THE PROCESS SCALE RELEASE OF INTRACELLULAR ENZYMES FROM FILAMENTOUS MICROORGANISMS

by

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To

My Parents

ABSTRACT

The impact of fermentation and processing conditions on the disruption of a strain of *Aspergillus niger* for the release of intracellular enzymes has been examined. High pressure homogenisers were used, and the objective was to seek the optimisation of the fermentation, harvesting and disruption stages to maximise the production of intracellular enzymes. One major variable studied was the effect of cell morphology on enzyme release.

The control work centred on filamentous and highly entangled or "clumped" microorganisms and here, the release of intracellular protein and enzymes was shown to be highly pressure dependent, and a weak function of the number of passes (after the first pass) through the homogeniser. Complete release of protein and glucose-6-phosphate dehydrogenase (G6PDH), a freely soluble cytoplasmic enzyme was achieved after 2 or 3 passes at 80 MPa. Glucose oxidase (G.O.), a peroxisomally (membrane bound organelle) located cytoplasmic enzyme follows similar kinetics but was only released fully at 100 MPa.

The morphology was changed to pelleted forms of up to 1.8 mm diameter by a decrease in spore inoculum concentration. This change in morphology from the "clumped" form did not alter the shapes of the disruption curves although there was a trend towards greater rate of release with increasing pellet size: e.g. 30-40 % more protein was released for pellets of 1.8 mm diameter at 60 MPa for a single pass, compared to the filamentous "clumped" morphology.

"Junlon", a polyacrylic resin, incorporated in the growth medium, led to a more freely filamentous morphology and a 20% increase in G6PDH release. However, filamentous growth arising from a reduction in agitation speed did not result in any change in the rate of release.

The effect of fermentation broth conditions on protein and enzyme release was also studied, the most important result being the effect of a change in the resuspending buffer for disruption from Tris HCl at pH 7.5 to an acetate buffer at pH 5.2. This led to the precipitation of 90% of the released protein, but not any of the glucose oxidase, and hence a considerable increase in the specific activity of the released enzyme.

A mechanism of disruption for filamentous microorganism is postulated. During the first pass, there could be disentanglement of the mycelia resulting in disruption. At subsequent passes, the disruption kinetics appears to follow first order kinetics. This two stage process adequately describes the release of protein, G6PDH or G.O., the model equation being:

$$(R_1 / R_m) = k_1 . P^a \qquad \text{(model for N=1)}$$

where R_1 is the amount of product released at the first pass (N=1); R_m is the maximum product released; k_1 is a rate constant; P is pressure and "a" is the pressure exponent. For subsequent passes (N > 1) the disruption can be described by a first order rate equation:

$$\log_{10} [(R_m - R_1) / (R_m - R)] = k_2 . P^b .(N - 1) \qquad \text{(model for N > 1)}$$

where R is the product released at N number of passes; k_2 is another rate constant, and "b" another pressure exponent. These two models are applicable to all the various morphologies, thus indicating a similar mechanism of disruption. It is the rate constants and pressure exponents which change with the different morphologies.

The implications of this study on further purification and process development are discussed.

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1.0 INTRODUCTION

1.1 Project Objective and scope

The recovery of biological products from fermentation broths of filamentous microorganisms strongly depends on the interaction between the morphological properties of the organism and the process used for microbial recovery. Such products can either be extracellular or intracellular. This will determine the process choice. In general the recovery of extracellular products is simpler than intracellular products. The latter has to be extracted, which tends to result in the release of other unwanted intracellular components such as nucleic acids and proteases, if the cell wall is broken. This creates problems in further purification steps. Therefore this work will focus on the isolation of intracellular products at the first step - cell disruption.

The objective of this project is to examine how fermentation conditions can best be manipulated to facilitate the mechanical release of enzyme product from the cells. This will examine the sensitivity of the cell disruption process in relation to the operation of a fermenter, and identify the properties of the fermentation broth which determine the resuspension and disruptability of the organism. The *Aspergillus sp.* has been chosen as the model organism because of its established industrial importance and its increasing use as an expression system for foreign gene products.

1.2 Impact of fermentation conditions on the properties of filamentous microorganisms.

1.2.1 Morphology of filamentous microorganisms

Moulds, some yeasts and actinomycetes usually grow by a process called hypha chain elongation, and branching, as shown in fig. 1.1. The hypha is a branching tubular structure, of 2-10 µm diameter for fungi, and 0.5-1.5 µm diameter for actinomycetes such as *Streptomyces sp.* The intertwining strands of hyphae are called mycelium. The hypha itself can be divided into individual cells, by septa, such hypha being termed septate, as seen in actinomycetes and fungi in the classes Ascomycetes, Basidiomycetes and Deuteromycetes, which includes *Penicillium sp.* and *Aspergillus sp.* Hypha without septa are termed aseptate, and is found in fungi such as *Rhizopus sp.* A septate hypha is composed of an apical compartment and a series of intercalary compartments. The septa can have pores which allow the passage of cytoplasm, nuclei, and other organelles from one compartment to the next.

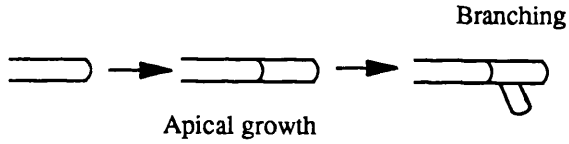


Fig. 1.1.: Schematic diagram of mycelial growth by branching and chain elongation.

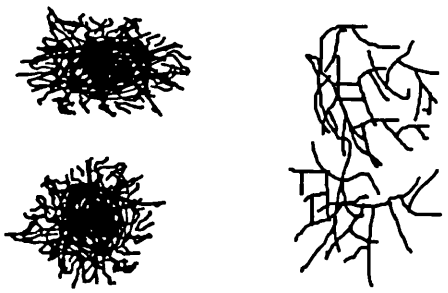


Fig. 1.2.: Forms of fungal growth : (a) pelleted growth ; (b) filamentous growth

There are two forms of mycelial growth in submerged cultures. Each form depends very much on the growth environment, inoculum size and other factors. The first is a network of disperse, filamentous mycelia, and the second is a pelleted form (Fig. 1.2). Filamentous growth is usually exponential since all the hyphae are exposed to medium, and the length of hyphae varies depending on the shear forces present in the environment. Filamentous growth can also be divided into long filamentous and short fragmented mycelium.

However, pelleted growth is not exponential because nutrients cannot diffuse into the pellet fast enough to maintain growth of the whole biomass. In general, pelleted growth is undesirable, due to the mass transfer problems, which cause extracellular product release to be more difficult. Cells within the pellet experience a different environment than those on the periphery. This results in formation of nutrient and product profiles within the pellet, as shown in fig. 1.3. At the centre there is little or no nutrient, but product concentration is high. As a consequence, there is oxygen and possibly other nutrient limitations, resulting in cell death and possibly autolysis, giving rise to an empty space at the centre. However this limitation depends on the size of the pellet, because extremely small pellets will not be affected. *(Clark 1962)*

In order to characterise morphology of filamentous microorganisms, a number of morphology indices have been introduced by *Metz. et al (1981)* These are (fig. 1.4.) :

(a) length of main hypha or effective length, L_e

(b) total length of all hyphae of a particle, L_t

(c) mean length of branches, L_b ; (d) mean length of the segments, L_s

(e) number of branches, n ; (f) hypha diameter, d

(g) hyphal growth unit : the length of hyphae per growth tip, L_{hgu} :

$$L_{hgu} = L_t / (n + 2)$$

(h) dimensionless effective length, $L_e^* = L_e / d$

The dimensions that were actually measured were :

(i) length of main hypha; (ii) hyphal diameter; (iii) length of branches;

(iv) number of branches; (v) length of segments (length from tip to first branch point)

Other indices were calculated from these dimensions.

Recent work by *Adams et al. (1988)* and *Packer et al.(1988)* have shown that characterisation of mycelial morphology using an image analysis method has a great advantage over the digitising table used by *Metz et al (1981)*, in terms of increased speed and potential for complete automation. The image analysis method has been used to characterise *Streptomyces sp.* morphology and estimate clumping of mycelia ie. aggregates of entangled mycelia, in *Penicillium chrysogenum.* With simple filamentous structures such as *Streptomyces sp.* which have few branches of short length and are not extensively entangled, the characterisation performs well. However with highly pelleted or entangled mycelia such as found in *Aspergillus sp.*, it may present difficulties in distinguishing separate branches.

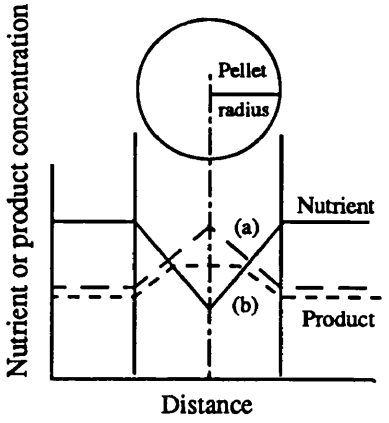


Fig.1.3.: Nutrient and product concentration profile in a mycelial pellet. Product made in the centre of the pellet will appear as (a). If cells in the centre are dead or starved, the profile will appear as (b).

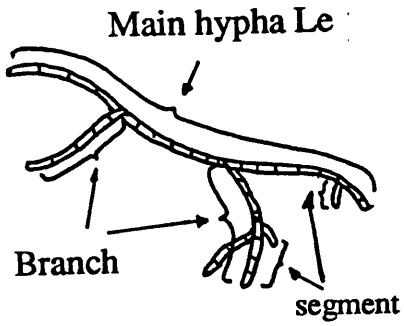


Fig. 1.4.: Schematic representation of a mycelial particle.

1.2.2 Effect of fermenter design on morphology

1.2.2.1 Agitation and aeration

The effect of these two parameters on the morphology of filamentous microorganisms is significant, but there have been few studies on concentrating on this topic. Several workers have noted changes in morphology caused by variations in agitator speed and aeration.

Dion et al (1954) studied the changes in hyphal dimensions of *Penicillium chrysogenum* under different agitation and aeration conditions. Two types of mycelia were distinguished:

1. At **low agitator speeds**, mycelia of a **"long, filamentous"** type were observed. The hyphae were long with few branches, a tapered apex, and the main hypha length was greater than 250 µm, with a diameter of 2-3 µm.
2. At **higher agitator speeds** (greater than 600 rpm), **"short, fragmented"** mycelia were observed. These were short, highly branched, thick hyphae, forming small compact units which increased in compactness with increasing agitation intensity. The length of the main hyphae were about 40-250 µm and the diameter 2.5-4 µm, with a strongly tapered apex. Autolysis, and fragmentation also increased with agitator speed : in 10 litre fermenters, autolysis was observed at 24 hours, at 800 rpm, whereas this only took place after 70-80 hours at 350 rpm.

An increase of scale from 10 to 3000 litres and 12000 litres, at low agitation of 350 rpm (for 10-

3000 L) and at the same power per unit volume for the 12000 L fermenter, resulted in no significant change in the hyphal type of long filamentous mycelia.

Other workers have found similar effects. *Carter and Bull (1971)* showed that *A.nidulans* had shorter more branched hyphae at high agitator speeds : in a 1 litre fermenter at 900 rpm, the mean hyphal segment length was 210 rpm, compared to 50-70 μm in a 3 litre fermenter at 1440 rpm. *Righelato et al (1968)* found that under continuous culture *P.chrysogenum* underwent fragmentation in a 2 litre vessel at 1440 rpm, when nutrients were supplied at a maintenance rate to keep it in a non-growing state. Both *Dion et al (1954)* and *Morrison and Righelato (1974)* have found that hyphae length in mechanically agitated fermenters were shorter than those in shake flasks, which indicate that shear damage is likely in the former.

More recent work by *Van Suijdam and Metz (1981)* examined the effects of agitation and aeration on *P.chrysogenum* by developing a semiautomatic method to measure the hyphal lengths and then calculating morphological indices such as hyphal growth unit length. (more details in Section 2.1) They also tried to fit the data to models based on dispersion of physical systems. Results showed that an increase in the energy dissipation per unit mass (which represents shear stress), caused a decrease in mean length of hypha, mean total hyphal length, mean hyphal growth unit length and mean dimensionless length. This agreed with the models qualitatively but failed quantitatively, possibly due to variation of tensile strength of hyphae with age and culture conditions. It was also noted that, the influence of shear stress on hyphal length was limited : a great increase in energy input was necessary to cause a significant decrease in hyphal length.

Mitard and Riba (1988) studied the effect of shear rate on the morphology and growth of *A.niger* in an annular reactor. They observed that growth at rotational speeds below 1500 rpm was initially in a pellet form which subsequently broke up as the shear stress reached 5 Pa, to liberate filaments which grew separately. However, at 1500 rpm, no initial pellet growth was noticed, but after a certain time, there was formation of highly compact pellets of about 50 μm diameter with highly branched filaments on the surface. They interpreted this structure as a growth form which offers good resistance to high shear damage. In addition, a study of the specific growth rates at various rotational speeds, showed two peaks of high growth rate. These peaks were related to the formation of pellets initially, which formed the first growth rate peak; followed by breakage of these pellets, resulting in decrease of growth rate; and then a revival of growth, as the fragmented pellets grow, to form the second peak. Furthermore, with increasing rotational speeds, the first peak corresponding to pellet growth gradually "disappears" in comparison with the second, and does so after a shorter time too. This was attributed to earlier breakup of pellets at high shear rates, and eventually at 1500

rpm, no pellets are formed. At 500 and 745 rpm, there was very little decrease or stagnation of growth rate, with the second peak giving the highest growth rate (compared to lower and higher shear rates). So, it may be that at this speed, pellet formation is not hindered by shear stress effects, as observed at 1500 rpm, where growth rate was stagnant for a long period. This study provides some insight into the morphological development during growth, but a more quantitative method of defining morphology would have been better.

Further recent studies by *Belmar Campero and Thomas (1988)*, on the effect of stirrer speed from 490 to 1300 rpm, on the morphology of *Streptomyces clavuligerus* showed that the rate of change of morphology from long highly branched mycelia to shorter less branched fragments was increased by increasing stirrer speed.

Zetelaki and Vas (1968) found that *A.niger* grown in aerated medium had thicker cell walls with well defined outlines whilst oxygenated cultures seemed to have ill defined cell walls. In addition autolysis occurred earlier in oxygenated cultures, and at a much faster rate. This indicates weaker cell wall structure when aerated by oxygen. Studies by *Carter and Bull (1971)* on *A.nidulans* showed no effect of increased oxygen tension on mean hyphal segment length or branching frequency.

1.2.2.2 Medium

The morphology of filamentous microorganisms is known to be very dependent on the environmental conditions such as pH, and constituents of a growth medium. Especially with complex media, the variability of constituents in media such as molasses or yeast extract can have a significant effect on the morphology. Therefore selection of media constituents has become an empirical approach, and once a suitable media is found, any change is usually avoide.

Various studies have been made, some of which will be dealt here.

pH effects : *Galbraith and Smith (1969)* found that *A.niger* grown in shake flasks was only filamentous below a pH of 2.3 whilst pellets were formed in more alkaline conditions. They suggested that the pH influenced the coagulation of spores: coagulation was significant above pH 2, thus causing pellet growth. *Miles and Trinci (1983)* showed the hyphal growth unit length of *P.chrysogenum* grown in a chemostat varied with pH, attaining a maximum value of about 110 µm at pH 6, compared to about 50-75 µm at other pH values. They also showed that at 30 C mycelia had significantly thicker cell walls and longer hyphal growth units, than at lower temperatures. *Pirt and Callow (1959)* hyphal length decreases with increase in pH, but found that growth was filamentous at pH 6, and pelleted above this value, with swollen cells forming above pH 7, for *P.chrysogenum* in continuous culture.

