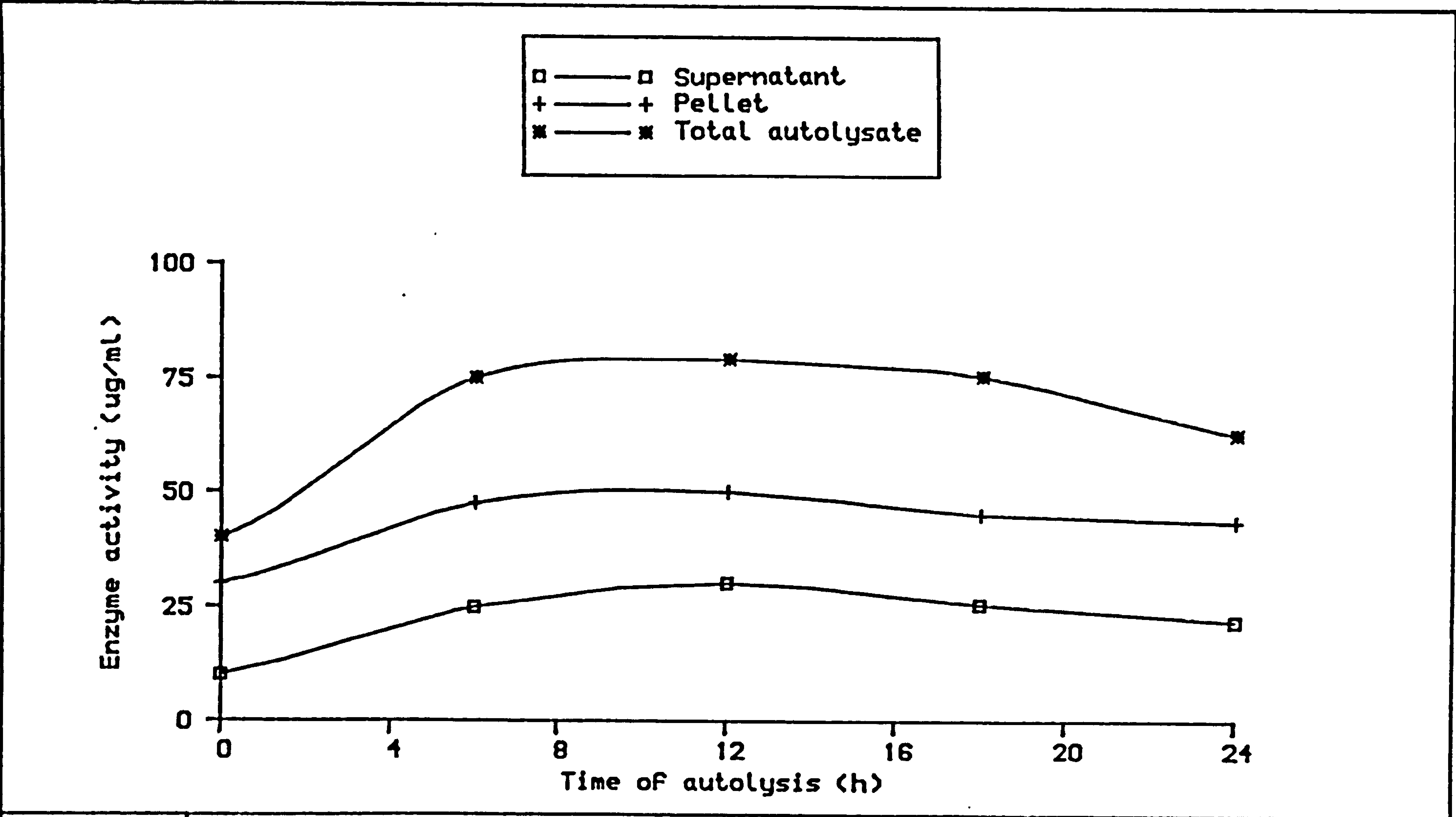


Fig. 4.5 β-1,3 glucanase activities during autolysis of yeast biomass at 40 °C.

Table 4.3 Release of ß-1,3 glucanase activity into the medium during autolysis of yeast biomass at 40 °C

ß-1,3 glucanase activity			
Time (h)	Pellet (Mean/sd.) ($\mu g\ ml^{-1}$)	Supernatant (Mean/sd.) ($\mu g\ ml^{-1}$)	Activity released (%)
0	30.33/3.01	3.58/0.88	10.6
6	47.40/1.65	25.50/0.87	34.9
12	50.10/2.35	30.16/2.00	37.6
18	45.00/1.50	25.40/0.96	36.0
24	43.50/3.03	21.80/1.56	33.4

4.3.3 ß-1,3 glucanase activity during autolysis at 50 °C

In these experiments the increase in ß-1,3 glucanase activity with autolysis at a temperature at which yeast killing is observed was studied (Fig. 4.6). Maximal activity (110.5 $\mu g\ ml^{-1}$) was observed following 6 h of autolysis with values of 62.5 $\mu g\ ml^{-1}$ and 45.5 $\mu g\ ml^{-1}$ being obtained for pellet and supernatant activity, respectively. By 12 h of autolysis at pH 6.5, glucanase activity was decreasing, and minima of 49.8 and 20.5 $\mu g\ ml^{-1}$ for pellet and supernatant were obtained after 24 h of autolysis.

Analysis of these experimental results (Table 4.4) also showed that the highest solubilisation of 42.1% total activity being in the medium after 6 hours of autolysis. Further incubation appeared to decrease the soluble activity so that after 24 h of autolysis, only 29.2% of glucanase activity was found in the medium, suggesting preferential breakdown of free enzyme.

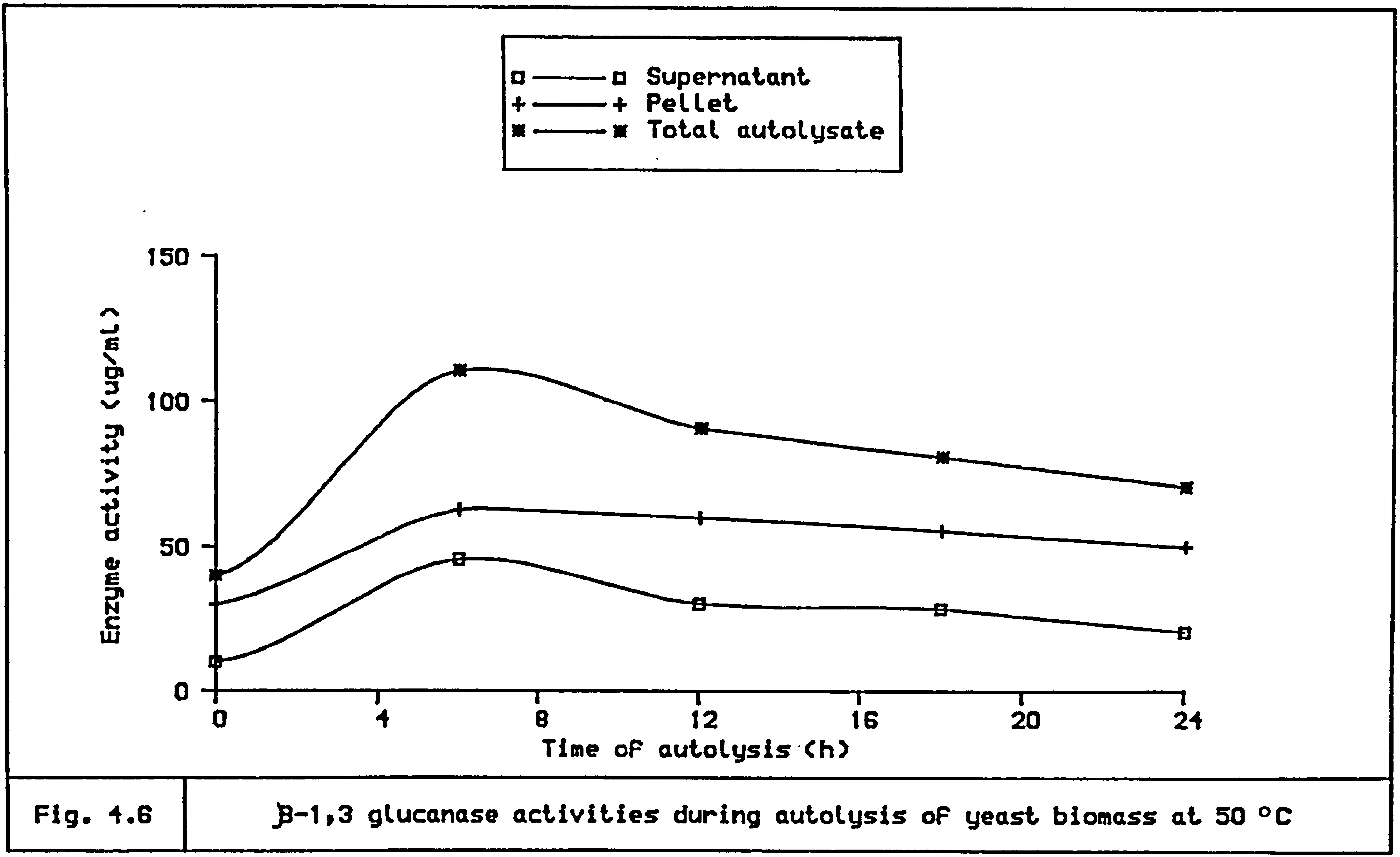


Fig. 4.6 β-1,3 glucanase activities during autolysis of yeast biomass at 50 °C

Table 4.4 Release of ß-1,3 glucanase activity into the medium during autolysis of yeast biomass at 50 °C

	ß-1,3 glucanase activity		
Time (h)	Pellet (Mean/sd.) ($\mu g\ ml^{-1}$)	Supernatant (Mean/sd.) ($\mu g\ ml^{-1}$)	Activity released (%)
0	30.33/3.01	3.58/0.88	10.6
6	62.50/2.00	45.50/0.87	42.1
12	59.16/0.72	30.20/3.65	33.7
18	55.30/1.49	28.50/3.05	34.0
24	49.80/2.20	20.50/2.00	29.2

4.3.4 ß-1,3 glucanase activity during autolysis at 60 °C

Autolysis of cells at temperatures significantly higher than that of cell death resulted in appearance of maximal glucanase values (79.5 $\mu g\ ml^{-1}$) after 6 h of incubation with 50 and 28.5 $\mu g\ ml^{-1}$ in pellet and supernatant, respectively. Following 24 h of autolysis, glucanase activity was estimated as 32.5 ug ml^{-1} in the pellet and had fallen to 12.6 $\mu g\ ml^{-1}$ in the cell supernatant (Fig. 4.7).

Recalculation of data showed that the highest amount (36.2%) of the enzyme activity was in the soluble fraction after 6 h of autolysis and little further changes in relative activity was observed on extending the incubation so that after 24 hours of autolysis, soluble activity was 28% of the total (Table 4.5).

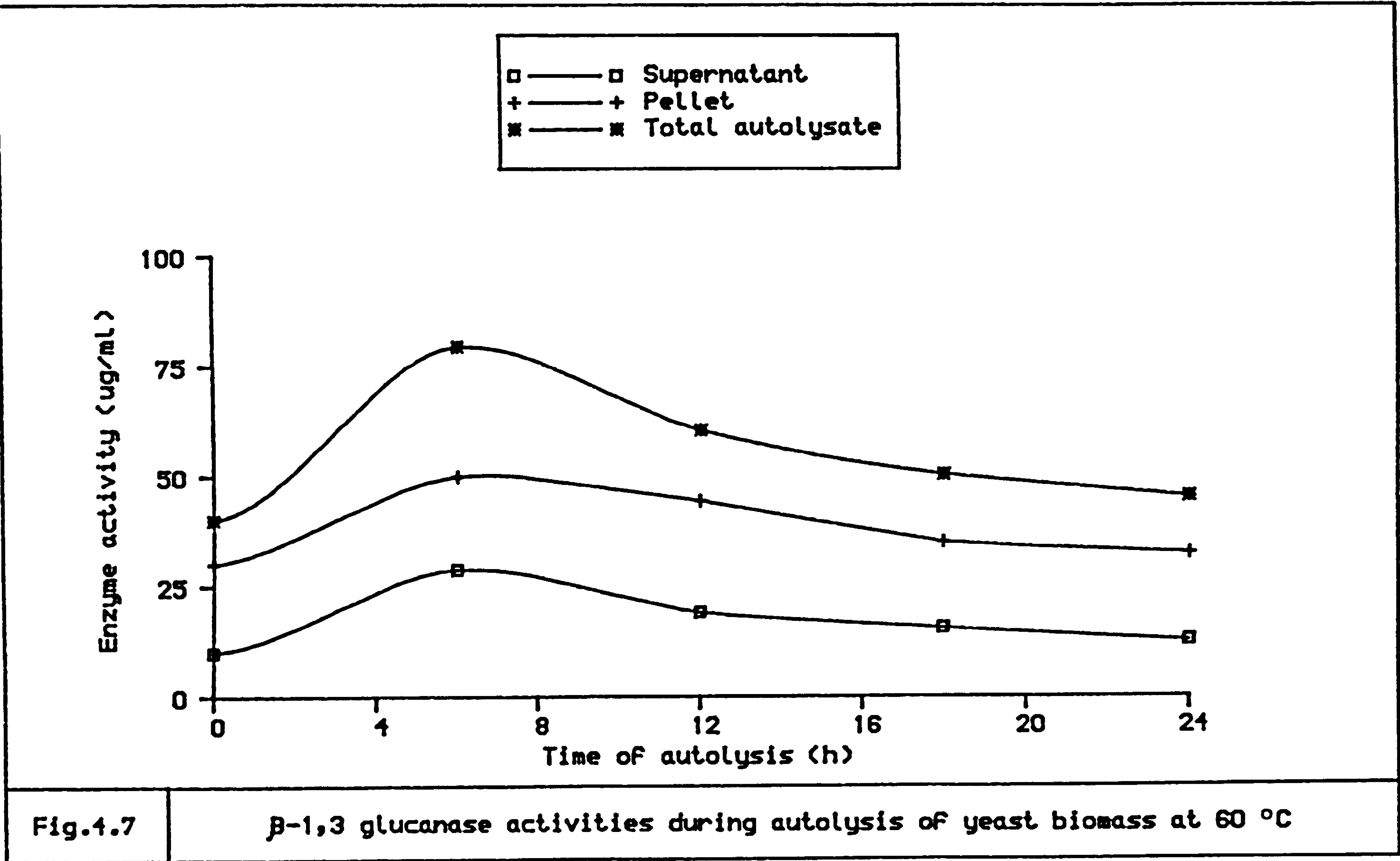


Fig.4.7 β-1,3 glucanase activities during autolysis of yeast biomass at 60 °C

Table 4.5 Release of ß-1,3 glucanase activity into the medium during autolysis of yeast biomass at 60 °C

	ß-1,3 glucanase activity		
Time (h)	Pellet (Mean/sd.) ($\mu g\ ml^{-1}$)	Supernatant (Mean/sd.) ($\mu g\ ml^{-1}$)	Activity released (%)
0	30.33/3.01	3.58/0.88	10.6
6	50.00/0.50	28.43/1.95	36.2
12	44.50/0.66	19.10/1.34	30.0
18	35.10/0.38	15.50/1.14	30.6
24	32.50/2.00	12.60/2.17	27.9

4.4.1 ß-1,6 glucanase activities during autolysis

Since ß-1,6 glucans also contribute to the structure of the yeast cell walls, ß-1,6 glucanases are also important contributors to cell wall solubilisation during autolysis. Thus the total activity of these enzymes was determined after periods of autolysis at a range of temperatures using pustulan (ß-1,6 glucan) as assay substrate.

4.4.2 ß-1,6 glucanase activity during autolysis at 30 °C

Glucanase activities were determined at the normal growth temperature of yeast growth, 30 °C. The results obtained (Fig. 4.8), demonstrated that ß-1,6 glucanase activity reached a maximum (45.5 $\mu g\ ml^{-1}$) after 12 h autolysis with values of 31.5 and 12.6 $\mu g\ ml^{-1}$ for pellet and supernatant respectively. No appreciable reduction in glucanase activity in the cell wall pellet was observed on extended autolysis so that by 24 h of autolysis 22.3 $\mu g\ ml^{-1}$ of activity was measured in the pellet although soluble glucanase activity had decreased to 5.5 $\mu g\ ml^{-1}$.

On recalculating this experimental data (Table 4.6), it was determined that only 24.2% of ß-1,6 glucanase activity was in the soluble fraction after 6 h of autolysis. Further incubation, however, was found to promote enzyme release so that after 12 h of autolysis 28.6% of activity was soluble.

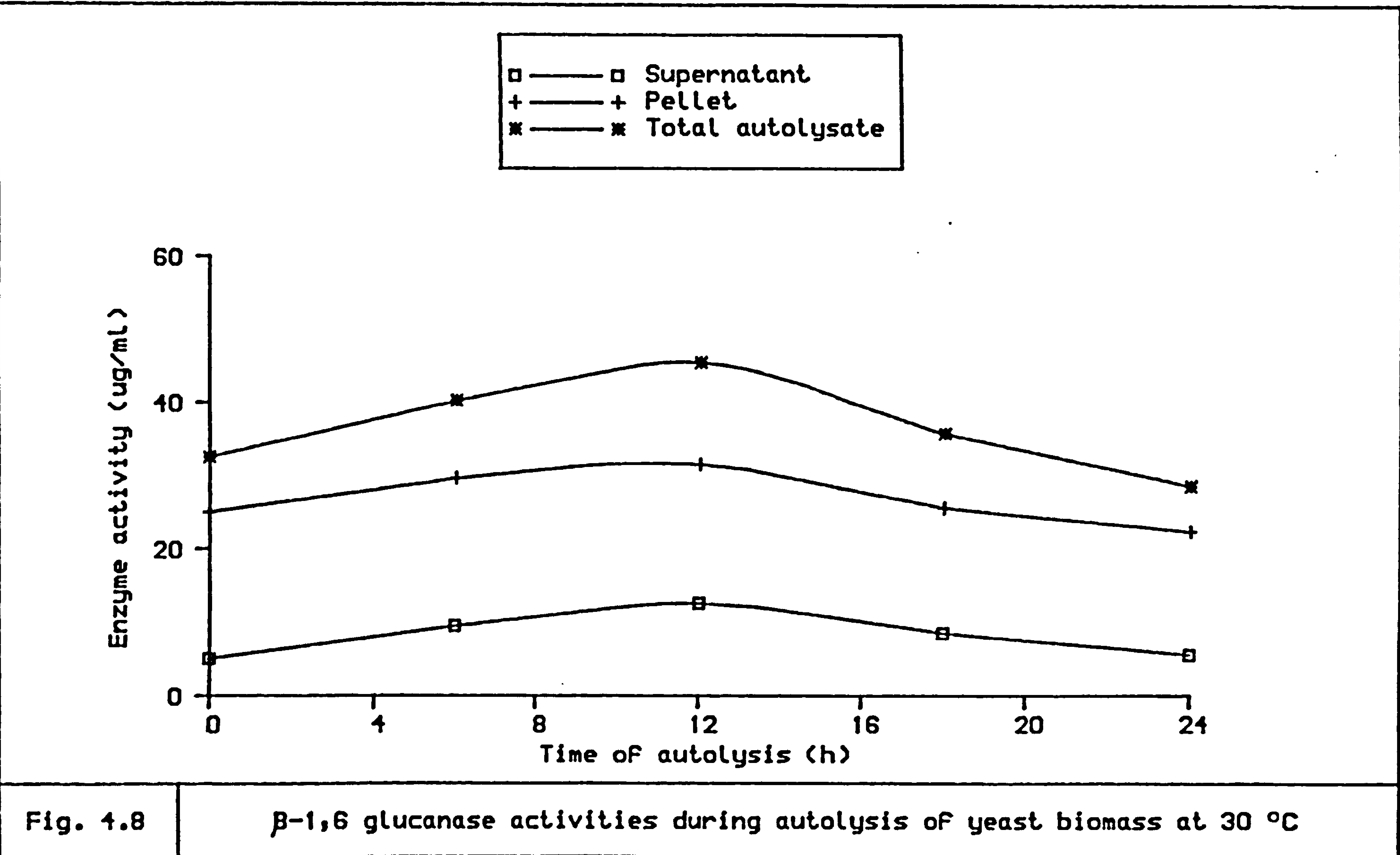


Fig. 4.8 β-1,6 glucanase activities during autolysis of yeast biomass at 30 °C

Following 24 h of incubation the soluble proportion had decreased to 19.8% suggesting rapid breakdown of soluble enzyme in the later stages of autolysis.

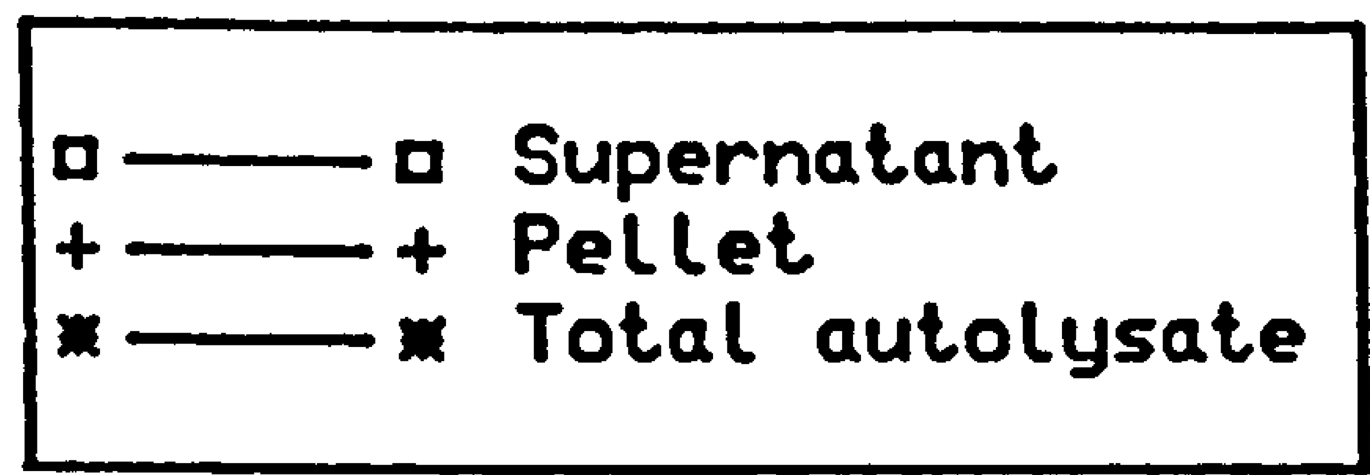
Table 4.6 Release of ß-1,6 glucanase activity into the medium during autolysis of yeast biomass at 30 °C.

ß-1,6 glucanase activity			
Time (h)	Pellet (Mean/sd.) ($\mu g\ ml^{-1}$)	Supernatant (Mean/sd.) ($\mu g\ ml^{-1}$)	Activity released (%)
0	25.50/0.88	2.00/0.40	7.2
6	29.60/1.64	9.50/1.00	24.2
12	31.50/1.15	12.60/2.74	28.6
18	25.60/1.64	9.16/1.52	26.4
24	22.30/1.11	5.50/1.00	19.8

4.4.3 ß-1,6 glucanase activity during autolysis at 40 °C

Repetition of ß-1,6 glucanase activity determinations during autolysis at 40 °C yielded the results shown in Figure 4.9. These indicated that total, pellet and supernatant glucanase activities reached maximal activities of 55.2, 37.4 and 15 $\mu g\ ml^{-1}$ after 6 h of autolysis. This was followed by a relatively slow decrease in activities so that following 24 h of autolysis pellet activity had decreased to 25 $\mu g\ ml^{-1}$ while only 5.7 $\mu g\ ml^{-1}$ of glucanase activity was observed in the supernatant at this time point.

Recalculation of results (Table 4.7) showed that after 6 h incubation 28.5% was in the soluble fraction and this decreased 24% after 12 h autolysis. The proportion of soluble activity declined so that after 24 h of autolysis only 18.6 % activity was soluble.



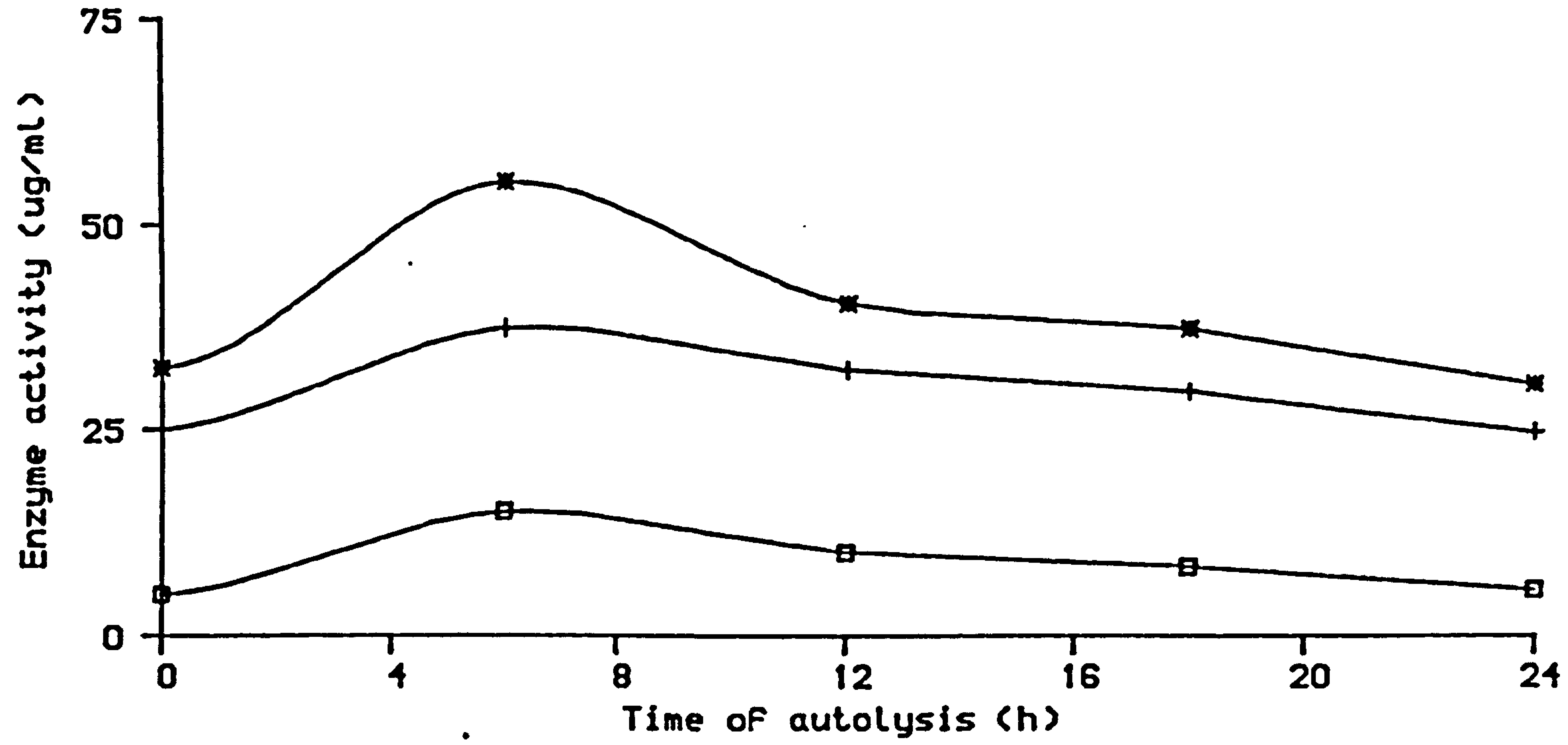


Fig. 4.9 β-1,6 glucanase activities during autolysis of yeast biomass at 40 °C

Table 4.7 Release of ß-1,6 glucanase activity into the medium during autolysis of yeast biomass at 40 °C

	ß-1,6 glucanase activity		
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	25.50/0.87	2.00/0.40	7.2
6	37.40/1.90	14.91/0.14	28.5
12	32.40/2.10	10.80/0.38	25.0
18	29.80/1.21	8.50/0.75	22.2
24	24.90/1.10	5.70/0.18	18.6

4.4.4 ß-1,6 glucanase activity during autolysis at 50 °C

Incubation of yeast biomass at 50 °C, a temperature sufficient to kill yeast biomass (Fig. 4.10), showed ß-1,6 glucanase activities reached maximal values after 6 h autolysis. At this time total, pellet and supernatant activities were estimated as 72.5, 45.5 and 25.5 µg ml^{-1}, respectively. After this a slow decline in enzyme activity was observed so that after 24 h autolysis values of 35.5, 25 and 7.5 µg ml^{-1} were obtained.

Recalculation of experimental data (Table 4.8) showed that 35.9% of activity was soluble after 6 h of autolysis but that the soluble portion decreased with time so that after 24 h of autolysis, only 22.9% of glucanase activity was present as as the soluble enzyme.

Table 4.8 Release of ß-1,6 glucanase activity into the medium during autolysis of yeast biomass at 50 °C

	ß-1,6 glucanase activity		
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	25.50/0.88	2.00/0.40	7.2
6	45.58/0.14	25.50/2.00	35.9
12	33.50/1.32	13.82/1.69	29.2
18	30.20/2.00	10.25/1.14	25.3
24	25.10/1.81	7.45/0.58	22.9

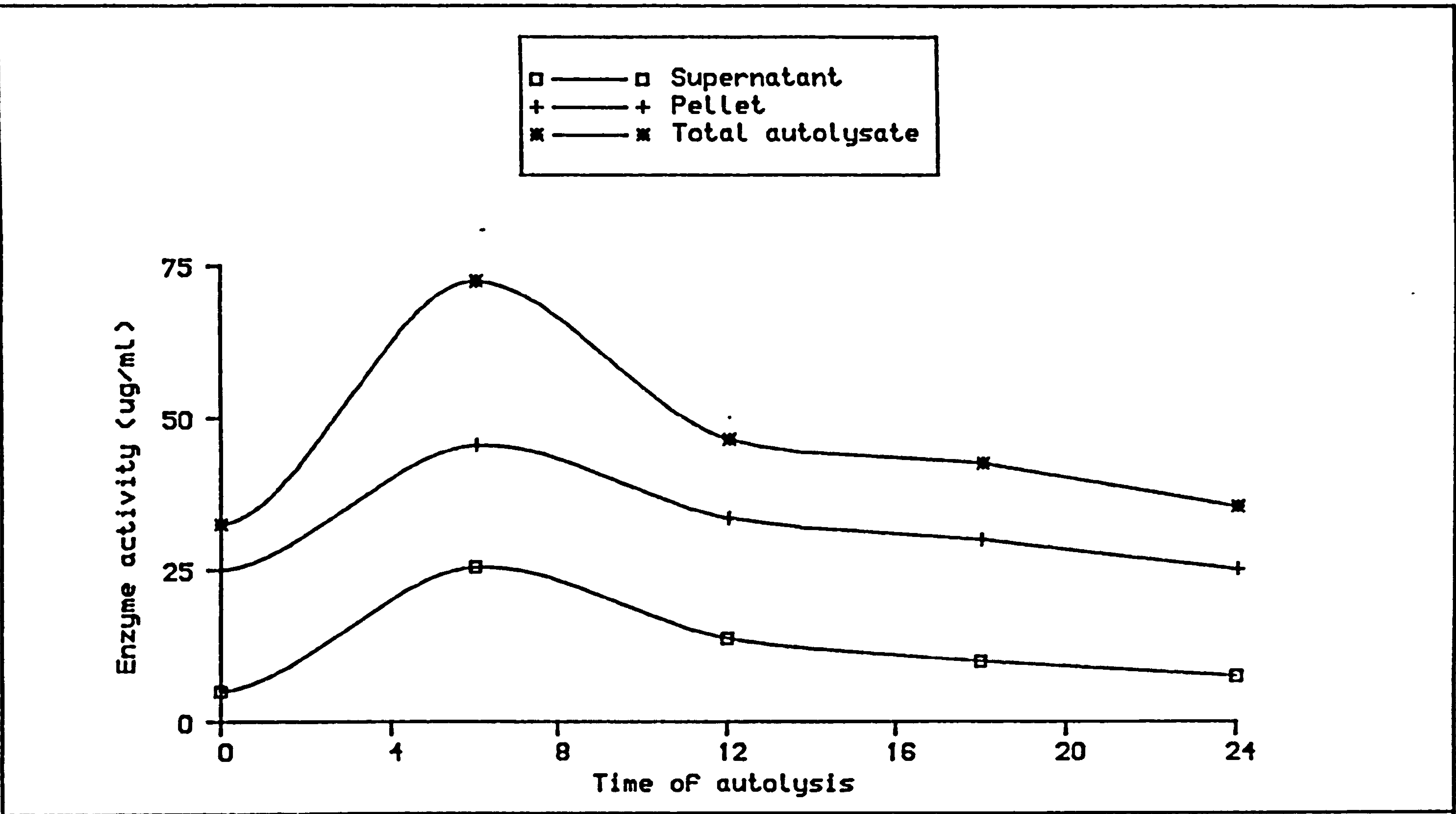


Fig. 4.10 β-1,6 glucanase activities during autolysis of yeast biomass at 50 °C

4.4.5 ß-1,6 glucanase activity during autolysis at 60 °C

Estimation of 1,6 glucanase activities during autolysis at 60 °C (Fig. 4.11) showed that incubation at a temperature above that of cell killing resulted in maximal glucanase activities of 52.4, 35.5 and 15 µg ml^{-1} for total autolysate, pellet and supernatant respectively, after 6 h autolysis, continued autolysis resulted in decreases in glucanase activities so that after 24 h incubation, pellet activity had been reduced to 20.5 µg ml^{-1} whereas soluble activity had declined to 4.8 µg ml^{-1}.

The proportion of soluble enzyme was maximal at 29.7% after 6 h of autolysis but this was observed to decrease rapidly so that by 24 h only 19.0% was observed in the soluble fraction (Table 4.9).

Table 4.9 Release of ß-1,6 glucanase activity into the medium during autolysis of yeast biomass at 60 °C

ß-1,6 glucanase activity			
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	25.50/0.87	2.00/0.40	7.2
6	35.50/1.88	15.00/2.29	29.7
12	30.60/2.00	9.58/0.88	23.8
18	24.80/1.53	7.66/0.29	23.6
24	20.50/2.02	4.83/0.29	19.0

4.5.1 Mannanase activity during autolysis at different temperatures

Although yeast cells walls are frequently considered to be composed mainly of glucan, it has been reported that S.cerevisiae cell walls contain 40% glucan and 40% mannan (Hough and Maddox, 1970) with mannan predominantly

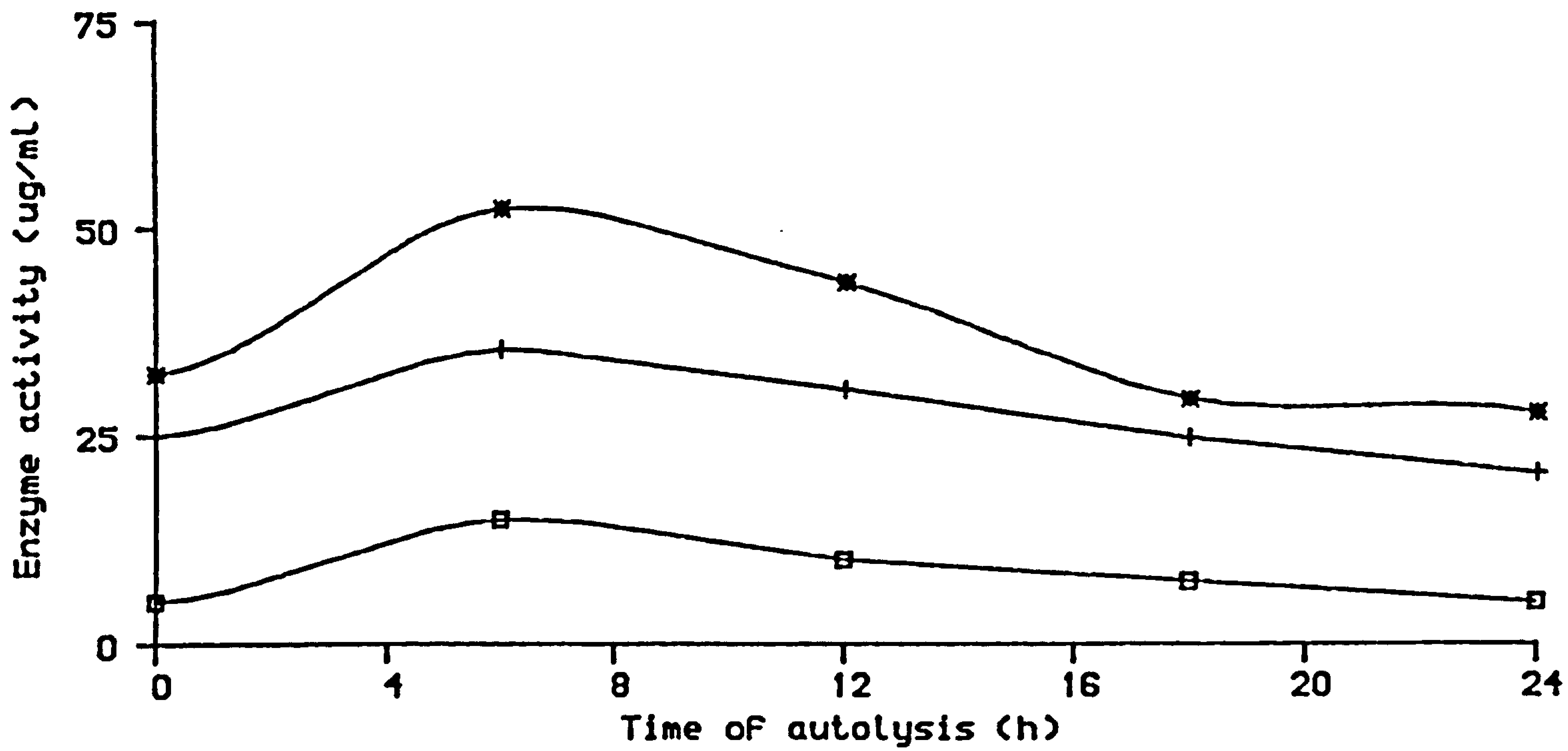


Fig. 4.11 β-1,6 glucanase activities during autolysis of yeast biomass at 60 °C

associated with protein on the exterior of the wall. Mannanases appear to be associated with the polymer in this outer layer and mannanase activity has been reported to be present in yeast autolysate (Hough and Maddox, 1970). Consequently changes in mannanase activity during autolysis were studied.

4.5.2 Mannanase activity during autolysis at 30 °C

Mannanase activities in total yeast autolysates, cell pellets and cell-free supernatants were determined over a 24 h period for biomass incubated at the growth temperature of 30 °C. The results (Fig. 4.12), showed total autolysate and pellet mannanase activity reached maximal values after 12 h of autolysis and decreased after this point to a minimum after 24 h. Although the mannanase activity in the total autolysates was found to be slightly higher than that in the cell wall pellet, no mannanase activity was detected in supernatant fractions under the experimental conditions used in this project.

4.5.3 Mannanase activity during autolysis at 40 °C

The results obtained in these experiments (Fig. 4.13) indicate that mannanase activity in the pellet fraction rapidly reached a maximum (27.5 µg ml^{-1}) after 6 h of autolysis and thereafter decreased so that a minimum (15.5 µg ml^{-1}) was reached after 24 h of autolysis. As previously, no mannanase activity was detected in autolysate supernatants.