Media constituents : A change in nitrogen source from ammonium sulphate to cornsteep liquor in the growth of *P.chrysogenum* in continuous culture resulted in an increase in hyphal length, reduced frequency of branching and prevented formation of swollen cells at pH 7.4 *(Pirt and Callow 1959)*. Significant work has been done on citric acid production by *A.niger*. It is well known that, only when the fungus grows in a hard, compact pellet form with short, branched, knotty hyphae, that citric acid is produced in high quantities. This morphology is formed under conditions of very low pH (1.5-2.8), a temperature of 28-33°C, and a deficiency of certain trace elements such as manganese *(Sodeck et al 1981)*. A study by *Kisser et al (1980)* showed that manganese deficiency (less than 10^{-7} M) resulted in abnormal morphology having squat, bulbous hyphae which formed smooth, dense pellets. This same effect was also produced by an excess of copper ions. Both trace elements, caused an increase in chitin and a reduction in B-glucan contents in the cell wall. *Byrne and Ward (1989)* found that peptone at 10-20 g L^{-1} caused clumped coalesced growth ie. no dispersion on dilution, whilst below 5 g L^{-1} caused loose, fluffy pellets of less than 1 mm diameter, but the reduced peptone also resulted in decreased biomass. They also found that decreasing glucose concentration with peptone at 5 g L^{-1} retained the pelleted growth but increased the number of pellets and did not affect biomass production significantly.

Other substances such as polymers have been observed to affect morphology. *Elmayergi et al (1973)* found that an addition of 'carbopol' caused a more filamentous dispersed growth of *A.niger*, possibly by the induction of electrostatic repulsion among the spores caused by ionised carboxyl groups. Less agglutinated spore clusters seemed to give more dispersed growth. Similarly, *Morrin and Ward (1989)* changed the morphology of *Rhizopus arrhizus* from clumped pellets to dispersed mycelia and clumped mycelia using carboxymethyl cellulose (CMC) and carbopol-934 respectively. This morphology change resulted in increased and decreased conversions of progesterone by the CMC and carbopol grown cells, respectively. Similar trends were also observed for fumaric acid production.

Hermersdorfer et al (1987) found that slowly metabolised carbon and nitrogen sources such as sugar beet, potassium nitrate or urea, caused pellet formation whereas quickly metabolised ones such as glucose and ammonium ions produced more filamentous growth. (Fig. 1.5)

1.2.2.3 Inoculum

In general it is known that a high inoculum concentration of spores gives filamentous mycelium , while low concentrations produce pellets. The cause is not known, but it may be due to the interaction of hyphae in early stages of growth could be important in determining final morphology. Therefore with high concentration there is this interaction earlier on in growth, but not so with a low concentration of spores.

Recent work by *Gomez et al (1988)* on pellet growth of *A.niger* and the citric yield in a 5 L fermenter, showed that an inoculum of spores gave rise to cultures with a filamentous morphology, either long filaments or a mixture of filaments with a small proportion of fluffy loose pellets, depending upon whether, the stirrer speed during the first 48 hours of culture was 450 or 1000 rpm. A stirrer speed of 1000 rpm maintained throughout the fermentation produced loose pellets. On the other hand an inoculation with a pre-culture of pellets gave pellets at all stirrer speeds. For stirrer speeds of 450 rpm and an initial speed of 450 rpm for 48 hours, followed by 1000 rpm, the morphology was large, gelatinous pellets and small compact smooth pellets. However for a stirrer speed of 1000 rpm, only small, compact, smooth pellets were formed. Therefore it seems that the form of inoculum and agitation conditions in the initial stages of fermentation can affect morphology.

A.niger producing polygalacturonase was studied by *Hermersdörfer et al (1987)*, who confirmed that in shake flask cultures, a low density of spores stimulates the formation of few but large pellets, and an increase in spore concentration leads to large number of small pellets and favours diffuse mycelium. Fig 1.5 shows various effects of culture conditions on morphology.

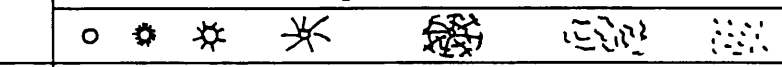
Parameters of cultivation	Morphological variance		
			
Strain material	*Aspergillus niger* strain		
	J_{36}	J_{41}	$R_{1/214}$
Density of inoculation (spores ml^{-1})	$10 - 10^2$	10^3	10^5
Carbon and nitrogen sources	metabolisation		
	slow: sugar beet, KNO_3, urea		fast: glucose, NH_4^+
	concentration		
	low		high
Temperature (°C)	25	30	35
Shaking frequency (rpm)	140	180	220
Cultivation time (h)	48 - 96	120 - 168	192 - 360

Fig. 1.5 : Dependence of pellet mycelium disintegration into diffuse mycelium on certain culture conditions (Hermersdörfer et al 1987)

1.2.2.4 Fermenter scale

This parameter has not been studied, when it comes to its effect on morphology. Morphology changes may be associated with the rheological properties exhibited by the form of growth. In the case of filamentous growth, the broth is usually non-Newtonian (pseudoplastic and very viscous). In a large fermenter, the mixing may not be so extensive, thus generating areas of intense mixing or high shear rates at the impeller region, and areas of low shear rates or poor mixing, close to the fermenter wall. Therefore filamentous growth near the impeller may form short, thick hyphae or even pellets, as a resistant form to shear, while growth near the wall could be more dispersed filaments with longer hyphae. On the other hand, pelleted growth is close to Newtonian behaviour and far less viscous, leading to easy mixing with no stagnant zones, and possibly resulting in no variation of morphology in the fermenter itself.

The development of morphology depends on so many factors, that it is very difficult to resolve effects to any particular source, since interactions of conditions will have combined effects. Fig. 1.6 shows the several factors. *(Metz and Kossen 1977)*

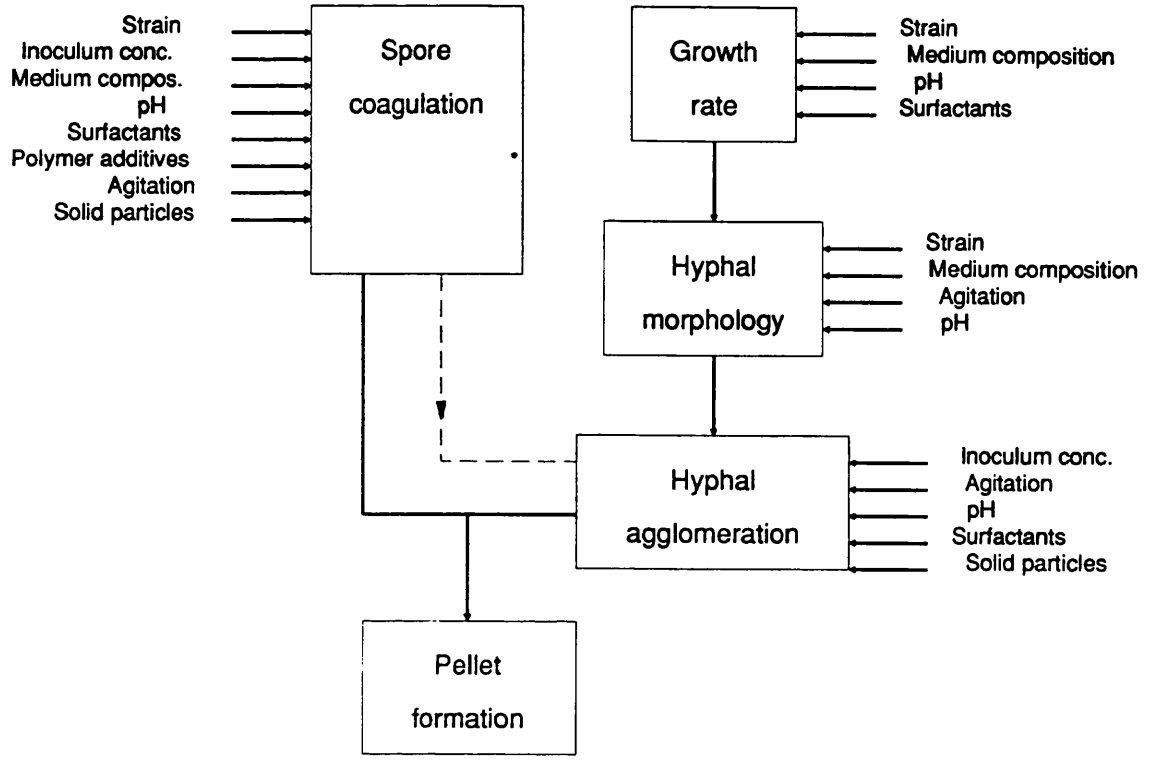


Fig. 1.6 : Block diagram representing factors influencing pellet formation (Metz and Kossen 1987)

1.2.3 Effect of morphology on the rheological properties of fermentation broth

It is well known that filamentous morphology contributes to a non-Newtonian, highly viscous broth, whereas pelleted morphology gives a Newtonian, less viscous broth. The former due to its pseudoplastic rheology leads to regions of low viscosity at high shear rates around the impeller and high viscosity in regions with low shear rates, near the wall. Highly viscous broths cause significant problems in mass transfer of oxygen and other substances, heat transfer and bulk mixing. Therefore it is important to control the rheology by controlling morphology. Many studies on the rheology of mycelial broths have been made, and some have related morphology to rheology. Fig. 1.7 shows the numerous factors affecting the rheology of mycelial suspensions, and the complexity of the interactions.

Metz et al (1979) made a detailed study on the various properties of filamentous mycelia which influence the rheological behaviour, and found that the Casson equation gives a good description of the rheology. A relationship was shown between the constants in the Casson equation and the morphological properties of *Penicillium chrysogenum*, in terms of a dimensionless length of hypha (length of main hypha/diameter), which can be thought of as a measure of "stringiness", and the hyphal growth unit (total length of hyphae/number of growth tips) which is a measure of branching frequency.

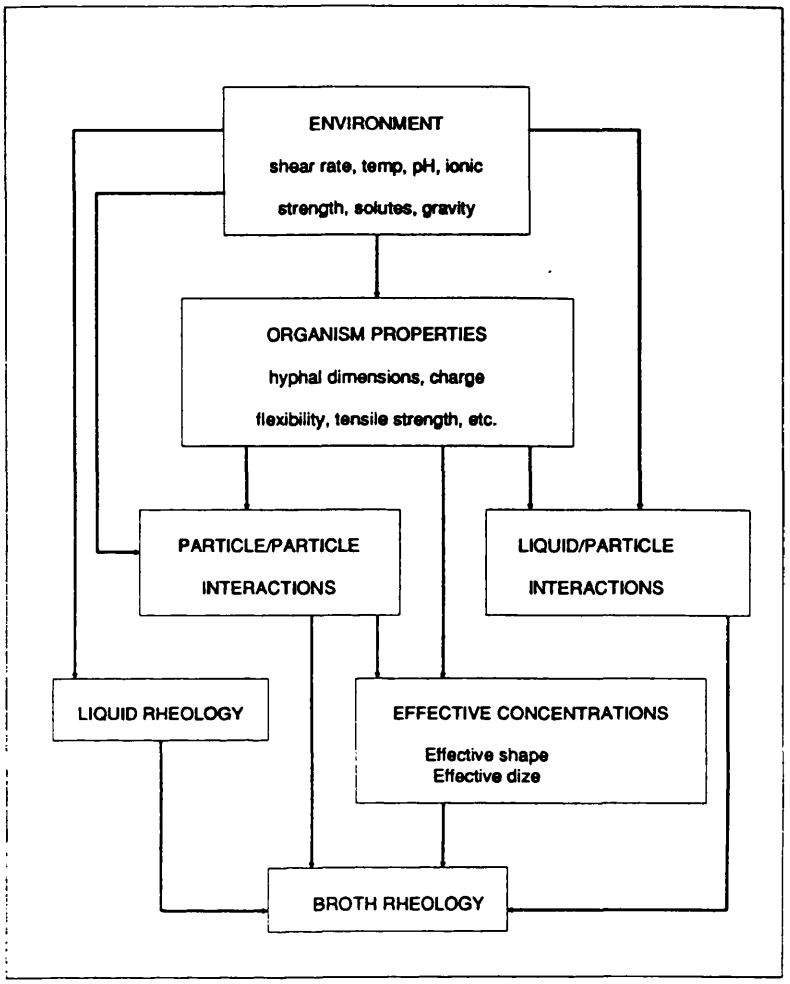


Fig. 1.7.: Factors affecting the rheology of suspensions of mycelial microorganisms.

Roels et al (1974) presented a model for the rheology, which included the influence of morphology and biomass concentration based on the Casson model. However, assumptions such as there is negligible interaction between branched hyphae, and the hyphae are flexible chains forming a spherical coil have met objections. Nevertheless *Fatile (1985)* used the model of Roels et al (1974) to measure the shape of the mycelial aggregate, and showed that *A.niger* suspensions were non-Newtonian and obeyed the power-law equation, with the consistency index increasing with the increase in size of the aggregate.

A comparison of the rheological properties of the filamentous and pelleted forms of *Absidia corymbifera (Kim et al 1983)* showed that the former behaved as a pseudoplastic fluid whereas the latter behaved as a Newtonian fluid up to a mycelial concentration of 28 g/L (dcw). For the filamentous form, non-Newtonian behaviour predominated at low shear rates and high mycelial

concentrations. This was attributed to the need for greater energy for deforming highly entangled mycelia, and breaking up the flocs of mycelial networks at lower shear rates but at higher shear rates, this was not necessary. Pelleted structures at higher than 28 g/L (dcw) mycelial concentration, exhibited non-Newtonian shear thinning type behaviour but at high shear rates, Newtonian behaviour resumed, which was thought to be due to complete breakdown of loose flocs of pellets to form single, rigid particles. The pseudoplastic model, $\tau = K.\ \gamma^m$ (where τ = shear stress, γ = shear rate, K = consistency index and m = flow behaviour index), used for both pelleted and filamentous forms where non-Newtonian behaviour was observed, gave values of K which were higher for the filamentous form, than the pelleted form, indicating that pelleted suspensions are far less viscous than the filamentous ones.

Work by *Mitard and Riba (1986)* on the rheology of pellet suspensions of *A.niger* showed that at low pellet concentrations, Newtonian behaviour was observed, but an increase in concentration, resulted in the appearance of a yield stress, therefore behaving as a Bingham plastic, and further increase in concentration caused pseudoplastic or plastic behaviour to form. With variations in pellet diameter, smaller pellets gave higher apparent viscosities (for any concentration) than larger pellets, since for a given dry weight or pellet volume fraction, the number of particles is higher for the smaller particle size. With different aged pellets, the "younger" pellets were smoother, whilst the "older" pellets were fluffy, and this difference resulted in the latter having higher yield stresses than the former. This was attributed to the interaction of filaments leading to entanglement in the fluffy pellets. Therefore the outer structure of pellets can also influence rheology.

1.3 Disruption of microorganisms

This section will review some studies on the various methods of cell disruption and the interactions between cell properties and disruption. Several of them have indicated the complexity of these interactions and the variability of disruption between different microorganisms and even between the same microorganisms grown under different conditions. The development of a generic model to predict cell disruption is therefore needed.

1.3.1 Requirements of the disruption process

Objective: To disrupt the microorganism so as to achieve maximum release of product(s).

The requirements of the process are may be:

1. Maximum product release. As this is a primary stage in the recovery of the intracellular product it is essential to achieve a very high step yield of 95-98 % to maintain an acceptable overall yield.
2. Maximum activity yield of product. It is essential to maintain a high activity of the product, by minimising denaturing conditions such as high temperature rises or air-liquid interfaces.
3. A process whereby the product can be released, but at the same time minimising disintegration of the entire cell. This is to avoid very small sized cell debris which will make clarification of homogenate and further purification a problem.
4. A relatively fast process to avoid possible degradation of product by exposure to proteases in the broth, or from disruption.
5. Reliable and consistent process to ensure uniform disruption.
6. Flexible enough to handle variability in feed composition.
7. Containment of system for hazardous products, and ability to sterilise.
8. Preferably a continuous operation
9. Easily scaled up
10. Economical ie. low capital and running costs.

1.3.2 Resuspension of cells

Fermentation broths usually contain a considerable amount of suspended solids as part of the growth media or from the fermentation process. Therefore, to minimise the need and cost of removing such impurities in further downstream purification operations, it is necessary to remove residual fermentation liquor which is associated with the cells. In cross flow membrane separation methods, it is possible to incorporate a diafiltration step into the cell concentration/harvesting procedure whereby liquor is removed and then replaced simultaneously with wash solution and then concentrated. However, centrifugation does not allow washing of the sedimented microbial paste. Therefore, it is necessary to remove the cells and resuspend it in the wash solution and then re-centrifuge the resuspended cells. This introduces an additional step in the recovery process, which is time consuming and may not be beneficial to the product. In order that the cells can be easily resuspended. it may be necessary to obtain a sedimented or filtered cake which is not too densely compacted by the initial recovery step.

Another reason for resuspension is the disruption of cells to release an intracellular product. Unless the disruptor can handle highly concentrated suspensions, or the recovery process gives a suspension of suitable concentration, it may be necessary to resuspend cells to the required concentration for optimal disruption. The solution used for resuspension can be an appropriate buffer which may contain substances to maintain the activity of the released product.

1.3.3 Mechanical methods of cell disruption

There are several mechanical methods for disruption of microbial cells. These usually involve applying high stresses produced by high pressure, rapid agitation with glass beads or ultrasonication. Fig. 1.8 illustrates the various mechanical methods. As will be evident in the upcoming sections, there are several interactions between disruption and cell properties.

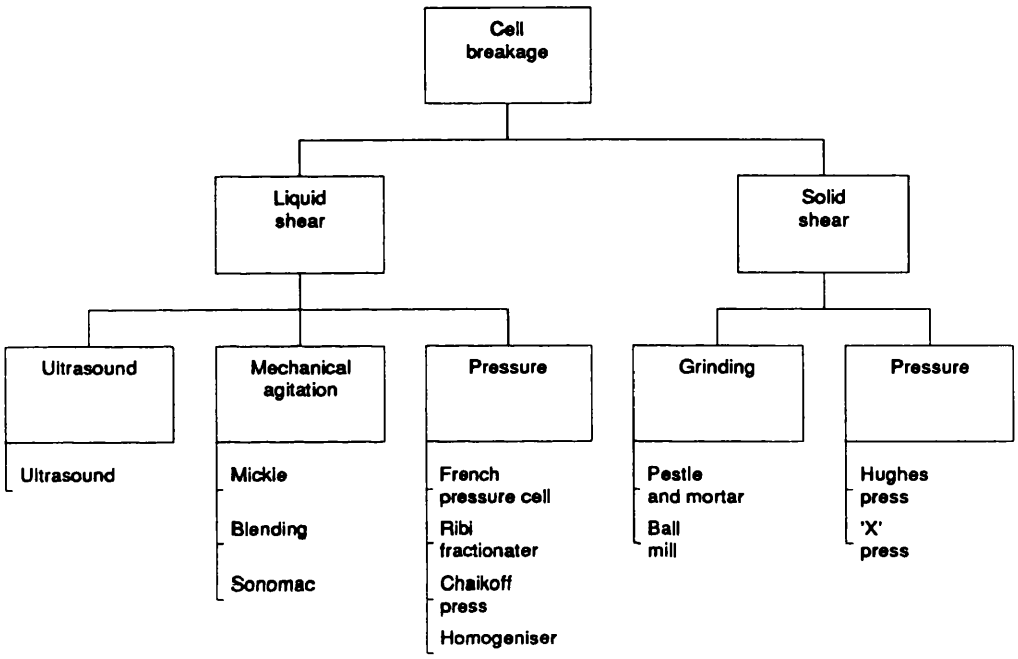


Fig. 1.8.: Mechanical methods of cell disruption (Wimpenny 1967)

The rigid cell walls of microbial cells need to be broken in order to release the intracellular products. In bacteria, the cell wall is composed primarily of peptidoglycans, whereas in yeast and fungi, it is of polysaccharides which form polymer networks causing the cell wall to be very strong and resistant to disruption. The strength and shape of microbial cell walls depend on the structural polymers within the wall and the degree to which they are cross-linked to one another and to other wall components. In addition, the structure is also dependent on the growth environment, and with fungi, on the development stage, and also the mechanical effects of agitation in a fermenter. Therefore, the extent of disruption will depend very much on the condition of the feed material, even if it is the same organism.

Table 1.1 shows the susceptibility of cells to disruption *(Edebo 1969)*. It indicates that there is a wide variation of susceptibility for mycelia between the different methods of disruption. Therefore there may be other factors apart from cell wall strength, such as morphology, which play a part in the extent of disruption.

	Sonic	Agitation	Liquid pressing	Freeze pressing
Animal cells	7	7	7	7
Gram-negative bacilli and cocci	6	5	6	6
Gram-positive bacilli	5	(4)	5	4
Yeast	3.5	3	4	2.5
Gram-positive cocci	3.5	(2)	3	2.5
Spores	2	(1)	2	1
Mycelia	1	6	(1)	5

The numbers indicate relative susceptibility: 7 = highly susceptible, 1 = resistant to disruption. Parenthesis indicates that the number is very uncertain.

Table 1.1 : Susceptibility of cells to disruption (Edebo 1969)

1.3.3.1 Small scale mechanical methods

Some workers have examined the disruption of *Aspergillus* cells by laboratory scale methods. Extraction of organelles such as mitochondria, from filamentous fungi, was reported to only require a few breaks or tears in the hyphal walls such as for *Aspergillus oryzae* using a roller mill *(Coakley et al 1977). Zetelaki (1969)* compared the disruption of *A.niger* by various methods : hand grinding and X-press released high amounts of the intracellular enzyme glucose oxidase, whilst the bead mill varied in performance depending on operating conditions, but the high speed vibratory disintegrator, ultrasonicator and laboratory homogenisers only released 40-50 % of the yield from hand-grinding. The ultrasonicator yield was low because of temperature inactivation (55°C). *Ishimori et al (1982)* used mild ultrasound waves (20 kHz, 15W) to release glucose oxidase from *A. niger* cells without breakage of the cell wall. However only 10 % of the total was released after 5 hours.

A study of the forces required for cell disruption was made by *Kelemen and Sharpe (1979)* who used a high pressure disruptor (by Stansted Fluid Power Ltd) based on the laminar flow of a cell suspension through a inverted conical shaped "funnel" with a ball valve forced against the inlet and expanding as it enters the conical section from the narrow orifice, causing high shear forces thus breaking the cells. The pressure applied was estimated through a relationship between the temperature rise and the pressure for water. Disruption was measured by microscopy and staining, by ultraviolet absorption at 260 and 280 nm of intracellular material, or by cell viability counts. By the cell viability count, a pressure of 15 MPa was required to achieve 50 % disruption of *E. coli*;

24 MPa for *B. subtilis*; 31 MPa for *Lactobacillus casei*; 150 MPa for *Streptococcus facealis* and *S. cerevisiae*; and 190 MPa for *Streptococcus aureus*. However 95 % disruption was achieved by 120 MPa for *E. coli, B. subtilis* and *E. coli*, whilst the larger *Streptococcus sp.* and yeast required about 270 MPa. Other cells such as *Aspergillus fumigatus* and a *Fusarium sp.* were disrupted at 68 MPa (determined by microscopy). These pressures are extremely high in comparison with the APV Manton-Gaulin high pressure homogeniser (sect. 1.3.3.2) which operate up to 124 MPa and achieve close to 100 % disruption of yeast cells, after 3 or 4 passes at 50 MPa.

Although there are several mechanical methods, most of them are laboratory scale techniques which have been difficult to scale up for industrial use. For example, it is difficult to provide adequate cooling for sonication at high power inputs. Therefore, for large scale operation, it has been more suitable to adapt commercially available high speed bead mills and the high pressure homogeniser, both of which are used for other purposes, for cell disruption. The former is used in the paint and dye industry and the latter in the dairy industry.

1.3.3.2 The high pressure homogeniser (APV Manton-Gaulin)

General design and operation

Of all the high pressure flow devices that have been studied, the APV-Manton Gaulin homogeniser is the most widely used for large scale disruption *(Scawen 1980)*.

This device has a high pressure, positive displacement pump, incorporating an adjustable restricted orifice discharge valve. The principle of operation is the same for all models, although there are variations in design parameters such as capacity, number of discharge valves, and pressure ranges. Feed material is drawn through a check valve into the pump cylinder and on the pressure stroke, is forced through the discharge valve assembly (fig.1.9). Operation can either be on a recycle mode or through several discrete passes where the feed is collected and passed through the homogeniser again. To achieve protein release of over 90 % of maximum release, for bakers' yeast, at 50 MPa pressure, at least 5 passes or the equivalent recycle time of 35 minutes is required *(Hetherington et al 1971)*.

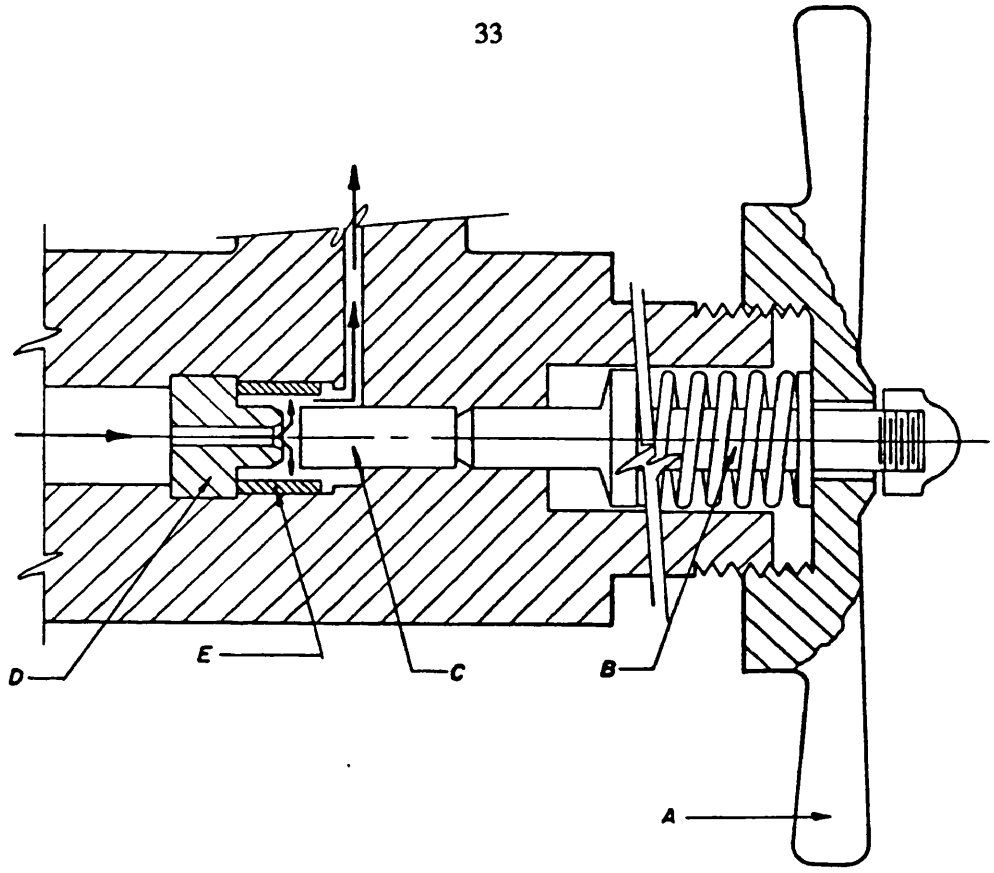


Fig. 1.9 : Schematic diagram of a APV Manton-Gaulin homogeniser valve assembly. Handwheel assembly A positions the valve C through valve rod B against the valve seat D. During discharge the material passes between the valve and its seat and impinges on impact ring E.(as shown by arrows).

Kinetics and effects of operating parameters.

The performance of a high pressure homogeniser is affected by process variables such as pressure, number of passes or recycle time, cell concentration and temperature, all of which have been studied by *Hetherington et al (1971)* who described the disruption process of bakers' yeast (up to a pressure of 50 MPa) as a first order rate kinetics using the equation:

$$\log_{10} (R_m / R_m - R) = K.N = (k.P^d).N \qquad \textbf{Eqn.1.1}$$

where R_m = maximum protein available for release

R = protein release for a number of discrete passes N through the valve.

K = a dimensionless constant, mainly dependent on the pressure drop P across the valve seat

k = a constant which is a function of temperature and possibly cell concentration;

The pressure exponent "d" is a function of cell type.

Follows et al (1971) also found that the rates of enzyme release from bakers' yeast were first order, and similar to the release to the release of protein. However, the rates of release of different enzymes were not the same. Enzymes located in the cell wall were released at a faster rate than soluble protein those in the cytoplasm at about the same rate, and those in subcellular particles at a slower rate. Location of product in the cell therefore determines the rate of disruption. For most enzymes there was no loss of activity after many passes through the homogeniser, at temperatures below 30°C, except for invertase and fumarase, which are membrane associated (cell wall and mitochondria respectively). This is in line with work by *Talboys and Dunnill (1985)* who found that membrane associated enzymes were highly shear sensitive. *Augenstein et al (1974)* also found that an enzyme complex forming the gramicidin antibiotic from *Bacillus brevis* degraded rapidly. A possible reason was by Keshavarz *et al* (1987) who suggested that the gramicidin synthetase was a membrane associated enzyme.

The pressure exponent "d" was reported was reported to be 2.9 for bakers' yeast *(Hetherington et al 1971)* and 2.2 for β-galactosidase release from *E.coli* mutant *(Gray et al 1972)*. This exponent does not vary between 10-30 MPa for bakers' yeast *(Hetherington et al 1971)*, but decreases for pressures higher than 30 MPa and cell concentrations over 210 kg dry weight m^{-3} and the rate is no longer first order. Other workers, *Dunnill & Lilly (1975)*, also reported a decrease in "d" for bakers' yeast above 70 MPa, and *Engler and Robinson (1981a)* for *Candida utilis* at pressures between 50 and 125 MPa. The former attributed this to exceeding the optimal range of pressures for which the homogeniser valve was designed, whilst the latter suggested that the exponent "d" may depend on the range of operating pressures.

N.B.: All disruptions by Engler and Robinson were done in a high pressure flow device by impingement of a high velocity jet through a nozzle of 80 μm, on a stationary surface which was capable of operating up to 300 MPa. Although for a different equipment, Hetherington's (1971) model was applicable and so this work is included here.

Another significant effect on disruption kinetics is the conditions of cell growth. Several studies have shown differences, even with the same species of organism:

1. *E. coli* cells grown on a synthetic medium were easier to disrupt than those grown in a complex medium *(Gray et al 1972)*.
2. *C. utilis* cells with a higher growth rate (in a cyclic batch culture) were easier to disrupt than those at a lower rate (continuous culture), and that out of both *C. utilis* and *S. cerevisiae* cells grown in identical continuous culture conditions, the latter was more difficult to disrupt *(Engler and Robinson 1981 a & b)*.

3. The exponent "d" varied from 3.1 to 1.6 from the exponential phase to late stationary phase for a Gram-negative bacteria, *Alcaligenes eutrophus (Harrison et al 1990)*, indicating a greater susceptibility of exponential phase cells to pressure.

From this data, it is not possible to optimise disruption because there are too many factors which affect the susceptibility to disruption. It is necessary to study each individual organism in order to determine the effects of growth conditions.

The effect of cell wall strength on disruption has been investigated. Results show that Gram negative bacteria (e.g. *E. coli* and *P. putida*) are easiest to disrupt, followed by Gram positive bacteria (e.g. *B.brevis*), yeasts (e.g. *S. cerevisiae*) and lastly fungi, by the decreasing value of the proportionality constant k *(Keshavarz et al 1987)*. However *Edebo (1969)* found that mycelial organisms were more susceptible to disruption than other microorganisms, when using agitation or freeze-pressing methods (Table 1.1). In this case, despite the high strength of the cell wall, it is likely that the morphology of mycelia, being long and branched causes entanglement of the mycelia, which tears apart and breaks the cells when shear forces are applied. In addition, non-septate fungi such as *Rhizopus sp.* do not need each individual cell to be broken to release the contents. With a high pressure homogeniser, it is not known whether morphology of filamentous microorganisms affects disruption, but it would be reasonable to suspect that it does.