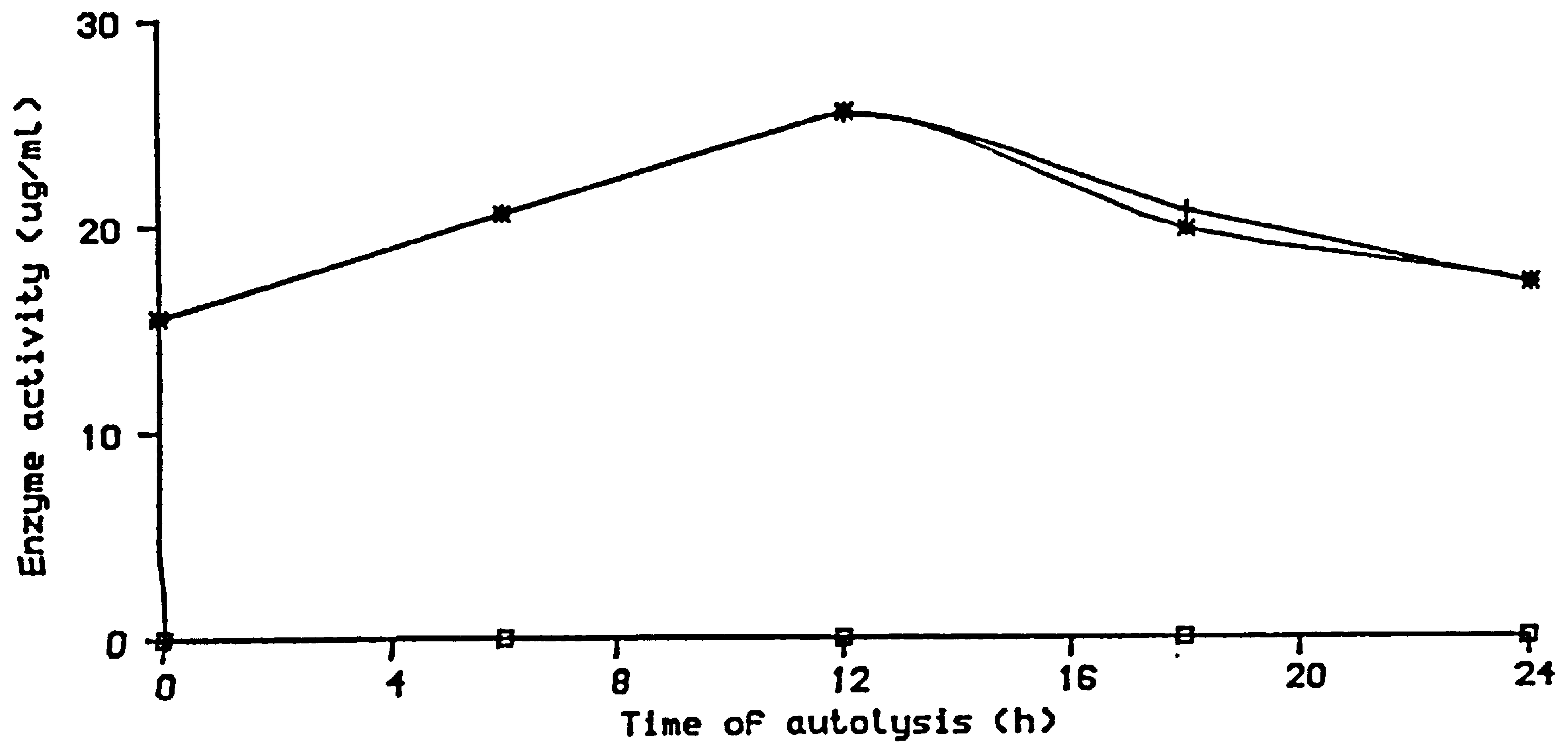


Fig. 4.12 Mannanase activity during autolysis of yeast biomass at 30 °C

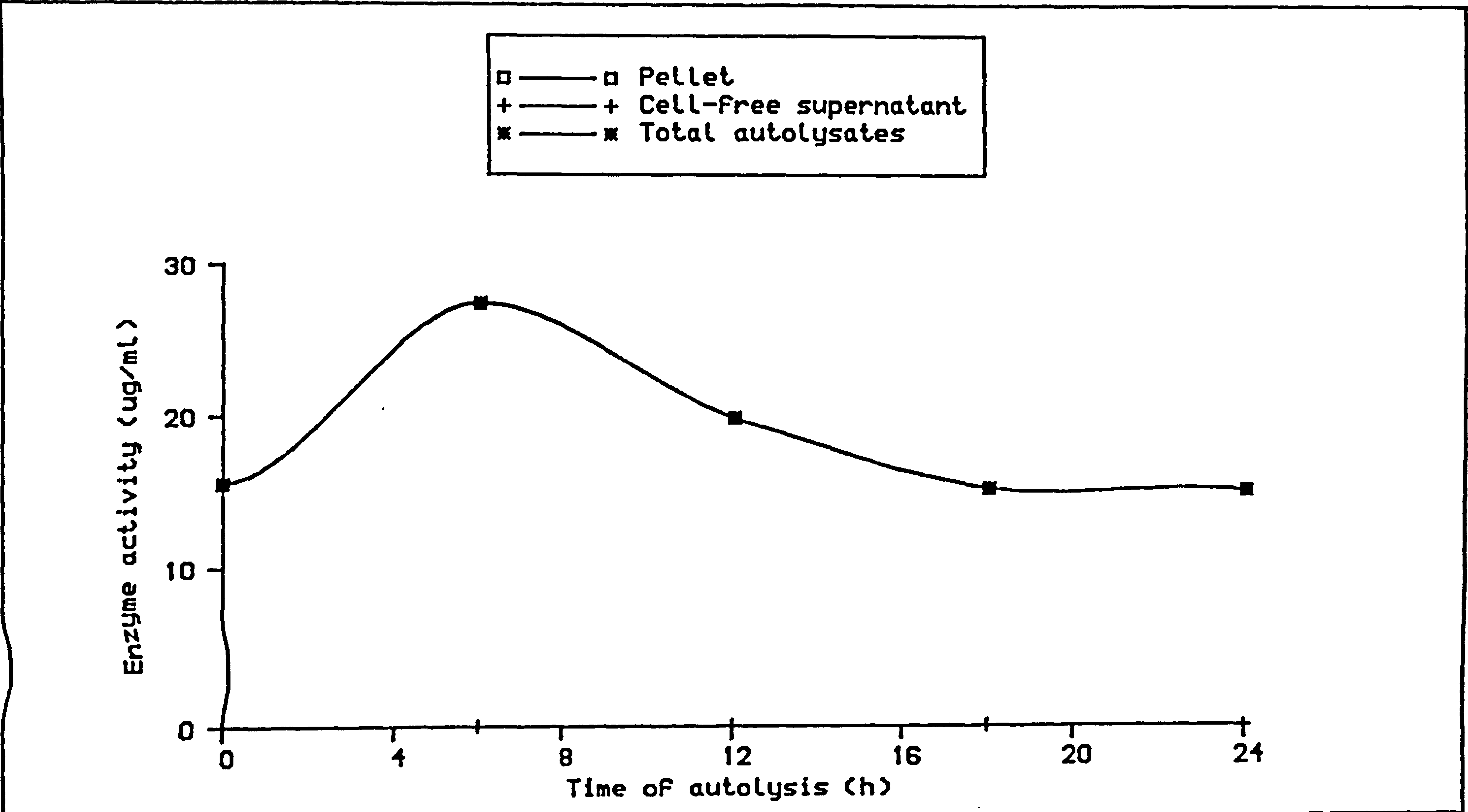


Fig. 4.13 Mannanase activity during autolysis of yeast biomass at 40 °C

4.5.4 Mannanase activity during autolysis at 50 °C

In this series of experiments, with incubation of yeast biomass at a lethal temperature, activity in the cell wall pellet reached a maximum of 39.5 $\mu g\ ml^{-1}$ after 6 h of autolysis and after this declined to a minimum (15.5 $\mu g\ ml^{-1}$) after 24 h of autolysis (Fig. 4.14). No mannanase activity was detected in cell supernatants.

4.5.5 Mannanase activity during autolysis at 60 °C

Mannanase activities in biomass autolysing at this relatively high temperature (Fig. 4.15) reached a maximum (28.5 $\mu g\ ml^{-1}$) in the cell wall pellet fraction after 6 h of autolysis and decreased significantly to a minimum of 7.5 $\mu g\ ml^{-1}$ after 24 h of autolysis. No activity could be detected in supernatant fractions.

4.6.1 Estimation of chitinase activity in yeast autolysates

Although chitin is restricted to bud scars in the S.cerevisiae cell wall, chitinase activity has been proposed to be important in the disruption of the continuity of the wall during autolysis. To investigate this hypothesis further, chitinase activities in biomass autolysing at differing temperatures were determined. Chitinase activities were determined using N-acetylglucosamine as standard (Fig. 4.16).

4.6.2 Chitinase activity during autolysis at 30 °C

The results from these series of experiments (Fig. 4.17) showed that chitinase activity could be detected both in cell pellets and in cell-free supernatants during incubation at 30 °C. It was found that chitinase activities

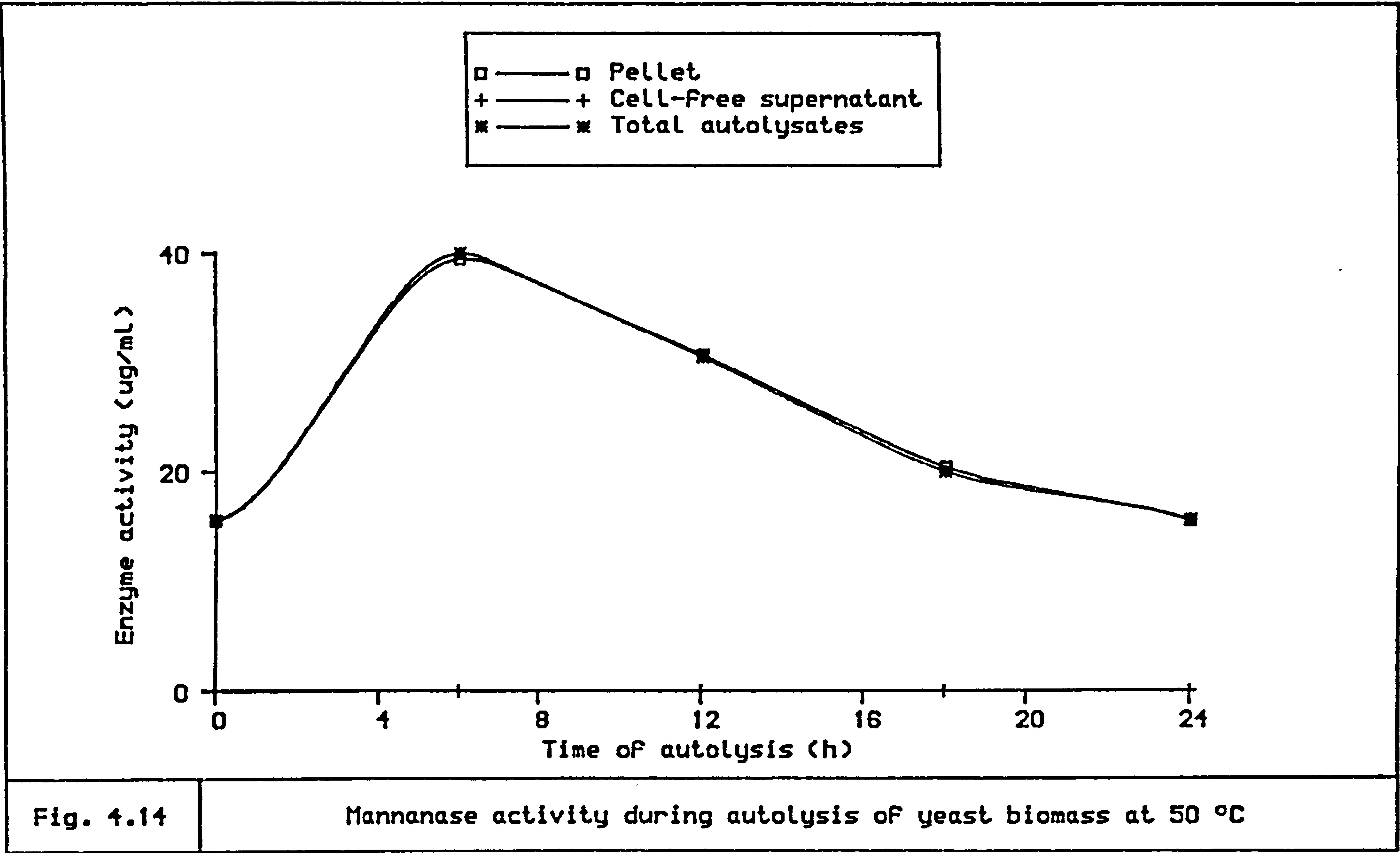


Fig. 4.14 Mannanase activity during autolysis of yeast biomass at 50 °C

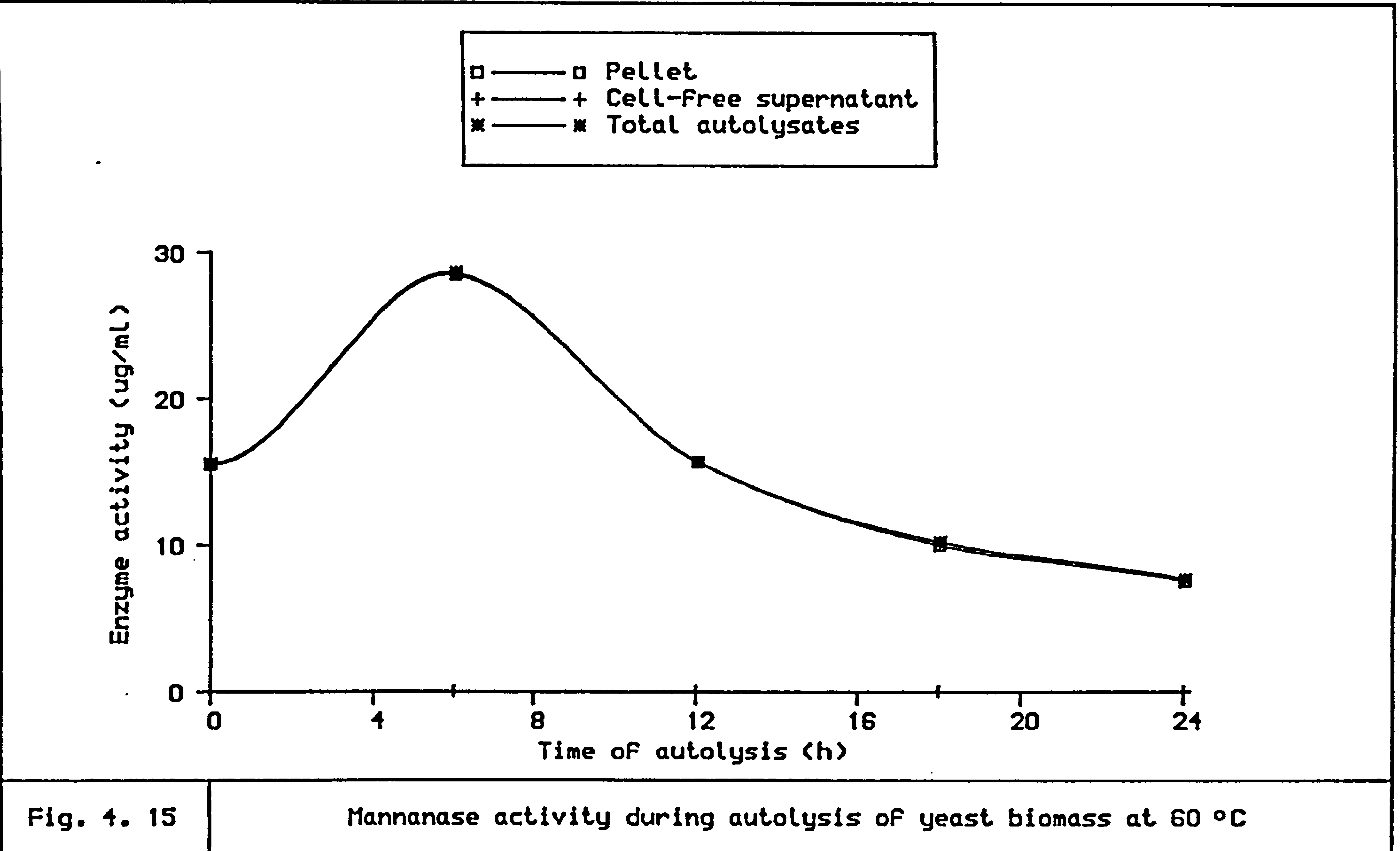


Fig. 4. 15 Mannanase activity during autolysis of yeast biomass at 60 °C

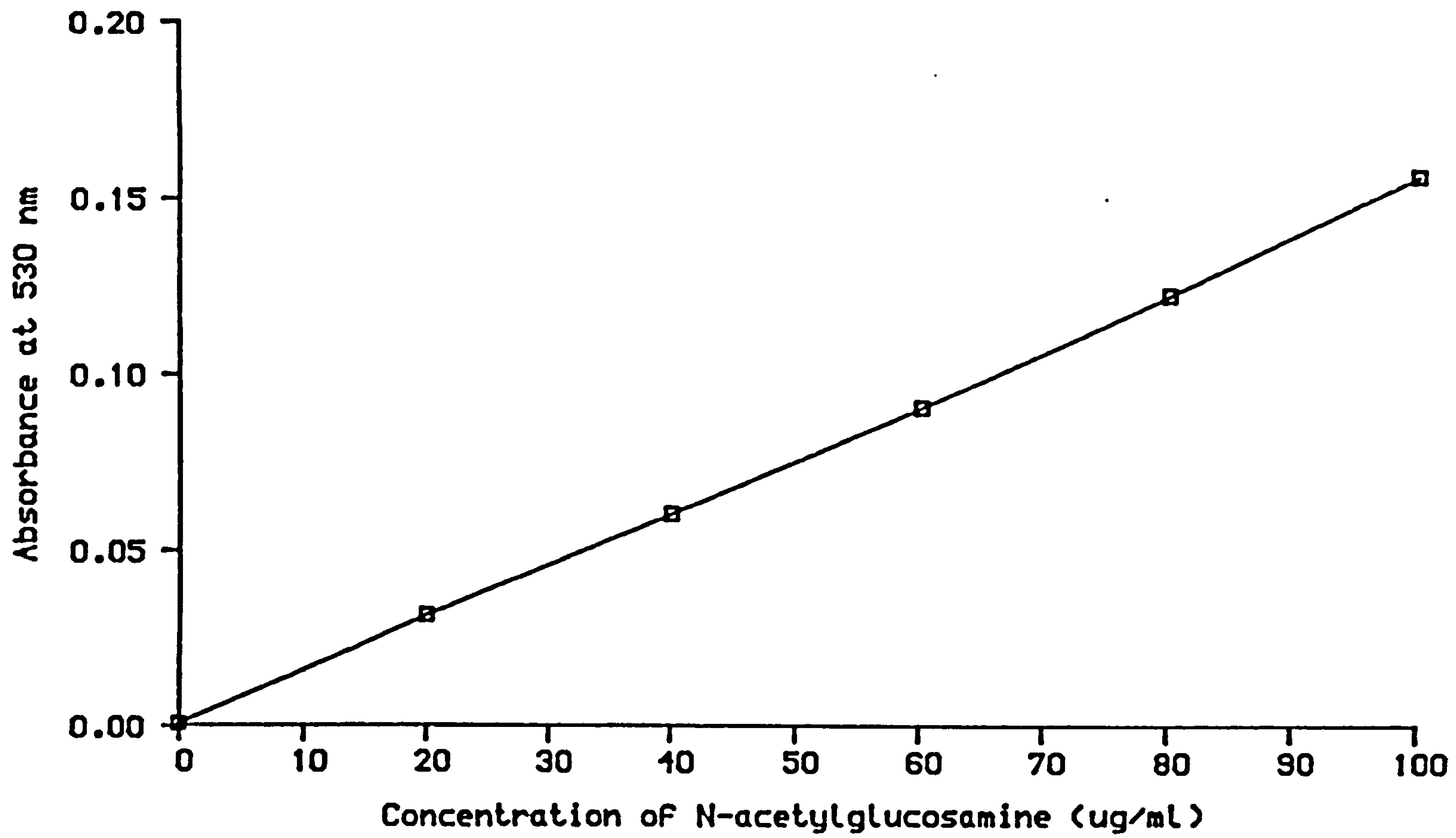


Fig. 4.16 Standard curve for N-acetylglucosamine (Rondle-Morgan, 1955)

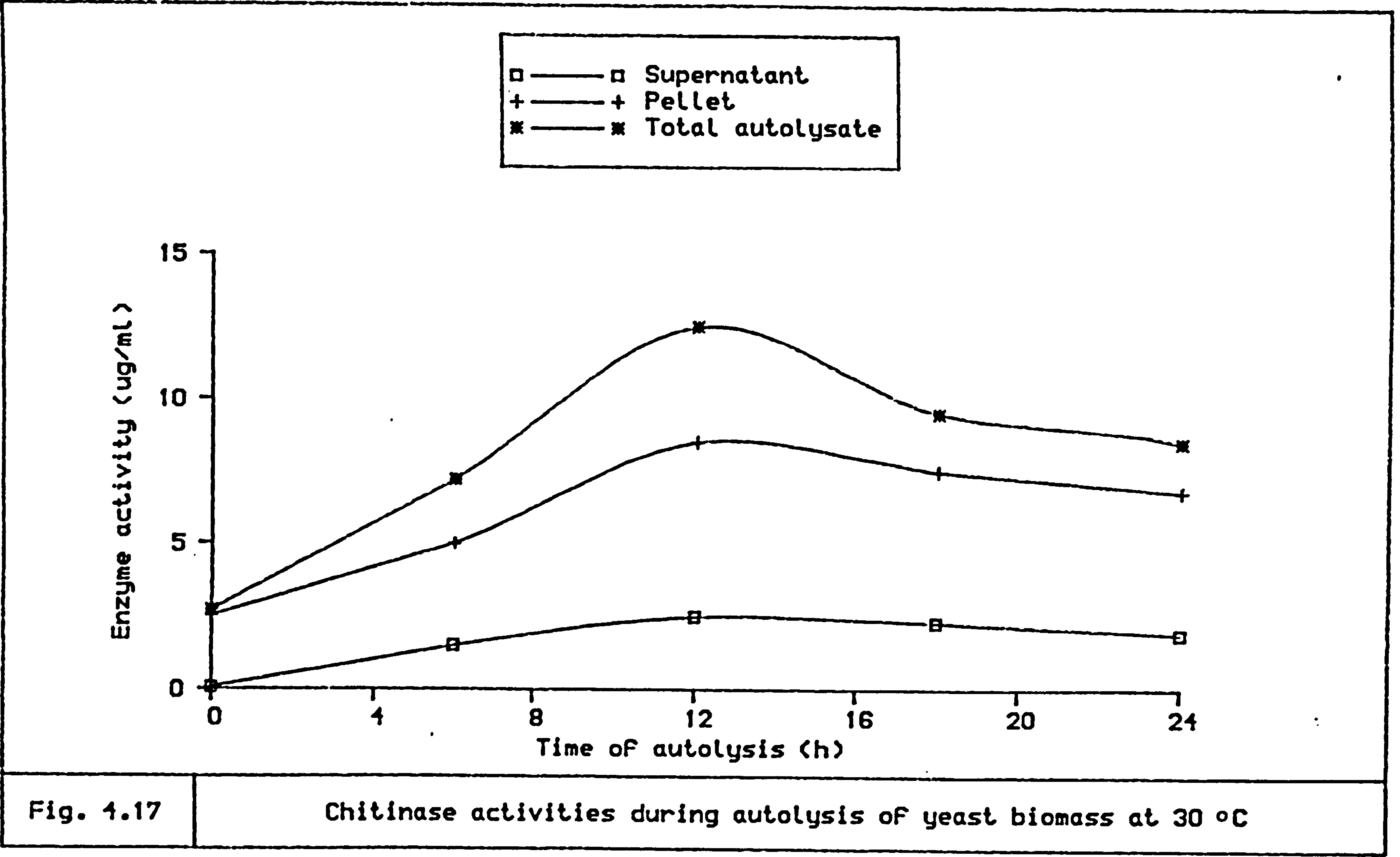


Fig. 4.17 Chitinase activities during autolysis of yeast biomass at 30 oC

reached maximal values after 12 h of autolysis (8.5 μg ml^{-1} in pellet; 2.5 μg ml^{-1} in supernatant) and reached minima after 24 h of autolysis (6.8 μg ml^{-1} in cell pellet; 1.9 μg ml^{-1} in cell-free supernatant).

Recalculation of these results (Table 4.10) reveals that the maximal proportion of soluble activity (24.1%) is present after 18 h of autolysis. Continued incubation gradually reduced the proportion of soluble activity until at 24 h only 21.8% was in this fraction.

Table 4.10 Release of chitinase activity into the medium during autolysis of yeast biomass at 30 °C

	Chitinase activity		
Time (h)	Pellet (Mean/sd.) (ug ml^{-1})	Supernatant (Mean/sd.) (ug ml^{-1})	Activity released (%)
0	2.50/0.50	0.05/0.03	2.0
6	5.00/0.25	1.50/0.23	23.0
12	8.50/0.25	2.50/0.44	22.7
18	7.33/0.28	2.33/0.10	24.1
24	6.80/0.41	1.90/0.36	21.8

4.6.3 Chitinase activities during autolysis at 40°C

The results of these experiments (Fig. 4.18) showed that chitinase activities reached maxima of 9.2 μg ml^{-1} in the pellet fraction and 3.2 μg ml^{-1} in the supernatant after 6 h of autolysis. After this both pellet and the supernatant chitinase activities decreased to minima at 24 h of 7.0 μg ml^{-1} for pellet and 1.8 μg ml^{-1} for cell-free supernatant.

The maximum proportion of soluble activity (25.8%) was observed after 6 h of autolysis and this declined with continued incubation to a minima of 20.5% after 24 h autolysis (Table 4.11)

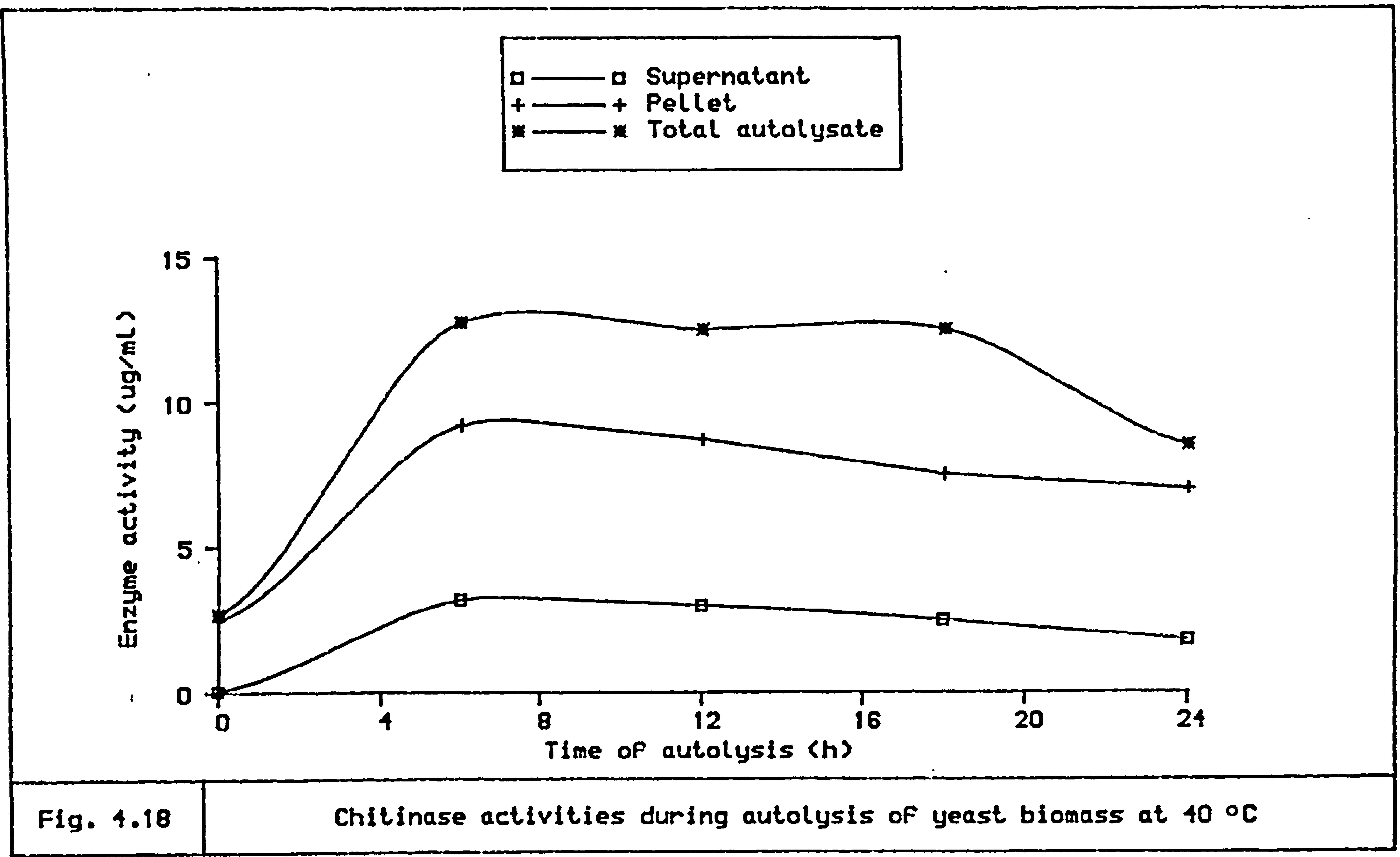


Fig. 4.18 Chitinase activities during autolysis of yeast biomass at 40 °C

Table 4.11 Release of chitinase activity into the medium during autolysis of yeast biomass at 40 °C

Chitinase activity			
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	2.50/0.50	0.05/0.03	2.0
6	9.20/0.67	3.20/0.46	25.8
12	8.70/0.72	3.00/0.48	25.6
18	7.50/0.30	2.50/0.46	25.0
24	7.00/0.43	1.80/0.26	20.5

4.6.4 Chitinase activities during autolysis at 50°C

This experiment was designed to measure the chitinase activities during autolysis at the lethal temperature of 50°C. These results (Fig. 4.19) showed that chitinase activities reached maxima of 22.5 µg ml^{-1} in pellet and 8.5 µg ml^{-1} in supernatant after 6 h of autolysis. Both pellet and supernatant related activity then declined until after 24 h minimal values of 6.5 µg ml^{-1} for pellet activity and 1.7 µg ml^{-1} for supernatant were obtained.

The maximal proprtion of soluble enzyme (27.5%) was observed after 6 h of autolysis and by 24 h this had decreased to 21% (Table 4.12).

Table 4.12 Release of chitinase activity into the medium during autolysis of yeast biomass at 50 °C

Chitinase activity			
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	2.50/0.50	0.05/0.03	2.0
6	22.50/1.00	8.50/0.87	27.4
12	15.50/0.90	5.50/0.23	26.1
18	7.80/0.38	2.50/0.15	24.2
24	6.50/0.90	1.70/0.17	20.7

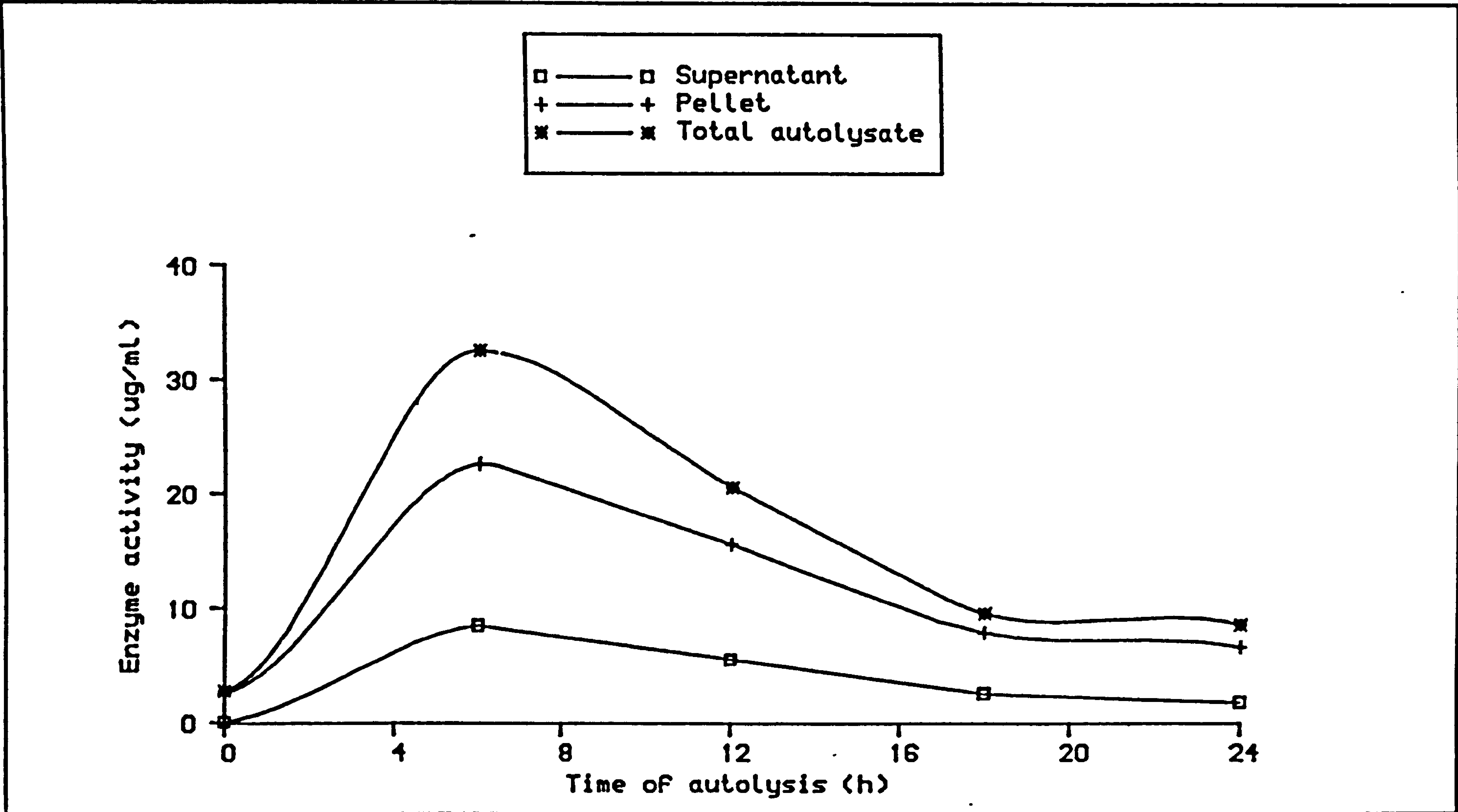


Fig. 4.19 Chitinase activities during autolysis of yeast biomass at 50 °C

4.6.5 Chitinase activity during autolysis at 60 °C

These experiment was carried out to estimate chitinase activities during autolysis at a relatively high temperature. The results (Fig. 4.20) showed that chitinase activities in both pellet and supernatant reached maxima of 15.2 µg ml^{-1} and 5.5 µg ml^{-1}, respectively, after 6 h of autolysis. With continued incubation minima of 6.0 µg ml^{-1} (pellet) and 1.5 µg ml^{-1} (supernatant) were reached after 24 h autolysis.

After 6 h of autolysis a maximum of 26.6% activity was in the soluble fraction and thereafter the proportion dropped until after 24 h a value of 20% was determined (Table 4.13).

Table 4.13 Release of chitinase activity into the medium during autolysis of yeast biomass at 60 °C

	Chitinase activity		
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	2.50/0.50	0.05/0.03	2.0
6	15.20/1.55	5.50/0.90	26.6
12	9.75/0.40	3.20/0.26	24.9
18	7.50/0.66	2.30/0.30	23.5
24	6.00/0.43	1.66/0.29	21.6

4.7.1 Protease activities during autolysis at different temperatures

These experiments were performed to estimate proteolytic activities during incubation at a range of sub-lethal and lethal temperatures. Protease activities of total autolysate, cell-wall pellet and supernatant were determined using casein as protein substrate by estimation of product

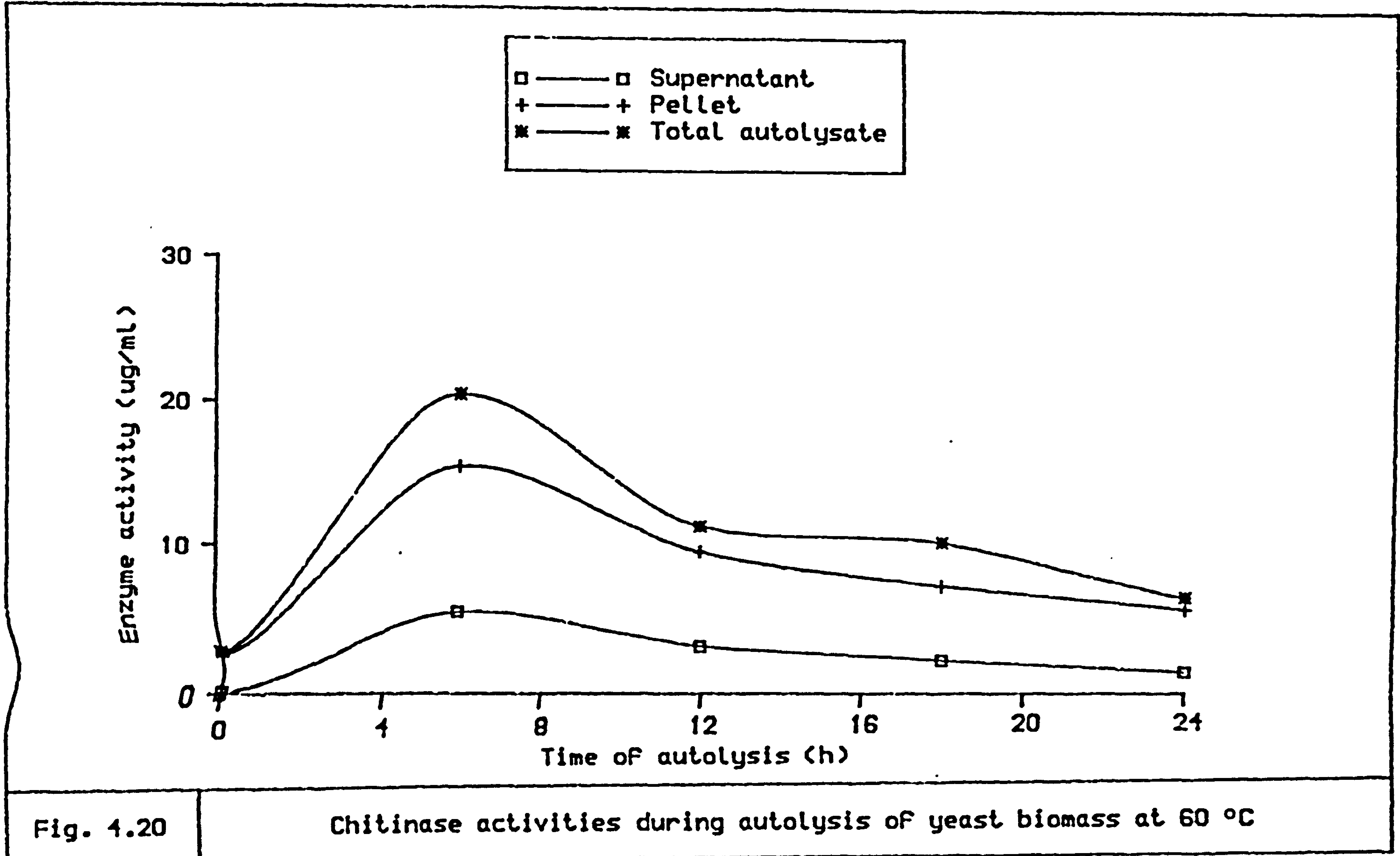


Fig. 4.20 Chitinase activities during autolysis of yeast biomass at 60 °C

α-amino nitrogen (Fig. 4.21).

4.7.2 Protease activity during autolysis at 30 °C

During incubation at the normal growth temperature of 30 °C, protease activities increased during autolysis until maximal values of 15.7 µg ml^{-1} and 5.5 µg ml^{-1} were determined after 12 h autolysis (Fig. 4.22). Thereafter protease activities decreased until after 24 h pellet values of 7.4 µg ml^{-1} and 2.5 µg ml^{-1} were determined for pellet and supernatant respectively.

It was found that the maximum of 25.8% of protease activity was present in the soluble fraction after 12 h and after 24 h this had decreased to 25% (Table 4.14).

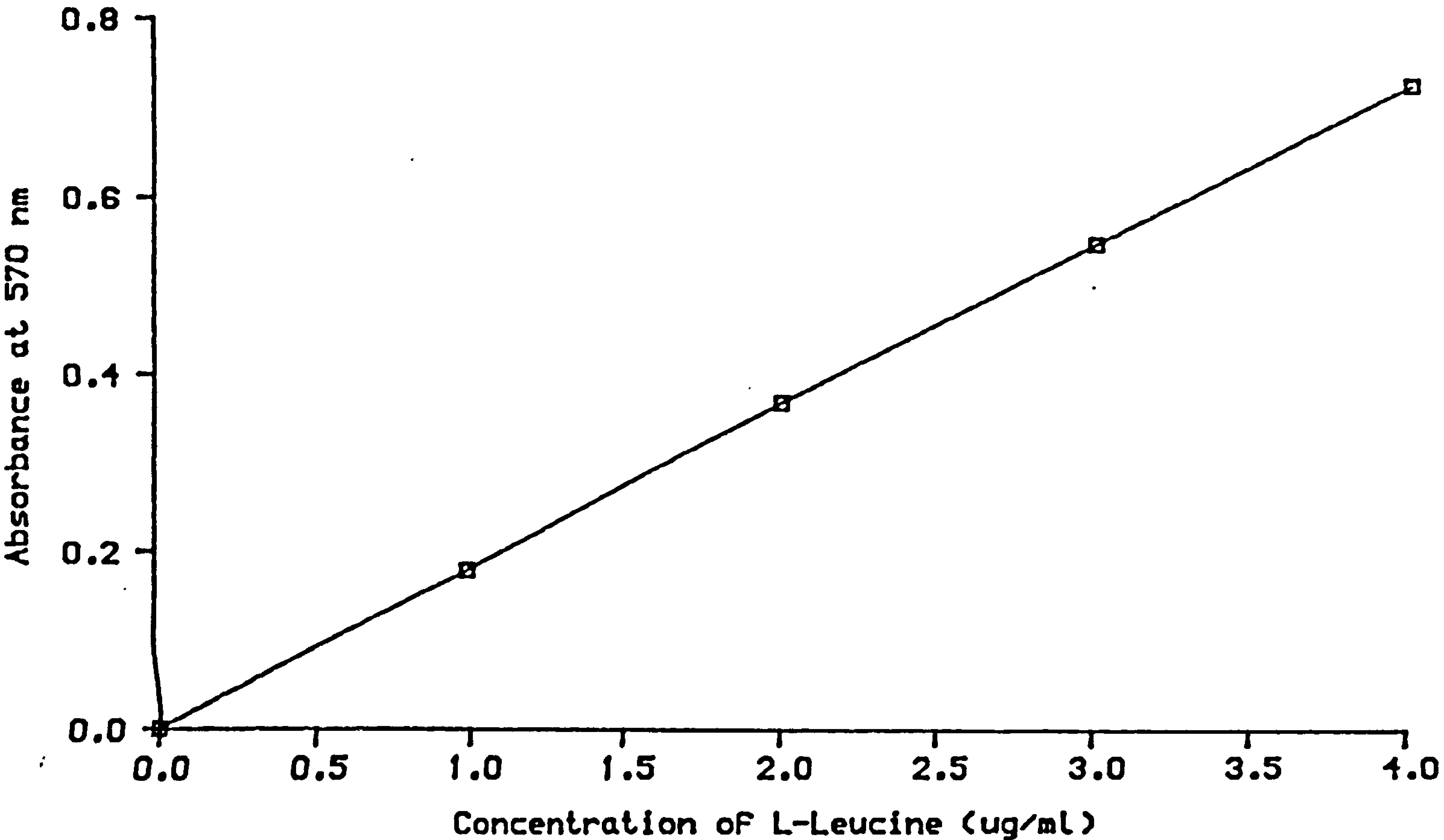
Table 4.14 Release of protease activity into the medium during autolysis of yeast biomass at 30 °C

	Protease activity		
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	5.00/0.50	0.15/0.25	2.9
6	12.50/1.89	3.20/0.26	20.4
12	15.70/1.35	5.45/0.83	25.8
18	10.50/0.25	2.54/0.44	20.2
24	7.40/1.01	2.50/0.23	25.2

4.7.3 Protease activities during autolysis at 40 °C

The results of these experiments (Fig. 4.23) show that maximal values of 17.5 µg ml^{-1} and 7.5 µg ml^{-1} for pellet and supernatant were determined after 6 h of incubation at 40 °C. Following 24 h of autolysis values had dropped to 10.7 µg ml^{-1} (pellet) and 2.4 µg ml^{-1} (supernatant).

It was found (Table 4.15) that the maximal soluble proportion of protease (30%) was observed after 6 h of



4.21 Standard curve for - amino nitrogen (Karkalas, 1988).

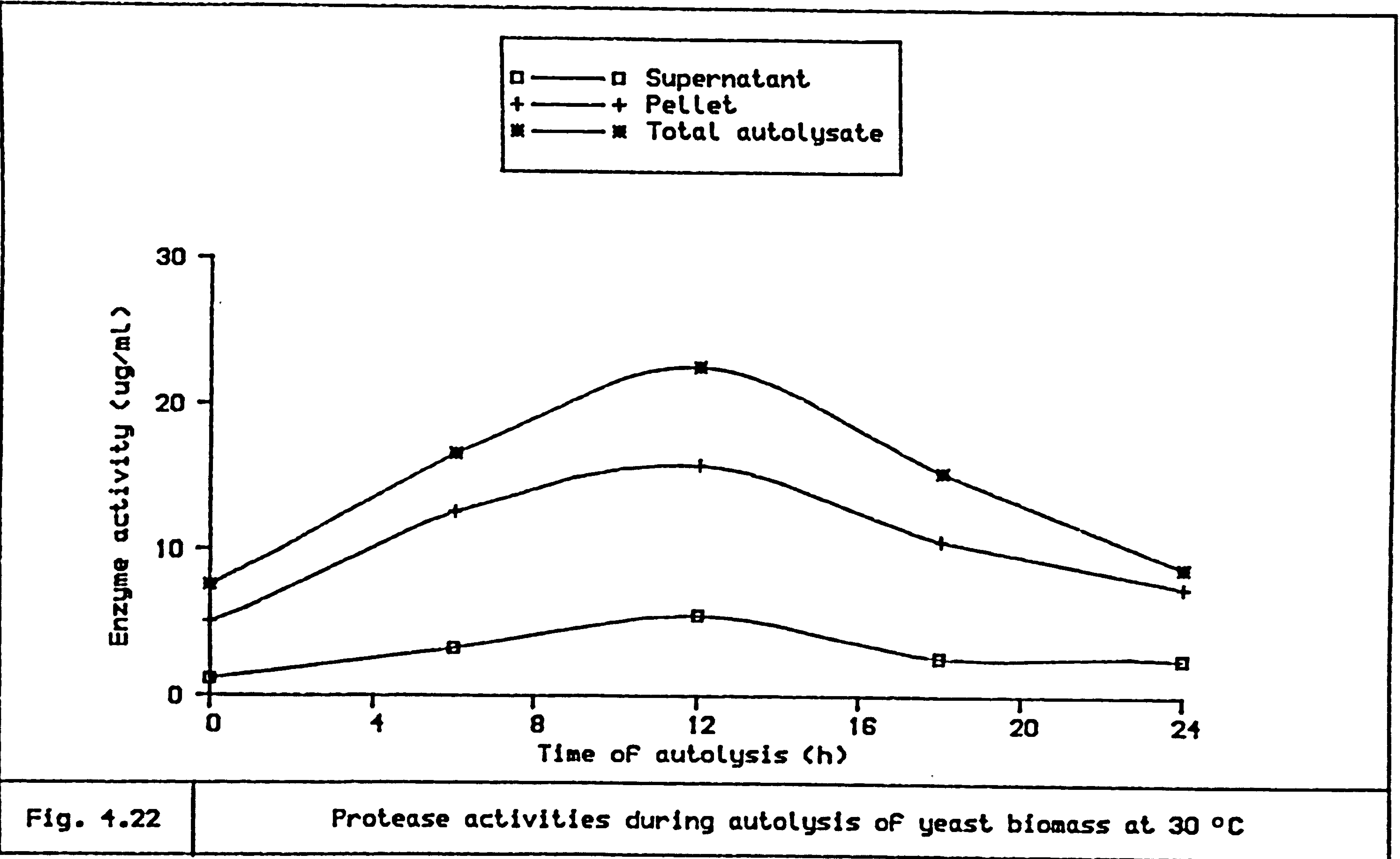


Fig. 4.22 Protease activities during autolysis of yeast biomass at 30 °C

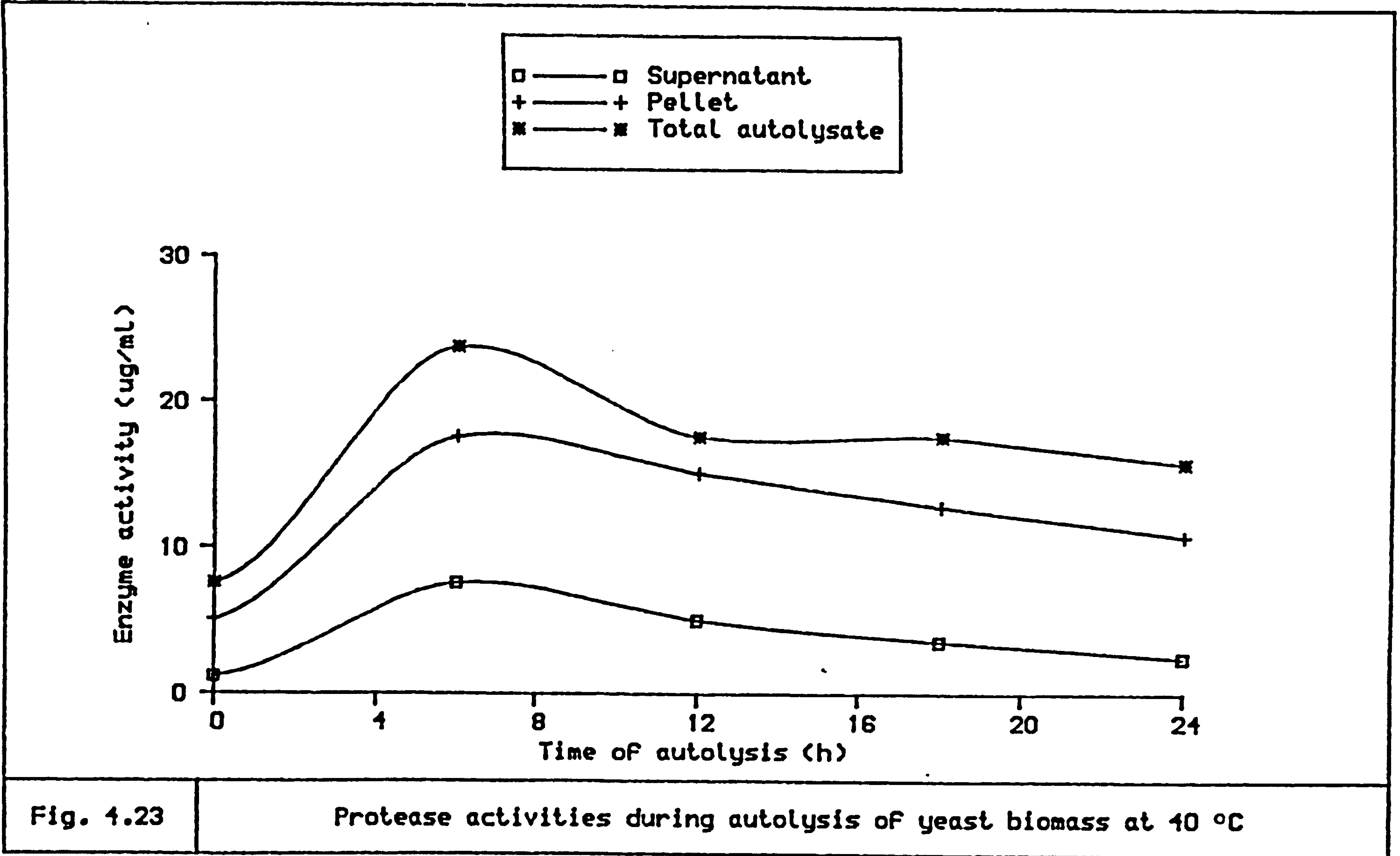


Fig. 4.23 Protease activities during autolysis of yeast biomass at 40 °C

autolysis. Thereafter, the proportion of soluble activity decreased to a minimum of 18.4% after 24 h incubation.

Table 4.15 Release of protease activity into the medium during autolysis of yeast biomass at 40 °C

Time (h)	Protease activity: Pellet (Mean/sd.) (ug ml^{-1})	Supernatant (Mean/sd.) (ug ml^{-1})	Activity released (%)
0	5.00/0.50	0.15/0.25	2.9
6	17.50/1.00	7.50/0.90	30.0
12	15.00/1.56	4.95/0.43	24.8
18	12.70/2.17	3.50/0.25	21.1
24	10.70/0.18	2.40/0.17	18.3

4.7.4 Protease activities during autolysis at 50°C

These experiments was carried out to measure protease activities during autolysis at a lethal temperature. The results (Fig. 4.24) demonstrated maximal values for pellet and supernatant after 6 h of autolysis 24.8 and 15.5 µg ml^{-1}. Further incubation led to decreases in both activities until minimal values of 12.7 µg ml^{-1} and 3.5 µg ml^{-1} were reached after 24 h autolysis.

A maximal for soluble protease activity of 38.5% was observed after 6 h of autolysis and by 24 h this had decreased to a minimum of 21.6% (Table 4.16).

Table 4.16 Release of protease activity into the medium during autolysis of yeast biomass at 50 °C

Time (h)	Protease activity: Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	5.00/0.50	0.15/0.25	2.9
6	24.80/2.42	15.50/0.25	38.5
12	19.50/1.95	6.50/0.90	25.0
18	15.50/2.53	5.00/0.43	24.4
24	12.70/0.18	3.50/0.43	21.6

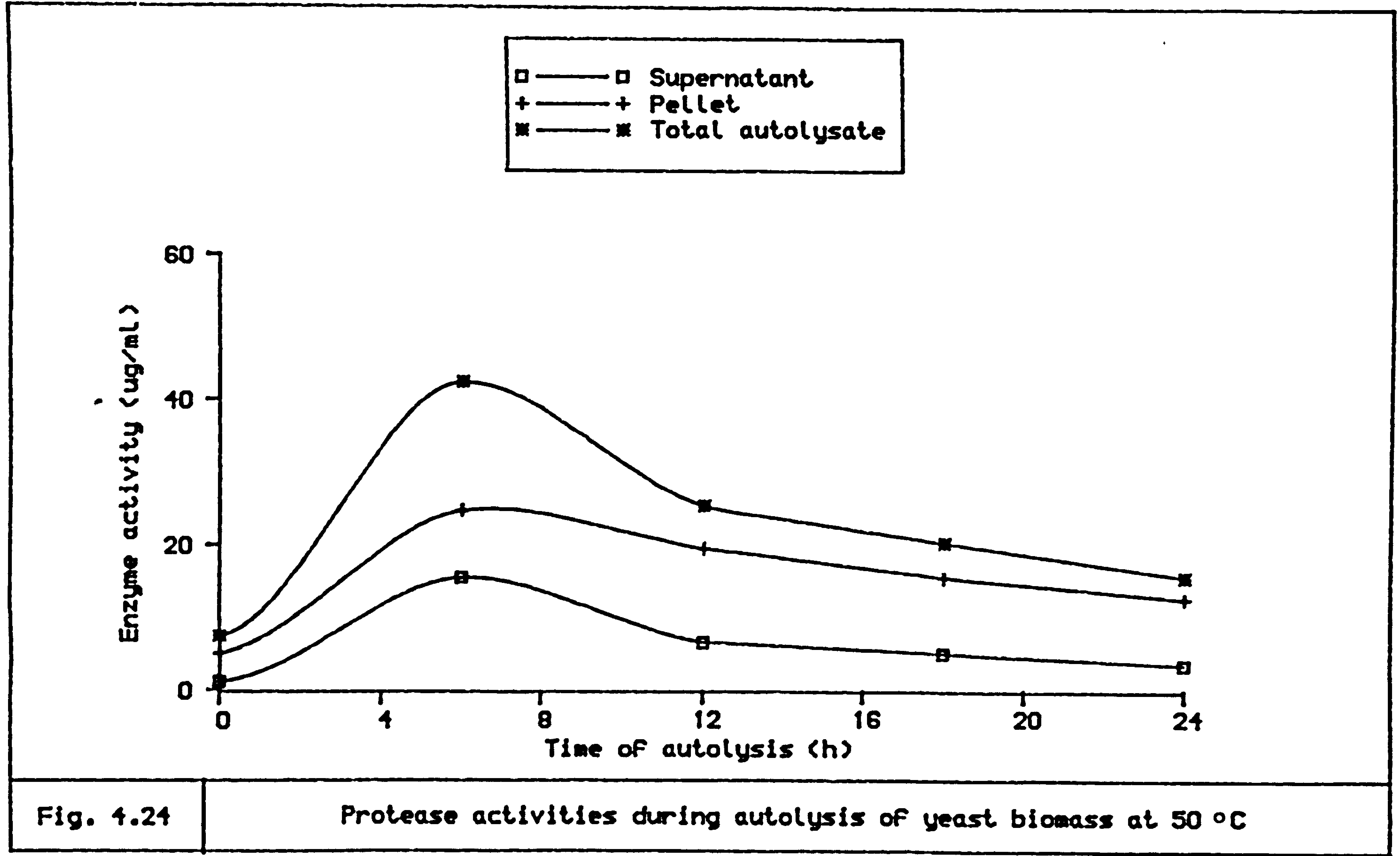


Fig. 4.24 Protease activities during autolysis of yeast biomass at 50 °C

4.7.5 Protease activity during autolysis at 60°C

This experiment was designed to determine the protease activities during autolysis at high temperatures. The results obtained (Fig. 4.25) showed maximal values of 20.2 µg ml^{-1} and 8.5 µg ml^{-1} for pellet and supernatant after 6 h of autolysis. Minima of 8.5 (pellet) and 2.1 µg ml^{-1} (superntant) were determined after 24 h.

Recalculation of these results (Table 4.17) showed that a maximum of 29.6% activity was in the soluble fraction after 6 h and this decreased to a minimum of 19.8% after 24 h.

Table 4.17 Release of protease activity into the medium during autolysis of yeast biomass at 60 °C

	Protease activity		
Time (h)	Pellet (Mean/sd.) (µg ml^{-1})	Supernatant (Mean/sd.) (µg ml^{-1})	Activity released (%)
0	5.00/0.50	0.15/0.25	2.9
6	20.20/1.47	8.50/0.05	29.6
12	17.20/1.47	6.50/1.17	27.4
18	12.50/1.25	3.50/0.25	21.9
24	8.50/0.66	2.10/0.10	19.8

4.8.1 Solubilisation of yeast biomass during autolysis at a range of temperatures

The objective of this set of experiments was to analyse how solubilisation of yeast biomass varied in response to temperature of yeast autolysis. The method used in these assessments was typical of those used in industrial analyses of autolysis and consisted in assessing the distribution of biomass dry weight between solubles (cell-free supernatant)

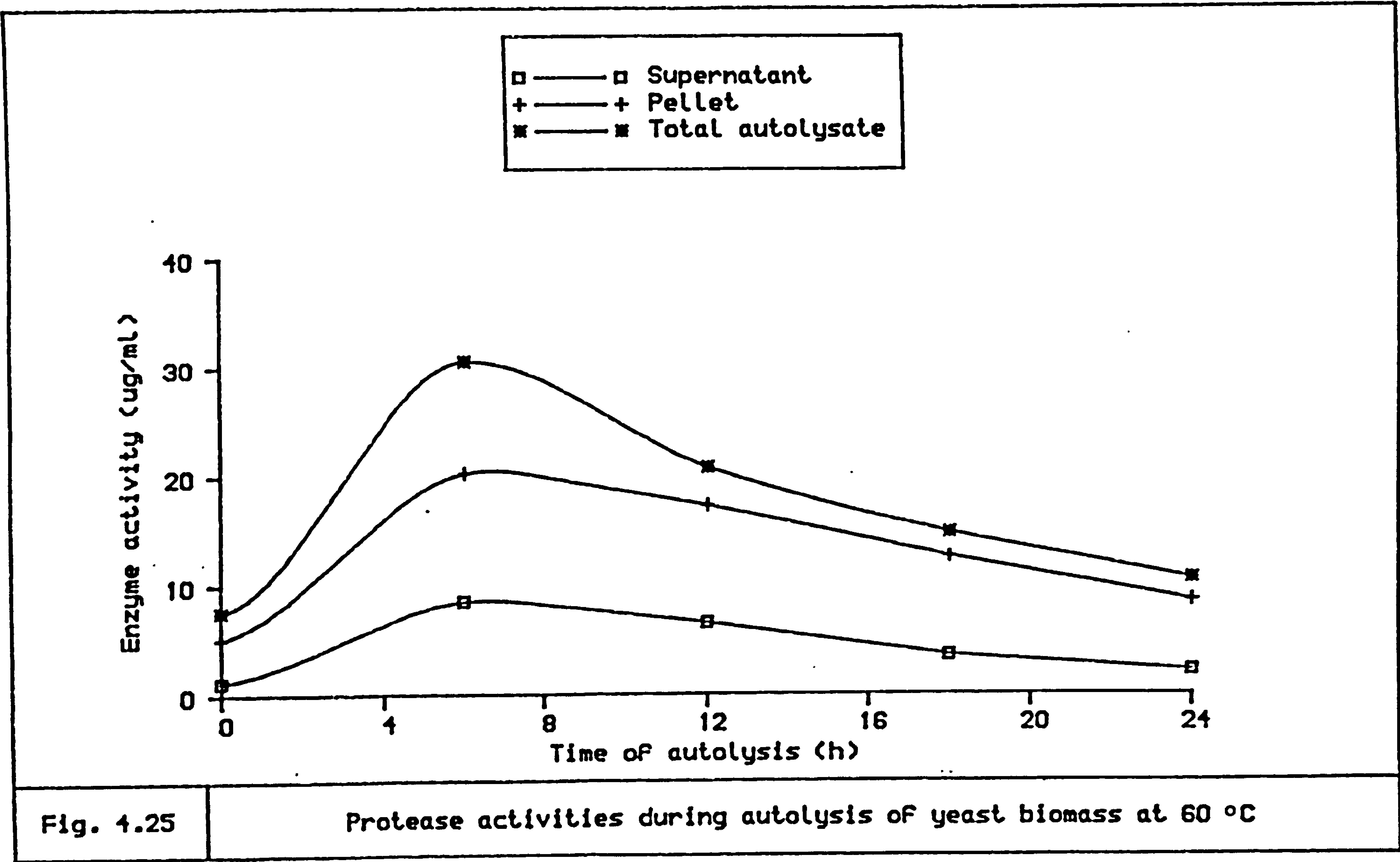


Fig. 4.25 Protease activities during autolysis of yeast biomass at 60 °C

and pellet (insolubles).

4.8.2 Solubilisation of yeast biomass during autolysis at 30 °C

Solubilisation of biomass during autolysis was studied during incubation at the normal yeast growth temperature. The results obtained for the autolysis timecourse (Fig. 4.26) showed that the proportion of biomass in the pellet decreased significantly with incubation time. Within 6 h an initial pellet dry weight of 6.6 g l^{-1} had decreased to 5.5 g l^{-1} while the dry weight in the supernatant increased by 1 g l^{-1} in this period. A decrease in dry weight loss of pellet was observed on extended incubation so that after 24 h the value reduced to 4.4 g l^{-1}. On the other hand, the dry weight of the supernatant increased to 1.6 g l^{-1} during the same period. The proportion of biomass solubilised was calculated and found to be 15.2% after 6 h increasing to 26.7% after 24 h (Table 4.18).

Table 4.18 Solubilisation of yeast biomass during autolysis at 30 °C.

	Dry weights		
Time (h)	Pellet (g l^{-1}) (Mean/sd.)	Supernatant (g l^{-1}) (Mean/sd.)	Solubilisation (%)
0	6.6/0.5	0.1/0.03	1.5
6	5.5/0.3	1.0/0.05	15.2
12	4.9/0.3	1.4/0.05	23.8
18	4.4/0.5	1.5/0.05	25.5
24	4.4/0.5	1.6/0.08	26.7

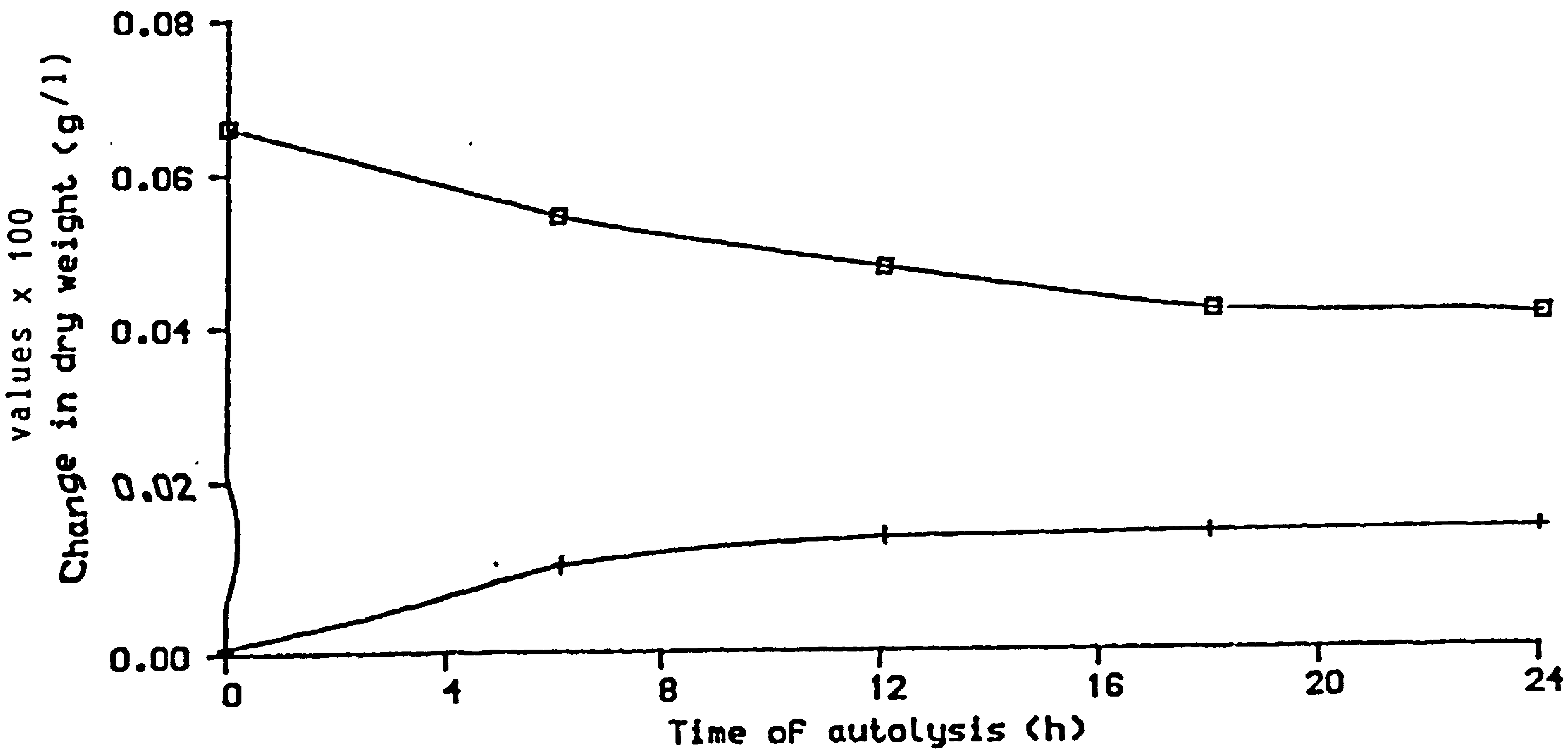


Fig. 4.26 Solubilization of yeast biomass during autolysis at 30 °C

4.8.3 Solubilisation of yeast biomass during autolysis at 40 °C

In this series of experiments, yeast biomass was found to be solubilised during autolysis at 40°C (Fig. 4.27). It was found that the dry weight of pellet reduced to 4.9 g l^{-1} from the initial value of 6.6 g l^{-1} after 6 h of autolysis while the dry weight of supernatant increased to 1.4 g l^{-1} during the same period. With longer incubation time, the pellet dry weight further decreased so that after 24 h the value of 4 g l^{-1} was observed.

Recalculation of the data (Table 4.19) suggested that 21.9% of the yeast solids was solubilised after 6 h of autolysis and this value further increased so that after 24 h of autolysis 33.4% solubilisation was observed.

Table 4.19. Solubilisation of yeast biomass during autolysis at 40 °C.

	Dry weights		
Time (h)	Pellet (g l^{-1}) (Mean/sd.)	Supernatant (g l^{-1}) (Mean/sd.)	Solubilisation (%)
0	6.6/0.5	0.1/0.03	1.5
6	5.0/0.2	1.4/0.05	21.9
12	4.5/0.3	1.8/0.05	28.6
18	4.3/0.5	1.8/0.03	29.5
24	4.0/0.5	2.0/0.08	33.4

4.8.4 Solubilisation of yeast biomass during autolysis at 50°C

These experiments were carried out to assess biomass solubilisation in autolysis at a lethal temperature. The results (Fig. 4.28) showed that a pellet initial dry weight of 6.6 g l^{-1} had been reduced to 3.4 g l^{-1} in the initial

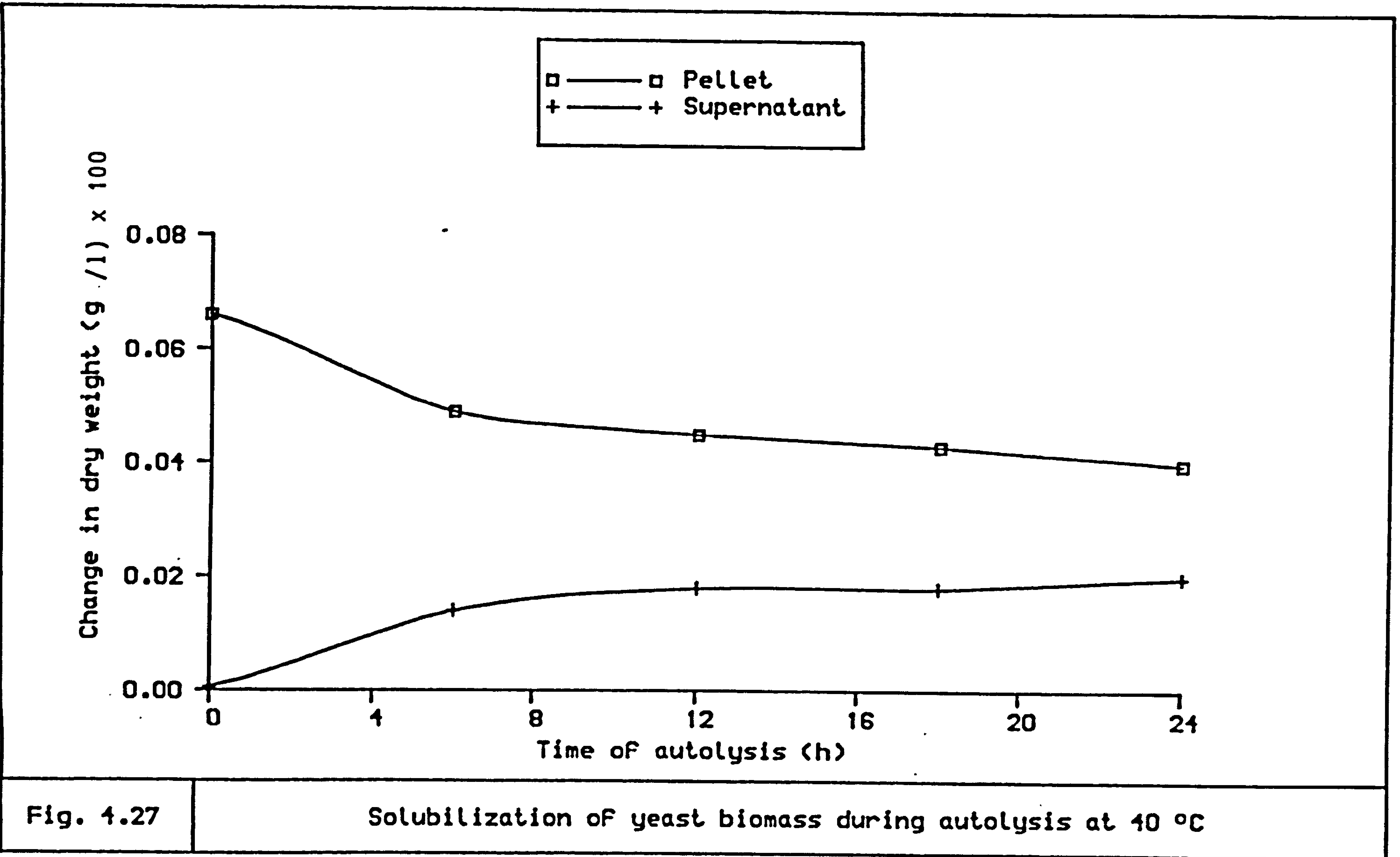


Fig. 4.27 Solubilization of yeast biomass during autolysis at 40 °C

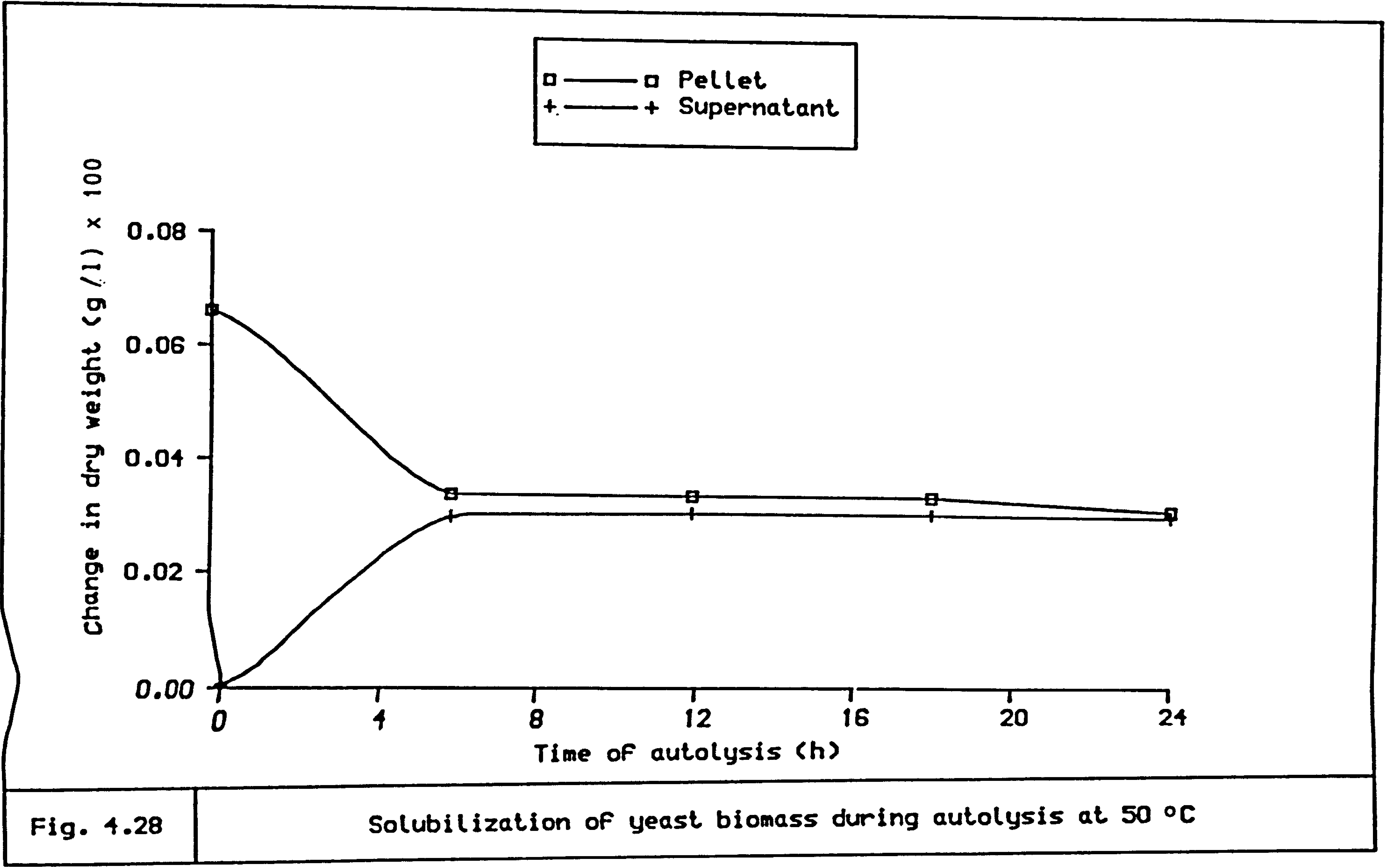


Fig. 4.28 Solubilization of yeast biomass during autolysis at 50 °C

6 h of autolysis. Over the same initial 6 h period the supernatant dry weight increased by 3 g l^{-1}. Subsequent reduction in pellet dry weight was minimal so that after 24 h hydrolysis the pellet dry weight had decreased to 3.2 g l^{-1}. In contrast, the dry weight of the supernatant increased to the highest value of 3.1 g l^{-1} under the same experimental condition.

Calculation of changes in yeast biomass during the autolysis period suggested that 46.9% of total solids were solubilised in the initial 6 h of autolysis. Further incubation was found to result only in minimal increases so that after 24 h only 49.2% was in the soluble portion (Table 4.20).

Table 4.20 Solubilisation of yeast biomass during autolysis at 50 °C

	Dry weights		
Time (h)	Pellet (g l^{-1}) (Mean/sd.)	Supernatant (g l^{-1}) (Mean/sd.)	Solubilisation (%)
0	6.6/ 0.5	0.1/0.03	1.5
6	3.4/ 0.5	3.0/0.05	46.9
12	3.4/ 0.7	3.1/0.05	47.7
18	3.3/ 0.5	3.1/0.07	48.4
24	3.2/ 0.8	3.1/0.05	49.2

4.8.5 Solubilisation of yeast biomass during autolysis at 60 °C

Results obtained in these experiments (Fig. 4.29) studying autolysis of biomass at high temperature indicated that pellet dry weight decreased from 6.6 g l^{-1} to 4.9 g l^{-1} in the initial 6 h of incubation whilst soluble dry matter

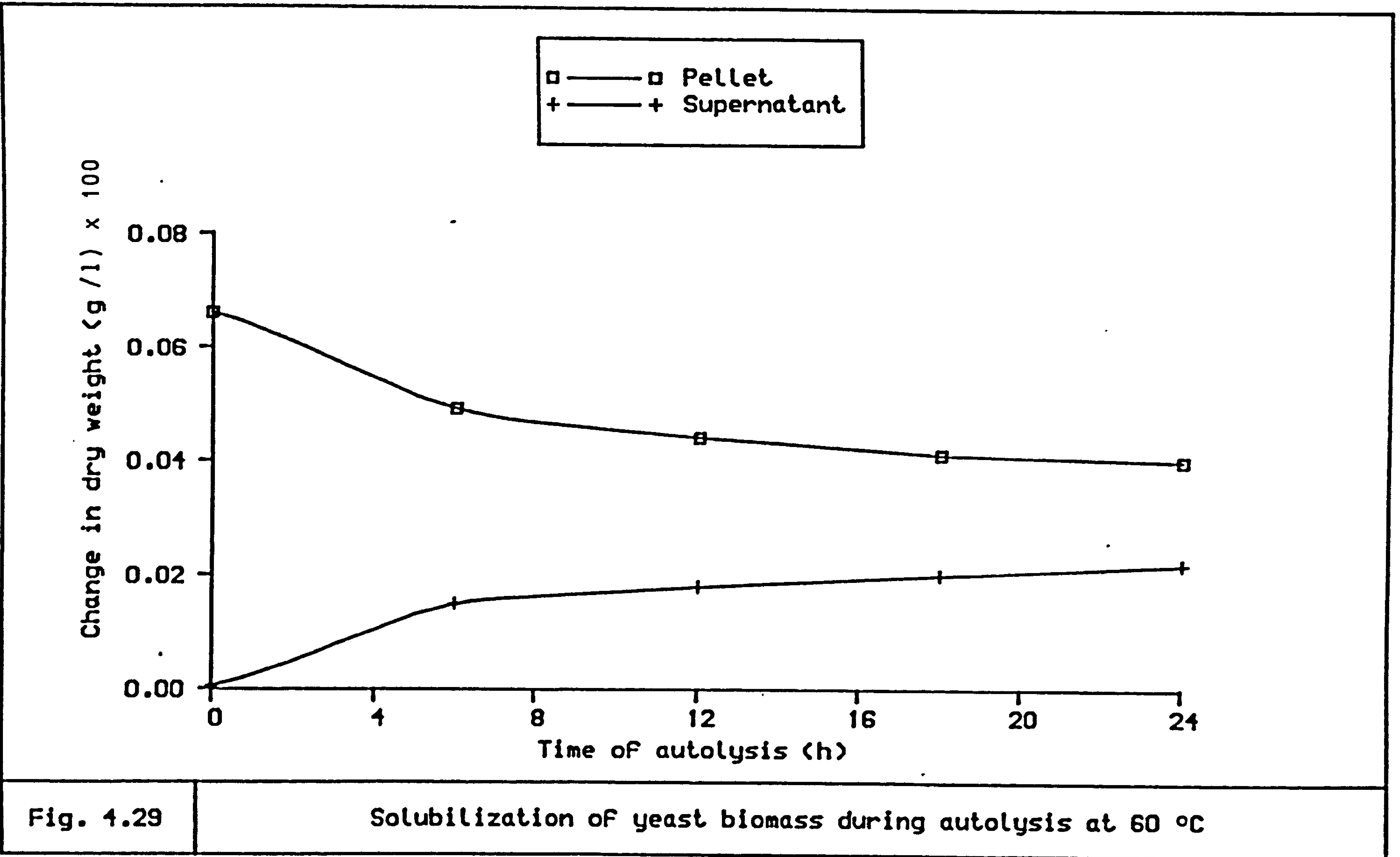


Fig. 4.29 Solubilization of yeast biomass during autolysis at 60 °C

increased by 1.5 g l^{-1} over this period. With continued incubation dry weight of pellet decreased and soluble fraction increased only slightly so that after 24 h values 4 g l^{-1} and 2.2 g l^{-1}, respectively, were recorded.