The pressure exponent "d" of 2.9 for bakers' yeast *(Hetherington et al 1971)* means that disruption rate is highly pressure dependent; and so instead of using several passes to achieve over 90 % disruption, it would be more efficient to increase the operating pressure and use fewer passes. Furthermore, the problem of further disintegrating cell debris, which makes subsequent clarification difficult, is alleviated. As the pressure dependence varies with cell type, and so far, values of "d" for other organisms have been less than 2.9, it may be that the value of "d" is lower or higher for filamentous microorganisms.

The homogeniser valve unit has various designs as shown in fig. 1.10. The CRF valve unit released more protein than the flat valve unit *Hetherington et al (1971)*. These studies have been carried out on bakers' yeast, and cannot be assumed to affect disruption of filamentous fungi in the same manner.

The mechanism of cell disruption is very much unresolved, because of the highly complex hydrodynamics occurring in the valve region. Cells are most likely subjected to turbulence, cavitation and liquid shear. Various attempts have been made: *Brookman (1974)* decided that the magnitude and rate of pressure drop are the major factors; and *Doulah et al (1975)* suggested that

turbulent eddies oscillates the cell liquid which disrupts the cell wall. A study *(Engler & Robinson 1981a)* of the disruption of *C. utilis* in a high pressure flow device indicated that impingement of a high velocity jet of cells on a stationary surface is the major cause of disruption. More recent evidence was provided by *Keshavarz et al (1990b)*, who varied the impact distance (as the cells exit the valve unit and impinge on the impact ring) and valve geometries or design, found that impingement was the main disruption mechanism. To disrupt bakers' yeast using a "cell disruption" type valve unit with a standard impact ring, about 20 % of disruption after the first pass was due to valve geometry, and the rest by impingement. A smaller impact distance led to increased disruption, as did a decreased valve gap width (between valve rod and valve seat). The knife edge unit gave highest disruption yields (at 46 MPa) followed by CR and CD units which had similar performance, and then the CRF unit and lastly the flat valve unit. This corresponds with the increasing gap width (from 12.55 to 21.61 µm), decreasing exit and entry velocity, and decreasing mean velocity gradient from the KE valve to the FV unit at 46 MPa.

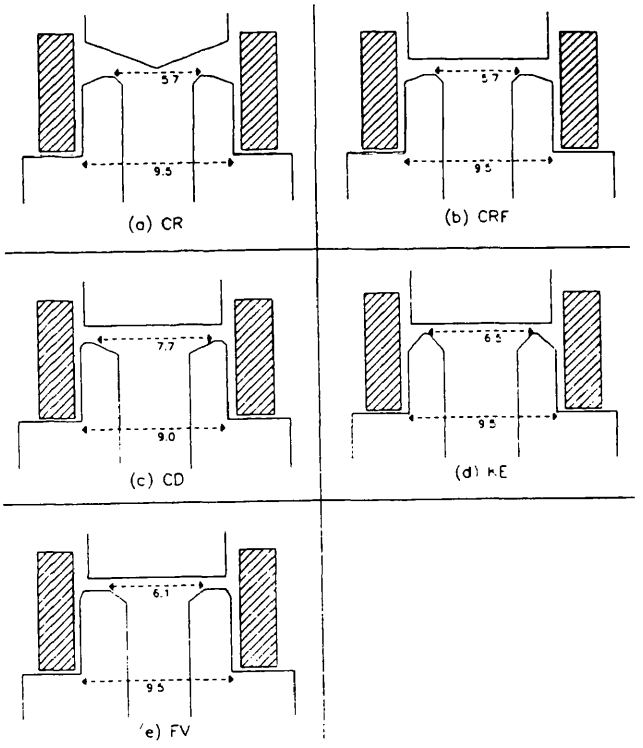


Fig.1.10 : Details of homogenising valve units - not to scale. (a) cell rupture unit (CR) with coned valve rod; (b) cell rupture unit (CRF) with flat valve rod; (c) cell disruption (CD) valve unit (d) knife edge valve (KE) unit and (e) flat valve (FV) unit. Distances shown in mm. (Keshavarz *et al* 1990b)

The use of high pressure homogenisers on filamentous microorganisms

There have been few published studies on the disruption of filamentous microorganisms using high pressure homogenisers. Two papers dealing with *Aspergillus niger* disruption will be discussed here, and two with *Rhizopus nigricans*.

Zetelaki (1969) made a comparison of the disruption of *A.niger* by hand-grinding, a bead-mill, ultrasonic disintegrator, the X-press and a laboratory homogeniser. Effectiveness of disruption was measured by the release of intracellular enzyme glucose oxidase in the supernatant.

A 5 % (wet weight/vol.) mycelial suspension was disrupted in a laboratory homogeniser capable of a maximum pressure of 533 MPa, and a throughput of 54 L/hr, releasing a glucose oxidase activity of 49 % of the activity found from disruption by hand-grinding with quartz sand. This gave the lowest glucose oxidase activity, out of all the other methods and clogging of the valve was also experienced even with very dilute samples. A microscopic examination of the homogenate showed many long and undamaged hyphae. This study indicates that the laboratory homogeniser is unsuitable, but since there is no data provided on the operating parameters, such as pressure and number of passes, it is not possible to draw any definite conclusions.

Lilly & Dunnill (1969) examined the disruption of bacteria, yeast and *A.niger* in an APV-Manton Gaulin homogeniser operating at about 35 MPa, on a recycle basis, at a throughput of about 800 mL/min. From fig. 1.11, the disruption curve for *A.niger* is similar to the *S.cerevisiae* one, which may indicate that *A.niger* disruption is a first order process (assuming that this *S.cerevisiae* data exhibits first order disruption kinetics). To achieve complete disruption of *A.niger*, a recycle time of about 25 minutes is required, which is equivalent to 3.5 discrete passes. The conversion from recycle time to number of passes is calculated using a calibration curve drawn up by *Hetherington et al (1971)*. Therefore the number of passes N can be superimposed on fig.1.11.

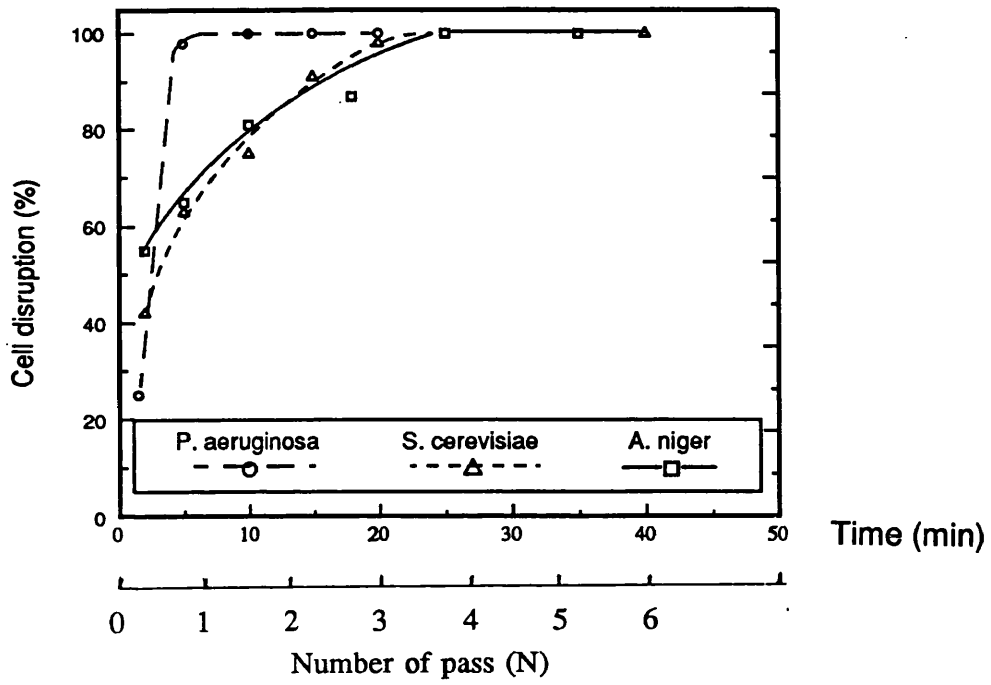


Fig. 1.11 : The disintegration of microorganisms by a Manton-Gaulin homogeniser at 34.5 MPa. 1 litre of slurry was recycled at 800 ml/min. *P.aeruginosa* 200 g/L ○, *S.cerevisiae* 600 g/L △, *A.niger* 700 g/L □ . Concentrations are on a wet weight basis. (Lilly & Dunnill 1969)

Then, if the disruption is a first order process, then equation 1.1. applies:

$$\log_{10} [R_m /(R_m - R)] = K.N = (k.P^d).N \qquad \text{Eqn 1.1}$$

and fig. 1.11 can be translated into fig. 1.12. This assumes that R_m = 100 % and therefore percentage cell disruption is also R, which allows $\log_{10} (R_m / R_m - R)$ to be calculated. Clearly from fig.1.12, *A.niger* is slightly easier to disrupt than yeast from the lesser slope.

So, it appears that the disruption of *A.niger* cells may not be very difficult, despite suspicions that highly filamentous microorganisms at high cell concentrations tend to block up the homogeniser valve. Disruption at 35 MPa for 3-4 passes to achieve 100 % protein release is certainly not a very high pressure nor too many passes, and if the pressure dependence for *A.niger* is as high as for yeast, (ie. the pressure exponent "d" is about 2.9), then, maybe at higher operating pressures, maximal disruption can be reached in 1 or 2 passes. Unfortunately, there was no evidence on the pressure dependencies of disruption, as only one operating pressure was examined.

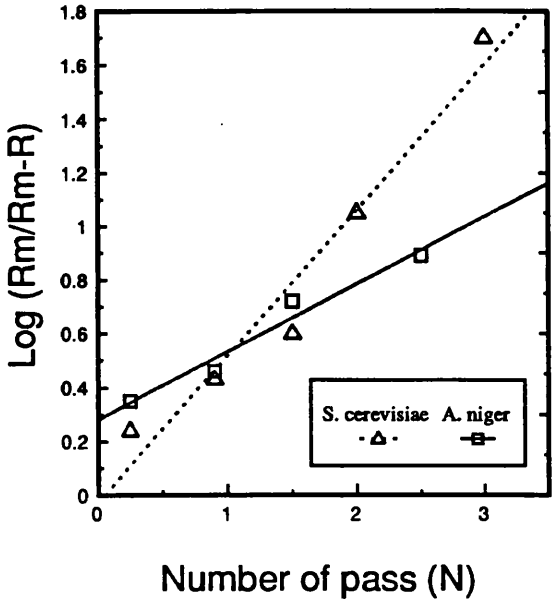


Fig.1.12 : Relationship between log (R_m/R_m-R) and N based on fig. 1.11

Thomas (1988) used the high pressure homogeniser to release a membrane-associated enzyme, progesterone 11α hydroxylase from *Rhizopus nigricans*. It was found that protein release could be described by the first order relationship by Hetherington et al (1971), with the exponent "a" being 0.57, thus indicating a very weak dependence on pressure. However, the maximum protein released R_m was found to increase with pressure according to the relation $R_m = 0.11.P^{0.14}$. In contrast the rate of enzyme release was slower than protein (proportionality constant k lower) and the exponent "d" being 0.55, but the maximum enzyme release was not a function of pressure.

Further work with this microorganism by *Keshavarz et al (1990a)* found similar disruption kinetics ie. soluble protein release was a weak function of pressure and number of passes, and the varying R_m was attributed to micronisation of the cell debris which may release insoluble complexed proteins and other compounds such as peptide, glycopeptide and amino acids giving positive results on the protein assay used, as first discovered by *Limon-Lason et al (1979)*. This likely cause was substantiated by measuring alcohol dehydrogenase (ADH) release as an independent measure of disruption. There was no variation in the maximum enzyme released from 10-50 MPa, thus indicating cytoplasmic proteins are released completely at low pressures (10 MPa) and therefore the apparent increase in R_m due to micronisation. It was also found that blockage of the homogenising valve occured beyond 22 g L^{-1} (dcw) for filamentous cells, because the intertwined cells tended to form cellular mats, whereas the pelleted cells formed homogeneous suspensions (much like unicellular organisms) which did not block until 30 g L^{-1} (dcw).

1.3.3.3 High Speed Bead Mills

General design and operation

Bead mills consist of horizontally or vertically mounted grinding chambers filled with glass or steel beads, acting as the grinding elements, which are agitated at high speeds by rotating disc impellers mounted either concentrically or eccentrically on a motor driven shaft (fig.1.13). On agitation, the beads, which typically occupy 80-85 % of the free working volume of the chamber causes disruption of the cells through the collisions, and the very high shear forces generated by the differential velocity profiles *(Rehacek & Schafer 1977)*. Each unit must be equipped with efficient cooling systems for processing temperature sensitive products. This requirement may restrict the size of the mill, but units from 0.1 to 20 litres are available. In general, the horizontal mills are preferred for cell disruption, as the grinding action in vertical mills is reduced due to fluidising effects of the upward fluid flow on the beads. The mode of operation can be batch or continuous.

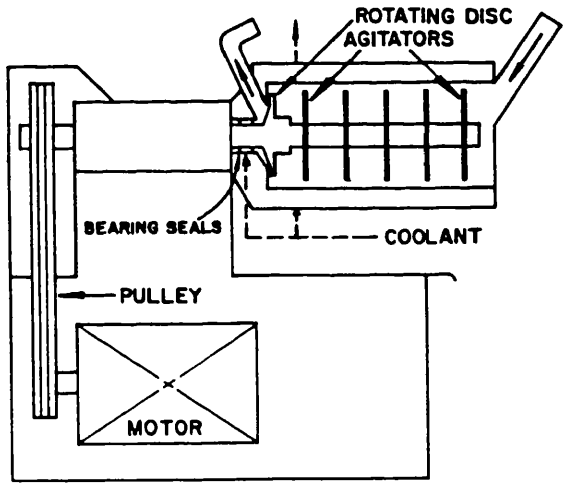


Fig. 1.13 : An example of an horizontal bead mill: The Dyno-Mill KD5

Kinetics and effects of operating parameters.

The performance of a bead mill is dependent on several process variable, including bead diameter and loading, cell concentration, temperature, flowrate of feed, agitator speed and design. The kinetics of disruption of bakers' yeast in a batch mode were found to be a first order rate process *(Currie et al 1972)*, where the rate of disruption as measured by the rate of soluble protein release was directly proportional to the amount of unreleased protein. This can be described by:

$$\text{Log}_e [R_m / (R_m - R)] = kt \qquad \textbf{Eqn.1.2}$$

where R = weight of protein released per unit weight of packed yeast

R_m= maximum protein release; k = first order rate constant; t = disruption time

Further studies by *Limon-Lason et al (1979)* confirmed the first order kinetics and showed that, for continuous disruption in a 5 L bead mill, the flow patterns behaved as a series of continuous stirred tank reactors, each impeller forming a reactor, whereas in a 0.6 L mill, there was a considerable degree of back-mixing between the reactors.