In the first 6 h of autolysis 23.5% of yeast biomass was solubilised and after 24 h this had increased to 35.5% (Table 4.21).

Table 4.21 Solubilisation of yeast biomass during autolysis at 60 °C

	Dry weights		
Time (h)	Pellet (g l^{-1}) (Mean/sd.)	Supernatant (g l^{-1}) (Mean/sd.)	Solubilisation (%)
0	6.6/0.5	0.1/0.03	1.5
6	4.9/0.7	1.5/0.04	23.4
12	4.4/0.8	1.8/0.05	29.0
18	4.1/0.6	2.0/0.08	32.8
24	4.0/0.5	2.2/0.05	35.5

4.9 Release of soluble protein during autolysis

The purpose of this work was to study the amount of soluble protein released during the autolysis at different incubation temperatures. Results (Fig. 4.35) showed that 21.3 μg ml^{-1} protein was released after the initial 1 h of autolysis at 30 °C whereas at 40 °C, this was increased to 23.7 μg ml^{-1} and with incubation at 50 °C this had increased to 39.5 μg ml^{-1} and 24.2 μg ml^{-1} was obtained after 1 h at 60 °C. Further incubation, showed that maximal (355 μg ml^{-1}) release of protein was obtained after 6 h of autolysis at 50 °C followed by 300 μg ml^{-1} at 60 °C. With incubation at 30 and 40 °C , 250.5 and 295.7 μg ml^{-1} of protein was found

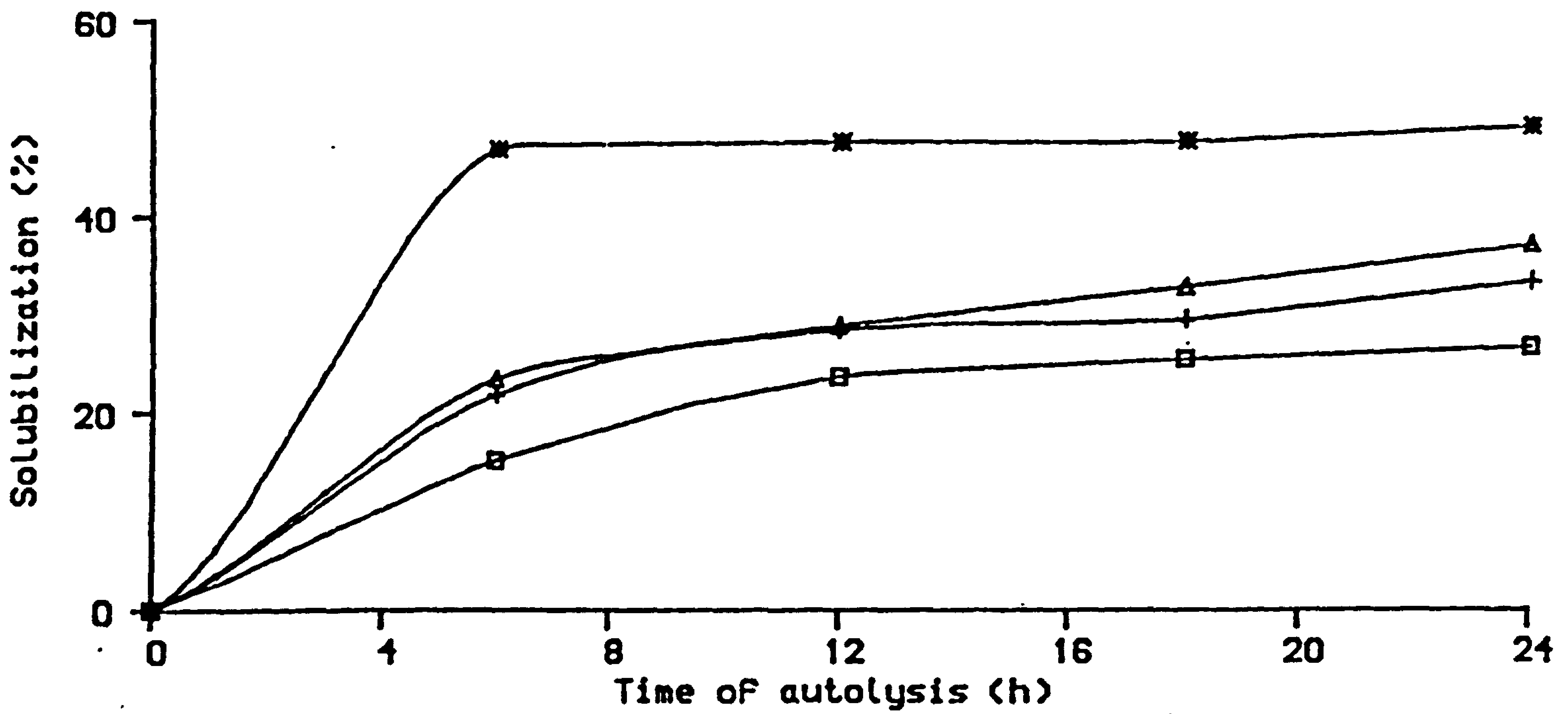


Fig. 4.30 Degree of solubilization of yeast biomass at different temperatures

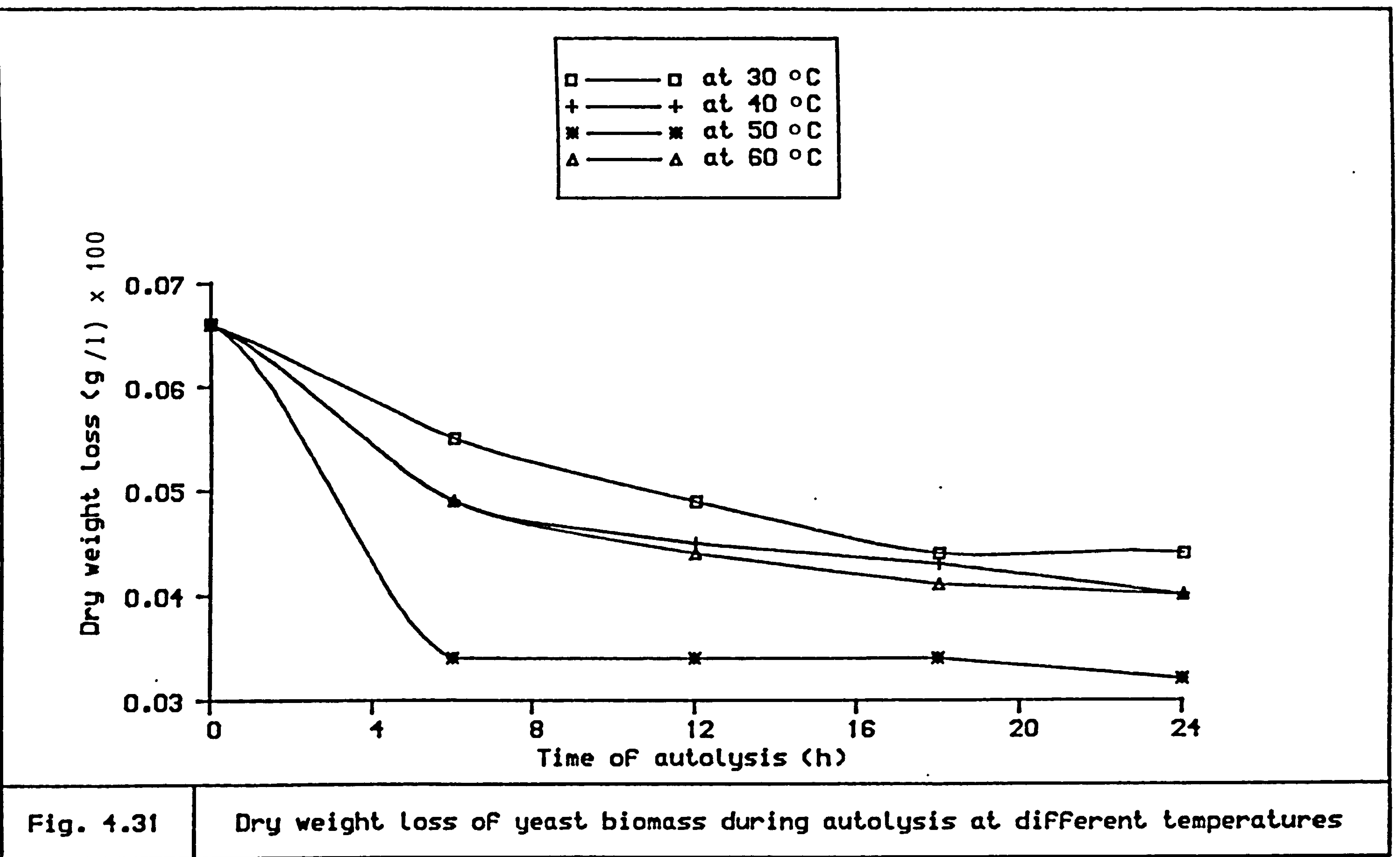


Fig. 4.31 Dry weight loss of yeast biomass during autolysis at different temperatures

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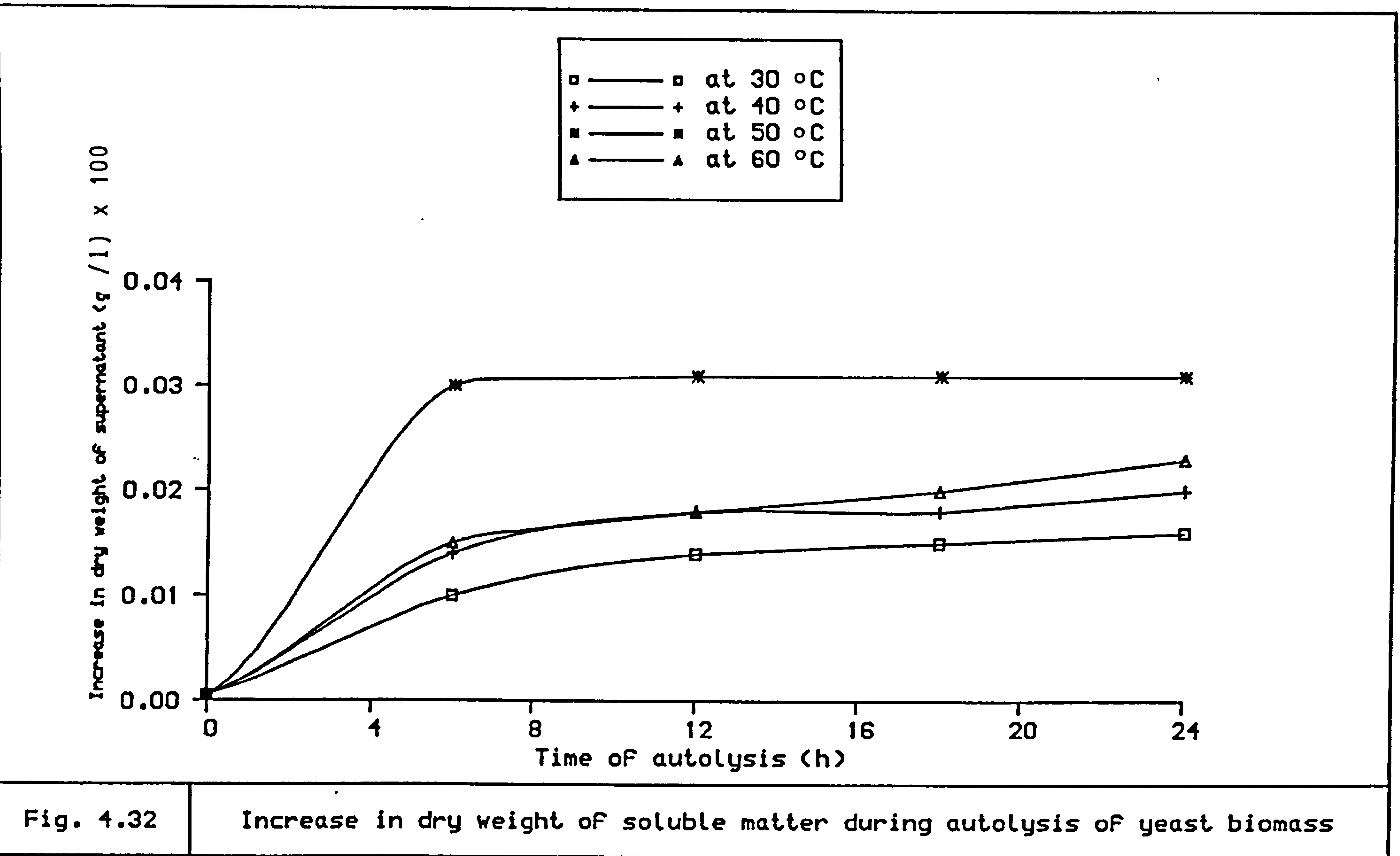

Fig. 4.32 Increase in dry weight of soluble matter during autolysis of yeast biomass

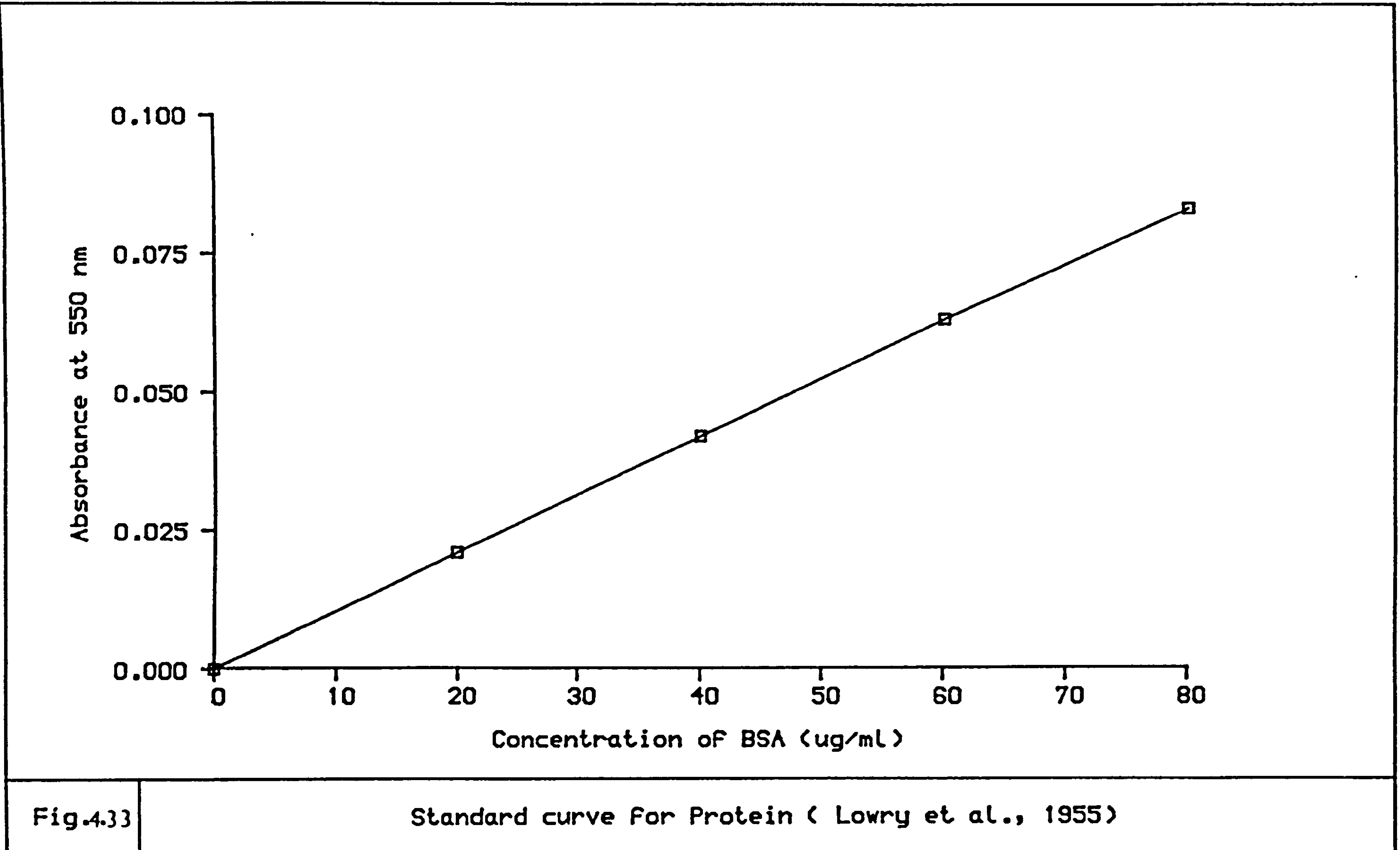

Fig.4.33 Standard curve for Protein (Lowry et al., 1955)

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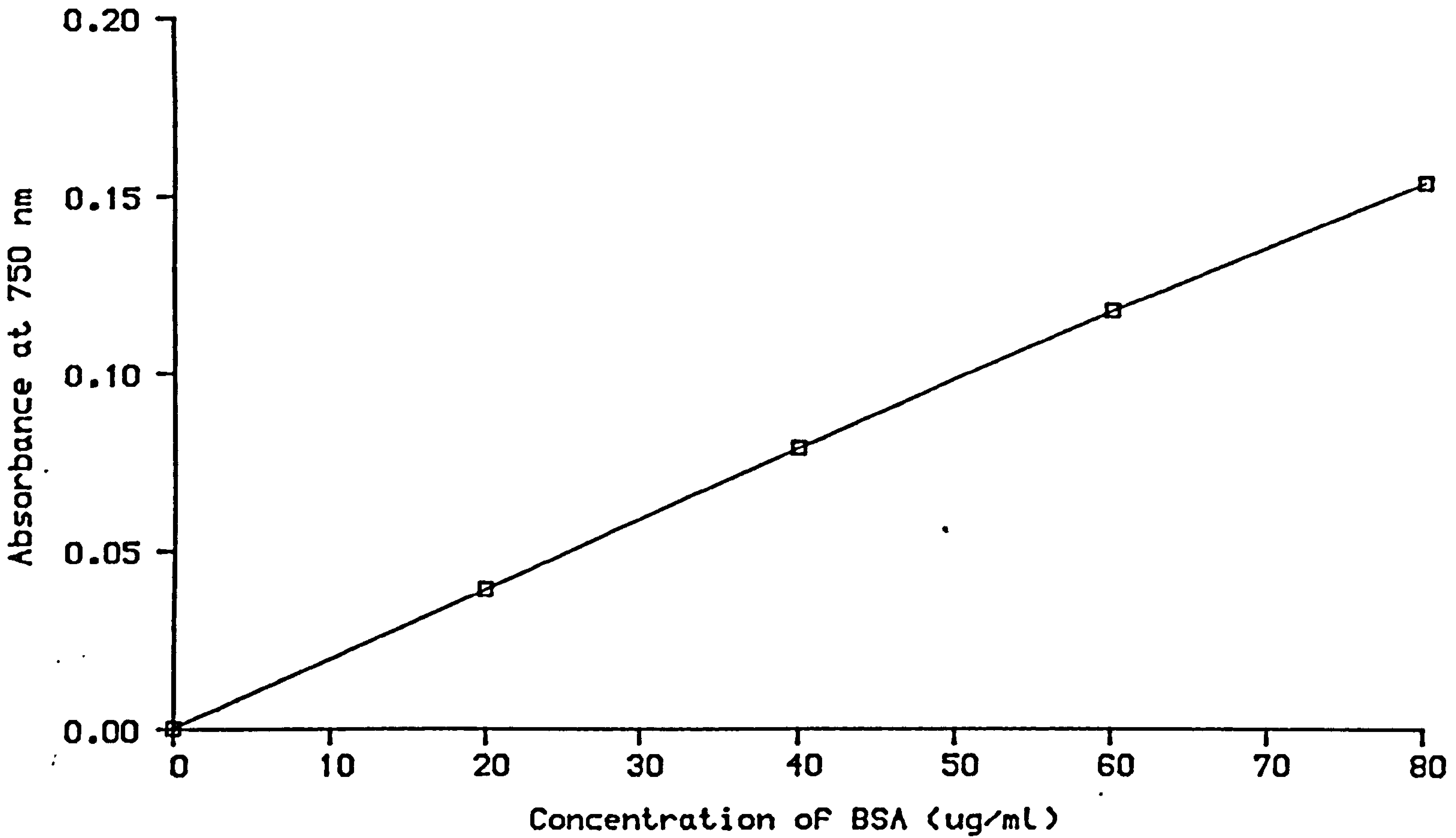

Fig.4.34 Standard curve For Protein (Lowry et al., 1955)

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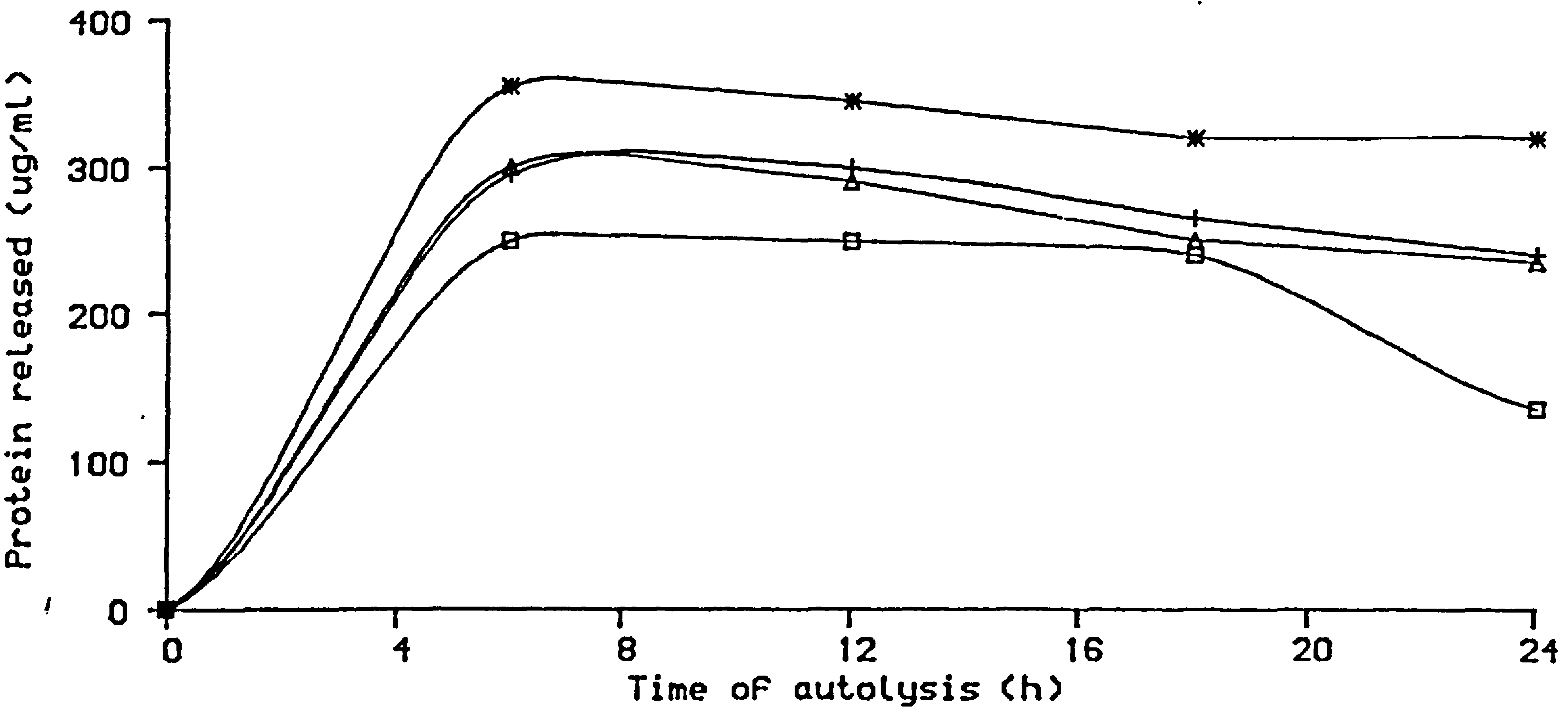


Fig. 4.35 . Release of protein during autolysis of yeast biomass.

to be released respectively. After 6 h of autolysis, the release of protein into the medium decreased slightly so that after 24 h of autolysis, the value of 235.5 and 240 µg ml^{-1} was achieved at 30 and 40 °C and at 50 and 60 °C, 320 and 235 µg ml^{-1} of protein released, respectively, into the medium.

4.10 Release of glucose during autolysis

The objective of these experiments was to determine the rate of release of glucose, assayed using the enzyme glucose oxidase, during autolysis of biomass produced by 18 h, 36 h and 72 h growth. Glucose is the product of breakdown of glycogen and trehalose, storage polymers in the cell cytoplasm, and glucan in the cell wall. Glucose was determined using glucose oxidase method (Fig. 4.36). Results obtained (Table 4.22) showed that very small amount of glucose was released into the medium after 1 h of autolysis of 18 h cells at 30 °C whereas 8.26 µg ml^{-1} of glucose was released during incubation of similar cells at 40 °C. For cells grown for 36 and 72 h only trace amounts of glucose were released.

However, 1 h of incubation at 50°C solubilised 11.6 µg ml^{-1}, 4.1 µg ml^{-1}, and 3.8 µg ml^{-1} glucose from 18, 36 and 72 h cells. This showed further increases with incubation at 60 °C so that 45.6 and 42.2 µg ml^{-1} were observed for 18 and 36 h cells although 72 h cells gave a value of 38.4 µg ml^{-1}.

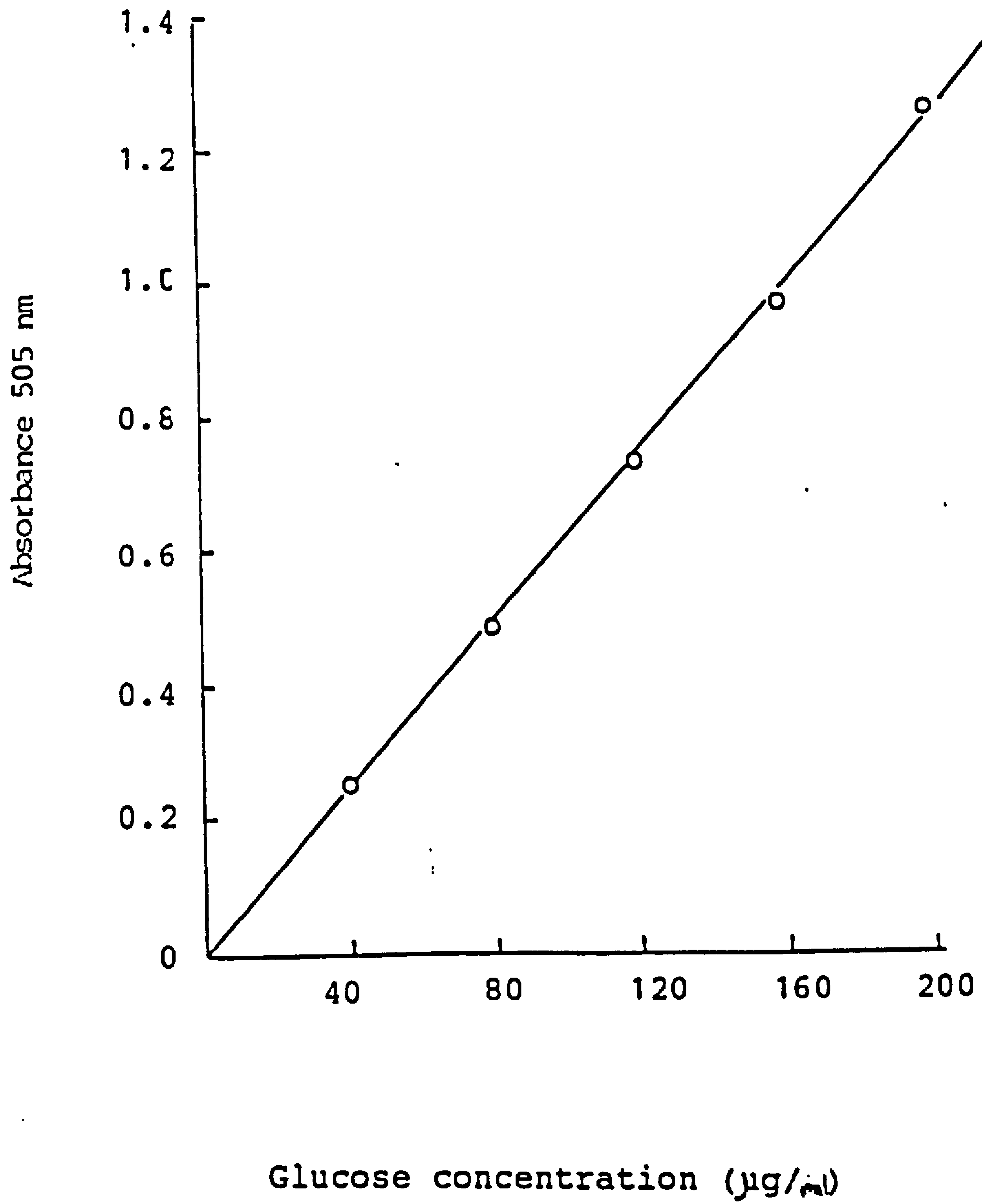


Fig.4.36 Standard curve of glucose by glucose oxidase method

Table 4.22 Glucose released into the media during autolysis of yeast biomass.

Age of cells (h)	Time of autolysis (h)	Temp. of autolysis (°C)	Glucose released (μg/ml) (Mean/sd.)
18	1	30	0.04/0.00
18	1	40	8.26/0.25
18	1	50	11.60/1.50
18	1	60	42.20/3.50
36	1	30	0.05/0.00
36	1	40	0.05/0.01
36	1	50	4.10/0.50
36	1	60	45.60/2.80
72	1	30	0.02/0.00
72	1	40	1.20/0.08
72	1	50	3.80/0.20
72	1	60	38.40/3.00

5. Morphology and physiology of autolysed yeast cells

5.1 Viability of cells

The loss of cell viability of cells during autolysis at different temperatures was studied. Aliquots of cells were stained with methylene blue, spread on to glass slides and examined under a light microscope. The results (Fig. 5.1) that after 1 h of autolysis at 30 °C, cell viability had decreased to 88% whereas with incubation at 40 °C, 50 °C, 60 °C reductions of viability to 82, 64.3 and 0% respectively were recorded.

Cell death rates increased in an inverse relationship with incubation temperature so that only 38 and 20% cells were found to be viable after 24 h of autolysis at 30 °C and 40 °C respectively while reductions to zero viability was observed at both 50 °C and 60 °C in the same period.

5.2 Fluorescence microscopy of yeast cells

The objective in these studies was to study changes in cell wall chitin particularly at bud scars. Normal and autolysing cells were stained with the fluorescent dye Calcofluor and examined using an epifluorescent microscope. In these experiments autolysis was carried out at 50 °C, since preliminary studies indicated that this was the optimum temperature for autolysis of the yeast strain studied.

Typical changes in yeast cell walls associated with autolysis are shown in Plates 5.1 - 5.5. In each micrograph chitin-rich bud scars can clearly be differentiated from the glucomannan cell wall. In Plate 5.1. 18 h cells are shown

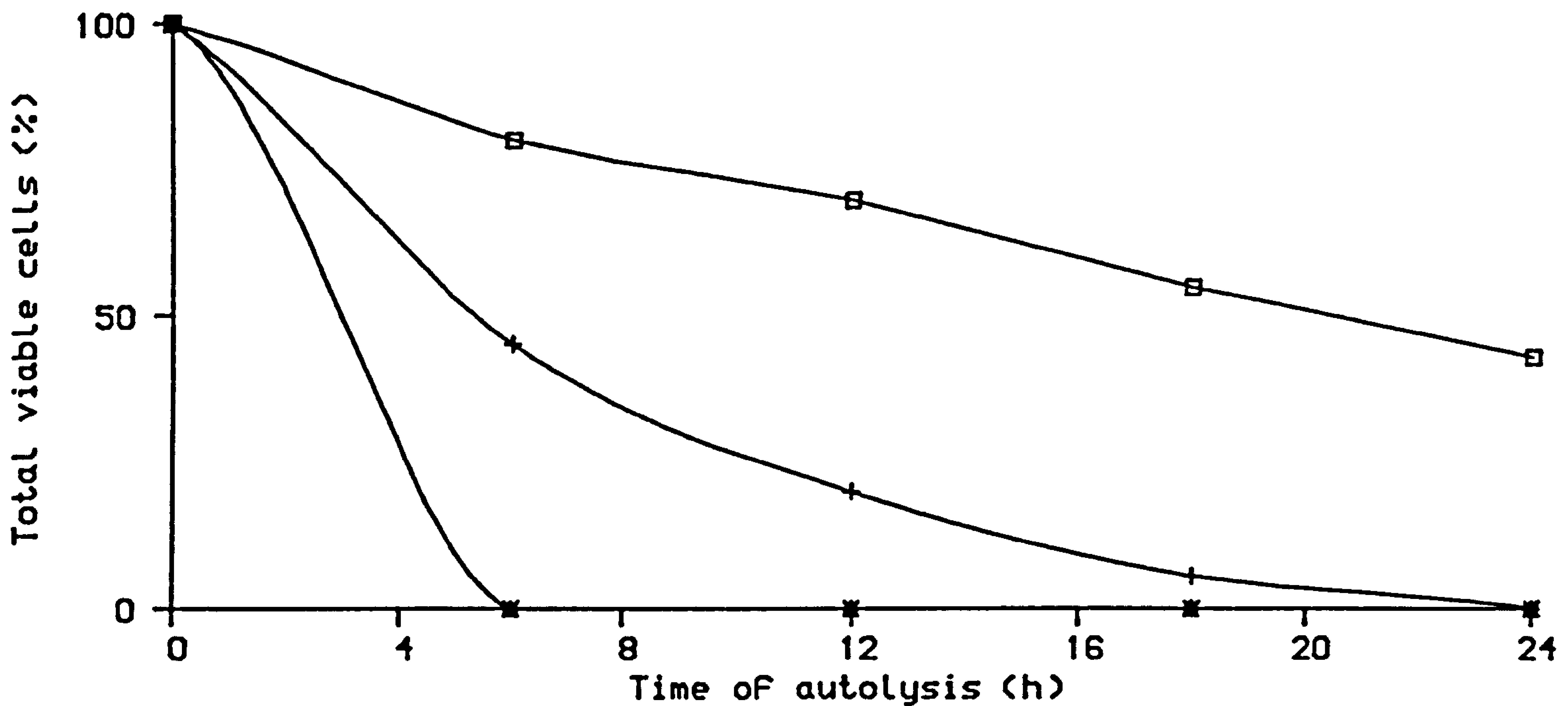


Fig. 5.1 Viability of yeast cells during autolysis at different temperatures

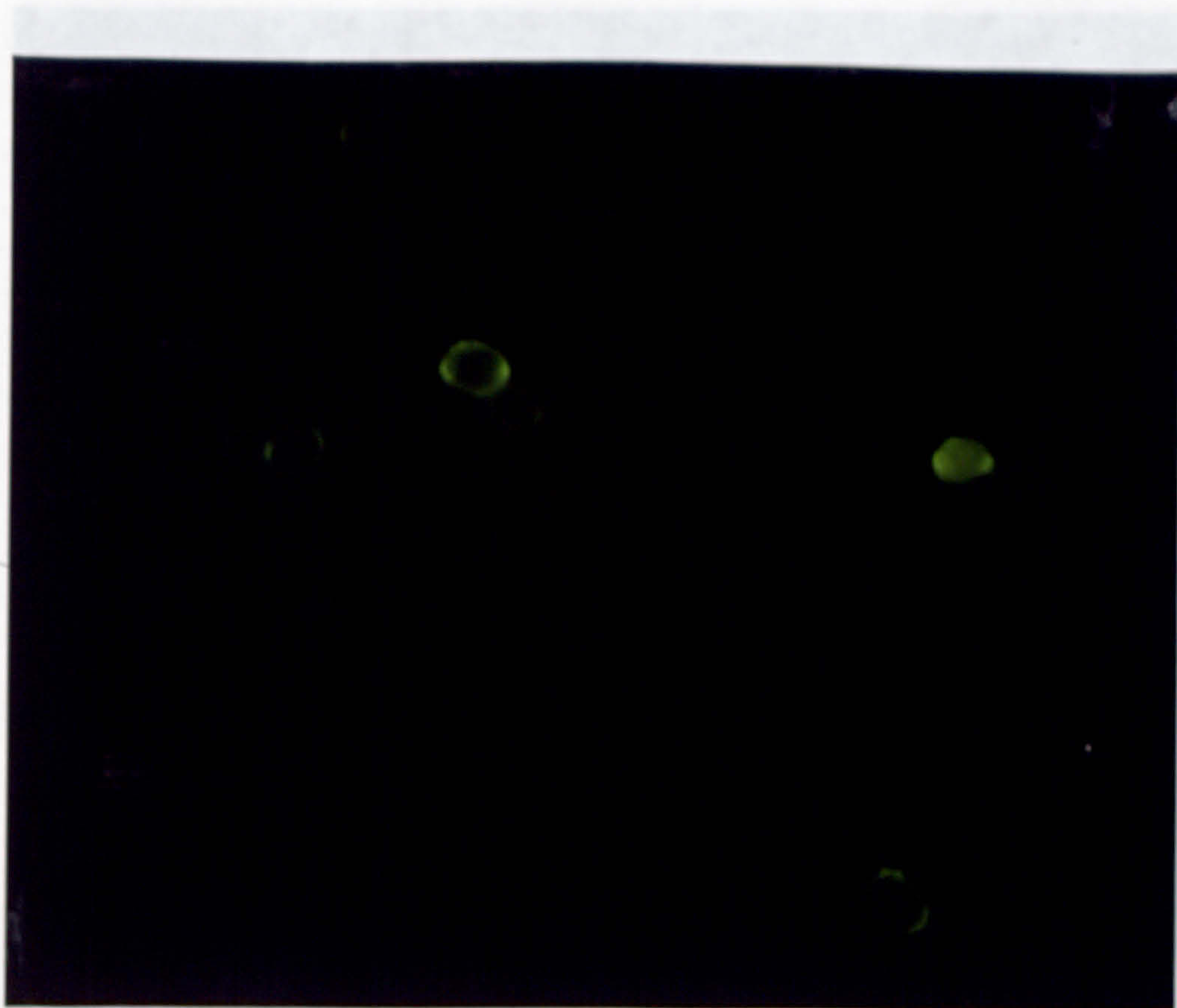


Plate 5.1 Yeast cells grown on YEPG medium for 18 h at 30 °C. Magnification x 500.

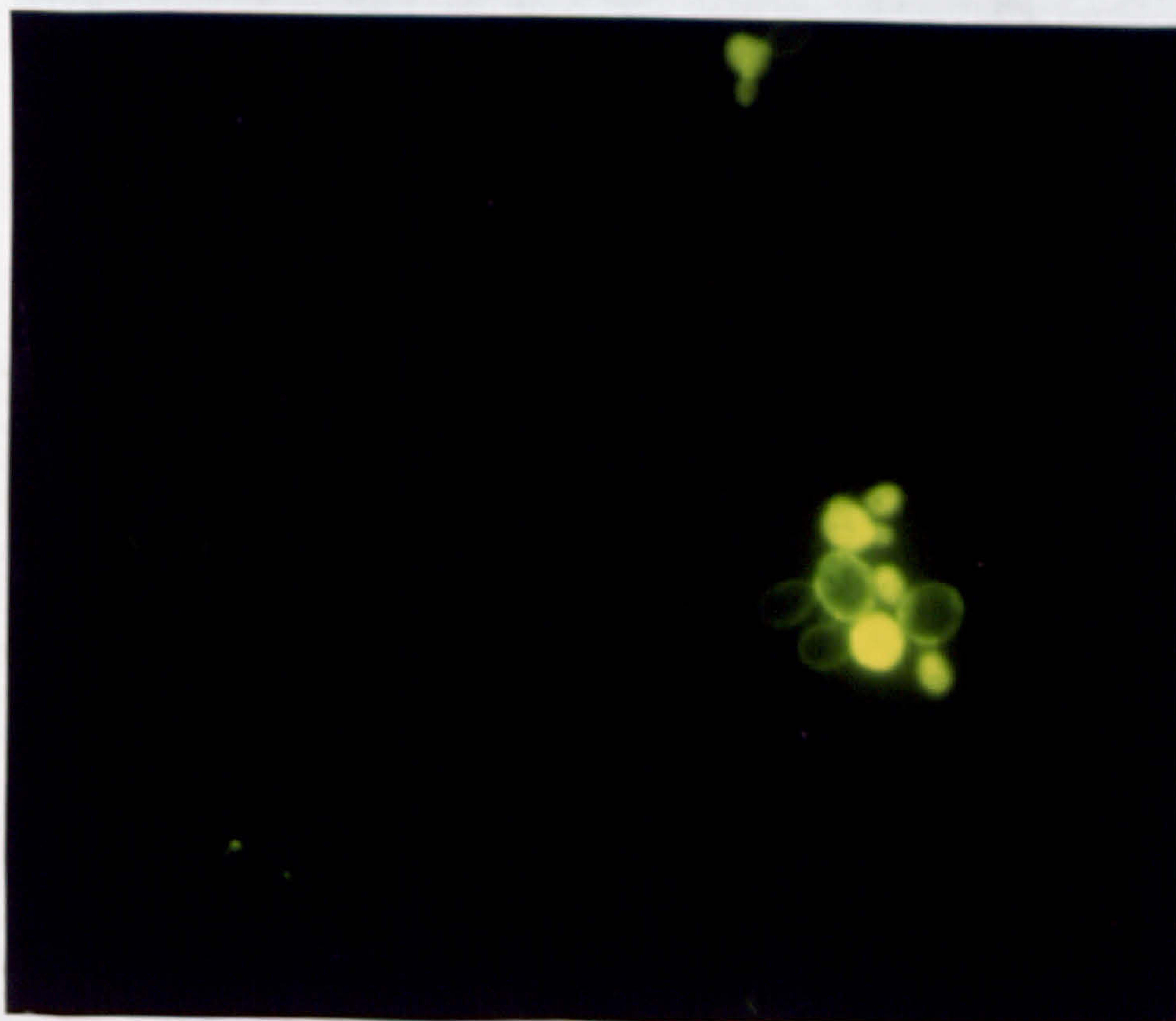


Plate 5.2 Fluorescence micrograph of autolysing yeast cells after 6 h of autolysis at 50 °C. Magnification x 500.

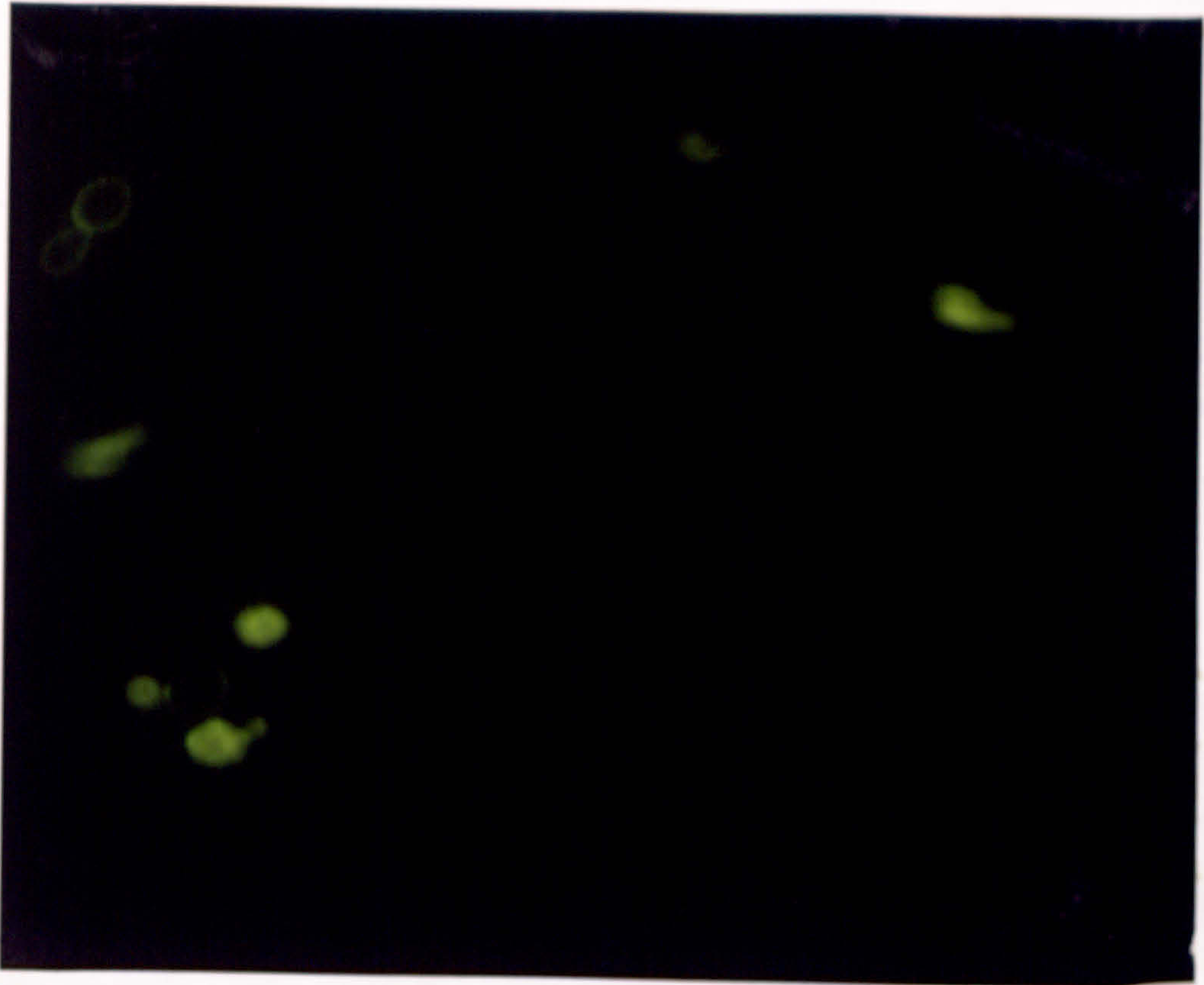


Plate 5.3 Fluorescence micrograph of autolysing yeast cells after 12 h of autolysis at 50 °C. Magnification x 500.

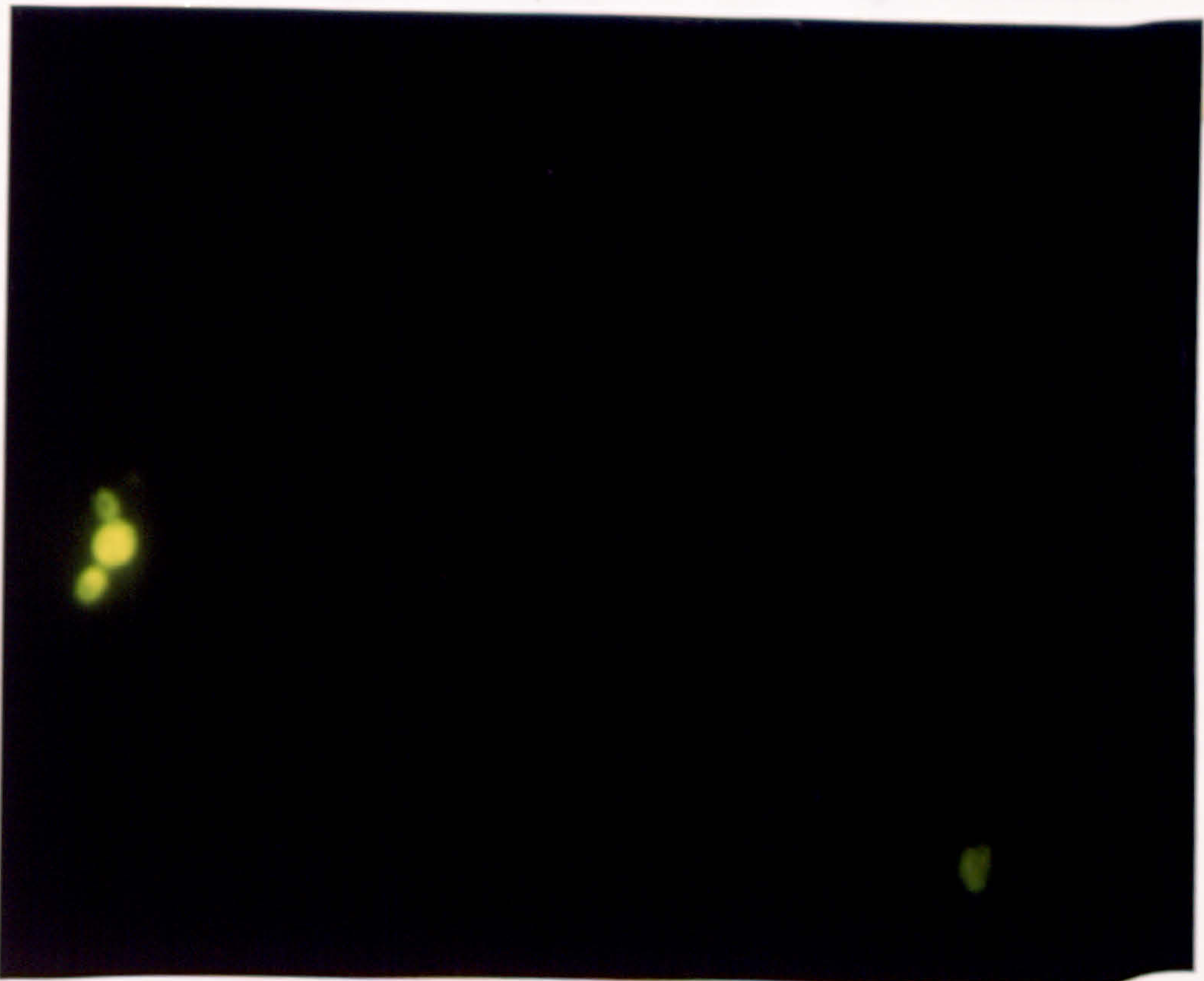


Plate 5.4 Fluorescence micrograph of autolysing yeast cells after 18 h of autolysis at 50 °C. Magnification x 500.

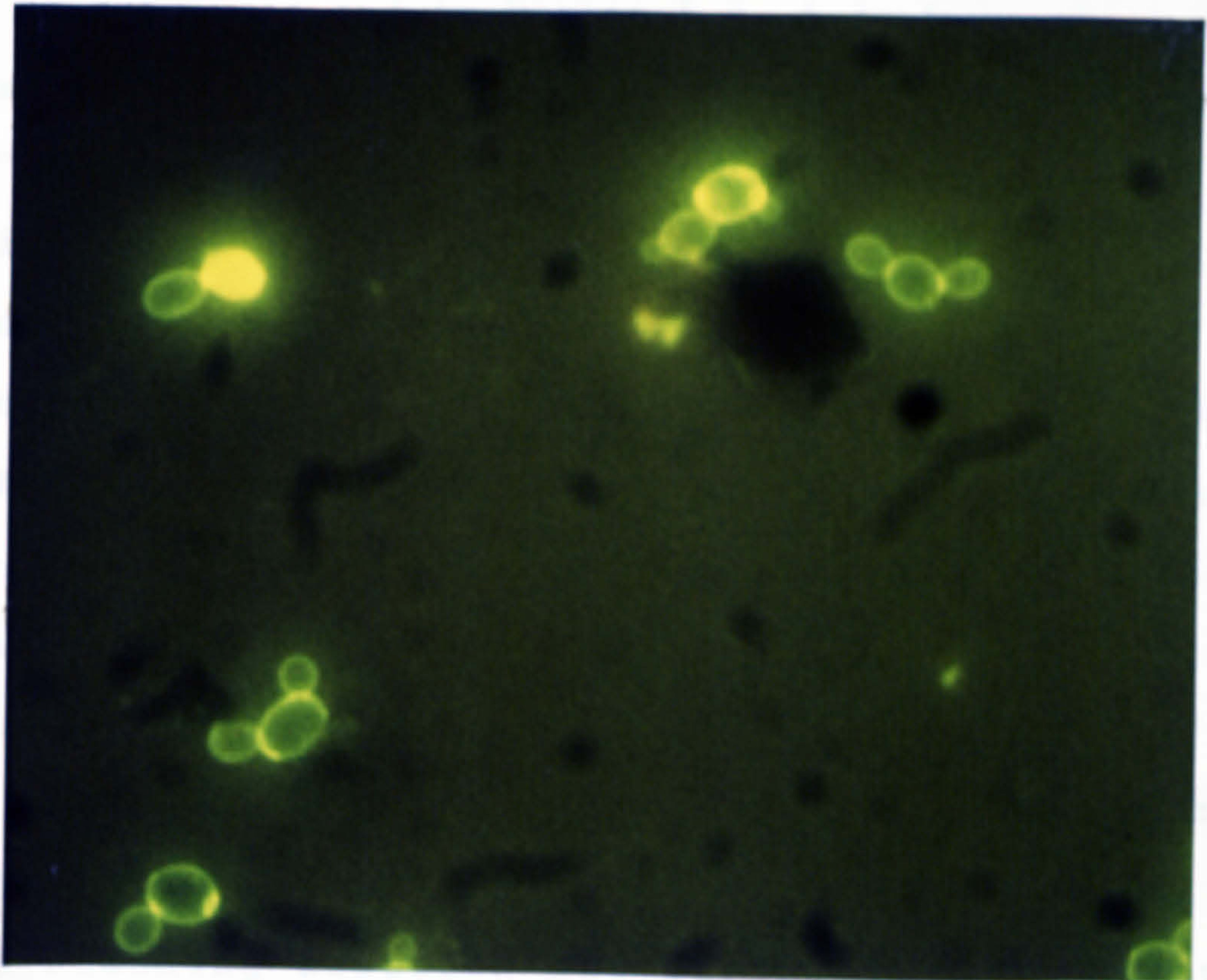


Plate 5.5 Fluorescence micrograph of autolysing yeast cells after 24 h of autolysis at 50 °C. Magnification x 500.

prior to autolysis and in Plates 5.2, 5.3, 5.4, and 5.5 are fields of similar cells after 6, 12, 18 and 24 h autolysis at 50°C respectively. In these micrographs it is clear that with increasing time of autolysis the bud scars become more prominent in the cell walls.

5.3 Scanning electron microscopy of autolysing yeast cells

The main objective of this phase of the project was to study, in some detail, morphological changes on external surfaces of the cell wall in autolysing yeast. In particular, examination at a greater magnification of bud scars and associated cell wall would be possible for cells before and during autolysis at 50 °C.

A scanning electron micrograph of 18 h cells is presented in Plate 5.6 and similar cells after 6, 12, 18 and 24 h autolysis are shown in Plates 5.7, 5.8, 5.9, 5.10 respectively. Cells prior to autolysis exhibited smooth external surfaces and rounded, protruding bud scars. With increasing autolysis the cell surface began to appear rougher and bud scars became deformed.

5.4 Transmission electron microscopy

Since scanning electron microscopy only provides data on the external surface of the cell wall, transmission electron microscopy was carried out to study changes in cell wall and bud scar structure. Once again, 18 h cells were studied before and during autolysis at 50 °C, after fixing with glutaraldehyde and staining with osmium tetroxide and lead acetate.

Cells prior to autolysis are shown in Plate 5.11 and

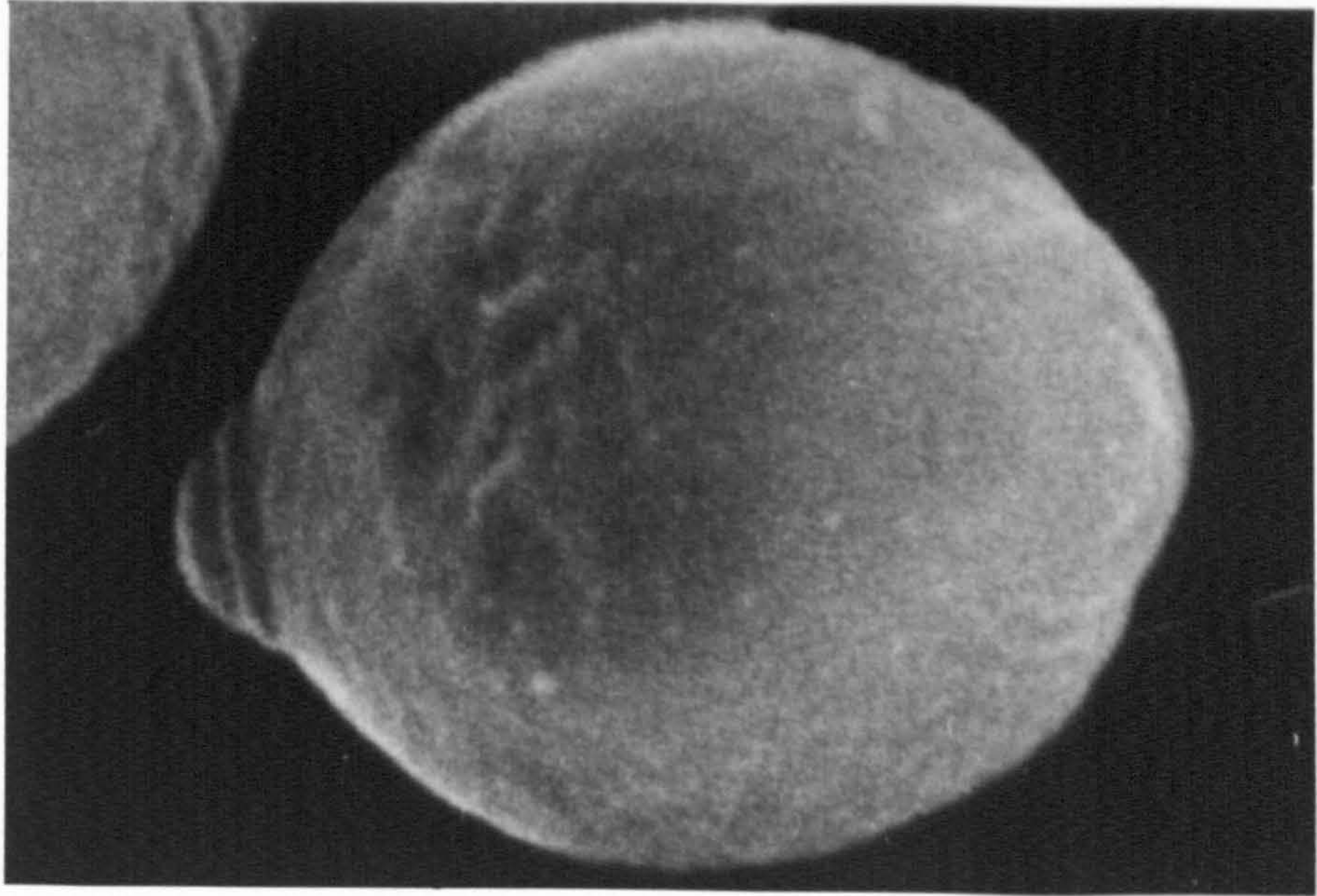


Plate 5.6 Scanning electron micrograph of 18 h grown yeast cells prior to autolysis.

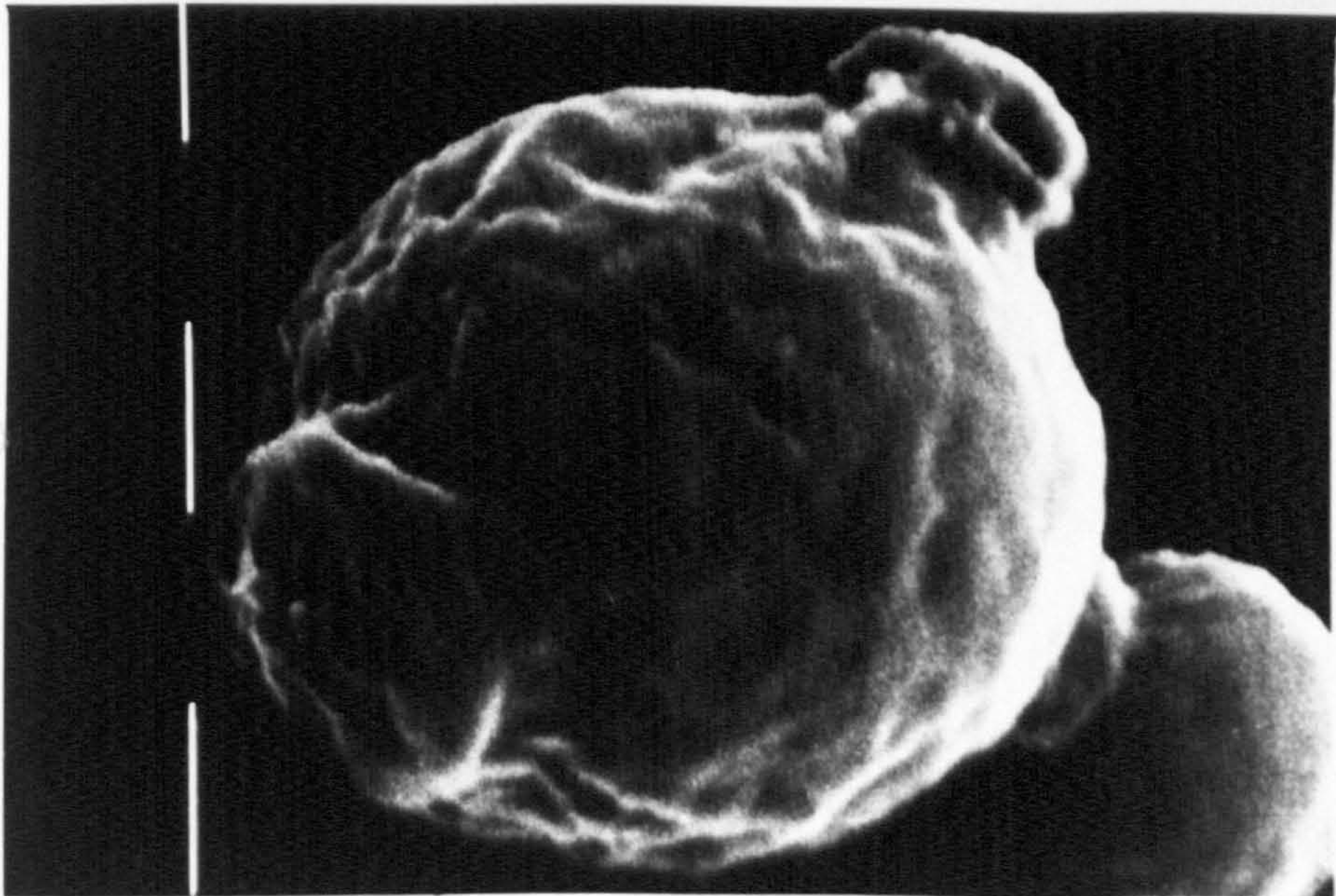


Plate 5.7 Scanning electron micrograph of autolysing yeast cells after 6 h of autolysis at 50 °C.

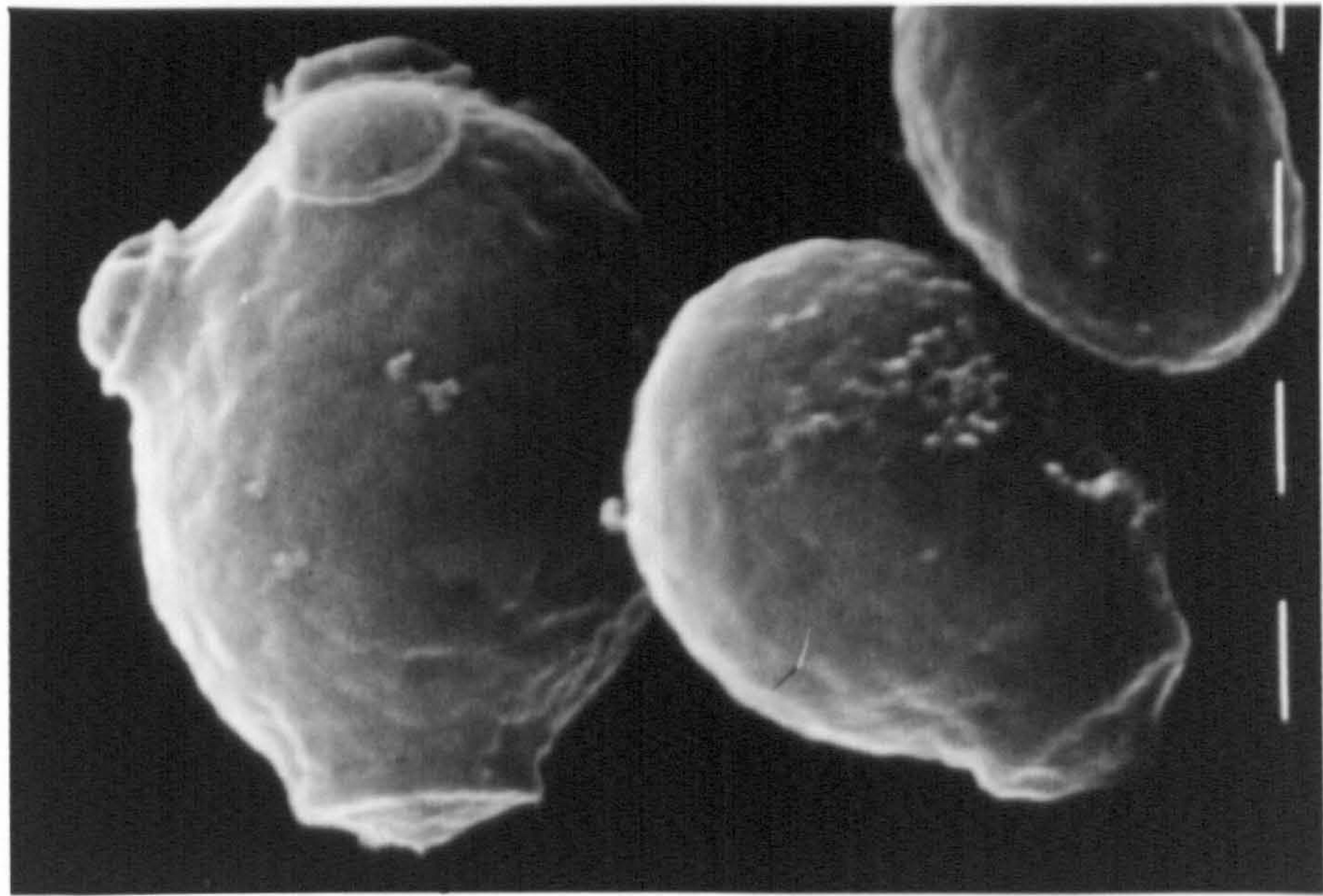


Plate 5.8 Scanning electron micrograph of autolysing yeast cells after 12 h of autolysis at 50 °C.

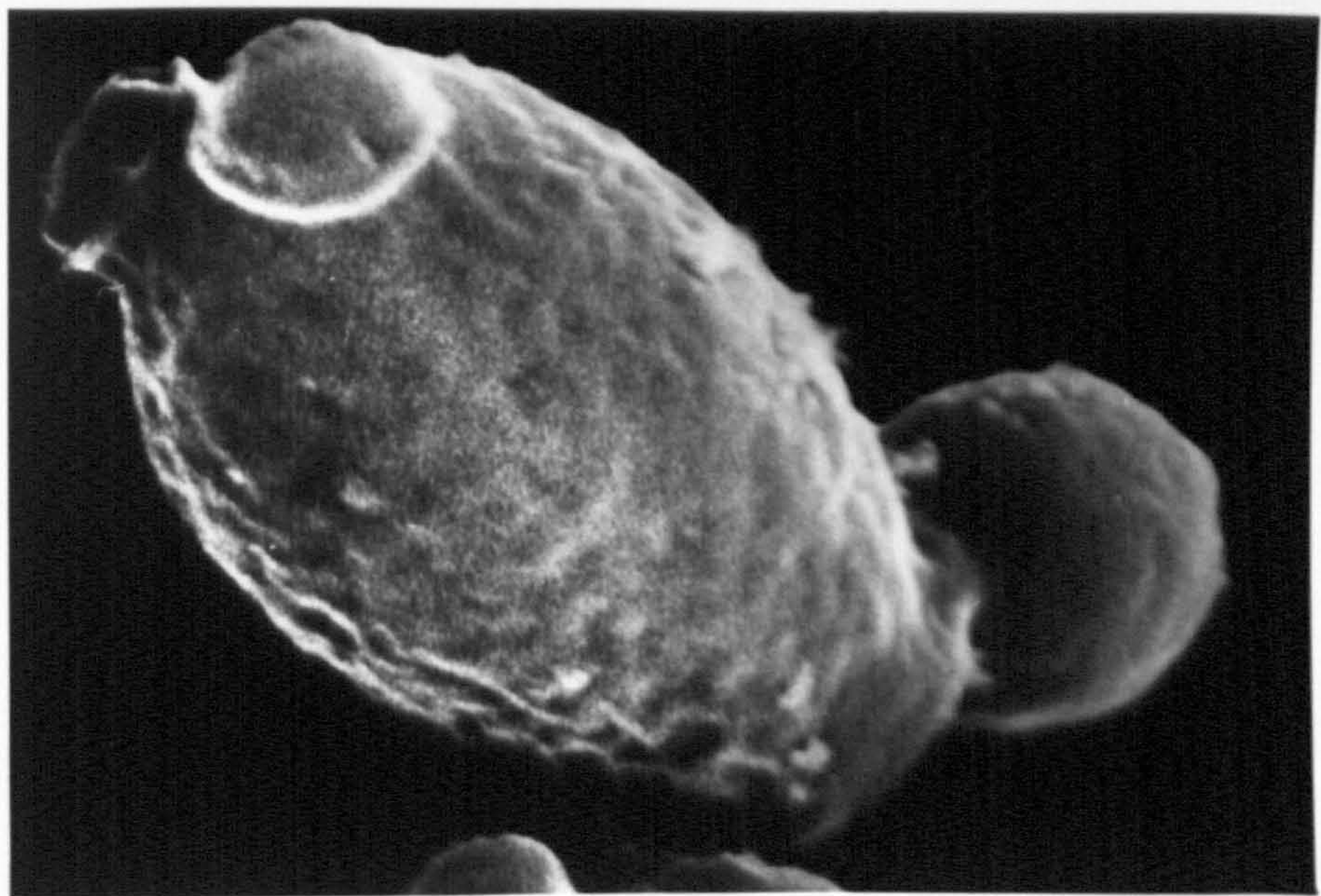


Plate 5.9 Scanning electron micrograph of autolysing yeast cells after 18 h of autolysis at 50 °C.

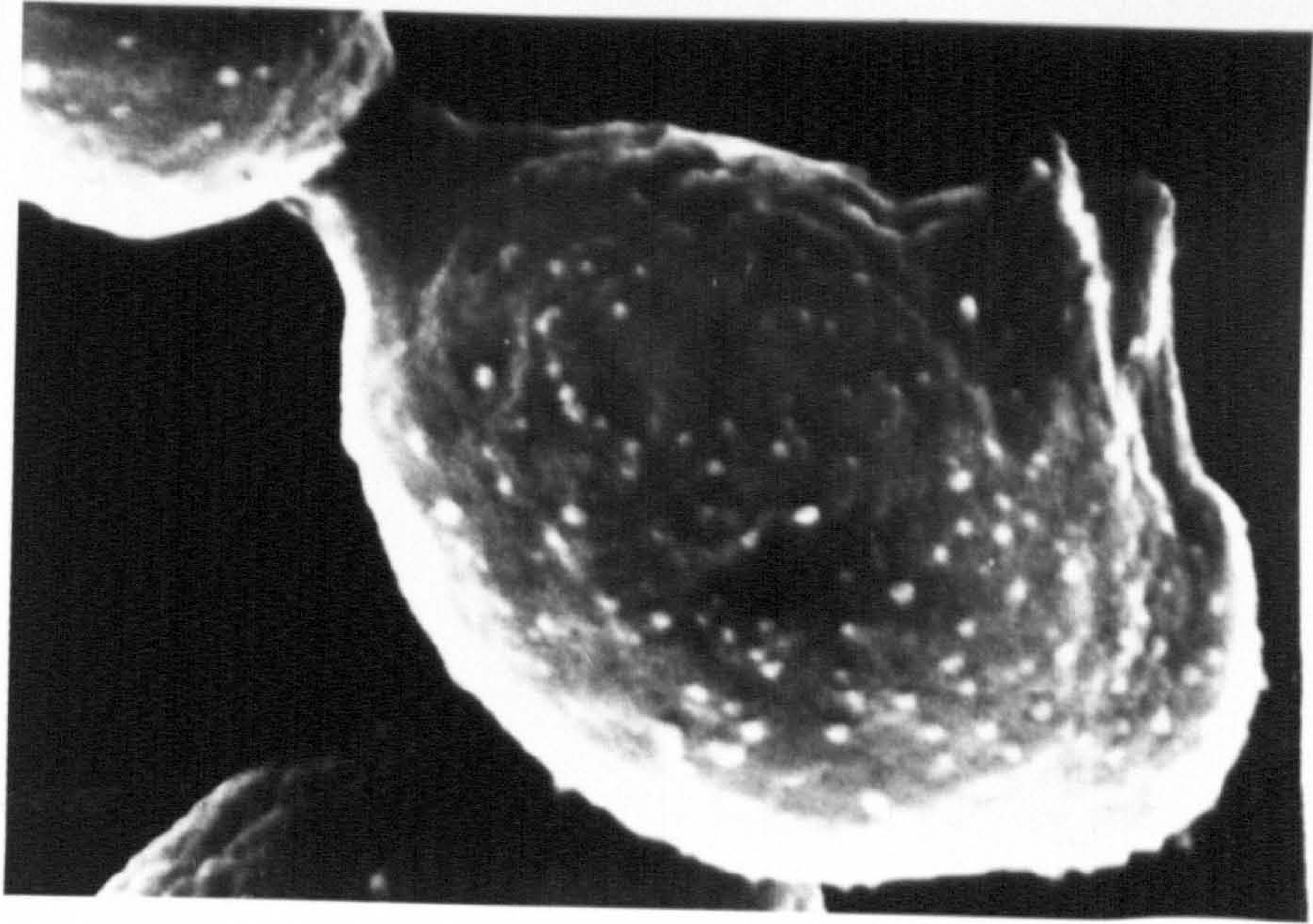


Plate 5.10 Scanning electron micrograph of autolysing yeast cells after 24 h of autolysis at 50 °C.

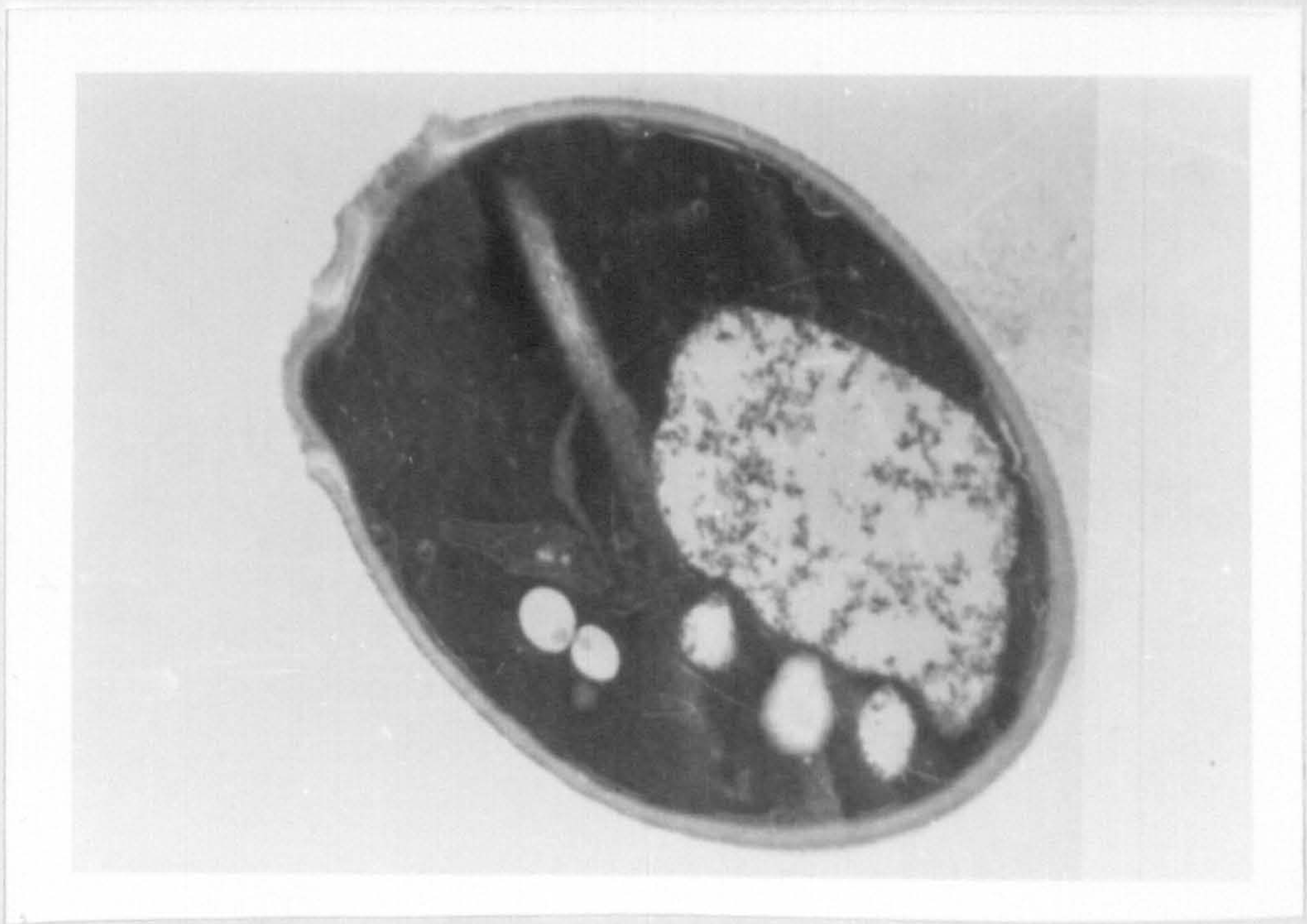


Plate 5.11 Transmission electron micrograph of 18 h grown cells prior to autolysis.