Bead diameter: The effects of bead size reported, have been highly variable. For bakers' yeast at concentrations between 30 and 60 % w/v packed yeast, *Currie et al(1972)* reported that the smaller beads tested in the range of 0.5-0.28 mm diameter were more effective, but, at concentration above 60 % w/v, smaller beads did not give as much an increase in rate constant. However, for brewers' yeast in a horizontal mill, the disruption rate was shown to increase with decreasing diameter from 0.6-0.35 mm, then fall as it was decreased to 0.2 mm *(Marffy & Kula 1974). Schutte et al (1983)* found an optimum bead diameter of 0.7 mm for bakers' yeast, disrupted in a 20 L horizontal mill; and also that higher release of periplasmic enzymes was obtained with larger beads of 1.0 mm, whilst for cytoplasmic enzymes, smaller beads were more suitable. This indicates that smaller beads are more effective for complete cell disintegration. Furthermore, it was suggested that for bacteria which are about one-tenth the size of a yeast cell, much smaller beads are likely to be optimal for its disruption. The existence of an optimum bead size may be caused by the positive effect of increased collision frequencies, due to the higher bead density, but negated by the effects of lesser kinetic energy of the smaller bead, and possibly bead fluidisation. *Woodrow & Quirk (1982)* found that 0.1 mm diameter beads were the most effective for disrupting a variety of bacteria, but also found some loss of enzymatic activity. For continuous disruption, bead size will be limited to the efficiency of retention of the beads by the separator.

Bead loading: Bead loading is usually expressed as volume percentage relative to the free volume of the grinding chamber. *Currie et al (1972)* reported very significantly increased disruption with increased bead loading, and *Rehacek & Schafer (1977)* obtained similar trends, but recommended that a loading of more than 88 % was not suitable. An optimum bead loading of about 80 % was recommended for bakers' yeast, but can vary slightly depending on the bead size *(Schutte et al 1983).* In addition an increase in bead loading will necessitate more cooling, due to higher temperature rises.

Cell concentration: Effects of cell concentration are inconsistent. Within a range of 40-200 kg m^{-3}, *Mogren at al (1974)* found no effect of cell concentration. However, *Marffy & Kula (1974)* found that the rate of disruption increased with cell concentration in a 0.6 L "Dyno-mill". This was attributed to the effect of viscosity on power input, assuming that flow in the mill was laminar. In contrast, the release of enzymes from bakers' yeast in a 20 L horizontal mill showed an optimum cell concentration of 40 % wet weight/volume, with 30 or 50 % w/v having much lower values. This is most noticeable for a tip speed of 5.1 m s^{-1}, and much less for 10 m s^{-1}. *Limon-Lason (1979)* found that the rate constant is independent of cell concentrations above 30 % w/v for only the stainless steel, "closed disc" type impeller. For the polyurethane "open disc" impellers, the rate constant decreased with increasing yeast concentrations. This effect was due to the changes in rheology and impeller type during disruption. The impellers are shown in fig. 1.15.

Temperature: Operating temperature was reported to have little effect on the rate constant, with only a small decrease of about 20 %, when the temperature increased from 5-40 °C. However, the rise in temperature during disruption is certain to cause product inactivation. Therefore it is vital to maintain the temperature at about 5 °C. This may be a significant problem in much larger units.

Agitator speed: In general, increasing the agitator or tip speed, causes a very rapid increase of the rate constant *(Currie et al 1972, Marffy & Kula 1974, Mogren at al 1974, Rehacek & Schaefer 1977)*. The degree of disruption levelled off at high tip speeds *(Schutte et al 1983)* and there was significant temperature rise, particularly at very high bead loading, which is undesirable, as well as increased power consumption.

LImon-Lason et al (1979) found that in a 5 L horizontal mill with polyurethane impellers, the disruption rate constant increased with tip speed up to 10 m s^{-1} and then stayed constant for higher speeds, whereas, with stainless steel impellers, an expected increase of rate occurred. With the polyurethane impellers, at higher tip speeds, the backflow created is greater than for the stainless steel impellers. So although the rate constant does increase with tip speed for both impeller types, in the polyurethane type, the backflow (which lowers the disruption rate - see flowrate effect) is "high enough" to negate the increase, whereas the backflow-induced mixing in stainless steel impellers is less, resulting in an effective increase in rate constant. The different impellers are shown in fig. 1.14.

Agitator design: The above mentioned effect of agitator speed already shows that impeller types can affect the disruption kinetics *(Limon-Lason 1979)*. Furthermore, it was found that in a 5 L mill, the disruption rate constants were always higher, when using polyurethane impellers, than for the

stainless steel ones, but maximum protein release was always lower for the polyurethane ones. This difference was attributed to the more open design of the polyurethane impellers, which creates a greater degree of mixing at a distance from the impeller, but lower shear rates close to the impeller. The higher degree of mixing explains the greater rates of disruption, whilst the lower shear rates, which controls maximum protein release gave lower release. The converse happens with the stainless steel impellers, where there are very high shear rates close to the impeller but lower degree of mixing. Eccentrically mounted circular discs in a helical array cause movement of beads opposite to the suspension flow direction, thereby preventing compaction of the beads *(Schutte et al 1983)*.

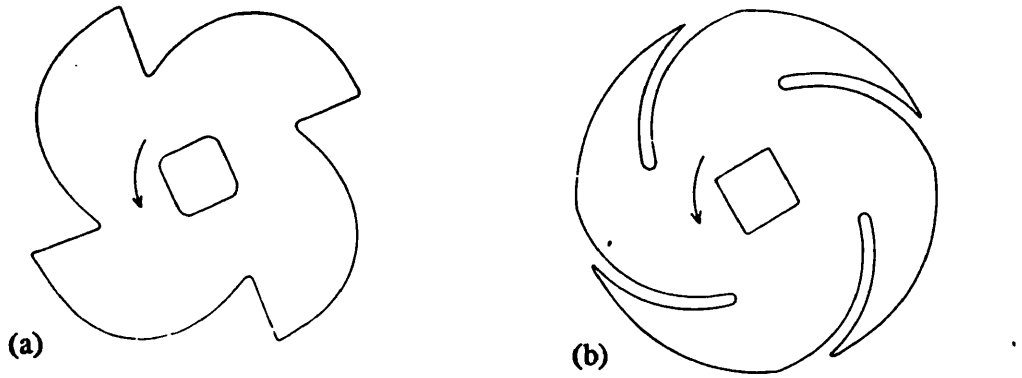


Fig. 1.14 : Details of (a) Polyurethane "open" type impeller (b) stainless steel "closed" type impeller *(Limon Lason et al 1979)*

In contrast with most bead mills, which have agitator discs mounted perpendicular to the drive shaft, oblique mounting by inclining the discs, gave better results at low agitator speeds, but with higher power consumption and higher temperature rise of product *(Rehacek & Schafer 1977)*. Orientation of the grinding chamber, ie. whether it is horizontal or vertical also has an effect. *Rehacek et al (1969)* found the horizontal one to be more effective, whereas *Currie et al (1974)* found the vertical type performed the disruption very well. However, vertical mills need very fine sieve plates for the bead retention, which are susceptible to blockage and need to be sealed for biosafety.

Flowrate: The effect of flowrate was shown to lower the disruption rate, due to the shorter residence time at higher flowrates *(Currie et al 1972, Marffy & Kula 1974)*. As previously mentioned *Limon-Lason et al (1979)* found that the flow pattern in a 5 L bead mill is equivalent to a series of continuously stirred tank reactors (CSTR's) but this model is further complicated in the 0.6 L mill, due to considerable back-mixing or longitudinal dispersion, which reduces the number of effective CSTR's, thus reducing the efficiency of the mill. Any type of impeller which causes backflow will reduce the efficiency of disruption.

Another type of bead mill reported recently by *Mao and Moo-Young (1990)* utilises a vertical cylindrical rotor, instead of impellers to agitate the beads. This mill operates up to 6300 rpm and the flow pattern was plug flow, with operation either in continuous or batch mode. It can achieve 76, 80 and 90 % protein release from bakers' yeast after the first pass at 2000, 3000 and 4000 rpm, respectively at 225 g L^{-1} (packed yeast) with a flowrate of 6 L h^{-1}. No dependency of protein release on cell concentrations between 250 and 600 g L^{-1}, but there is non-linear decrease in protein release when the flowrate was increased to 18 L h^{-1}. This was explained treating it as a fluidised chamber, where there will be an optimum flowrate when the beads and cells are homogeneously fluidised resulting in maximum collisions and hence maximal disruption. Higher and lower flowrates cause the cells to be suspended at the bottom and the top of the chamber respectively with the beads at the opposite end.

Disruption of filamentous microorganisms using bead mills

There is only one published study on the disruption of *Aspergillus* using a bead mill. *Zetelaki (1969)* has shown that an increase in the agitator speed of a 5 L bead mill, from 800 to 1200 rpm will result in increased glucose oxidase release (from *Aspergillus niger)*. The highest release at 800 rpm (≈ 6.5 SU/mL) occurred after 60 minutes disruption, whereas the highest at 1200 rpm (≈ 10 SU/mL) required 40 minutes at 1200 rpm. However, accelerated disruption only took place when the temperature was kept low, at about 16-17 °C for 1200 rpm with chilled water (fig. 1.15: curve 3b). Otherwise, cooling by tap water (curve 3a) resulted in a decreased activity due to heat denaturation at almost 40°C. Therefore at high agitator speed, temperature control is essential.

Further studies by the same worker also showed that when the cell concentration was halved, there was a small increase in the total glucose oxidase released. This is insufficient data to suggest any trends. An increase in bead loading (double) resulted in greater release of the enzyme, and for the higher load, an increase of agitator speed from 800 to 1200 rpm also gave a higher total release. A study using a 5 L continuous bead mill *(Zetelaki 1969)* operated at 1800 rpm showed that disruption was best at the lowest flowrate (10.8 L/h). For two different cell concentrations at 4 times and 5 times dilution, the latter gave higher total enzyme release. It was suggested that the more dilute suspension provided a better extraction effect produced by the larger quantity of water.

This work has shown that the effect of bead loading and agitator speed are the same as for disruption of yeasts, but it would be necessary to have more extensive data to conclude more definitely. No results on the change in disruption rate constants with these parameters were presented. Further work will need to be done to determine if filamentous microorganisms have similar disruption kinetics as yeast or bacteria.

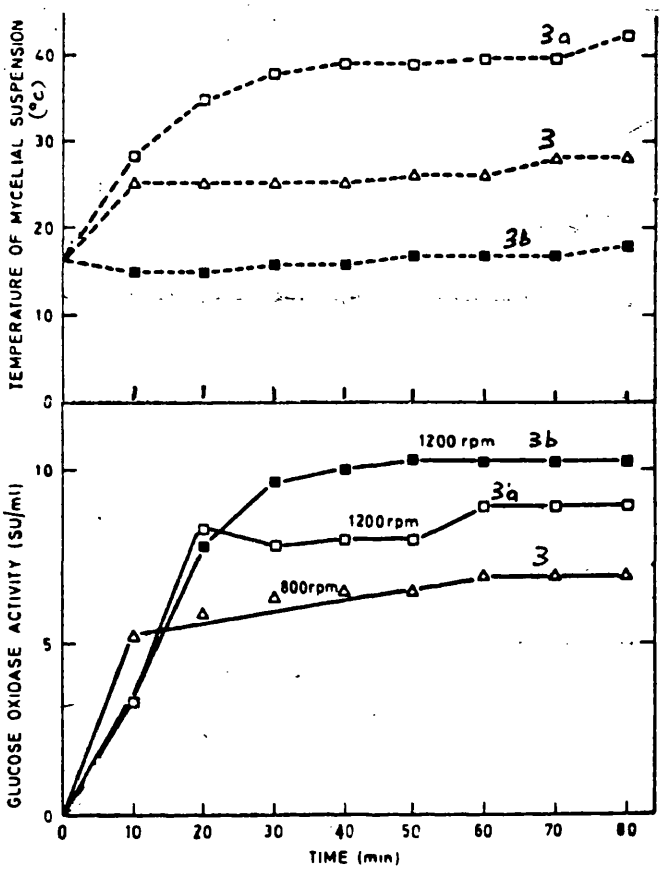


Fig. 1.15 : Glucose oxidase activities per ml of supernatant after disruption in a 5 litre batch type bead mill (3 & 3a - cooling by tap water; 3b - cooling by chilled water) (Zetelaki 1969)

1.3.3.4 The "Microfluidizer®" - a new high pressure homogeniser

This homogeniser consists of an high pressure pump which pressurises the cell suspension stream which enters an "interaction chamber" where it is directed into precisely defined microchannels. In these channels, the stream is accelerated to very high velocities (up to 450 m s^{-1}) and intense cavitation occurs within a controlled area resulting in cell disruption. Various models have been designed from small scale ones operating down to 10 mL at 6-12 L h^{-1} to production scale models operating with a minimum batch of 20 L and 11000 L h^{-1}. Operating pressure can be up to 158 MPa. The microfluidizer can be linearly scaled up from small scale through to pilot and production scale. This homogeniser is claimed to produce no aerosol, can be operated aseptically, is sterilisable by steam, and a blocked disruption chamber can be cleaned by reversing the direction of flow. For the laboratory scale microfluidizers a jacketed cooling coil which immediately cools the exit stream from the interaction chamber is incorporated and the whole machine can be immersed in an ice bath to enhance cooling. Temperature rise is about 2.5°C per 10 MPa pressure drop.

It is claimed that over 99 % rupture can be achieved after 1 pass at 75 MPa for *E. coli,* and a 40-200% increase in enzyme activity over a conventional homogeniser after 1 pass at 90 MPa for filamentous fungi *(Technical Bulletin, Microfluidics Corp., 1989).* For the latter organism type, since the type of conventional homogeniser and rupture efficiency with respect to the fungi used was not specified, this evaluation is unclear, and so the microfluidizer may not even achieve 100 % disruption for filamentous fungi.

Sauer et al (1989) tested the laboratory scale "M-110T microfliudiser" with native and recombinant strains of *E. coli.* Fig.1.16 shows the schematic diagram of this machine. As with the APV Manton-Gaulin type homogeniser, the extent of disruption was highly dependent on both the operating pressure and number of passes. The disruption kinetics could be described using equation 1. by *Hetherington et al (1971)* but with an added exponent d, on the number of passes, N :

$$\log_{10} [R_m / (R_m - R)] = k.\ N^c.P^d \qquad \textbf{Eqn. 1.3}$$

The exponent c, was found to vary between 0.28 and 0.94, depending on the specific growth rate of the cells in continuous culture, the concentration of cells being disrupted, and the type of cell (recombinant or native *E. coli*). Similarly these factors have contributed to a variation in both the proportionality constant K, and the exponent d. Disruption concentrations ranged from 4 to 175 g(dcw/L).

In continuous culture, both strains of cells grown at a higher specific growth rate were more readily

slower growing cells; and the recombinant strain was disrupted more readily than the native one. However, in batch culture, there was no appreciable difference between the two strains. this work exemplifies the impact of growth conditions and strain type on cell disruption. Therefore any predictive model may only be suitable for the system from which it was developed.

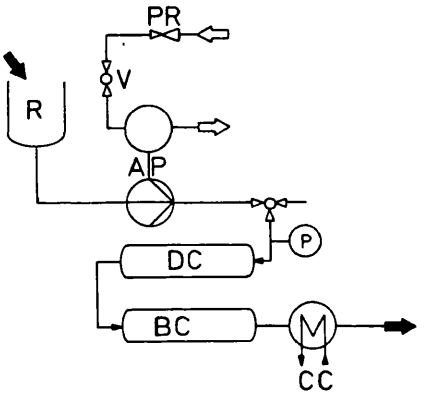


Fig. 1.16 : Microfluidizer M-110T set-up: AP, air-driven pump; BC, back-pressure chamber, CC, cooling coil; DC, disruption chamber; P, pressure gauge; PR, pressure regulator; R, reservoir; V, valve. (Sauer et al, 1989)

Further work with the "M-110T microfliudiser" on bakers' yeast *(S. cerevisiae)* by *Baldwin and Robinson (1990)* found that only 32 % disruption was achieved after 4 passes at 95 MPa. Using a lytic enzyme preparation called "Zymolyase" to pre-treat the cells, which only resulted in 5 % disruption after 2 hours, the microfluidizer was able to achieve 100 % disruption at 95 MPa after 4 passes. To describe this combined disruption, a factor was introduced into the Hetherington model (Hetherington et al 1971) to give :

$$\log_{10} [(R_m - R_x) / (R_m - R)] = k.N^c.P^d \qquad \textbf{Eqn. 1.4}$$

where R_x = cells disrupted by enzymatic treatment alone

R = total of cells disrupted after both enzymatic lysis and homogenisation ie. $R_x + R_h$, where R_h is the cells disrupted in the homogenisation step.

The exponent d was 3.03 and exponent c, 1.3. Cell concentrations of 0.6 and 15 g dcw/L did not affect the disruption kinetics of both steps. These are relatively low concentrations compared to at least 84 g dcw/L used by *Hetherington et al (1971)*. The outlet temperature was maintained below 18°C, with the machine immersed in an ice bath. Therefore, for yeast disruption, the microfluidizer does not seem to be effective enough on its own. In practice, the use of these two disruption steps

may be too time consuming and costly, especially on a large scale.

1.3.4 Non-Mechanical : Enzyme and Chemical Lysis

Other non-mechanical methods of cell disruption such as sonication, osmotic shock, freezing and thawing, are mainly suitable for specific applications and small scale use only *(Wang et al 1979 pp 247-249; Scawen et al 1980).* Enzyme and chemical lysis and represents two of the non-mechanical methods w4hich has commercial potential. Some advantages of enzyme lysis are the mild conditions and very specific action of the enzyme. However, costs of lytic enzymes are high and mostly unavailable commercially. Unless immobilisation is feasible, the enzyme is usually lost after disruption. Chemical lysis tends to use rather harsh chemicals such as guanidine HCl and Triton X-100 which can denature proteins. Both these techniques are also very time consuming, lasting for several hours.

Large scale work has been conducted by *Asenjo et al (1981)* who extracted yeast-lytic enzymes from *Cytophaga* cells produced on a 900 l scale and used it to disrupt yeast cells. The protein release from the cells which were initially disrupted in a high pressure homogeniser at 400 bar for 4 passes, and then treated with lytic enzymes was substantially more than for cells being treated with enzymes only. Immobilisation of the enzyme onto a linear soluble carbohydrate retained activity but not with a globular soluble carbohydrate. Furthermore, recovery of the enzymes from the support would require sophisticated technology, thus favouring free enzyme systems.

A scheme for the differential product release (DPR) from bakers' yeast *(S. cerevisiae)* using a combination of a lytic enzyme from *Oerskovia xanthineolytica* and 0.25 % Triton X-100 has been reported by *Andrews et al (1990).* They found that a mixture of 0.6 M KCl or sorbitol acting as osmotic supports, 20 mM $ZnSO_4$ as membrane stabilisers and the lytic enzyme, when incubated with whole yeast cells for 60 minutes at 30°C, only lysed the cell wall to release wall associated enzymes like invertase (83 % of total). The lysate was then centrifuged to isolate the intact spheroplasts, which were disrupted with the same preparation for 15 minutes but also with DEAE-Dextran and glucose, which lysed the cell membrane and released cytosolic proteins such as alcohol dehydrogenase (ADH-60 % total). Again after separation of cytosol products, the still intact organelles such as mitochondria were disrupted with Triton X-100 releasing such enzymes as fumarase (94 % of total). Almost 50 % of total protein was released in the second step to release cytosol products. In comparison with DPR, a mechanical bead mill released twice as much protein, 70 % for invertase and ADH, and similar amounts for fumarase. In contrast enzyme lysis on it own released 60 % protein, 70 % invertase, 86 % ADH and only 10 % for fumarase in comparison with DPR. Therefore DPR results in less protein contaminants and more enzyme products. This scheme

would seem to be time consuming and will be costly unless the enzymic preparation can be recovered for the cytosolic lysis step and other batches.

Chemical permeabilisation of *E. coli* using a combination of guanidine-HCl (Gu-HCl) and Triton X-100 was reported by *Hettwer and Wang (1989)*, who found that protein release yield was high (about 50 %) at two regions: 0.1 M Gu-HCl and 2 % Triton and G-HCl greater than 2M. The higher Gu-HCl concentration was shown to cause severe loss of activity for 5 enzymes examined. Therefore for the same yield the combination of the two reagents would be more acceptable, although the protein release rate was 15 times slower. It was also shown that permeabilisation involves a solubilisation of the inner membrane. This means that there is no cell fragmentation and nucleic acid release, which will enhance the cell debris removal and eliminate or simplify the nucleic acid removal step. However it was noted that the chemicals must be removed, irreversible loss of product by denaturation can occur, and suboptimum yields. In the case of recombinant inclusion bodies the Gu-HCl must be concentrated enough to solubilise it.

Further work by *Naglak and Wang (1990a)* showed that β-lactamase expressed by a recombinant *E. coli*, 96 % of which is produced in the periplasmic space, could be selectively released by permeabilisation with 0.2 M guanidine-HCl alone, but with only 4 % protein release. The purification was 40 fold compared to whole cell extract and 15 fold only for lysozyme/EDTA treatment. Therefore, expression of foreign products into the periplasmic space may be a strategy which will simplify downstream processing considerably. More work by *Naglak and Wang (1990 b)* achieved similar levels of protein release with *Pichia pastoris* using 2M guanidine-HCl and 0.5% Triton X-100; mechanical "bead-beater" (bead mill) and enzymatic lysis ("lyticase" preparation from *Arthrobacter luteus*): 46 % in 24 h, 53 % in 4 minutes and 69 % in 2 h, respectively. Clearly, the time factor is the main disadvantage, and also the high concentration of G-HCl causing denaturation of products, but the cells are not lysed. The simplicity of using a stirred vessel for such a technique will obviously save capital costs.

An enzyme which had to be extracted by chemical lysis on a large scale from a 1000 L fermentation was cholesterol oxidase from a *Nocardia sp. (Buckland et al 1974)*. The APV Manton-Gaulin homogeniser released it very slowly with a rate constant of 0.0085 (cf. 0.23 for *S. cerevisiae*) at 50 MPa. With a 0.5 % Triton X-100, only 40 % of the total (as determined by sonication) could be released, and this had to be done at the end of the growth phase because the yield would drop to 12 % if harvested 6 hours after growth stopped. However only 2 % of total protein was released, thus facilitating rapid further purification. As secondary damage of the cell wall was needed for full release, (by prolonged sonication) the enzyme was thought to be associated with the cell membrane.

1.3.5 Effect of cell morphology on disruption

In contrast with organisms such as bacteria and yeast which have fairly definite sizes and shapes, and are therefore easily characterised, filamentous microorganisms form structures which vary from several hyphal units with innumerable branches entangled to form a complex mycelial network such as in *Aspergillus*, to a few hyphal units with few branches as in *Streptomyces*. This presents a different aspect to its disruption as the effects of entangled filaments may cause interaction and perhaps easier disruption despite the high strength of the cell wall. Few studies have related morphology directly to variations in disruption kinetics; the following mention the morphology changes as possible reasons.

Only recently *Sauer et al (1989) and Harrison et al (1990)* have suggested that the cell morphology was a possible cause of a change in disruption kinetics. Sauer et al, using a microfluidizer (sect. 1.3.3.4) to disrupt both a native and recombinant *E. coli* found that the latter was always easier to disrupt. Initially, it was thought that when the recombinant strain was heat induced (for expression) the change in morphology to short "fat" chains of 3-4 cells resulted in easier disruption because of the linkage points between the cells may be weak. The heat induced native cells did not undergo a change in morphology. However, an experiment without heat induction resulting in no change in morphology for both strains, still resulted in greater disruption for the recombinant strain. Therefore it was noted that it is uncertain whether morphology change is the cause; it is likely that physiological differences also plays a part.

Harrisson et al (1990) found that there was an increase in sphericity from a small short rod as growth of a Gram-negative bacterium *Alcaligenes eutrophus* progressed, due to accumulation of an intracellular storage product poly-β-hydroxybutyrate (PHB). They also found that exponential phase cells were disrupted with only one pass at 60 MPa (Manton-Gaulin high pressure homogeniser), whilst stationary phase were disrupted only after 2 or 3 passes at the same pressure. However, disruption of stationary phase, more rounded cells seem to be initiated at a lower pressure than exponential phase, smaller, rod shaped cells ie. fractional protein release was higher for pressures below 60 MPa, although the comparison was only based on a single pass run. It was postulated that this was due to the increased cell size.

Whitworth (1974) found incomplete release of only 30 % of total protein from *Candida lipolytica* in a Manton-Gaulin homogeniser after 6 passes at 55 MPa, and non-first order rate of protein release. It was suggested that the heterogeneous culture of elongated-ovoid and filamentous cells may be responsible.

It is well known that morphology of filamentous microorganisms are significantly affected by growth conditions such as stirrer speed and medium composition (section 1.2.2), and since these conditions cause differences in disruption kinetics, morphology must therefore have an impact.

If the morphology is largely filamentous, it would be reasonable to surmise that on passing a suspension of these cells through the discharge valve of a high pressure homogeniser, there is considerable interaction between filaments by stretching of entangled hyphae. The degree of entanglement will affect the interactions. Consequently if a mycelial aggregate is stretched in one or more directions, the intertwined hyphae will eventually break up by the shear effects. This process of disentanglement leading to breakage is clearly observed in a bead mill.

On the other hand, if the morphology is pelleted, the "core cells" in the centre of the pellet may not be broken, depending on the size of the pellet. An additional step of "opening up the pellet" before actual disruption may be required, after which a filamentous structure may be obtained. The dense pellet would not allow a considerable amount of interaction between the filaments since these are packed tightly. However, small pellets may be beneficial because the viscosity is usually very much lower, and the whole pellet can possibly be broken easily.

It is therefore necessary to examine methods to change the morphology, observing and characterising the morphology and linking it to any changes in disruption kinetics.

1.4 Impact of disruption on product recovery

1.4.1 Cell debris removal

The process of cell recovery will not have a significant effect on cell debris removal unless the dewatering has conferred irreversible changes to the structure of the cells or the interaction between hyphae, which are carried through the disruption process to affect debris removal. However, it is best to remove as much media or solids in the media as is economical to minimise the load of impurities in further purification steps.

Disruption of cells has a very significant effect on the cell debris removal either by filtration or centrifugal sedimentation. Few studies have been made, but it is clear that cell debris removal is a problematic process. *Su et al (1987)* found that increased number of passes of yeast cells through a high pressure homogeniser increased product release but deteriorated filtration properties. Although there is a decrease in suspended solids concentration due to liquid released from disrupted cells, which improves specific cake resistance, the decreasing average particle size and increase in "fines" has a far stronger effect on increasing the filtration resistance. Filtration was by dead-end method through 0.2 or 0.45 membranes. It was also mentioned that pH changes during disruption due to cell contents released can affect the cake resistance unless buffering is used. This introduces the factor of ionic strength which can also affect filtration. They also found that a filter aid added as body feed improved the filtration rate up to a certain point, when the effect of increasing solids content counteracted the purpose of the filter aid and so filtration rate decreased.

Studies by *Mosqueira et al (1981)* showed that yeast debris from disruption in a high pressure homogeniser required a settling rate of 6×10^{-8} m s^{-1} to achieve a 95 % recovery compared to 10^{-6} m s^{-1} whole yeast cells. In pilot scale disc stack centrifuge, an increase of feed flowrate from 315 to 3500 L h^{-1} would mean a decrease in sedimented solids from 99.9 to 30 % and 70 to 15 % for *E. coli* whole cells and debris respectively compared to yeast debris which falls from 93 to 80 % *(Higgins et al 1978)*. This illustrates the impact of decreased particle size upon centrifuge operation. Much smaller *E.coli* debris will only settle at very low feed rates for efficient separation and so requires operation at low throughputs leading to undesirable temperature rises.

Further studies with a recombinant *E. coli* producing prochymosin inclusion bodies showed that the recovery of the inclusion bodies (IB) (after high pressure homogenisation) requires a balance to be established between high residence times necessary for high yields of IB (which settle faster than cell debris) and short residence times to prevent sedimentation of cell debris. An optimum flowrate 200 L h^{-1} recovered 90 % of IB as sediment and 80 % of cell debris in the effluent. Further cell

debris removal will involve wash stages or rehomogenisation of the sediment *(Hoare & Dunnill 1989).*

A pilot plant study of the cell debris removal of *E. coli* disrupted in an SHL-15A Bran & Lubbe high pressure homogeniser by a single pass at 73 MPa at 60 g L^{-1} (dcw) was made with an Alfa-Laval BTPX-205 disc stack centrifuge *(Sanchez-Ruiz 1989)*. The centrifuge feed solids concentration, C_o was 24 g L^{-1} and at 11°C. Flowrates ranged from 19-77 L h^{-1} and a solids discharge time of 3.75 minutes. The efficiency of cell debris removal is shown in table 1.3. To achieve a separation efficiency S_{eff} of 0.93, a flowrate of 19 L h^{-1}, representing a reduction of 98 % in its hydraulic capacity (1200 L/h) was necessary. Increasing the flowrate to about 40 L/h reduced S_{eff} to 0.88, but a further increase beyond 40 L/h caused a significant drop to 0.54 at 77 L/h. Therefore, the debris removal can only be efficient with very low feed flowrates.

Feed flowrate (L/h)	Solids concentration in light phase, C (g/L)	C/C_o	Separation efficiency S_{eff} $(1- C/C_o)$
19	1.7	0.07	0.93
38	2.98	0.12	0.88
49	5.63	0.23	0.77
58	8.5	0.35	0.65
77	11.08	0.46	0.54

Table 1.2 : Influence of the feed flow rate on the removal of *E. coli* cell debris in a disc stack centrifuge. (Sanchez-Ruiz 1989)

Kloostermann et al (1988) related an optimal residence time for disruption of yeast in a bead mill with the minimum filtration resistance on a precoat filter. They showed that the mean particle diameter decreased with increase in residence time of disruption, but is virtually independent of agitator speed. The minimum blockage filtration resistance was obtained for a mean residence time between 80-100 seconds. The occurrence of an optimum mean residence time was explained by the interaction of two effects: as the mean particle size decreased with disruption time, the filtration resistance increased due to blockage of precoat pores; but an opposing effect of narrowing particle size distribution which causes less severe blockage because the intraparticle space between the larger particles may not be filled completely by larger particles, decreases the blockage resistance. Also noted was that the mean residence time optimal for maximal protein release differed from the one optimal for minimal filtration resistance. Therefore, it is necessary to optimise the two processes, which depends on other factors such as the value of the product. A high value product would

necessitate complete release of the product, but the presence of fine solids in subsequent filtrate may be the penalty.

A rotary vacuum filter was used to remove 85 % of the cell debris of *E. coli* producing β-galctosidase, but the use of filter aid was 5 to 8 times higher than for mycelial filtration although the feed flow rate was comparable *(Gray et al 1972).*

It is therefore necessary to select a disruption method which minimises the formation of very small particles and if possible to selectively release the product without extensive damage to the cell.

1.4.2 Precipitation

Recovery of the product would be much easier if it is possible to extract the product by precipitation in unclarified post-disrupted liquor. This will avoid a cell debris removal stage. However, it may be necessary to precipitate out a lot of other impurities first before fractionating out the product, which means separation of those impurities by other means may be more economical. Precipitation, being such a complex process dependent on several factors means it is not possible to draw any general rules. For example, the precipitation of salicylate hydroxylase from a *Pseudomonas sp.* by ammonium sulphate and polyethylene glycol is affected by the presence of contaminating cell debris and/or culture media components. For ammonium sulphate as precipitant, the presence of culture supernatant caused comparable enzyme precipitation at lower precipitant concentration, but for each case of absence or presence of culture supernatant, clarification prior to precipitation did not make any difference. However, with polyethylene glycol, presence of culture supernatant gave lower enzyme precipitation, but in each case of absence or presence of culture supernatant, unclarified feed gave greater precipitation. This work was by *Russell (1980).*

Therefore it is not possible to predict the impact of dewatering or disruption on precipitation. Individual tests need to be carried out to determine such effects.

1.4.3 Chromatography

Chromatography is a process used for the final stages of purification and is usually relatively expensive. As far as possible it would be best to remove as much impurities as possible using less costly methods such as precipitation, thus leaving chromatography to deal with the impurities which are very difficult to remove. Impurities which cannot be removed by preceding methods or impurities in very small amounts are separated by chromatography. The sensitivity of resolution and flow through chromatographic columns are easily affected by several compounds. This means that compounds released in disruption or unremoved by cell separation and washing must be

subsequently removed prior to chromatographic purification if it is known to affect it adversely.