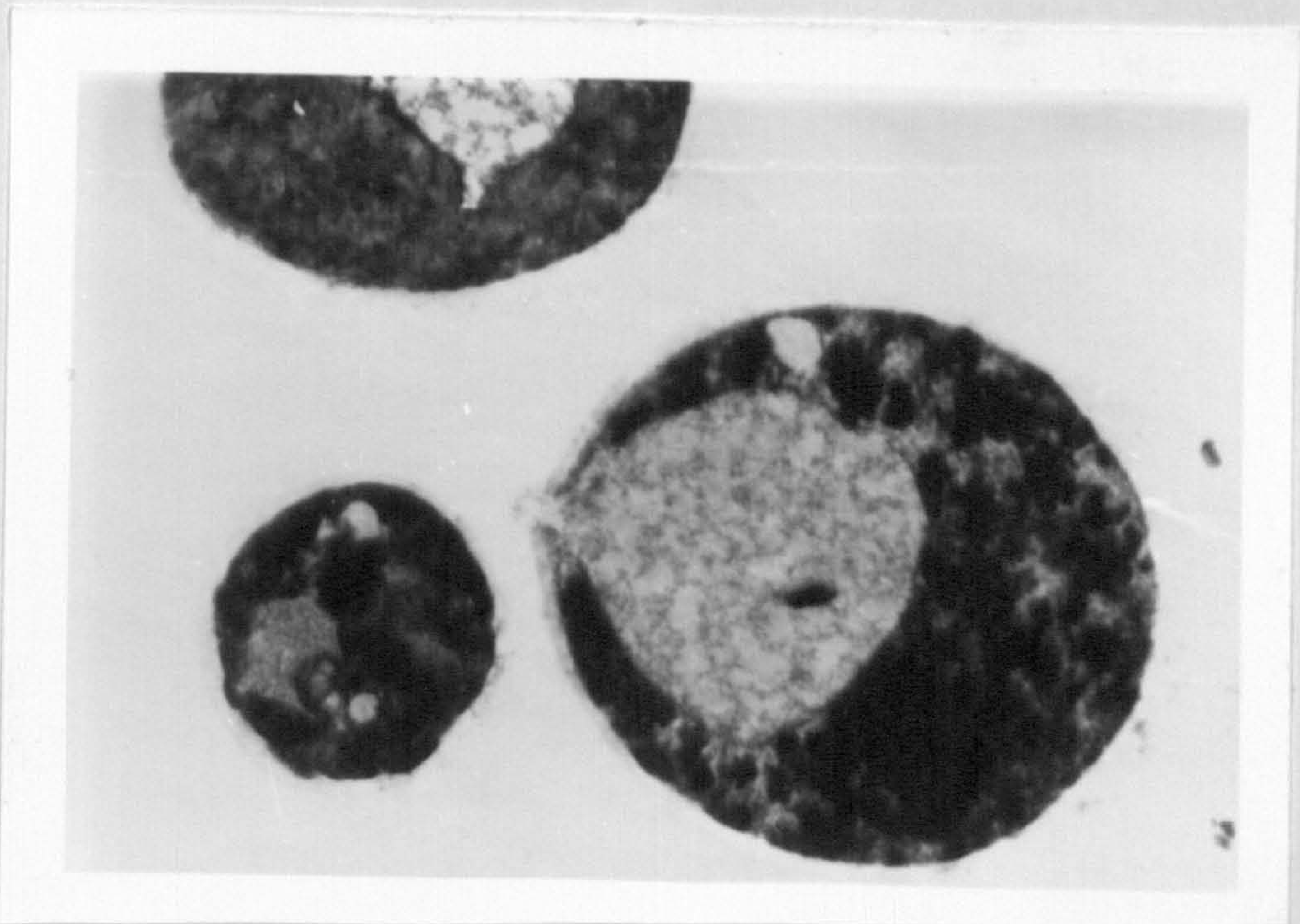


Plate 5.12 Transmission electron micrograph of autolysing yeast cells after 6 h of autolysis at 50 °C.

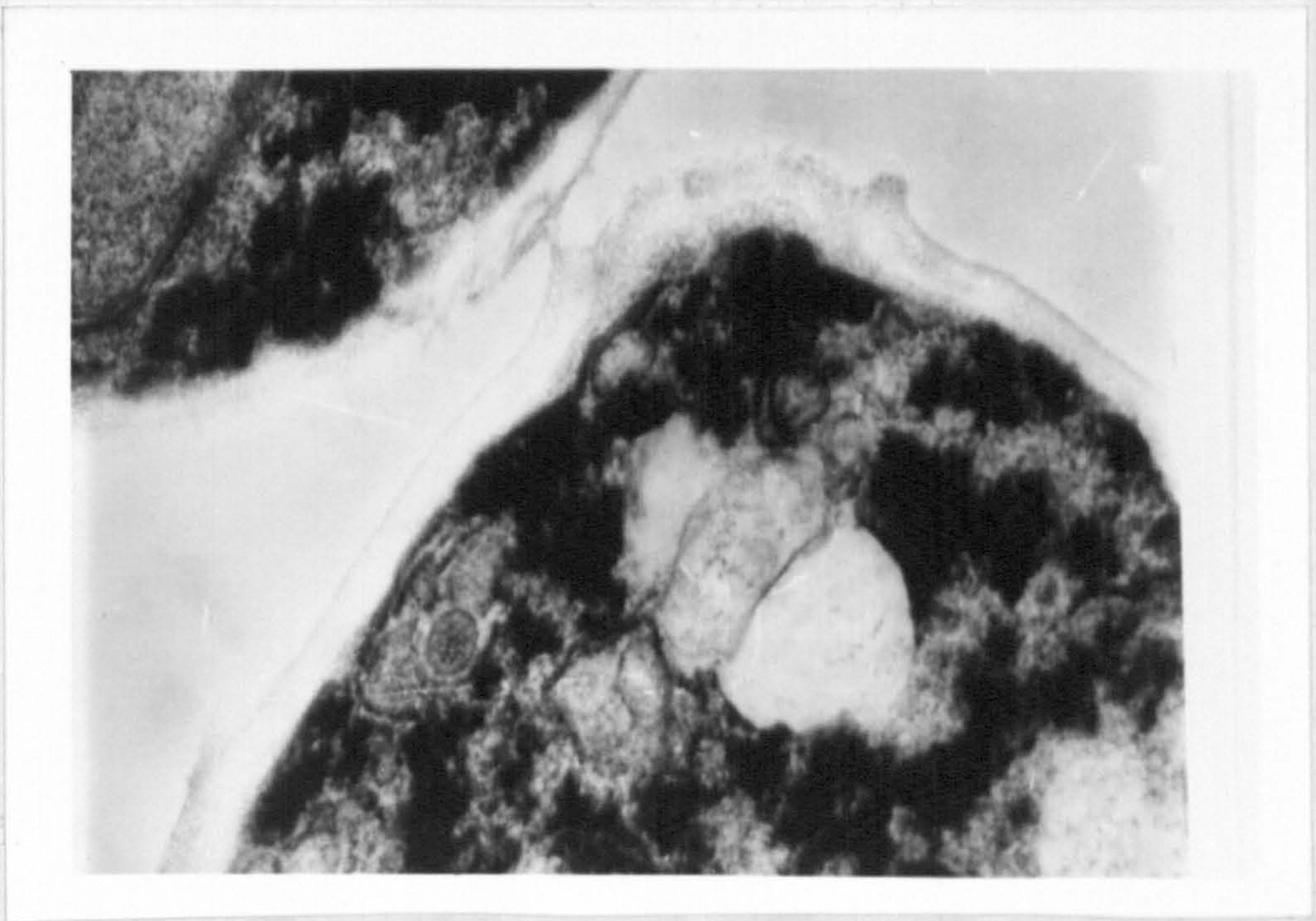


Plate 5.13 Transmission electron micrograph of autolysing yeast cells after 12 h of autolysis at 50 °C.

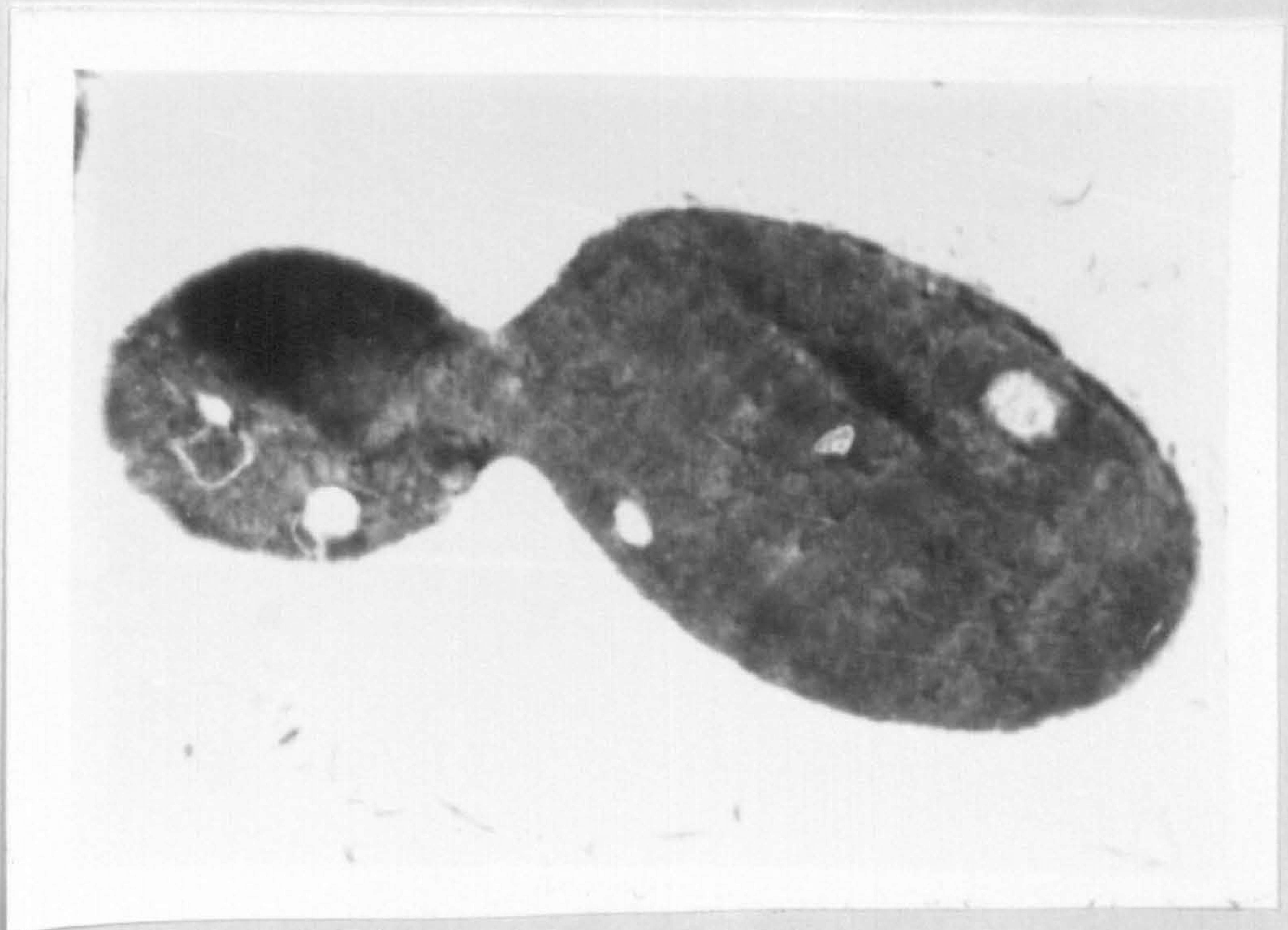


Plate 5.14 Transmission electron micrograph of autolysing yeast cell after 18 h of autolysis at 50 °C.

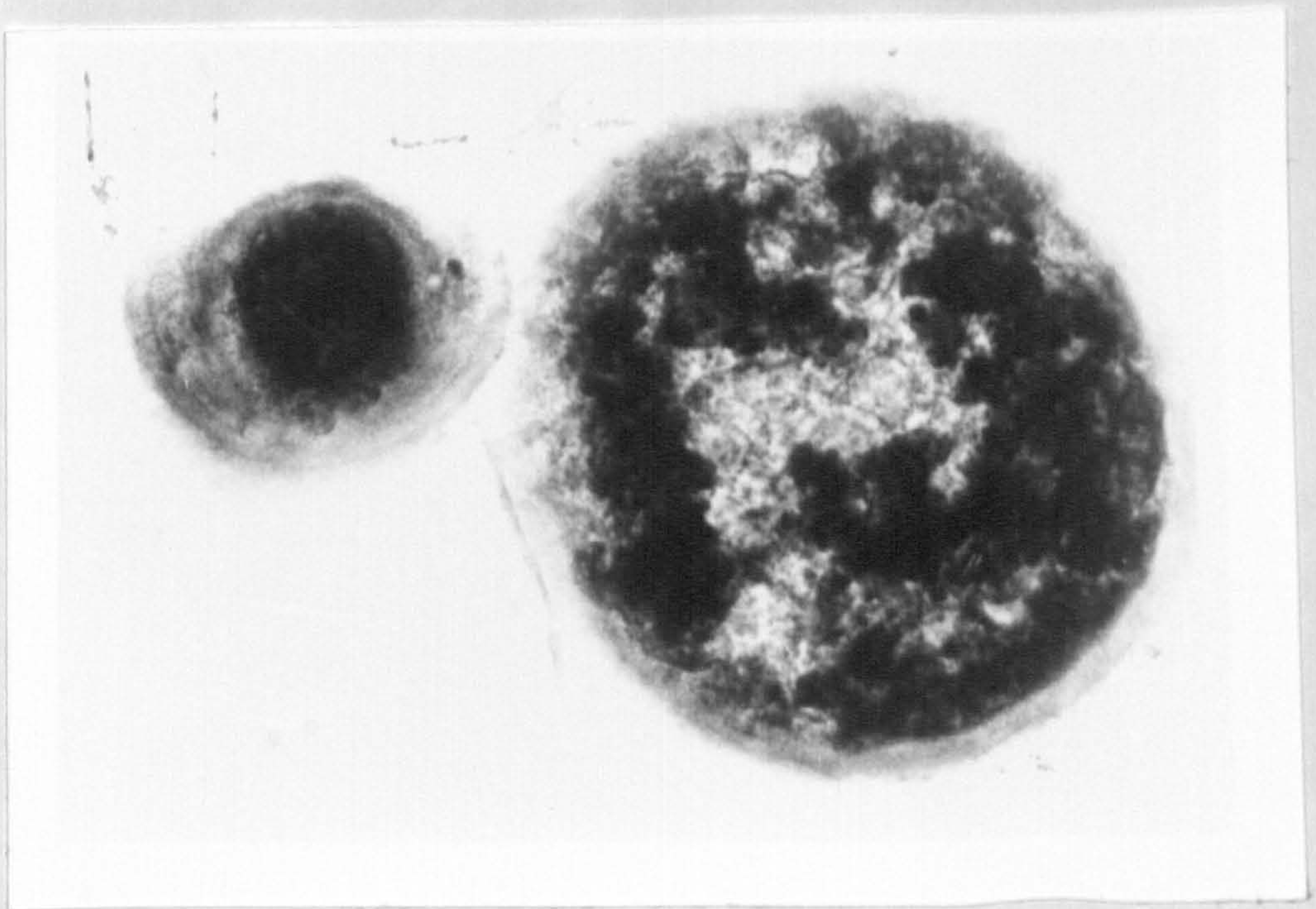


Plate 5.15 Transmission electron micrograph of autolysing yeast cells after 24 h of autolysis at 50 °C.

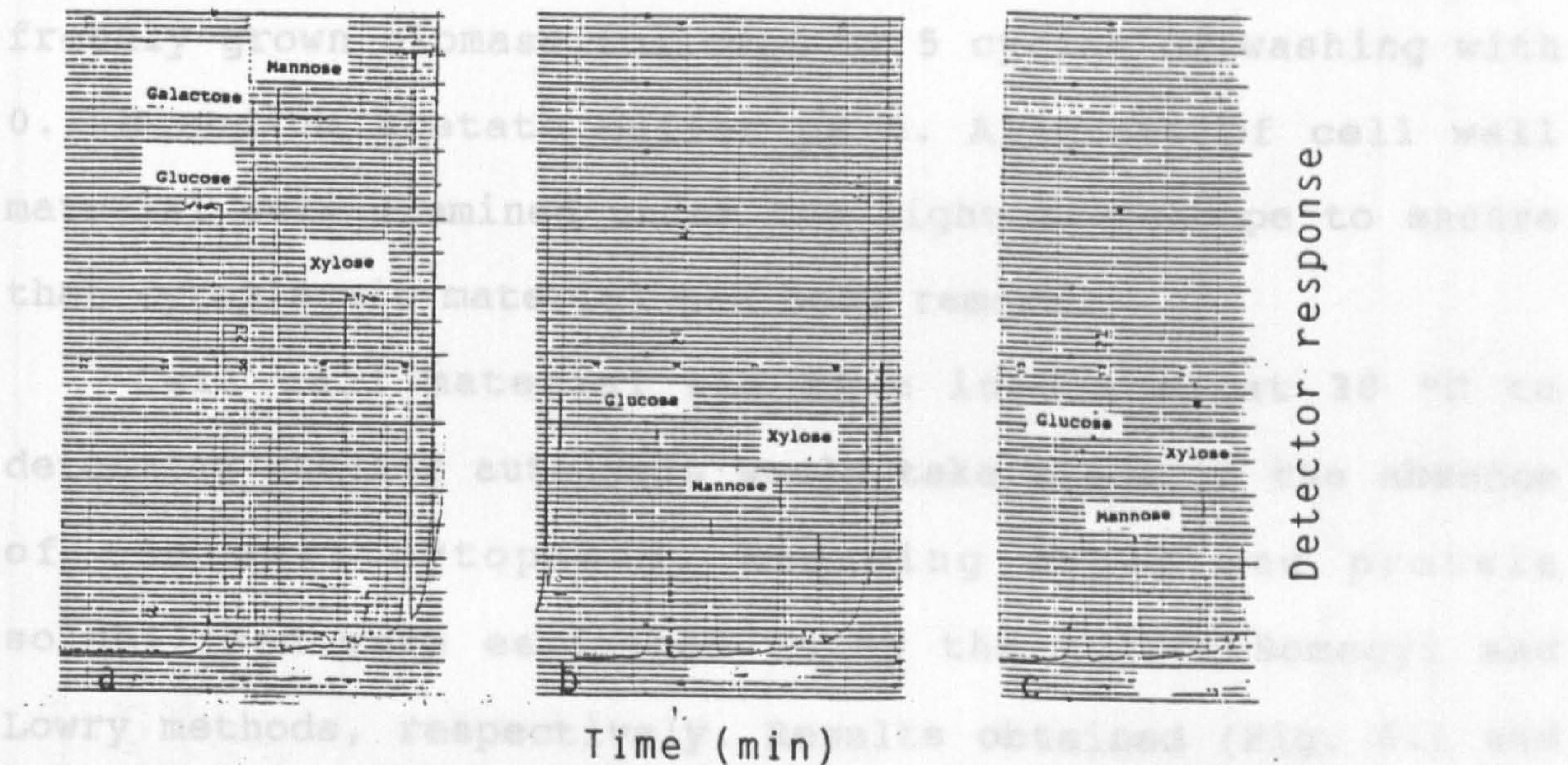
appear to have thick, well-defined cell walls with intensely stained bud scars. Plate 5.12, shows cells after 6 h of autolysis in which cell walls appeared thinner as if eroded and some cells were perforated, an observation not made in native cells. After 12 h of autolysis (Plate 5.13) cells had lost their normal regular, curved shape and walls appeared thin to the point of being almost absent in many cells. Bud scars appeared to be relatively denser than surrounding cell wall material but had lost the smooth exterior of native cells. Similar changes were observed after 18 h of autolysis (Plate 5.14).

After 24 h autolysis (Plate 5.15) further deterioration in the cell wall was observed and significant erosion of bud scars could be discerned.

5.5 Compositional changes in cell wall carbohydrates during autolysis

To study further the erosion of the cell wall during autolysis glucose and mannan contents of cell wall material prior to and during autolysis at 50 °C. Cell wall material was hydrolysed and the resulting monosaccharides were analysed by gas liquid chromatography.

The results (Fig. 5.2) showed slight reductions in glucose contents with autolysis so that after 6 h of autolysis 8 mg of glucose per 100 mg of biomass had decreased to 7.73, although values for mannose content of 3.58 mg were recorded for both samples. After 24 h of autolysis, glucose content had decreased to 6.19 mg, whilst mannose content had been reduced to 3.48 mg per 100 mg of



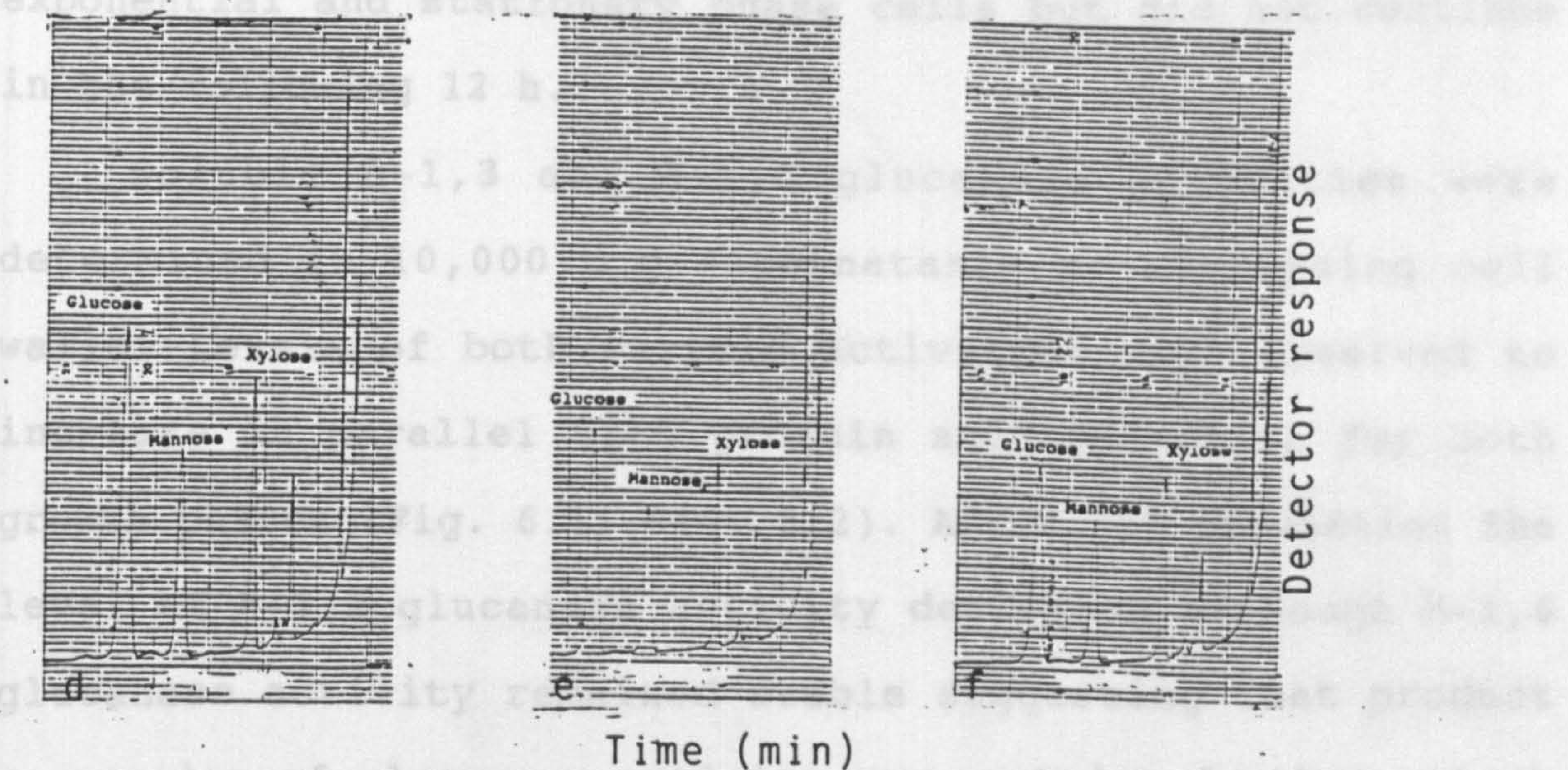


Fig. 5.2 GLC of constituent monosaccharides of the cell walls of autolysing yeast cells. a. standard, b. cells prior to autolysis, c. cells after 6 h of autolysis, d. cells after 12 h of autolysis, e. cells after 18 h of autolysis and f. cells after 24 h of autolysis.

biomass.

6.1 Study of purified cell walls

Cell wall fractions of 18 h log-phase cells and 72 h stationary phase cells were prepared by homogenization of freshly-grown biomass followed by 5 cycles of washing with 0.1 M sodium acetate buffer pH 5. Aliquots of cell wall material were examined under the light microscope to ensure that cytoplasmic material had been removed.

Cell wall material was then incubated at 30 °C to determine whether autolysis would take place in the absence of the cell cytoplasm. Reducing sugar and protein solubilised were estimated using the Nelson-Somogyi and Lowry methods, respectively. Results obtained (Fig. 6.1 and 6.2) showed that solubilisation of both protein and sugars took place in the first 12 h of the incubation for both exponential and stationary phase cells but did not continue in the following 12 h.

Soluble ß-1,3 and ß-1,6 glucanase activities were determined in 10,000 x g supernatants of autolysing cell walls. Levels of both enzymic activities were observed to increase in parallel with protein solubilisation for both growth phases (Fig. 6.1; Fig. 6.2). After 12h incubation the level of ß-1,3 glucanase activity decreased although ß-1,6 glucanase activity remained stable suggesting that product repression of glucanase activity may restrict further attack by these enzymes be present. ß-Glucanase, mannanase and chitinase activities in extracts reached maxima (Fig. 6.3) during exponential phase decreasing subsequently.

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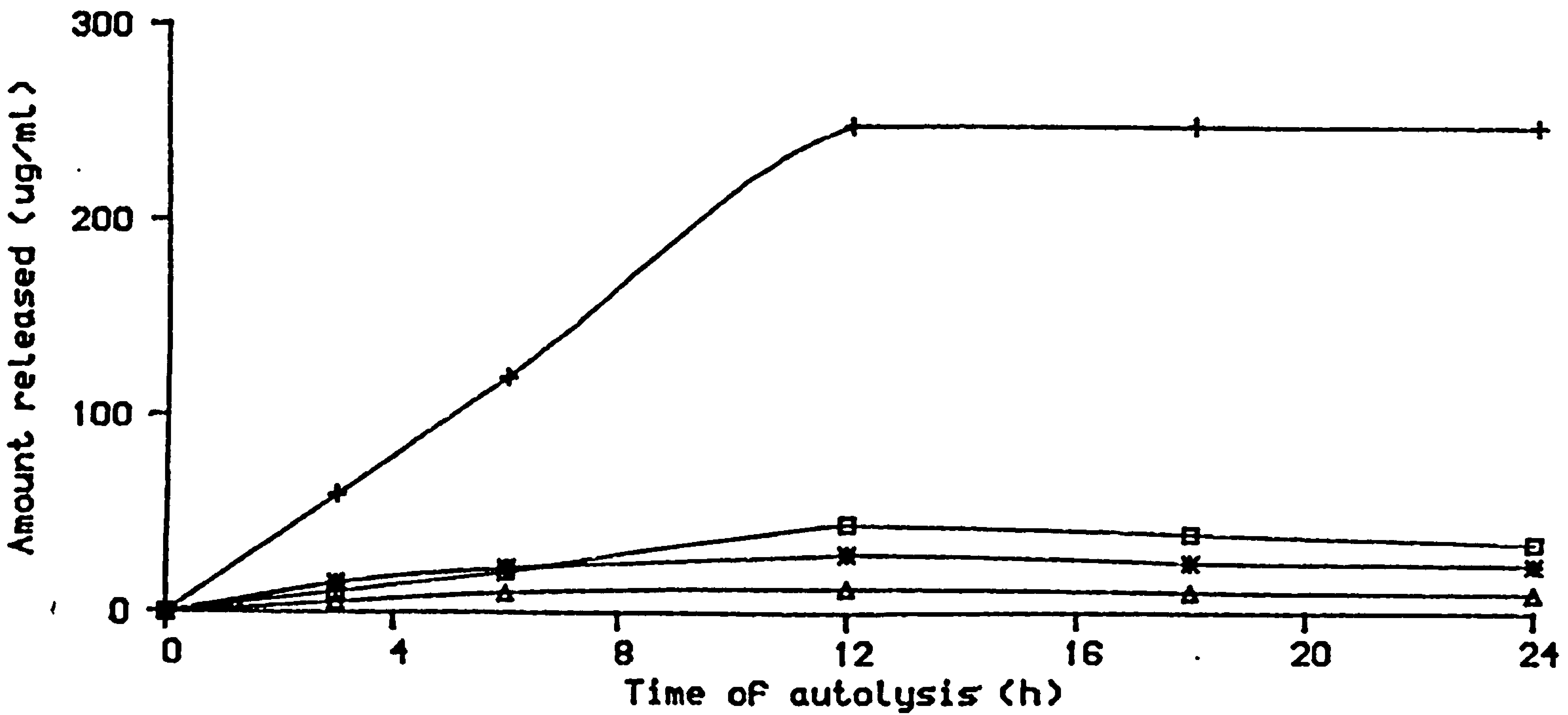
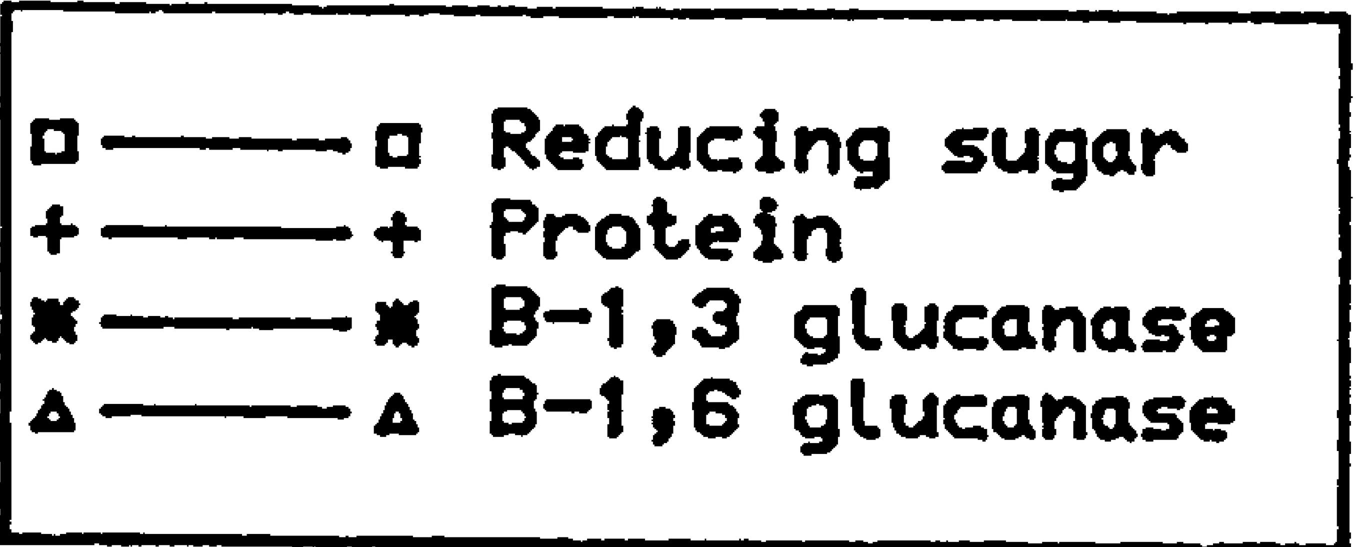


Fig. 6.1 Autolysis of isolated cell walls of yeast prepared from log phase cells.



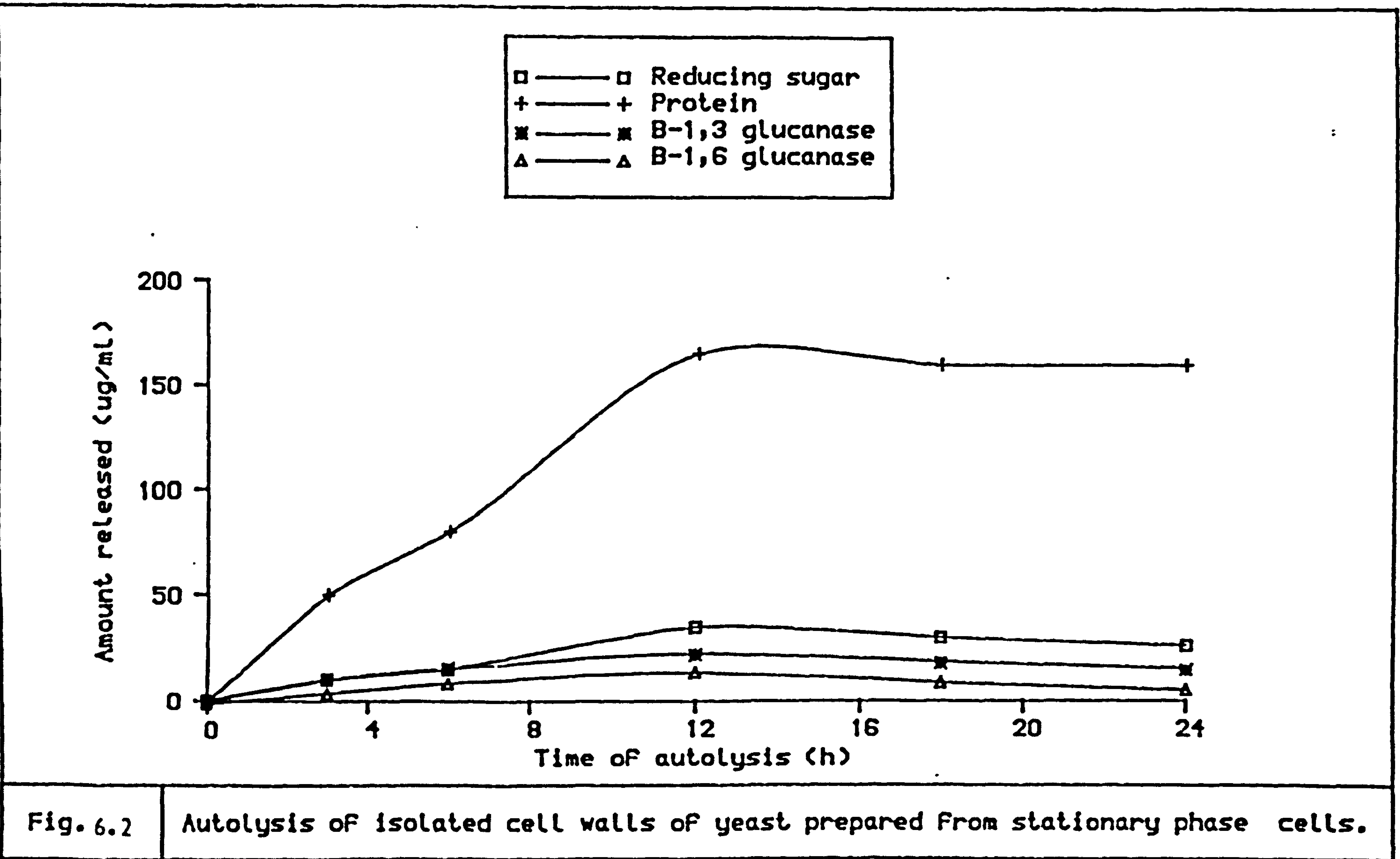


Fig. 6.2 Autolysis of isolated cell walls of yeast prepared from stationary phase cells.

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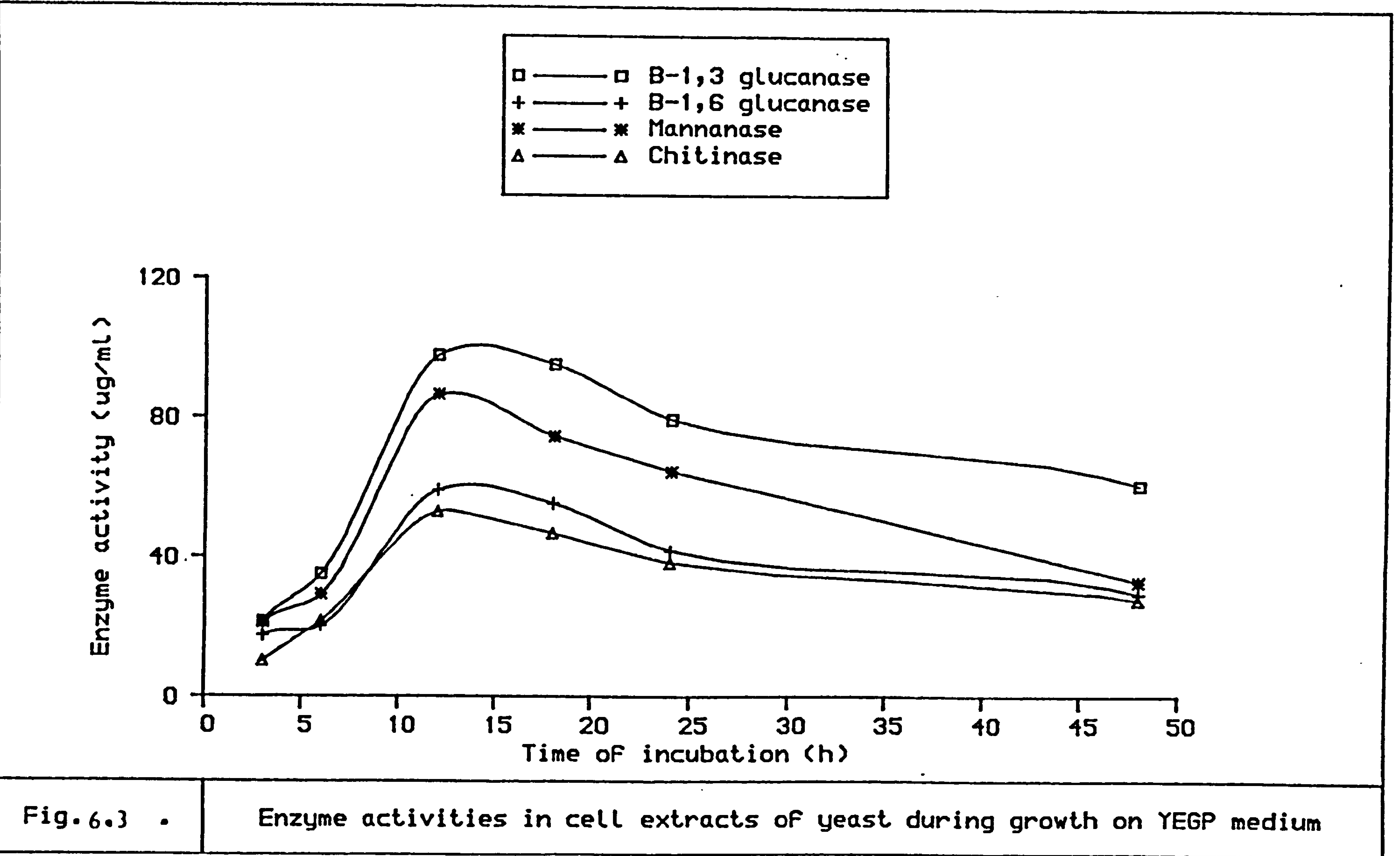

Fig. 6.3 . Enzyme activities in cell extracts of yeast during growth on YEGP medium

6.2 Electrophoretic analysis of cell wall proteins

To expand the characterisation of the autolysis of isolated cell walls, isoelectric focussing of proteins solubilised during the 24 h autolysis of 18 h purified cell wall fractions was carried out using ampholytes within the pH ranges 3.5-9.5 (Fig. 6.4) and pH 4.5-7 (Fig. 6.5). Analyses were carried out using polyacrylamide gels with 12.5 % cross-linking.

Proteins were characterised in soluble extracts of 12 h, 18 h and 24 h autolysing cell walls using isoelectric focussing in the ranges pH 3.5-9.5 and pH 4.5-7. In each gel 10 protein bands were observed. Samples containing total cytoplasmic proteins from autolysing cells were also characterised and contained a much larger population of proteins, with many bands present in cell wall preparations also appearing in total cell extracts.

6.3 Identification of glucanases on iso-electrophoretograms

To identify glucanases solubilised from cell-wall pre parations the methods described by Teather and Wood (1982) and Bartley _et al._ (1984) were employed . Duplicate IEF gels, after electrofocusing, were overlaid with agarose gels containing either laminarin (ß-1,3 glucan) or pustulan (ß-1,6 glucan) and incubated at 50 °C for 10 min in a waterbath. Gels were then stained with Congo red (1 mg ml^{-1}) to delineate zones of glucan depolymerisation or for total proteins with Coomassie brilliant blue. Clearing of laminarin (Fig. 6.6) and pustulan (Fig. 6.7) suggested that both ß-1,3 and ß-1,6-glucanases are present in autolysates

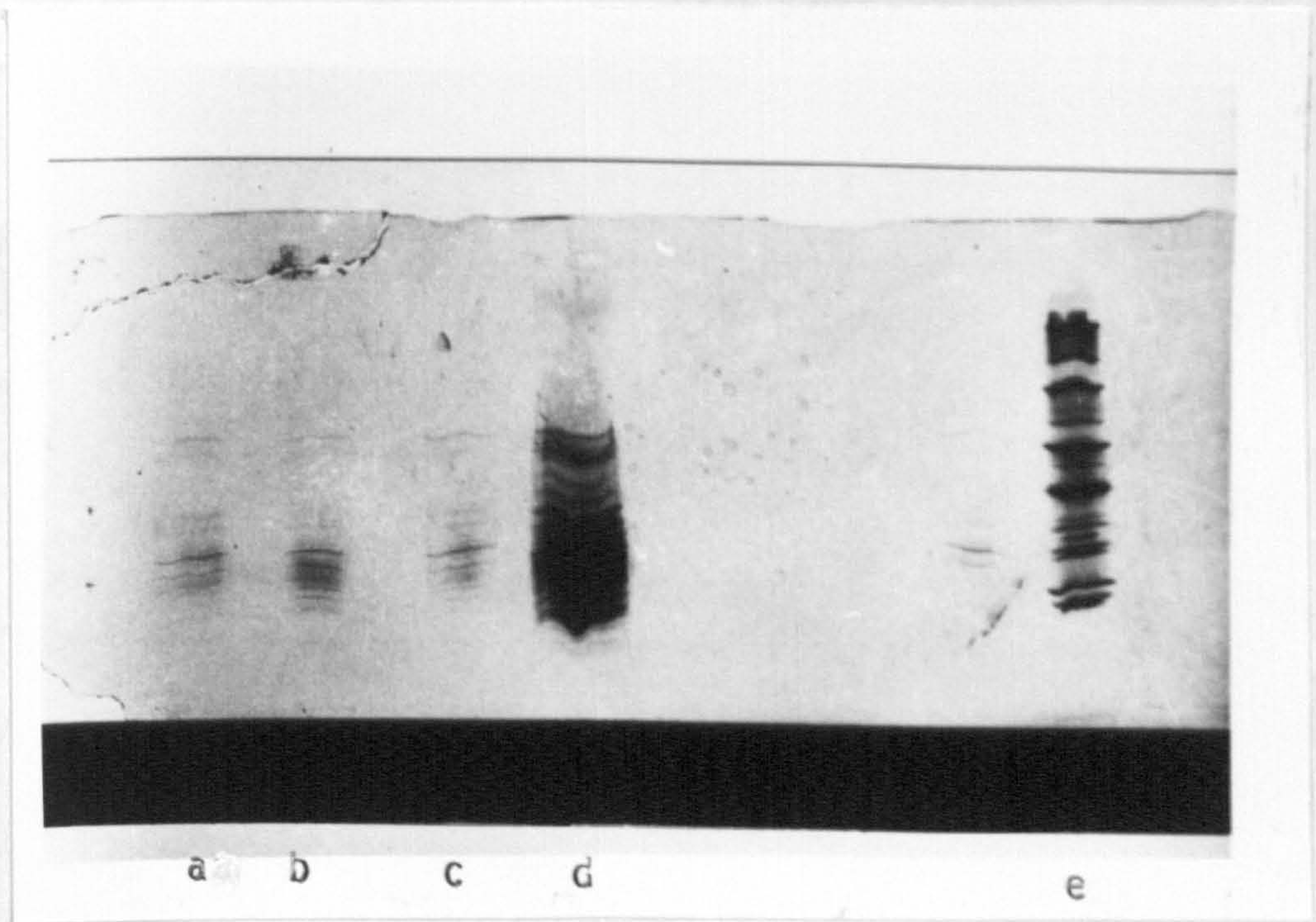

Fig. 6.4 Isoelectric focussing gel (pH 3.5-9.5).
a, b, c, supernatant after 12, 18 and 24 h of autolysis. d. cell extracts and e. IEF marker.

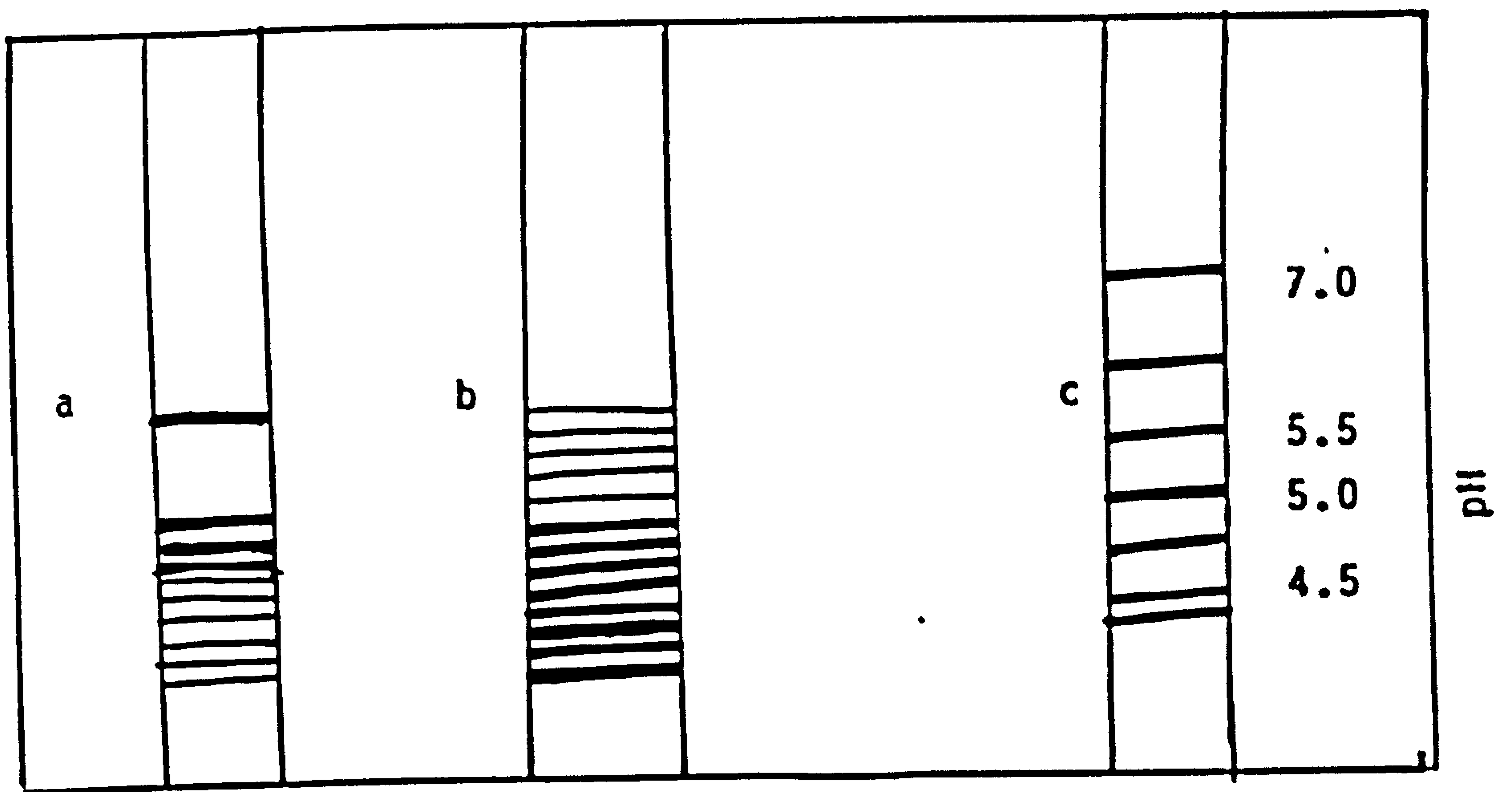

Fig. 6.4 Isoelectric focussing of soluble cell wall proteins
a. Supernatant after 24 h of autolysis, b. cell extracts,
c. IEF marker. (pH 3.5-9.5).

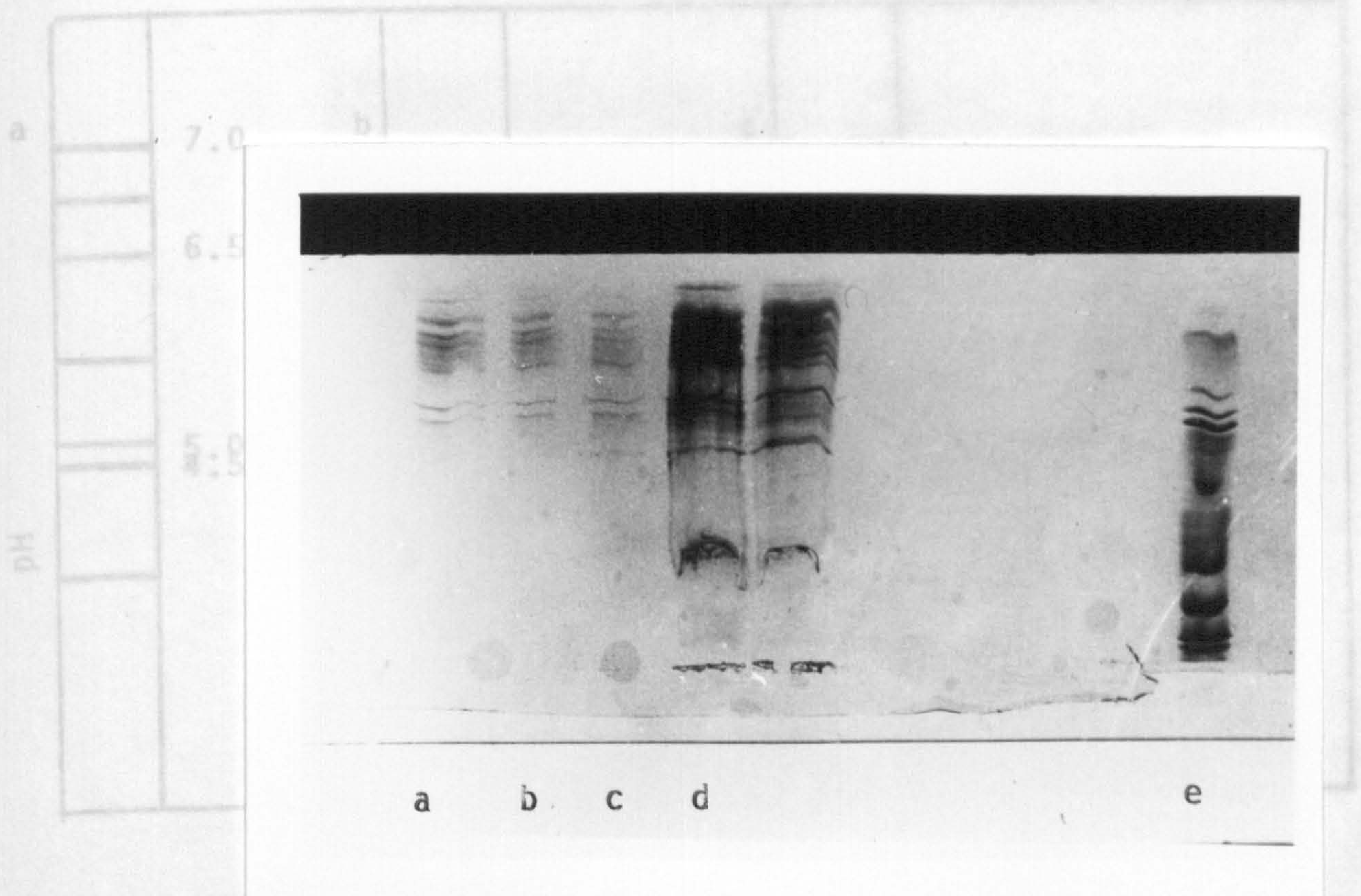

Fig. 6.5 Isoelectric focussing gel (pH 4.5-7.0). a, b, c, supernatant after 12, 18 and 24 h of autolysis. d. cell extracts and e. IEF marker.

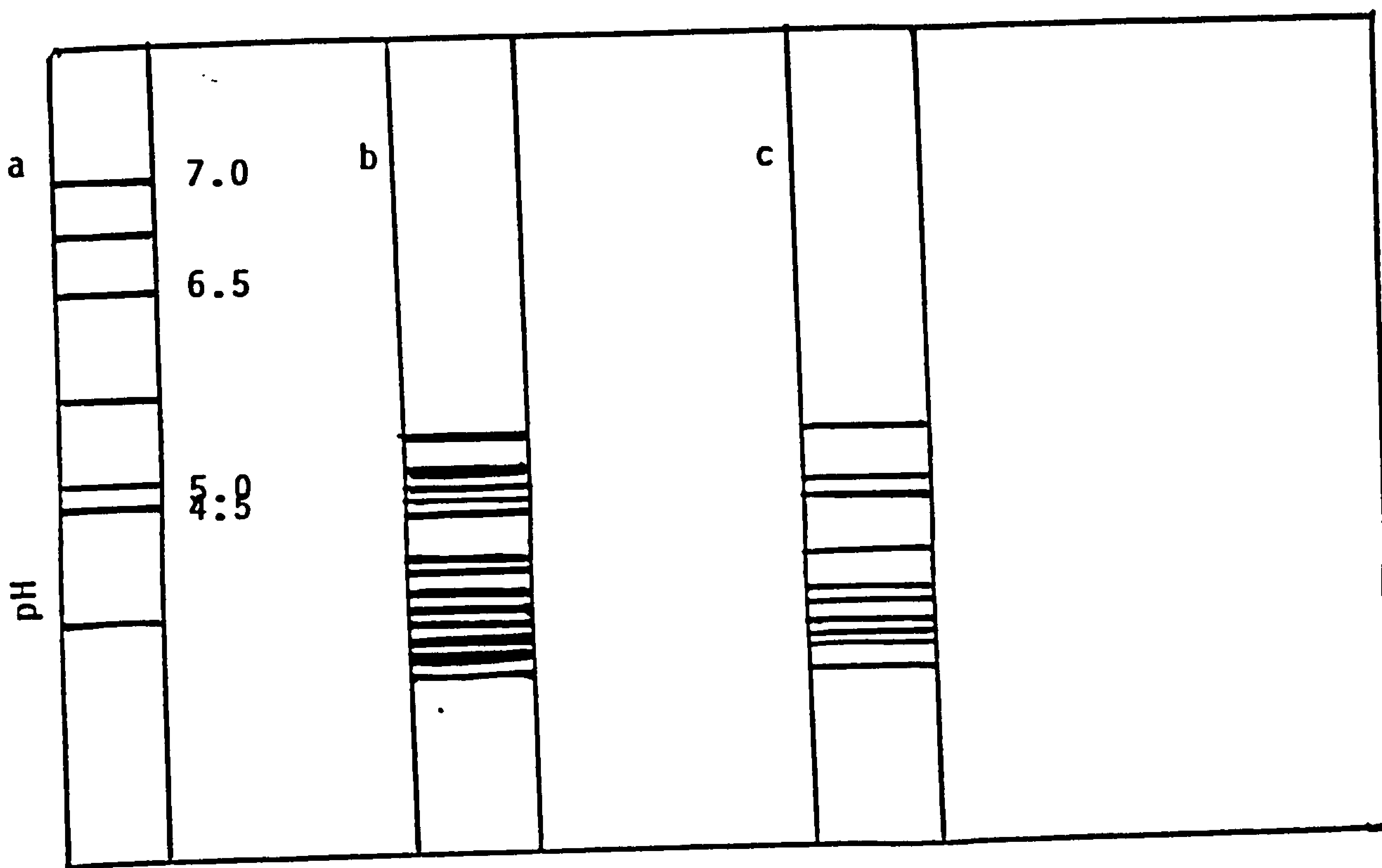

Fig. 6.5 Isoelectric focussing of proteins (pH 4.5-7.0). a. IEF marker, b. cell extracts and c, supernatant after 24 h of autolysis.

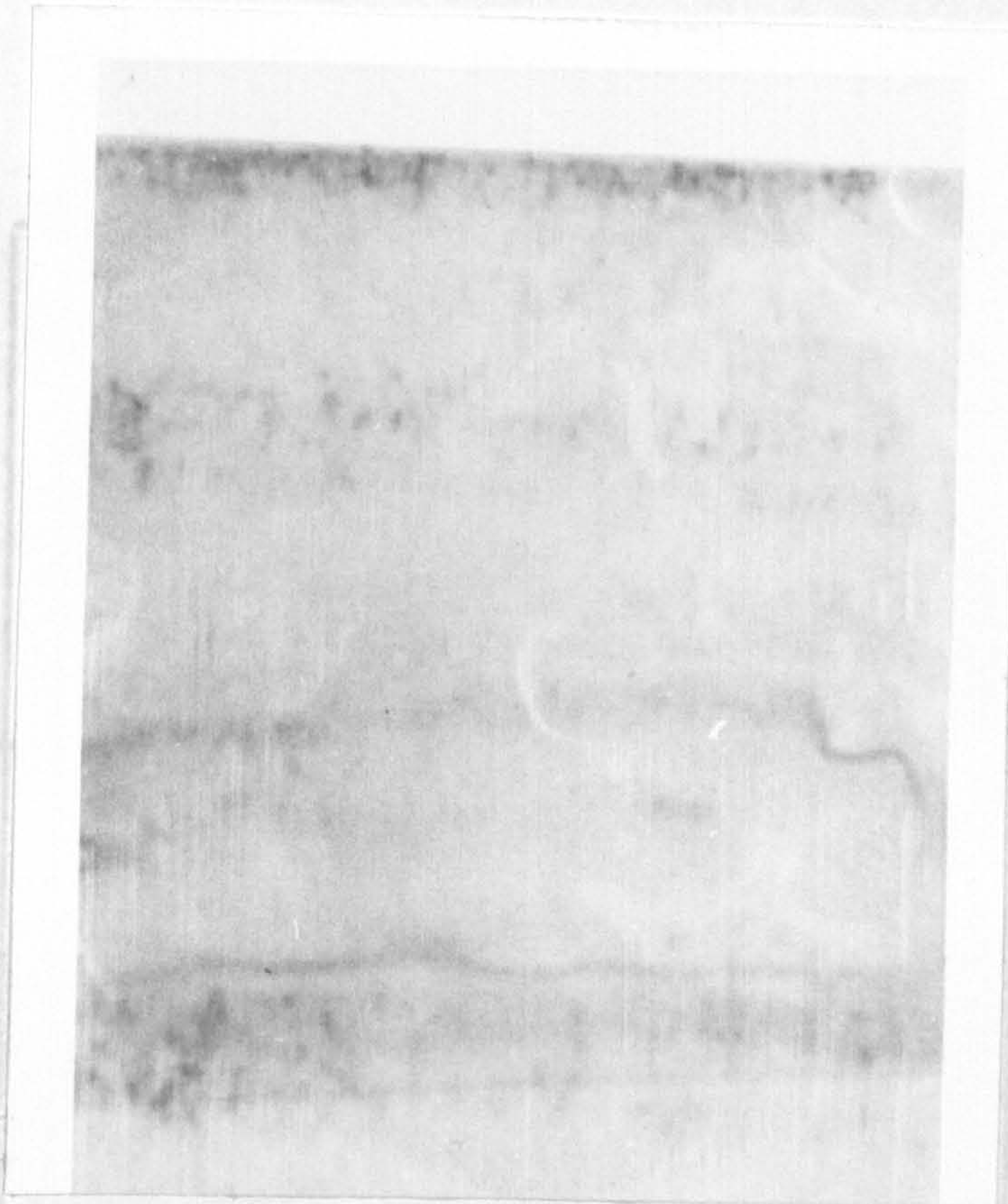

Fig. 6.6 Zone of laminarin hydrolysis in agarose gel

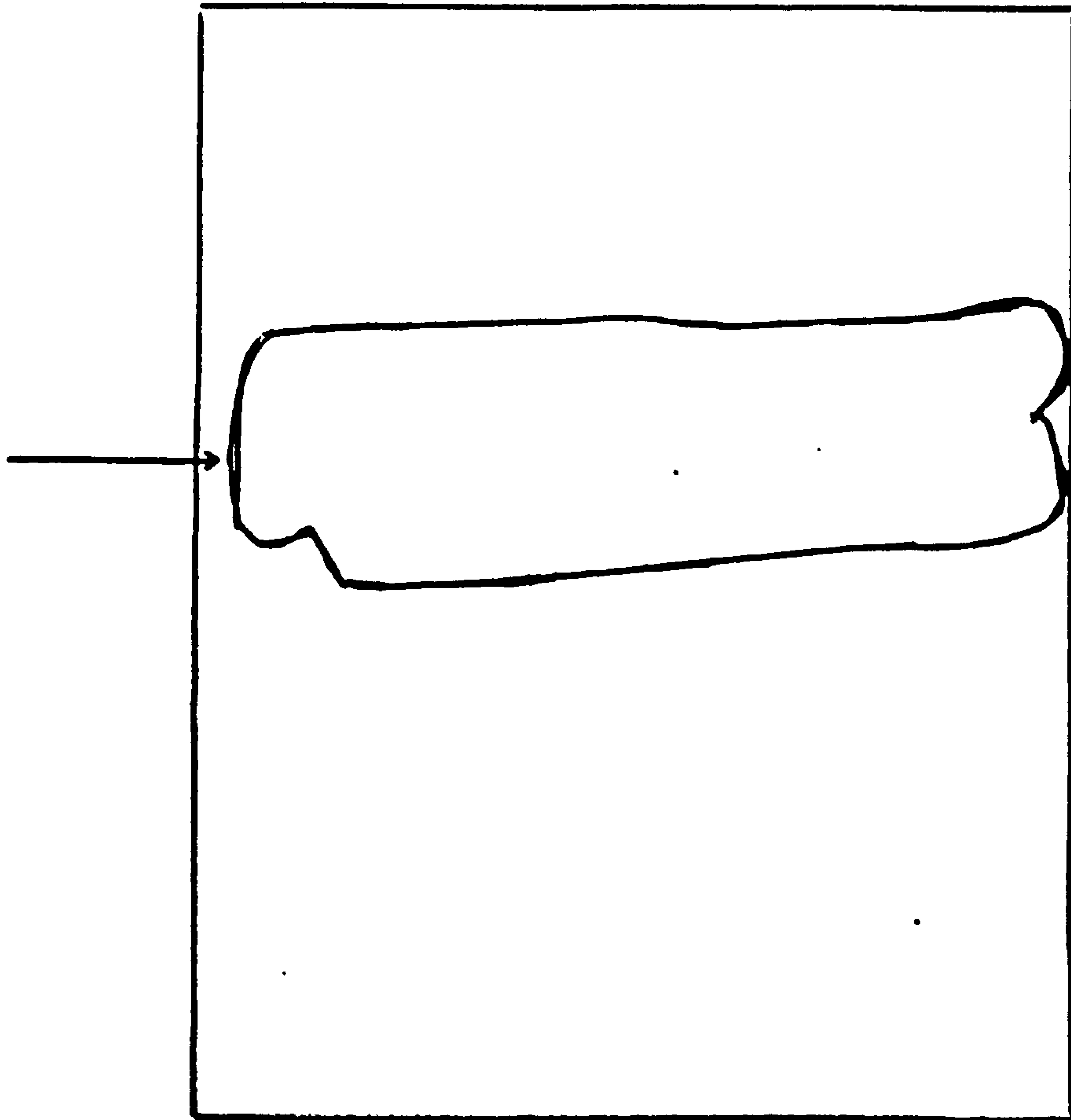

Fig. 6.6 Zone of laminarin hydrolysis in agarose gel.

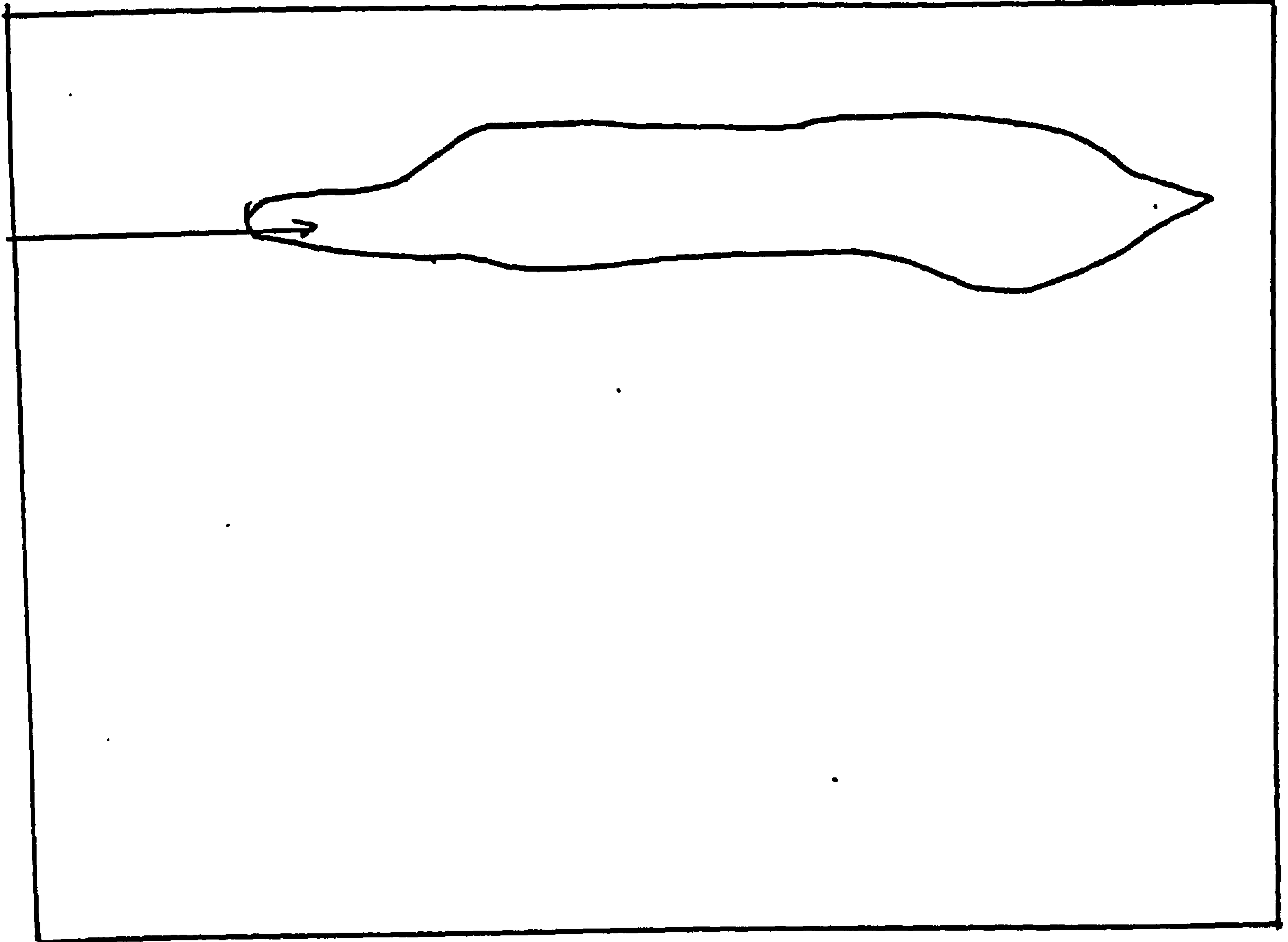

Fig. 6.7 Zone of pustulan hydrolysis in agarose gel.

of cell-free cell walls. However, the resolution of this approach was limited and it was not possible to identify which of the proteins hydrolysed individual glucans.

6.4 Analysis of proteins released during autolysis of purified cell walls by SDS polyacrylamide gel electrophoresis

Solubilised polypeptides were analysed utilising SDS gel electrophoresis using the method outlined by Laemmli (1970). The stained electrophoretograms (Fig. 6.8) confirmed the presence of 6 major protein bands in soluble fractions which are also present in total cell lysates. No attempt was made to study the rate of appearance of enzymes during autolysis.

7.1 Study of thermostability of cell wall degrading enzymes

ß-1,3 glucanases

These experiments were carried out to investigate the stability of ß-1,3 glucanases during autolysis at different temperatures. Cell-free extracts were incubated at a range of temperatures and after a defined period samples were removed for assay of release of reducing sugar from laminarin. Since maximal ß-1,3 glucanase was observed at 50 °C, activities were most conveniently expressed normalised to these values (Fig. 7.1)

Within 5 min of incubation at 60 °C, ß-1,3 glucanase activity had been reduced to 75 % of the initial value whereas at 70 °C and 80 °C enzyme activity had decreased to 30 and 20 % of initial values, respectively, over the same period. After this initial 5 min no further inactivation

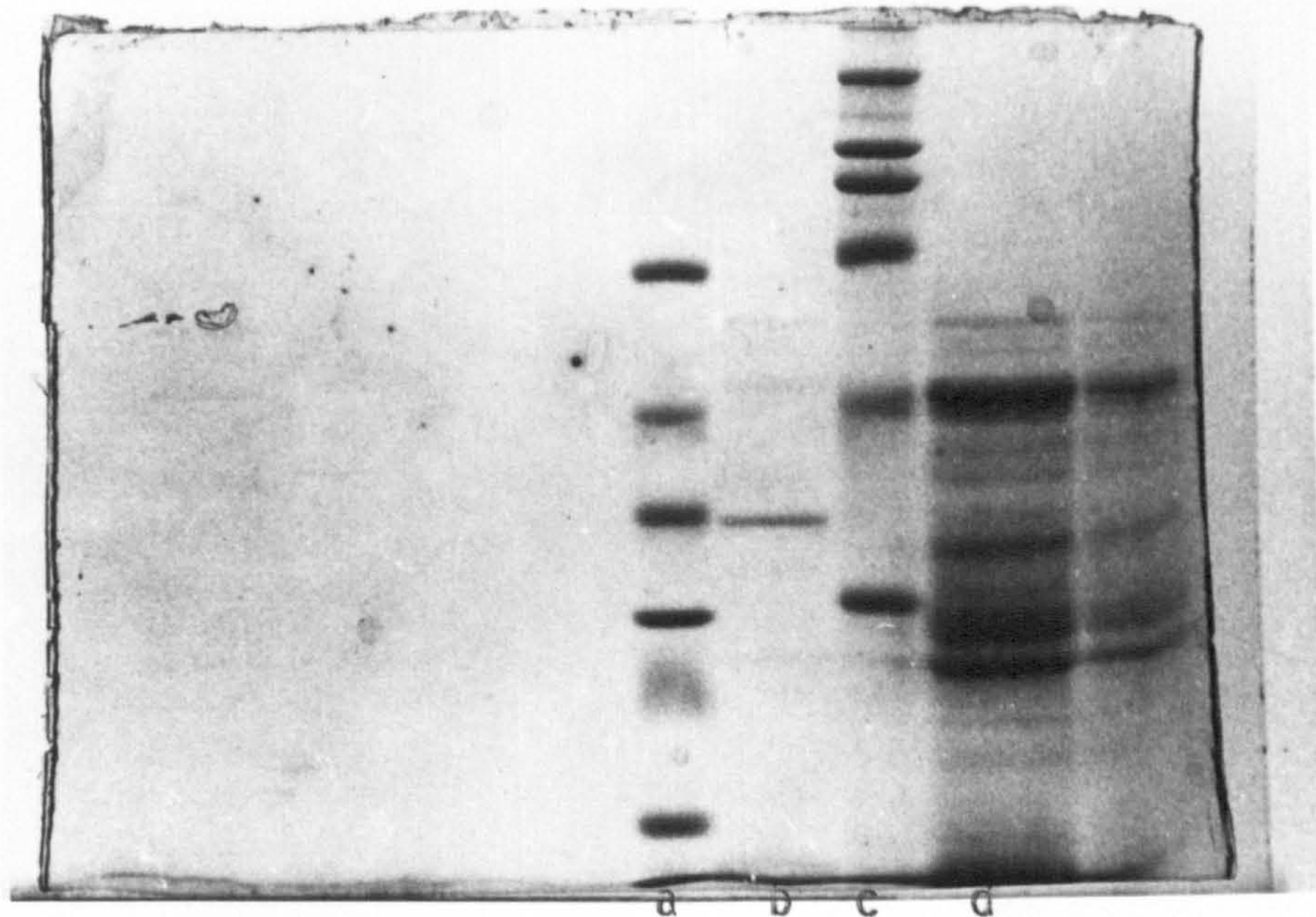

Fig. 6.8 SDS-polyacrylamide gel. a. low molecular weight marker, b. supernatant after 24 h of auto lysis, c. high molecular weight marker, d. cell extracts.

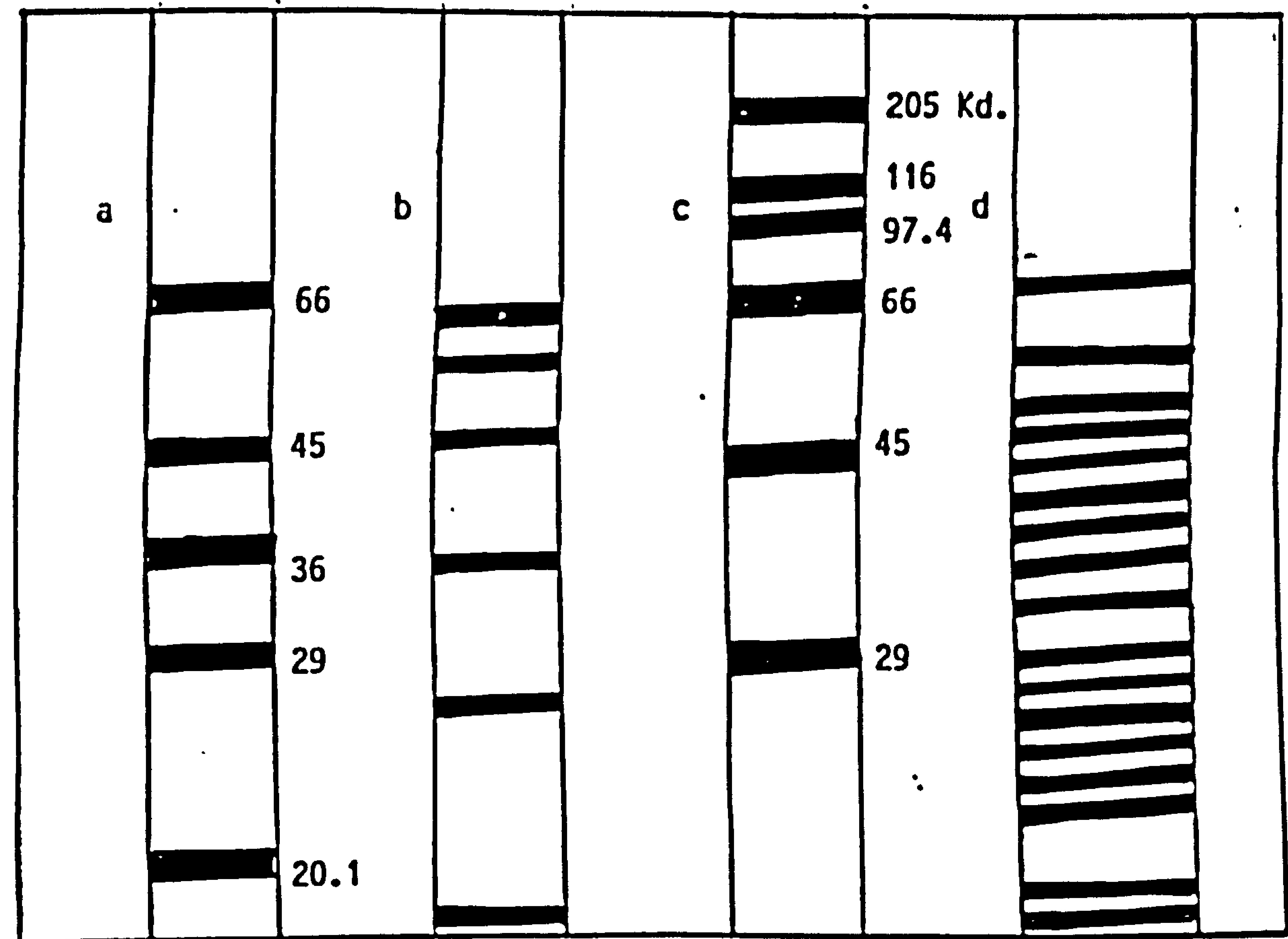

Fig. 6.8 SDS-polyacrylamide gel. a. low molecular weight marker, b. supernatant from 24 h autolysis, c. high molecular weight marker, d. cell extracts.

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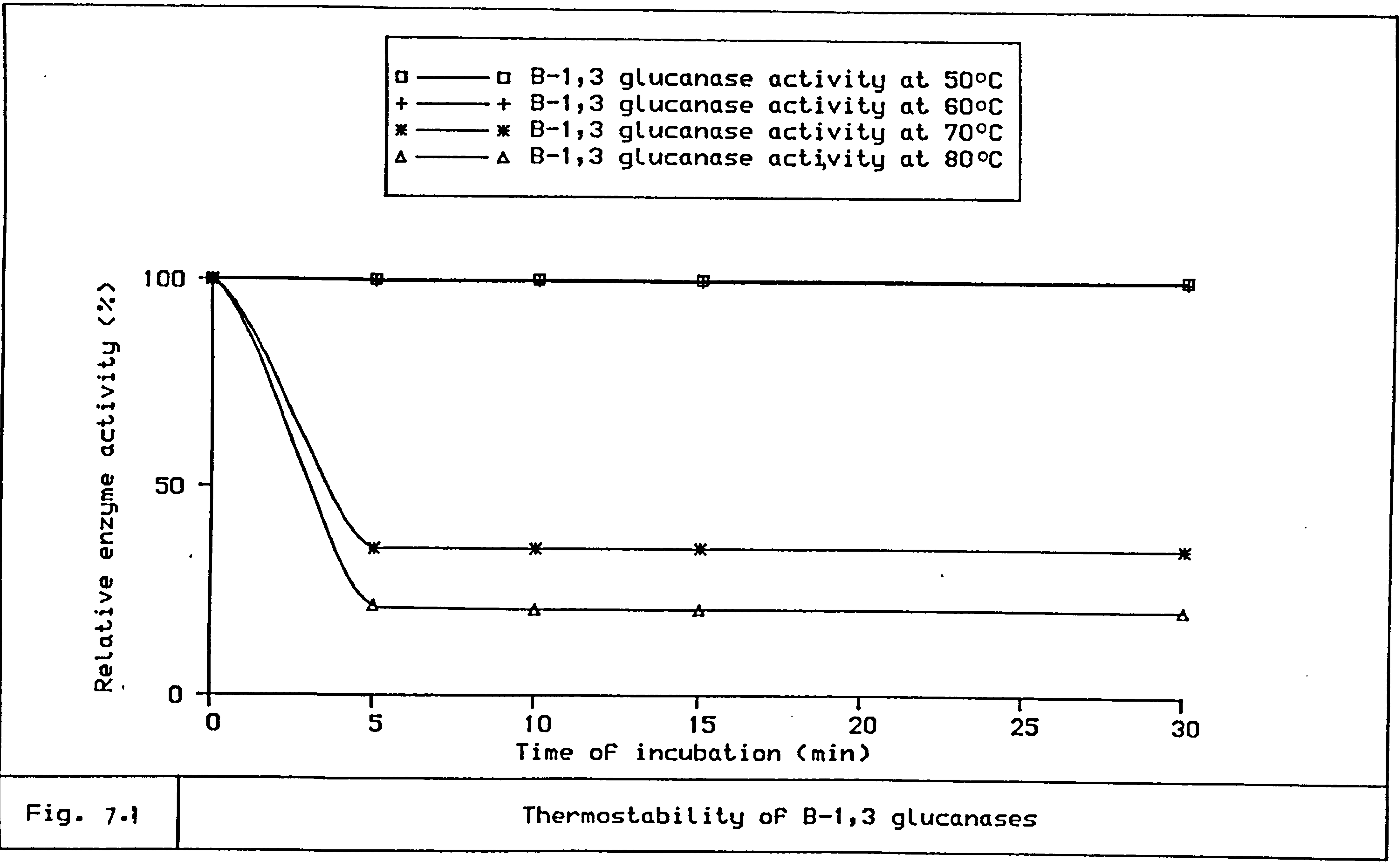

Fig. 7.1 Thermostability of B-1,3 glucanases

could be detected.