1.5 Overall requirements for downstream processing

1.5.1 Process efficiency: yield and purification

The recovery and purification of any product will always result in some loss of the product. Product loss may be due to physical damage (shear effects); irreversible chemical denaturation (heat, pH, high salt concentration); or physical loss of product from the process stream. It is therefore vital to minimise these losses which can render a process uneconomical.

The process efficiency can be considered in two parts:

1) Purification of product or product enrichment
2) Yield, which minimises product loss.

For each step of the process it is important to achieve a maximum yield in the order of 90 % efficiency, especially in the primary stages of recovery such as cell recovery and disruption. Otherwise, the overall yield of the process will decrease rapidly. Alternatively, the fewer steps involved in downstream processing can increase the overall yield. Fig.1.17 shows the relationship between step yields, number of steps and overall yield in a process *(Fish & Lilly 1984)*

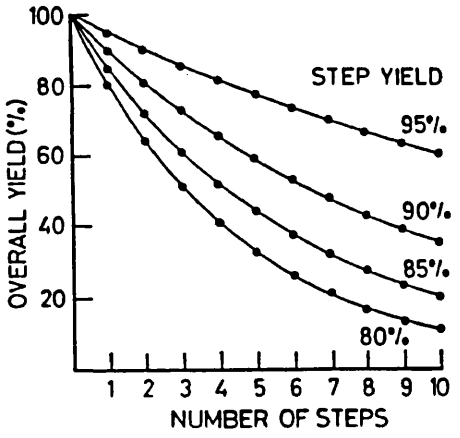


Fig.1.17 : Relationship between step yields, number of steps and overall yield in a process *(Fish & Lilly 1984)*

However, by maximising step yield, there is a negative effect on the purification of the product. In solid/liquid separation steps, a high step yield will result in lower product concentration because the recovery of most of the product also means some impurities will not be separated; and also operating

at lower flow rates.

Other unit operations such as disruption to extract the intracellular product will result in difficulties with cell debris removal due to increased disintegration of cell contents caused by maximising disruption to release or obtain maximum product recovery. The effect of a high step yield in final purification operations such as precipitation and chromatography is to reduce the purification factor, since to increase yield, it is inevitable that there is also increased impurities.

Therefore purification and yield must be considered with respect to each product to achieve economically acceptable final process efficiency.

The two requirements of purity and yield may be opposing each other, so that for example, for a human therapeutic drug, it would be very important to achieve close to 100 % purity despite lesser yields. Eventually it resolves down to the required purity of the product and then trying to maximise yield.

1.5.2 Interaction of fermentation with downstream processing

The interaction of fermentation and downstream processing and the integration of the two has not been studied very much, and certainly publications are few. Industry may have adopted this integrative approach but research leading to publications is scant. So far, the approach may be to optimise product yield in the fermentation and then it is up to the downstream processing to handle whatever comes out from the fermenter. This does not lead to high overall yields.

The effect of fermentation on downstream processing is more pronounced in the primary recovery stages of harvesting and extraction. Harvesting on an industrial scale may take several hours, but the enzyme titre in a batch culture may only be maximal for a much shorter period. This means that harvesting over a long period will have a range of cells having different enzyme titres. Therefore, unless the culture can be cooled rapidly to prevent further metabolism at the optimal enzyme titre, time of harvesting will need to be non-optimal. Furthermore, cells which are just suspended in broth, waiting to be harvested can autolyse, depending on the media constituents, shear effects and conditions of "storage". Cells grown in defined media or nitrogen or carbon limited media are more prone to cell lysis or increased protein turnover on exhaustion of media *(Gray et al 1972)*. Therefore such fermentations may require rapid processing. Cell lysis will not only cause problems in solid/liquid separation but, for an intracellular product, there will be loss of product in the overflow (supernatant) stream, unless another step is incorporated to recover this "extracellular" product. It also defeats the advantage of having an intracellular product: to process a smaller volume instead

of the far greater volume of media if the product was secreted. An alternative strategy may be to consider continuous flow processing.

In the case of filamentous microorganisms, the morphology and consequently the culture conditions which determine it, will decide the method of cell harvesting. Filamentous microorganisms can be harvested by means of a rotary vacuum filter or basket centrifuges because it forms a mycelial mat which also acts as a filter, and compacts well. However, if growth exists as pellets, the spherical form may not compact so well, which may lead to a wetter solids fraction. The use of complex media containing large number of suspended solids usually needs resuspension and washing of the cells to remove such components. Centrifugation does not allow washing of cells, and so an additional step of resuspension may be necessary but a method such as cross-flow filtration allows for in situ washing of cells by diafiltration. This additional step may cause changes in product yield. Media selection is therefore important in determining the downstream processing equipment. Choice of antifoam is also important because membrane filtration can be sensitive to adhesion of some antifoams, causing declines in flux rates.

The extraction of the product by mechanical or non-mechanical means depends a great deal on the fermentation conditions, since those conditions determine the strength and morphology of the cells. Disruption rates have been shown to vary depending on whether the cells have been grown on complex or simple media; whether the cells have been harvested and disrupted at an exponential or stationary phase of growth; whether the cells are grown at a high growth rate; the location of the product in the cell (which can possibly be controlled by genetic manipulation); and the mode of operation of the fermenter ie. continuous or batch. These effects can be found in section 1.3.3 and will not be further elaborated here. Of importance to filamentous microorganisms is the morphology of cells. It is reasonable to expect that the filamentous form and the pelleted form will possess different mechanisms of disruption and hence different disruption kinetics.

Other downstream processing steps are less influenced by fermentation conditions, since most of the impurities should be removed in the primary steps. However, as shown in section 1.4.2, precipitation can be affected by the presence or absence of fermentation media constituents. Antifoam selection is also important because non-metabolised ones can interfere with precipitation; may adsorb to ion-exchange resins or even cause problems in chromatographic separation.

1.5.3 Process control

Based on the knowledge of the interactions between fermentation and downstream processing, the yield and purification required, it should be possible to optimise the efficiency of the process. It is unlikely that fermentation batches are "identical" in producing identical enzyme titres or constant cell morphology, but if it is possible to determine certain limits or range of enzyme titres or morphological forms obtained, then it follows that downstream processing equipment can be selected to handle all these possibilities. This will mean that, if the fermentation produces a different cell morphology or quantity of enzyme, then the processing equipment selected can be adjusted to optimise recovery for that particular batch.

Therefore, it is hoped that eventually it is possible to examine the fermentation broth properties such as cell concentration, morphology, products levels, rheology, and perform some quick but essential off line tests to characterise the type of broth produced and then from previous studies on the behaviour of such a broth in cell harvesting, cell disruption and other recovery steps, it is then possible to decide how the downstream processing can be operated for maximum efficiency. Each performance run on different batches can then be used to give feedback information to fermentation process, as to how fermentation conditions can be altered to optimise downstream product recovery.

1.6. The use of *Aspergillus sp.* as a model system.

The fungi of the *Aspergillus* genus represent a very important industrial organism because it is used to produce several enzymes and organic acids. These products are primarily used in the food industry. Various enzymes such as amyloglucosidase, fungal amylase, pectinase and fungal protease, glucose oxidase, catalase are produced by *Aspergillus sp.* with production of amyloglucosidase amounting to about 300 tonnes per year, whilst for fungal protease it is less than 10 tonnes per year *(Barbesgaard 1977)*. Organic acids such as citric and gluconic acid are produced in vast quantities of 100,000 tonnes per year. Therefore *Aspergillus* represents an established industrial organism.

Further reasons for selecting *Aspergillus* is the increasing importance of fungi as recombinant organisms for the expression of foreign proteins *(Van Brunt 1986)*. As recombinant DNA technology improves, the selection of an appropriate host expression system has turned to yeast and filamentous fungi, from the traditional *E.coli*. Various reasons are presented for the preference for a change in host expression systems. Fungi are capable of performing post-translational events such as glycosylation, acetylation, protein refolding which seem to be necessary for the proper activation of eucaryotic proteins. Bacteria are unable to do this, although large quantities of protein can be made. Bacteria tend to produce recombinant products as inclusion bodies which require difficult and inefficient methods of re-activating the foreign protein. Inclusion bodies have not been found in

fungi yet. There are also problems of plasmid stability in bacteria. It is now possible to introduce foreign genes into fungi by integrating the plasmid containing the foreign gene into the host genome, thereby making it highly stable. The foreign gene is replicated together with the fungal genome and is carried through generations. *Aspergillus* as a host is convenient because it is an established industrial organism and *A.nidulans* is well characterised in terms of its genetics. Nevertheless, there are some products which cannot be secreted and will therefore rely on efficient extraction.

In addition, since *Aspergillus* is a septate fungi, it is likely to be more difficult to disrupt than an aseptate fungi such as *Rhizopus* where release of the alcohol dehydrogenase enzyme was complete by only 10 MPa *(Keshavarz 1990a)*. Therefore, the disruption of the *Aspergillus sp.* should be investigated as its disruption kinetics may be quite different.

In this research, *A.niger* producing intracellular glucose oxidase and catalase was used as the study organism. Additionally, a recombinant strain of *A. nidulans* producing intracellular alcohol dehydrogenase has been examined briefly.

2.0 MATERIALS AND METHODS

A process flowsheet of the system used and the various experimental "paths" are shown in fig. 2.1. Where mentioned, the address of common suppliers/companies are as follows : Fisons plc (Crawley, West Sussex, U.K.); Sigma Ltd. (Poole, Dorset, U.K.); and BDH Chemicals Ltd. (Poole, Dorset, U.K.); J.E. Sturge Ltd, (Selby, North Yorkshire, U.K.) which is now owned by Haarmand and Reiner.

2.1 Cultivation of *A. niger* strain

This section will cover the all the materials and methods used for the growth of the *A. niger* strain, from the maintenance and propagation of the master culture to its growth in the fermenter and any other techniques used before the downstream processing. Basically it will deal with the upstream side of the process.

2.1.1 Microorganism

A culture of strain 788 (non-recombinant) of *Aspergillus niger* producing high levels of intracellular glucose oxidase was a gift from J.E. Sturge Ltd.

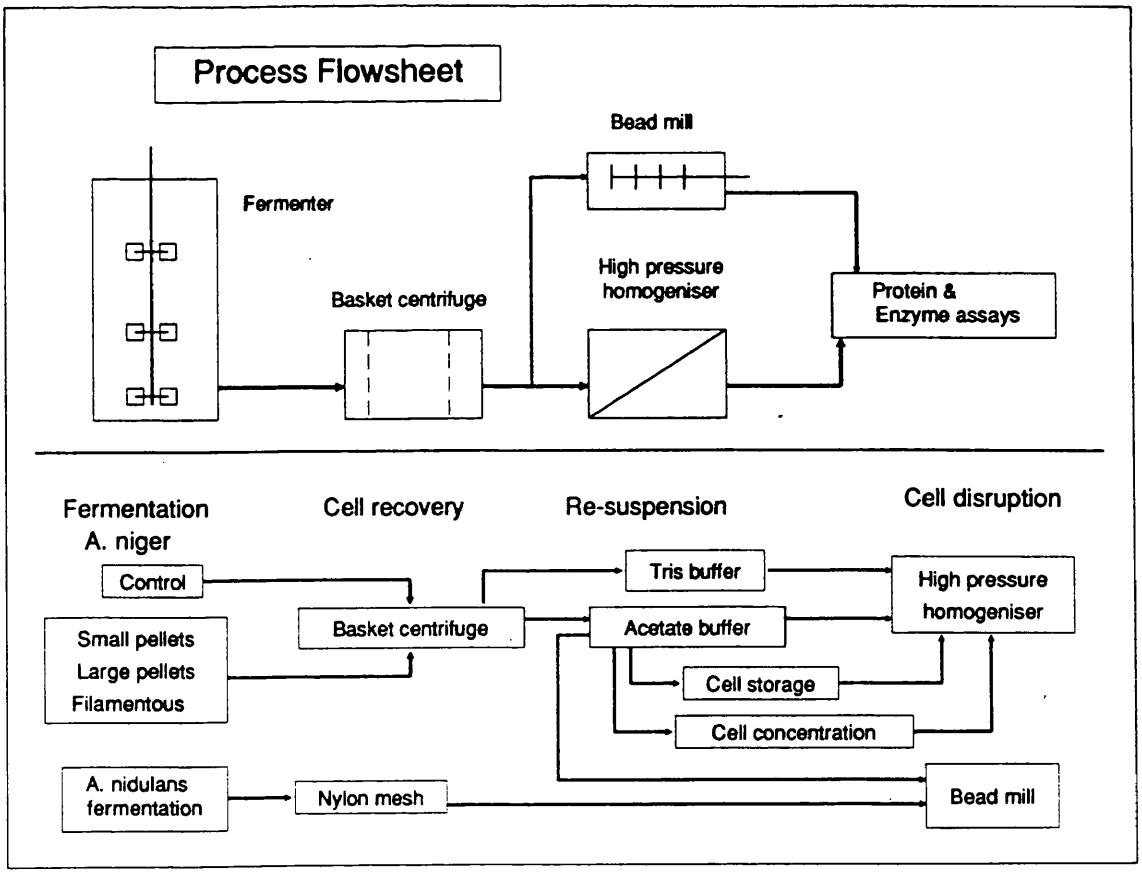


Fig. 2.1 : Process flowsheet and experimental programme

2.1.2 Culture maintenance and propagation

This concerns the preservation of the master culture and the propagation of the spores for fermenter cultivation.

2.1.2.1 Sporulation media and preparation

Beet molasses (gift from J.E. Sturge Ltd.) and of bacteriological agar (agar no.1) from Oxoid Ltd., (Basingstoke, Hampshire, U.K.) were dissolved in tap water to a final concentration of 300 g l^{-1} and 15 g l^{-1} respectively, and adjusted to a pH between 6.5-7 for preparing agar slopes either in 30 mL universal bottles or 500 mL medical flat bottles. This media was steam sterilised at 121°C, 0.1 MPa for 15 minutes. Sterile agar slopes can be stored at 4°C for several months.

2.1.2.2 Propagation of spores

All the procedures below were carried out under aseptic conditions. Some spores were transferred with a wire loop from the master culture on an agar slope, to 10 universal bottles with the sporulation media in order to obtain sub-master cultures. A large quantity of spores were grown on medical flats, using spores from one particular sub-master which were harvested with 10 mL 0.1 % (v/v) "teepol" detergent as a wetting agent (BDH Chemicals Ltd), diluted 10 fold and 0.5 mL used for each medical flat. These cultures were incubated at 30°C for seven days. The master and sub-master cultures were stored at 4°C. Fig. 2.2 shows a diagrammatic representation of the spore propagation.

The spores from the medical flats were harvested with 20 mL 0.1 % (v/v) teepol per medical flat, mixed together, and counted using a particle counting chamber (modified Fuchs Rosenthal ruling, double cell, standard platform, from Weber Scientific Int. Ltd, Teddington, Middx, U.K.). The spore suspension was then stored in a 20 % (w/v) sucrose (BDH Chemicals Ltd) solution at -70°C in 7 mL bijou bottles (containing 4.5 mL each) with a spore concentration of 2.2 x 10^8 mL^{-1}. For all fermentations, these were used as inoculum, thereby ensuring that they all originate from the same generation and batch of spores. This procedure also meant better reproducibility of fermentation characteristics.