Mannanases

Heat inactivation of mannanases was also analysed with activities determined being normalised with respect to that at 50 °C except that cell extracts were used instead of cell-free extracts. Results obtained (Fig. 7.2), showed significant decreases in enzyme half-life with increasing temperature. At 70 °C and 80 °C enzymic activities were reduced to 29 and 20 % after a 5-min incubation although at 60 °C, the activity appeared stable.

Chitinases

Data on thermal stability of chitinases (Fig. 7.3) showed that this enzymic activity was relatively stable at temperatures at or below 80 °C while at 90 °C, 50 % of the initial activity was lost in a 5-min incubation although no subsequent loss in activity was observed.

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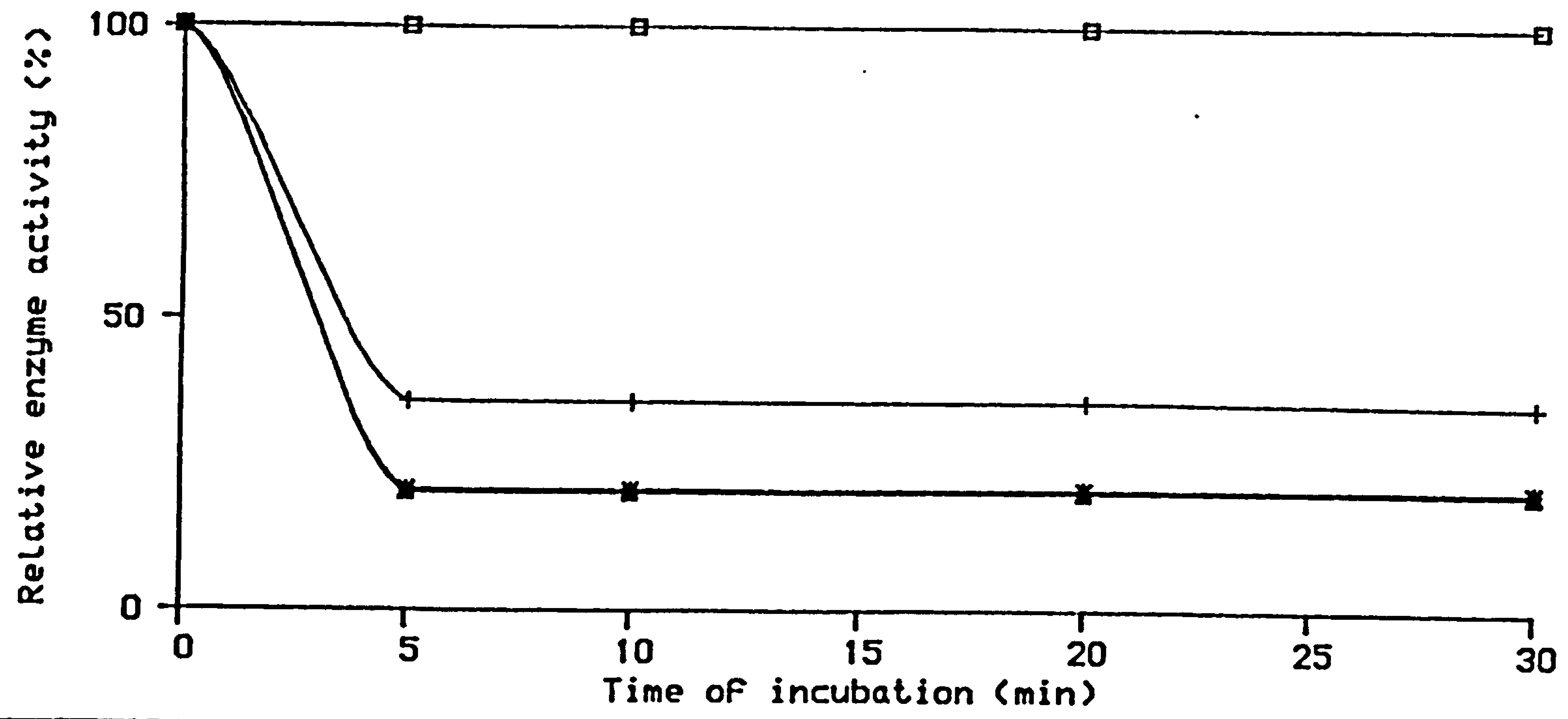

Fig. 7.2 Thermostability of Mannanases

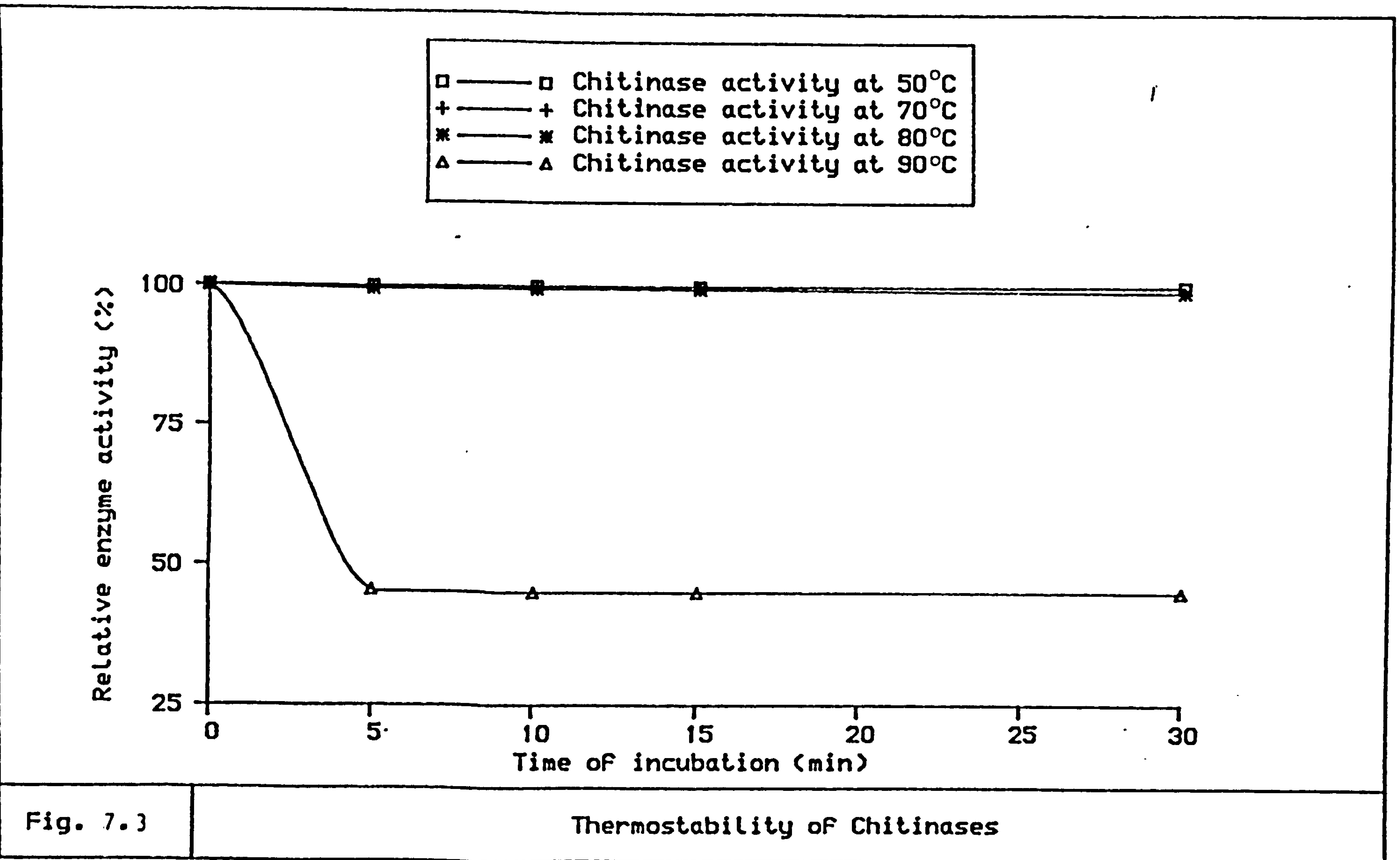

Fig. 7.3 Thermostability of Chitinases

DISCUSSION

the rate of solubilisation was greatly reduced.

Fluorescence microscopic analysis of normal and autolysing cells showed that in autolysing cells the chitinous bud scar became increasingly prominent. Autolysis was further characterized using both scanning and transmission electron microscopy. Compositional analyses showed that in autolysis whole cell glucose and mannose contents were markedly reduced while chitin depletion was insignificant.

Study of the effect of autolysis on purified cell walls showed that a considerable amount of ß-glucanase activity was released into the soluble fraction. Cell wall preparations from exponential and stationary phase cells showed different ratios of activities towards the glucan substrates, laminarin and pustulan. It was found that cell wall preparation from log phase cells showed a higher total glucanase activity than stationary phase cells. Both protein and glucose were found to be in the soluble fraction in significant proportion. Electrophoresis under native (IEF) and denaturing conditions (SDS) showed the presence of differing enzyme species present in soluble fractions. Staining for enzymic activity using overlaying of gels containing ß-1,3 and ß-1,6 glucans confirmed the presence of isoenzymes of ß-glucanases with pI ranging between 3.5 and 4.5.

Babayan et al. (1981) reported that yeast autolysis appears to be a two-step process. The first step consists of restructuring of the cell endostructures and activation of

lytic enzymes. The second step directly followed the first step and consisted of hydrolysis of cell components with release of products into the extracellular space. These authors confirmed the initial rearrangement of cell structures by microscopic examination which showed the absence of the normal cell turgor and loss of definition of cell organelles and compartments. They also found that although cell walls were thickened they remained intact after autolysis, which was consistent with previous findings of Arima et al. (1965). The products of hydrolysis (the second step of autolysis) were reported to diffuse through the cell wall into the extracellular space. In contrast lipid components in protein and nucleic acid hydrolysates released from the cell appear to coalesce into discrete droplets. Morphological changes appeared to consist of loosening and changing of the appearance of the polysaccharide coat that may be associated with partial hydrolysis of proteins incorporated into the cell wall (Babayan et al., 1981). Thus, the appearance of hydrolysis products in the extracellular space in this study is in agreement with the findings of Babayan et al. (1981).

Losses in yeast biomass dry weight during autolysis observed in this study support the hypothesis that intracellular materials were solubilised to appear in cell extracts. Hough and Maddox (1970) reported similar results in studies of autolysis of baker's yeast where the amount of DNA lost by the cell was approximately the same as that gained by cell extracts. However, RNA did not follow this

pattern suggesting differences in quantification where nucleotides were measured in the cell (McMurrough and Rose, 1967) and only the pentose sugar ribose was measured in extracts (Dische, 1949) would give artefactual values. On the other hand, it appears that in the absence of other suitable carbon sources, brewing yeasts will adjust their metabolism in order to utilise ribose although healthy cells do not normally catabolise nucleotides. This suggests that RNA is hydrolysed and products degraded during autolysis, in contrast to the situation with breakdown of DNA (Hough and Maddox, 1970). In this process RNases (Markham and Smith, 1952) yield nucleotides that are further attacked by phosphomonoesterases to give nucleosides and inorganic phosphate. Degradation of the nucleosides to ribose is catalysed by nucleosidases producing ribose; free base DNA is probably degraded in a similar fashion (Carter, 1951). Such ribose can be utilised by the yeast cell through the hexose mono-phosphate pathway. Phosphorylation to yield ribose 5′ phosphate is followed by conversion to hexose mono-phosphate via a transketolase-transaldolase series of reactions and hexose monophosphate is catabolised by glycolysis (Gibbs, 1955).

Release of protein and amino acids are considered by the yeast industries to be primary aspects of yeast autolysis. The release of active enzymes from cells during autolysis in this study suggesting that fairly extensive proteolytic attack on cell compartmentalisation occurs as reported previously (Hough and Maddox, 1970). During

autolysis, partial hydrolysis of cell cytoplasms takes place with both proteins and amino acids being released into the soluble fraction. Since proteins rather than amino acids were observed to accumulate in this study, it appears that hydrolysis into amino acids was the rate-limiting step (Vosti and Joslyn, 1954). Protease A (the dominant species; Maddox and Hough, 1969) may act to hydrolyse polypeptides produced through action of the other three isoenzymes (B-D). Each of these four glycoproteins may form part of the mannan-glucan-protein complex as proteases have been reported to be released from cell wall material (Hough and Maddox, 1970). However, synthesis new cell wall material, thought to take place in organelles referred to as sphaerosomes, is located immediately beneath the cytoplasmic membrane, a region which extracellular enzymes have to pass during secretion (Hough and Maddox, 1970).

The release of reducing sugars from biomass into cell extracts supports the notion that polymeric cell wall glucan and mannan are broken down by carbohydrases (Hough and Maddox, 1970) during autolysis. The release of reducing sugar, however, appears to be much lower than that of protein although some glucose and mannose is released from the cell as the carbohydrate moeity of glycoproteins and is subsequently split off, probably by carbohydrases, and released to form soluble sugars. Such enzymes, degrading both mannan and glucan to low-molecular weight sugars, have been detected in autolysates (Maddox and Hough, 1971).

Although yeast autolysis has been shown to involve the

breakdown of cell wall glucans and solubilisation of wall-associated ß-1,3 glucanases (Kroning and Egel, 1974), yeast cell-wall glucans are currently thought to undergo little turnover during vegetative growth (Budd, 1974; Kratky _et al._, 1975; Villa _et al._, 1980). However, these authors have concluded that limited site-specific hydrolysis of cell wall glucans by glucanases may take place during budding, wall growth, conjugation and ascus formation. This interpretation of data has also been supported by observations of Notario _et al._ (1982), Brock (1965), Crandall _et al_ (1977), Fleet and Phaff (1975), and Maddox and Hough (1971). Rapid liberation of ascospores from asci has also been reported to be associated with elevated levels of glucanase activity, suggesting that ß-glucanases may be involved in the release of spores from the ascus (Fleet and Phaff, 1975).

In this study, higher levels of glucanase activity were found in cell extracts of exponential phase as opposed to stationary phase cells which lends support to the concept that these glucanases may be involved in plasticizing and degradation of yeast cell walls during budding (Maddox and Hough, 1971; Barras, 1972; Hien and Fleet, 1983). Glucanase activities observed in this study of autolysis suggest that such enzymes may be involved in the degradation of cell wall glucans that takes place during autolysis although complete degradation is never obtained (Maddox and Hough, 1971).

During autolysis, the equilibrium of cell wall metabolism is shifted resulting in breakdown of walls and lysis of the cells (Arnold, 1981) rather than synthesis of

cellular material. Release of ß-glucanases into culture fluids during growth and cell-wall autolysis has been reported previously by Abd-el-al and Phaff (1968), Biely et al. (1972), Villa et al. (1975) and Hien and Fleet (1983).

Release of ß-glucanases into the extracellular space as observed in this study, suggests that such enzymes must be located in the periplasm or associated with cell walls as reported previously (Barras, 1972; Cortat et al., 1972; Fleet and Phaff, 1973, 1974, 1975). Cortat et al. (1972) suggested that vesicles in the endoplasmic reticulum might carry the enzyme to the site of bud formation, as enzymes are carried to the growing tip in filamentous fungi, and the enzyme subsequently diffuses through the membrane at the site of fusion. Thus, glucanase activity will be external to the cytoplasmic membrane and may be tightly associated with its substrate of cell wall (Villa et al., 1975).

Since both yeast and filamentous fungi contain mannans and a mixture of ß-1,3 and ß-1,6 glucan, reducing sugar found in the soluble fraction of autolysates could arise from the action of mannanases and ß-1,3 and ß-1,6 glucanases. Degradation of glycogen will also contribute to the overall concentration of low molecular weight sugars and autolysis may also yield oligomers derived from glucan and mannan (Ram et al., 1984). The washed cell walls produced autolysates containing enzymes capable of degrading laminarin, pustulan and mannan, indicates that ß-glucan and mannan are likely to be significant enzyme substrates during autolysis. Such enzymes may also be involved in normal wall

turnover since it is widely accepted that localized secretion of glucanases (Crandall *et al.*, 1977; Phaff, 1977) and indeed lytic enzymes, occurs during cell wall morphogenesis. Such lytic enzymes may effect limited hydrolysis of existing wall material before new growth; corresponding to enzymes activity during exponential phase. Similar findings have been reported for *C. albicans* (Notario *et al.*, 1982; Ram *et al.*, 1984).

Although mannan is a major component of yeast cell walls and is known to be subject to metabolic turnover (Pastor *et al.*, 1982) relatively little work on the occurrence of mannanases in yeasts and other fungi has been published (Fleet, 1984). In this study, higher levels mannanase activities observed in cell extracts during exponential growth phase supports the hypothesis that such enzymes are involved in normal metabolic turnover. No mannanase activity was detected in extracellular fluids in this study in contrast to the findings of Hough and co-workers (Maddox and Hough, 1971; Lyons and Hough, 1970) that mannanase activity is present in culture fluids and autolysates of *S. cerevisiae* and *S. carlsbergensis*. These authors noted a cyclical variation in activity, particularly associated with budding and a major increase in activity during the stationary phase.

The induction of mannanase activity at the onset of growth may be important for changing the structure of the cell wall to meet the requirements of the growth phase in which active cell wall plasticity is required for budding

and extension. Barrett-Bee *et al.* (1982) detected mannanase activity in cell extracts of *C. albicans* whilst Augustin *et al.* (1980) reported production of extracellular mannanase in *S. cerevisiae* and 47 other species of yeast grown in medium containing mannan as sole carbon source. However, several other workers were unable to detect mannase activity in either extracts of *S. cerevisiae* (Kratky *et al.*, 1975) or *C. albicans* (Gale *et al.*, 1980) and this has been suggested to be consistent with the observation that polymeric mannan, rather than mannose is released as the major product during autolysis of *S. cerevisiae* walls. However, other workers have drawn attention to the need for careful characterisation of substrate mannans, as minor levels of contamination with glucan, as found in crude mannans extracted from yeasts, may give misleading results in mannanase assays using crude enzyme preparations (Fleet, 1984). Mannanase activities determined in this study are in agreement with previously published reports of autolysis (Maddox and Hough, 1971; Lyons, 1970). Maddox and Hough (1971) found that complete degradation of mannan was not achieved and concluded that its interwoven lattice structure limits access of the enzymes to superficial layers or certain points in the matrix. On the other hand, Lyons (1970) reported that yeast mannan could be completely degraded by mannanases. In this project little turnover of mannan was observed although substantial mannanase activities were detected under experimental conditions similar to those described by Maddox and Hough (1971).

Barrett-Bee and Hamilton (1984) reported maximal chitinase activity in early exponential cells during different stages of growth of *C. albicans*. In this present study, higher chitinase activities observed in cell extracts prepared from exponential phase cells, supporting the proposal that chitinases may be involved in chitin remodelling during yeast growth (Barrett-Bee and Hamilton, 1984) and in cell division (Elango *et al.*, 1982).

Although chitinases have been proposed to have a role in fission of septa, leading to cell separation, it is not clear how the temporal and spatial regulation required for such a function would be achieved.

The parallel expression of both chitinase and chitin synthase activities during the growth cycle of filamentous cells of *M. rouxii* suggests a balance of such activities may be required for fungal growth (Lopez-Romero and Pedraza-Reyes, 1989). Moreover, a peak of chitinase activity early in growth of *M. rouxii* suggests these enzymes are involved in germination (Lopez-Romero and Pedraza-Reyes, 1989).

Since in conversion of *S. cerevisiae* cells to protoplasts, approximately half of total chitinase activity was liberated into the medium, Elango *et al.* (1982) concluded that a significant proportion of enzyme activity was located in the periplasmic space. As cycloheximide, which blocks secretion of mannan glycoproteins by protoplasts (Farkas *et al.*, 1970) and tunicamycin which inhibits glycosylation (Kuo and Lampen, 1974), had little effect on chitinase release, this enzyme appeared to be

normally present in the periplasm. Chitinases have been reported to be secreted and from their subcellular localization, may be initially sequestered in vesicles or vacuoles. Yet it is not clear what how chitinases are retained in the periplasmic space. Addition of digitonin is required for extraction of chitinases from intact cells but not when the cell wall has previously been destroyed with lytic enzymes. Furthermore the inability of periplasmic enzymes to act on the insoluble extracellular substrate would suggest that these enzymes act on nascent chitin secreted into the periplasm during septum formation.

Although chitinases have been isolated from the cytosolic fraction of M. rouxii, this does not mean these enzymes are cytoplasmic (Lopez-Romero and Pedraza-Reyes, 1989). Activity has been detected in the vacuoles and periplasmic space of S. cerevisiae (Elango et al., 1982). In N. crassa, chitinolytic activity was distributed in the cytosol (55%) and cell wall fractions (30%) with little activity in membrane fractions (Zarahin-Herzberg and Arroyo-Begovich, 1983). Polacheck and Rosenberger (1978) detected chitinolytic activity in walls of A. nidulans; Humphreys and Gooday (1984) observed activity in high speed supernatant and microsomal fractions from Mucor mucedo; and in mycelial cells of M. rouxii, activity was observed both in cell wall and mixed membrane fractions (Chagolla et al., 1987). Interestingly, particulate but not soluble chitinase required a phospholipid environment for maximum activity (Humphreys and Gooday, 1984). As chitin synthase behaved

similarly, these authors proposed that microsomal chitinase and chitin synthase may be co-regulated to play a morphogenetic role in hyphal growth.

However, Valerie Keer et al. (1989), studying C. albicans, did not find extracellular chitinases in growth media following 16 h of incubation suggesting that export of the enzyme to the periplasm was not followed by secretion. These authors, in studies with allosamidin an inhibitor of chitinase, found that concentrations of 30 µM had no effect on late exponential phase cultures suggesting that either it was not taken up by C. albicans or that in this organism the enzyme was not essential for growth. However, Humphreys and Gooday (1984) concluded that in M. mucedo. membrane-bound chitinase had a key role in growth and morphogenesis.

In the cell walls of filamentous fungi, chitin appears to be distributed more or less uniformly throughout the entire wall structure (Mahadevan and Tatum, 1967); in budding yeast, it is localized at the site of bud formation (Houwink and Kreger, 1953; Bacon et al., 1966). Bacon et al. (1969) reported problems in localizing chitin with specific enzymes although they used "cell ghost" preparations containing glucan and other polymers that may mask the effect of chitinases.

Chitin has been reported to be concentrated in a ring that surrounds the bud scar (Cabib and Bowers, 1971) which was confirmed in this study although the precise dimensions were difficult to determine. The polysaccharide appears to be accumulated in the crater ridge and extends for some

distance into the surrounding cell wall (Cabib and Bowers, 1971). This location suggests that the polymer is involved in the budding process (Marchant and Smith, 1968). These authors showed that during bud formation an electron transparent material accumulates around the neck or channel which connects parent and daughter cells and in later stages, the material appears to occupy more of the periplasmic space, squeezing the sides of the cytoplasmic membrane together. Finally a septum is formed, and the bud scar plug and most of the electron transparent material is seen in an annular space around the septum. This role of chitin in budding may be to provide a rigid ring to protect the channel between mother and daughter cells during the passage of the nuclear material and cytoplasm into the bud. Further production of chitin would assist in constricting the channel as the cytoplasms of the two cells become separated. The septa appears to be formed largely from glucan and perhaps mannan, interspersed with previously existing chitin as reported by Molano et al. (1980) for S. cerevisiae. After separation of mother and daughter cell, this region constitute the central plug of the scar with the chitinous ring preventing growth of the new wall into the mother cell (Cabib and Bowers, 1971).

The soluble chitinase activity in this study probably represents enzymes sequestered in lysosomal vacuoles (Iten and Matile, 1970) released during autolysis. Autolysis appears to be an extreme case of intracellular lysis, normally taking place in a specific cell compartment, the

lysosome (Matile, 1969). The limited chitinase activities observed in this study during autolysis supports the notion that these enzymes may have a restricted role in breakdown of cell wall material (Matile, 1969; Iten and Matile, 1970).

The existence of isozymic forms of chitinases has been described by different authors. In the hornworm *Manduca sexta*, chitinases have been separated by gel chromatography into three fractions with differing electrophoretic properties. Chitinase I and III were found to be glycoproteins while chitinases I and II were immunologically cross-reacting but chitinase III was not (Cabib, 1987). Tominaga and Tsujisaka (1976) purified two chitinases (I and II) from culture broths of *Streptomyces orientalis*. *Serratia marcescens*, a chitinase producing bacterium, has been shown to produce five chitinase species ranging from 21 to 57 kDa in molecular weight (Fuchs *et al.*, 1986). Purified chitinase preparations from *S. cerevisiae* have been resolved into several bands by PAGE (Correa *et al.*, 1982) which was attributed to differences in the carbohydrate moeity of these glycoproteins. Correa *et al.* (1982) reported *S. cerevisiae* chitinases were found to be composed of approximately 18% carbohydrate.

The temperature optima for *S.cerevisiae* chitinases has been reported to be 50 °C, which was confirmed in this study, and the optimum pH was 6.5, although chitinases have been found to act over a wide range of pH (Barrett-Bee and Hamilton, 1984). Dickinson *et al.* (1989) found an optimum temperature of 45 °C and of pH 6.5 for *C. albicans*

chitinase. Nascent chitin was found to be the preferred substrate for fungal chitinases (Lopez-Romero et al., 1982; (Chagolla et al., 1987) and those purified from wheatgerm (Molano et al., 1979), S. cerevisiae (Correa et al., 1982), N. crassa (Zarahin-Herzberg and Arroyo-Begovich, 1983) and S. marcescens (Vermeulen and Wessels, 1986). Other hydrolases such as chitin deacetylase (Davis and Bartnicki-Garcia, 1984) and ß-1,3 glucanase (Perez et al, 1984) also exhibit higher activities on chitin nascent polymers. The physiological implications, if any, of the preferential breakdown of nascent over preformed chitin by chitinases is still a matter of speculation (Cabib, 1987).

Proteolysis plays a major role in autolysis. In industry, the temperature of autolysis is usually greater than the death point of the yeast so that no proteinase synthesis can occur after the onset of autolysis. Maddox and Hough (1970), in studies of growth of yeast cells in different nitrogenous sources, found proteolytic activity present if proteins were used was absent when amino acids or ammonium salts were used as nitrogenous source. In industry, yeasts for autolysis are grown on largely utilising ammonium salts as nitrogen sources. In the model system as in this study peptones were used in growth media, in an attempt to ensure synthesis of the full complement of proteolytic enzymes. Protease activities observed during autolysis in this study supported the hypothesis that autolysis proceeds with the accumulation of both polypeptides and amino acids (Vosti and Joslyn, 1954; Hough and Maddox, 1970).

It is interesting to speculate on the origin of proteolytic enzymes involved in autolysis. If yeast protoplasts are prepared by degrading the cell wall with snail-gut juice, the protoplasts that emerge can be readily lysed to yield four particulate proteolytic enzymes (Maddox and Hough, 1969). However, these enzymes lack the carbohydrate moeity found on autolytic glycoprotein proteases. Matile and Wiemkem (1967) prepared intact vacuoles from yeast protoplasts and demonstrated that these contained high specific activities of proteases. The enzymes observed in these vacuoles were characteristic of those found in animal cell lysosomes, suggesting that these organelles have a similar function in yeasts.

It has been reported (Moor, 1967) that during the initial stages of asexual reproduction, vesicles accumulate at the site of budding and material is passed across the plasmalemma. These vesicles appear to be equivalent to the 'protease particles', described by Moor (1967), who suggested that these were derived from the endoplasmic reticulum and transport enzymes for weakening and dissolving the cell wall at the point of bud formation.

During autolysis at high temperatures, biomass will become a mixture of living, dying and dead cells. Initially, cells will be starved of nutrients and some will become moribund, leading to changes in plasma membrane permeability, releasing intracellular material into media. Living cells will respond to the appearance of such protein in the medium by secreting proteolytic enzymes (Maddox and

Hough, 1969). These proteolytic enzymes were probably derived from cell vesicles or lysosomes, released by reverse pinocytosis. A part of the proteolytic activity in the medium, however, will be derived from dead yeast cells and in parallel, other hydrolases will degrade macromolecules such as nucleic acids. This process will proceed within the yeast mass until eventually all cells will be dead and complete autolysis will take place.

Two proteases with optimum pH values of 3.7 and 6.2, liberated during autolysis of S. cerevisiae with chloroform, were described by Dernby (1918a,b). The former was extremely labile in urea solution and had no essential thiol groups whereas the latter was relatively stable. Two enzymes with similar pH optima were purified from autolysing yeast by Lenney and Dalbeck (1967) and Hata et al. (1967) described the purification of three such enzymes.

In autolysis four proteolytic enzymes are reported to be present (Maddox and Hough, 1970), generating a range of products, in yields differing in relation to temperature and pH. The optimal pH value for yeast extract manufacture is thought to be 6.0-6.5 which closely agrees with the autolytic condition used in this study.

Schulze and Colowick (1969) demonstrated that conditions of autolysis influence the nature of the hexokinase isolated from yeast and suggested that such isoenzymes may be artefacts resulting from differential action of proteases. This situation may be paralleled in other enzyme preparation involving yeast autolysis.

The proteolytic enzymes secreted by living yeast cells are glycoproteins, as in other higher organisms (Lampen, 1968), but in other respects these enzymes resemble those from lysed protoplasts rather than those from autolysing yeast, notably, in pH optima and temperature stability. This suggests that enzymes from autolysing yeast are of different origins from those of living yeasts or protoplasts. Where a cell wall is present, linkage of proteases to carbohydrates occurs (Maddox and Hough, 1970) and this may be either within the cell wall or in the region just inside the plasma membrane, where there is active synthesis of the cell wall material. Obviously in protoplasts, with no cell wall template, active synthesis of cell wall material does not occur. It is known that glycosylations of proteins take place during intracellular transit (Swenson and Kern, 1968). Vesicles accumulating at the site of bud formation and during enlargement of the bud wall (Sentandreu and Northcote, 1969), carry material for cell wall synthesis and may transfer proteases to the site of cell wall synthesis and the plasma membrane. Matile and Wiemken (1967), studying vacuoles isolated intact from yeast protoplasts, observed the presence of proteolytic and other enzymes at high specific activities which were released with the approaching death of cells. These enzymes could be distinguished from other extracellular enzymes by their prefered digestion of mother cells rather than potential nutrients in the media (Maddox and Hough, 1970).

However, proteases appear to have a number of specific

roles in living yeast. Cabib _et al._ (1974) and Cabib (1976) have shown that membrane-bound chitin synthase essential for yeast budding is activated by a proteases that convert an inactive precursor to the active enzyme. Proteases may also be involved in inactivation of such enzymes as malate dehydrogenase, alcohol dehydrogenase and hexokinases (Betz and Weiser, 1976) and such activities may be part of the normal cell cycle (Betz and Weiser, 1976; Esposito _et al._, 1969; Klar and Halvorson, 1975). A mutant low in proteinase A activity was found to be unable to sporulate suggesting that these enzymes are involved in this process (Betz and Weiser, 1976; Betz, 1976; Klar and Halvorson, 1975). Small vacuoles containing proteases (Holzer, 1976; Holzer and Saheki, 1976) may fuse with the plasmalemma prior to the appearance of activity and at sites certain enzymes may be activated by proteases (Matern and Holzer, 1977; Goerts, 1969). Another interesting process probably controlled by limited proteolysis is the assembly of cytochrome oxidase from precursor proteins (Poyton, 1977; Holzer, 1978). However, it is not known whether this limited proteolysis is catalysed by mitochondrial or vacuolar proteases.

In these studies, purified cell walls that had been thoroughly washed, were found to autolyse further in incubations for up to 24 h. It can be concluded that endogenous enzymes continued to act to solubilise a number of cell wall components. Similar observations have been reported previously (Fleet and Phaff, 1974; Reichelt and Fleet, 1981; Hien and Fleet, 1983; Notario, 1982). Such

results indicate that yeast glucanases may act to hydrolyse wall glucans "in vivo": these lytic enzymes are wall bound and contribute to wall breakdown. However, only a fraction of cell wall material was solubilised in autolysis. Pertinent questions can be asked about the nature of this association and the role of these enzymes _in vivo_. In particular, one might ask if the enzymes when associated with the cell wall were normally inactive, and were activated under certain circumstances. No clear answer to these questions is available at present. However, it is clear that the association is so stable that high salt treatment of isolated cell walls does not remove the enzymes that can solubilised by autolysis (Nombela and Santamaria, 1984; Fleet, 1984). However, isolated cell walls never completely solubilised during autolysis and continue to maintain their integrity. This observation, coupled to the observation that yeasts vary in the glucanases secreted in autolysis underscore the complexity of the ß-glucosidic linkages in yeast cell wall structure (Fleet, 1984).

Yeast cells undergoing autolysis at elevated temperatures, such as 50 °C, in nutrient deficient conditions are known to accumulate degradation products with granulation and globule formation in the cytoplasm (Vosti and Joslyn, 1954). However, these authors observed no lysis. Similar observations were reported by Shihata and Mark (1951) during digestion of baker's yeast by _Drosophila_. Despite partial hydrolysis, cell walls of _S. cerevisiae_ remained intact after autolysis (Babayan _et al._, 1981)

although low molecular products of protein and nucleic acid decomposition were released into the medium (Trevelyan, 1976). Thus, rigidity and resistance to lysis of the yeast cell wall has also been reported by Potgieter and Alexander (1965) and Houwink and Kreger (1953).

Reductions in cell viability observed in this study supports the notion that death must preceed autolysis (Vosti and Joslyn, 1954) although it does not necessarily follow that all dead cells will autolyse. In this study, fluorescence microscopic studies of normal and autolysing cells using calcufluor revealed chitin in, and around, the bud scars although no significant turnover of cell wall chitin during autolysis was observed. The presence of chitin in bud scars and the cell wall was consistent with the findings of Bauer *et al.* (1972), Cabib *et al.* (1974), Horisberger and Rosset (1976) and Horrisberger and Vonlanthen (1977). Horrisberger and Vonlanthen (1977) found that, in *C. albicans*, gold-linked wheat germ agglutenin reacted mainly with chitin and its oligomers and not with glycoproteins containing ß-(1,4)-N-acetyl-D-glucosaminyl residues. These authors observed chitin in bud scars and in cell walls adjacent to the plasmalemma. This confirmed that the presence of glucosamine in the yeast cell wall was not limited to the bud scars (Cabib *et al.*, 1974; Bauer *et al.*, 1972).

In this study scanning electron microscopy revealed significant differences between normal and autolysing yeast cells. Normal cells appeared to have smooth exterior

surfaces whereas autolysing yeasts were rougher and had distorted bud scars. Transmission electron microscopy revealed lysis was preceded by general reduction in electron density of cell walls in autolysing cells. Although it has often been suggested that bud scars will form weak elements in the cell wall, more susceptible to degradation, no preferred point of lysis was been observed in light and electronmicroscopy studies of autolysing cells. It was further confirmed by analysing cell wall carbohydrates of the normal and autolysing yeast cells that glucan and mannan were broken down during autolysis, as reported by Maddox and Hough (1971) while chitin breakdown was insignificant. This study however, contradicts the findings of other workers. Al-Shahwani (1979) suggested that the bud scar is the site of lysis and Bowers *et al.* (1974) reported rupture of cell walls at the site of bud initiation in *S. cerevisiae* as has been observed at hyphal apices of *M. rouxii* (Bartnicki-Garcia and Lippman, 1972a), in the presence of polyoxin D. 2-Deoxyglucose, an inhibitor of cell wall hexosan synthesis (Farkas *et al.*, 1969) in yeast was reported to cause cell lysis (Megnet, 1965) at the postulated original site of cell wall growth (Johnson, 1968).

To clarify this matter, microscopic observations must be discussed together with aspects of enzyme activity, bud scar morphology and cell wall structure.

Many authors have set out to study the relationship between enzyme activity and cell geometry in yeasts and other fungi. Weiss *et al* (1975) investigated the

relationship between activity of cell surface enzymes and cell fitness with respect to haploidy and diploidy, and concluded that the basic biochemical parameters of cells are determined primarily by cell geometry rather than ploidy level. Bartnicki-Garcia (1972b) and Bartnicki-Garcia and Lippman (1969) reported that the pattern of wall synthesis is probably the primary determinant of cellular shape. Cabib (1976) considered the role of chitin synthase central in the formation of the primary septum in S. cerevisiae, a model of cell wall morphogenesis, since septum initiation appears to take place by localized activation of enzyme attached to the plasmalemma. Altered morphology of autolysing cells in this study might involve the participation of a number of lytic enzymes such as ß-glucanases, mannanases, and possibly chitinases and proteases, although evidence for the latter was scant.

In budding, the mother cell is left with a bud scar and the daughter with a birth scar (Matile et al., 1969). Cabib and Bowers (1971) confirmed the presence of chitin in the bud scar and described as a shallow crater with a raised rim. Streiblova and Bevan (1963) investigated bud scar morphology using fluorescence microscopy. Aberrant raised curved structures found on the surface of the normal and autolysing cells in this study were probably the bud scars referred to by Al-Shahwani and Berry (1979).

At the time of separation of daughter from mother cell, a primary septum made of chitin is developed to delineate the cells (Cabib and Bowers, 1971; Cabib and Farkas, 1971).

This is followed by formation of a secondary septum resembling the remainder of the cell wall in appearance and composition, on either side of the primary septum (Cabib and Bowers, 1971; Cabib *et al.*, 1974). When cells separate, the primary septum is retained by the mother cell forming the exterior surface layer of the bud scar (Cabib *et al.*, 1974; Cabib and Bowers, 1975). In this extensive study, no evidence was observed for prefered sites of rupture associated with the bud scar, although some distortion of the bud scar structure was observed.

Hartwell (1974) showed that at an early stage in the growth cycle, a discrete morphological entity, the bud, appears on the yeast cell at a site which becomes a channel connecting mother and daughter cells. In formation of this structure local destruction of cell wall is necessary, involving such hydrolytic enzymes as ß-glucanases (Cortat *et al.*, 1972, 1973) and mannanases (Maddox and Hough, 1971; Phaff, 1971). Since ß-glucanases, mannanases (Maddox and Hough, 1971) and chitinases (Iten and Matile, 1970) are also involved in autolysis, it must be concluded that this study has shown that predominantly glucan and mannan, and relatively little chitin, is degraded during autolysis. This leads to general erosion of the cell wall followed by rupture at a point not predetermined by the structure of the yeast cell wall.

Yeast extract and autolysates contain proteins, amino acids, carbohydrates, lipids, vitamins and minerals (Davidek *et al.*, 1979). In current industrial practice, α-amino

nitrogen content is regarded as most useful index of flavour potential. Nucleic acid derivitives from RNA autolysis imparts characteristic "meaty flavour" to yeast products (Moore, 1977). The amino acids present in the yeast extract usually contribute to imparting flavour and aroma of the yeast extract (Albrecht and Deindoerfer, 1966). In particular, the balance of amino acids and mono- and disaccharides available for the Maillard reaction will have major impact on individual product flavours and characters.

Autolysis is reported to be induced by temperature (Peppler, 1967; Hough and Maddox, 1970; Reed and Peppler, 1973; Prescott and Dunn, 1982 and Hill, 1981) which was also observed in this study. Different temperatures appear to contribute to different functional properties for flavour development as they can influence the ratio of products of Maillard browning reactions (Darrington, 1986). Temperature and pH not only determine the rate and degree of autolysis but also the flavour potential of resulting autolysates (Hough and Maddox. 1970). Maillard reaction is known to be influenced by temperature with carbonyl groups, usually from reducing sugars, condensing with free amino groups, most commonly from peptides and proteins.

The extent of the Maillard reaction depends on temperature, as well as pH, moisture content and time. These factors are known to determine colour and flavour potential of processed foods (Ames, 1988). Although usually associated with application of elevated temperatures, non-enzymic browning can also occur at ambient temperature during

storage.

The aroma character of autolysates will be determined by the concentrations or relative concentrations of reaction substrates available for condensation. Thus, it is clear, from this study, that variations in time and /or temperature of autolysis will produce differences in yeast product aroma when autolysis is a component of the manufacturing process.

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9. REFERENCES

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