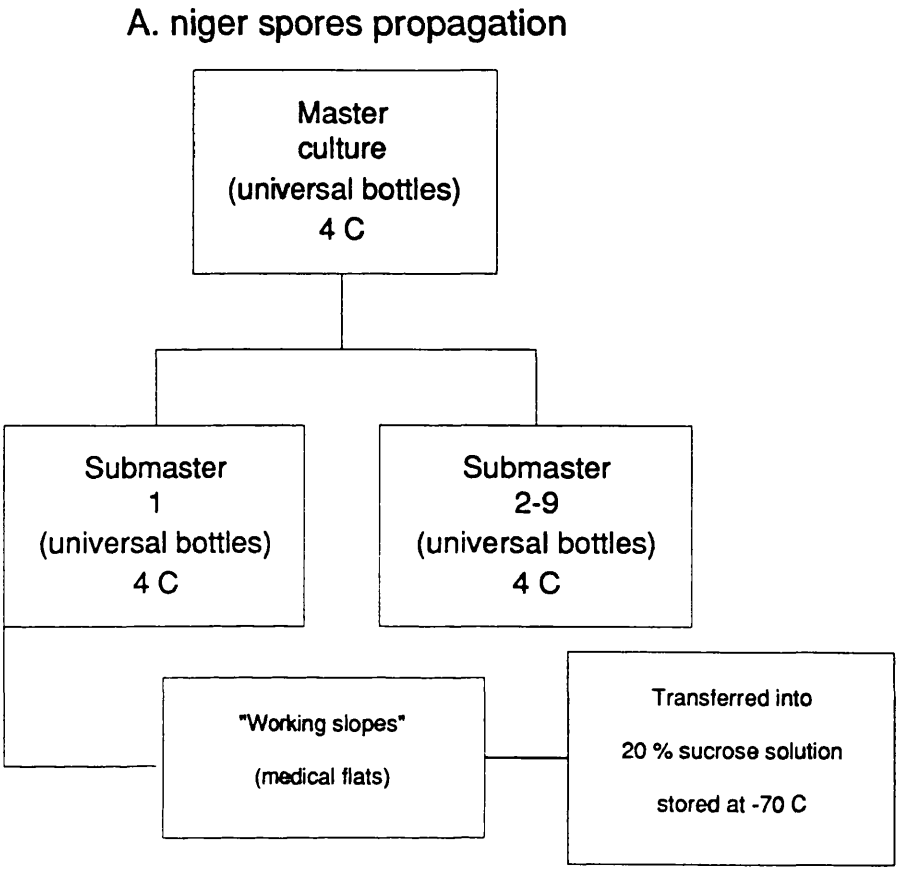


Fig. 2.2 : Propagation of *A. niger* spores

2.1.3 Submerged growth medium

The following recipe for the growth medium was provided by J.E. Sturge Ltd.

Constituent	Concentration (g L^{-1})	Supplier
Beet Molasses	100	J.E. Sturge Ltd.
Sodium Nitrate ($NaNO_3$)	5.2	Fisons plc
Magnesium sulphate ($MgSO_4.7H_2O$)	0.1	Fisons plc
Sodium dihydrogen phosphate (NaH_2PO_4)	0.05	Fisons plc
Calcium sulphate ($CaSO_4.2H_2O$)	2.6	Fisons plc

The medium was made up with tap water and adjusted to pH 5.2. This medium was used for all submerged culture growth.

2.1.4 Growth in fermenters

2.1.4.1 Fermenters

Most of the work was done in a 42 L fermenter from LH Fermentation Ltd. (Maidenhead, Berks. U.K.) which was operated in a batch mode with 30 L working volume. A schematic diagram of this fermenter is shown in fig. 2.3. One fermentation was done using a 1500 L fermenter from Chemap AG. (Männedorf, Switzerland) with 1000 L medium. All fermenters were stainless steel.

Instrumentation

The fermenters were equipped with Ingold steam sterilisable pH and dissolved oxygen tension (DOT) electrodes (Life Sciences Labs., Beds., U.K.). These parameters, including the temperature, stirrer speed and air flow rate have direct digital control. Foam control was detected by a foam probe consisting of a metal disc in the headspace, and earthed on the headplate. When foam touches the disc a circuit is completed which triggers the addition of antifoam polypropylene glycol 2000 (PPG) from BDH Chemicals Ltd. Exit gases were analysed by a microprocessor controlled VG MM-80 mass spectrometer (VG Gas Analysis, Cheshire, U.K.). Monitoring of all physical parameters was made by a micro PDP 11/73 MicroVAX 2 and VAXmate mini and microcomputers, which enabled calculations of OUR, CER and RQ during each fermentation.

Sterilisation

All the fermenters were jacketed for cooling by cold water or steam sterilisation. Medium was sterilised in situ for 0.5 h at 121°C 0.1 MPa, with 6 mL PPG antifoam (for 42 L fermenter) to prevent foaming and pH adjusted to 5.2.

2.1.4.2 Growth conditions

Control fermentations

The following physical parameters were set for the both fermenters : temperature 30°C, aeration rate 0.5 vvm and impeller tip speed 2.6 m s^{-1} (15 & 500 L min^{-1} and stirrer speed 500 & 200 rpm for 42 and 1500 L respectively). pH was controlled at 5.2 with 5M and 10M sodium hydroxide (Fisons plc) for the 42 and 1500 L fermenters respectively. No acid was necessary, since gluconic acid is produced. The spore inoculum size was 10^5 spores per mL of fermentation medium.

Pelleted growth

The medium and conditions were identical to that of the control fermentation, but the spore inoculum size was reduced to 10^4 mL^{-1} and 10^3 mL^{-1} for small pellet growth (0.5 mm diameter) and large pellet growth (0.9 & 1.8 mm diameter) respectively.

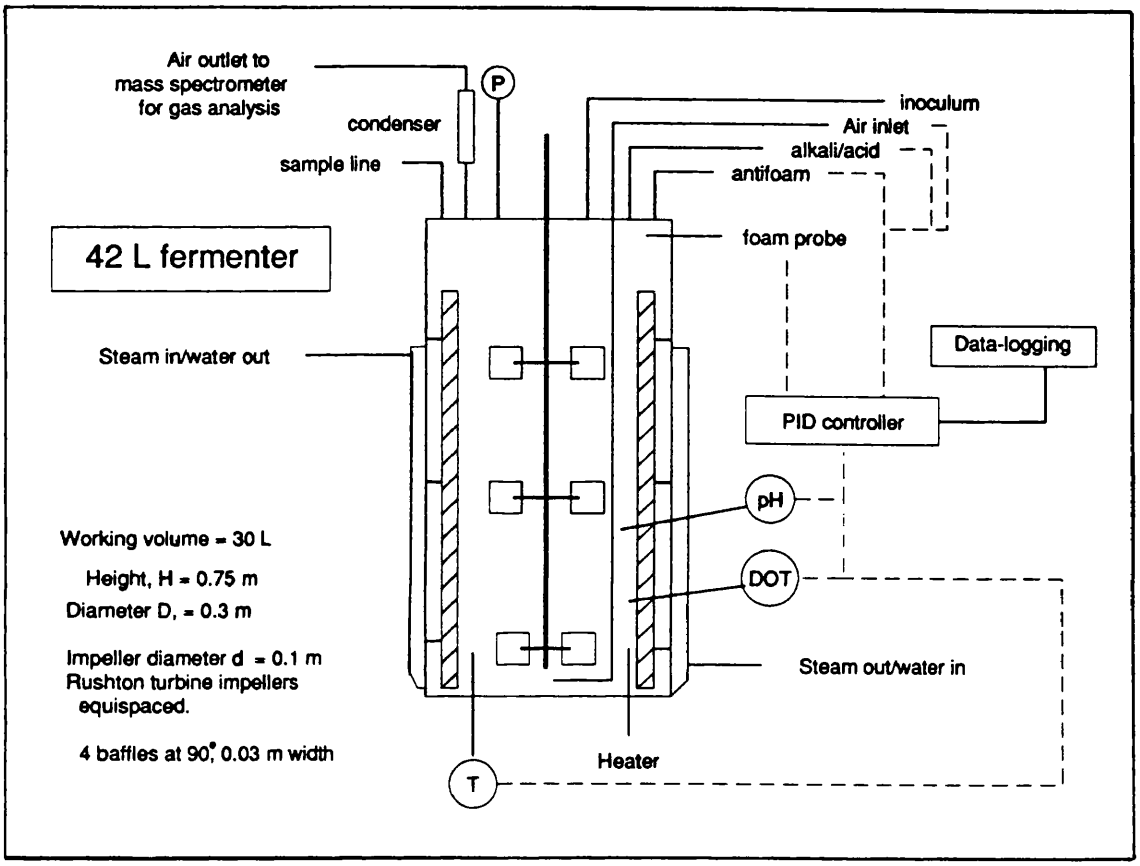


Fig. 2.3 : Schematic diagram of 42 L fermenter

Filamentous growth

Again the medium and conditions were identical as for control but in one case, a polyacrylic resin "Junlon PW110" (Honeywill & Stein Ltd., Sutton, Surrey, U.K.) was added at 1 g L^{-1} to the medium and sterilised together. In another case the stirrer speed was reduced in the 42 L fermenter to 300 rpm.

2.1.5 Dry Weight Measurement

For determining the growth curve and the cell disruption concentrations, the dry weight was obtained by filtering a known volume of sample through a pre-dried and pre-weighed glass microfibre filter, Whatman grade GF/A (Whatman Int. Ltd., Maidstone, U.K.). The sample was washed with twice the sample volume of distilled water before oven drying at 105°C to a constant weight.

2.1.6 Characterisation of Morphology

Two methods were used: the image analysis method and by photomicrographs. The former was only possible for the pelleted cells because it would not be able to distinguish individual hyphae in highly entangled filamentous mycelia and will therefore discard such results. Hence, photomicrographs were the only quick and convenient method for the filamentous morphology.

2.1.6.1 Image Analysis

Image analysis was used to characterise the pellet sizes. The equipment was a Magiscan 2A Image Analyser (Joyce Loebl Ltd., Gateshead, U.K.) attached to a Nikon Optiphot microscope (Nikon U.K. Ltd., Telford, U.K.) set at a magnification of 40 x . The software used was a general purpose commercial image analysis software called "MENU" (Joyce Loebl Ltd.).

A microscope slide of randomly chosen pelleted cells stained with trypan blue dye was placed on the microscope, and by a random but systematic (so as not to repeatedly examine the same pellets) manual movement of the microscope stage, an image was obtained. A live digitised image from the microscope via a television camera is sent to the image analyser where the image is captured and then a threshold made to give a binary image. A light pen was then used to select pellets for measurement. This selection was based on excluding small particles of media and other irrelevant particles. No pellets of atypical shape or size were excluded. The only intervention was to exclude non *A. niger* particles and to distinguish and separate pellets (by the light pen) which were so close, but different ones which may have been regarded as one large pellet.

Since the pellets were not entirely spherical, the machine was programmed to automatically measure the "length" which is the longest distance across the pellet, and the "breadth" which was the distance

perpendicular to the length. Approximately 100 pellets were measured to give a statistically valid estimation of pellet size. These measurements were stored in a data file for subsequent statistical analysis using proprietary software in the image analyser. The average length was then taken to be the approximate diameter of the pellet.

2.1.6.2 Photomicrographs

In addition to characterising the morphology of whole cells, this was also a relatively good method of observing the cell disruption. Homogenate was also photographed for this purpose. All photomicrographs were taken with an Olympus camera OM-2 (Olympus Optical Co., London, U.K.) attached to an Olympus BH2 stereoscopic light microscope. Only the cell samples were stained with trypan blue dye to enhance the contrast, before mounting on the slides. All samples were taken randomly.

2.1.7 Ultrasonication of cell samples

In order to monitor the glucose oxidase production during fermentation, cell samples were ultrasonicated to disrupt the cells so as to assay for the intracellular enzyme. The following procedure was used:

(a) A known volume of cell sample from the fermenter was filtered through a pre-dried and pre-weighed ashless filter paper (Whatman Int. Ltd., Maidstone, U.K.). The filtrate which is the growth media was collected for analysis of extracellular glucose oxidase, before the cells are washed with twice the sample volume of deionised water to remove any media. The cells were then gently peeled off the filter paper. To ensure easy removal, the sample volume should be such that enough cells are deposited to form a layer about 1 mm thick, and that the cells are relatively "dry".

(b) The cells were resuspended in cold pH 6 phosphate buffer. The volume of buffer depends on the cell concentration at the sampling time. At initial stages (after 12 hours) the volume should be equal to sample volume but at about 24 hours when the cell concentration was about 5-6 g L^{-1}, twice the sample volume was used. This is because of possible incomplete disruption due to a too concentrated sample.

(c) About 20 mL of the resuspended cells were sonicated for 15 minutes at an amplitude of 14 μm in a jacketed glass chamber cooled by cold water at 15°C ("Soniprep 150" from MSE Ltd., Crawley, Sussex, U.K.).

(d) The sonicated cells were then centrifuged at 16000 g to separate the cell debris and the supernatant assayed for glucose oxidase.

2.2 Cultivation of recombinant *A. nidulans* strain

Similar to section 2.1, this will cover the growth of the *A. nidulans* strain.

2.2.1 Microorganism

A culture of strain *C62 PABA* (para-aminobenzoic acid requirement) *A. nidulans* was a gift from Allelix Inc. (Mississauga, Ontario, Canada). This was a self-cloned strain producing high levels of intracellular native alcohol dehydrogenase (ADH). There are a multiple copies (unknown) of the stuctural gene *alcA* (codes for ADH 1) and 20 copies of the *alcR* gene cloned by random insertion into the host genome using the plasmid vector *pBR322*. The *alcA* gene is controlled by a system regulated by the positive regulatory gene *alcR* which codes for a product that, when coupled with the natural inducer acetaldehyde, elicits expression of the gene. A second mode of control is by carbon catabolite repression under the control of the negative regulatory gene *creA (Lockington et al 1985).*

2.2.2 Culture maintenance and propagation

2.2.2.1 Sporulation media and preparation

The following medium was used to make up the complete medium (CM) agar for sporulation.

Constituent	Concentration (L^{-1})	Supplier
Glucose	10.0 g	Fisons plc
Peptone (type 1 from meat)	2.0 g	Sigma Ltd.
Casamino acids (casein, acid hydrolysate)	1.5 g	Difco (East Molesey, Surrey)
Bacteriological agar (agar no. 1)	2.5 g	Oxoid (Basingstoke, U.K.)
Yeast extract ("Veeprex B800")	1.0 g	Champlain Industries Ltd. (Stanbridge, Quebec, Canada)
Solutions: See below for make-up		
Trace elements	1.0 mL	
Vitamins	1.0 mL	
Salts	10.0 mL	
Adenine	10.0 mL	

Adjust pH to 6.5 with NaOH and make up with deionised water. The whole medium (including vitamins) was then steam sterilised at 121°C, 0.1 MPa for 20 minutes and poured onto sterile petri dishes.

Trace elements solution		mg L^{-1}
Sodium tetraborate,	$Na_2B_4O_7.10\ H_2O$	40
Copper sulphate,	$CuSO_4.5H_2O$	400
Manganese sulphate,	$MnSO_4.4H_2O$	800
Iron II sulphate,	$FeSO_4.H_2O$	800
Sodium molybdate,	$NaMoO_4.2H_2O$	800
Zinc sulphate,	$ZnSO_4$	8000

Salts solution		g L^{-1}
Potassium chloride,	KCl	52
Magnesium sulphate,	$MgSO_4.7H_2O$	52
Potassium dihydrogen phosphate,		152

Adenine solution 7.5 g L^{-1} in 0.05 M HCl

Vitamins solution	mg L^{-1}
Putrescine dihydrochloride	2500
Para-aminobenzoic (free) acid	400
Thiamine HCl (Aneunine HCl)	50
D-Biotin	1.0
Myo-Inositol	400
(Hemi) Calcium D-pantothenate	200
Pyridoxine monohydrochloride	250
Choline chloride (99 % crystalline)	1.4
Nicotinic (free) acid	100

All "vitamins" and adenine were from Sigma Ltd. Trace elements and salts were from BDH Chemicals Ltd.

Sterilisation : Trace elements - steam sterilise 121°C, 0.1 MPa, 15 minutes.
Vitamins, salts and adenine - filter sterilise by 0.2 µm "Acrocap" filter (Gelman Sciences, Northampton, U.K). Salts and vitamins stored with 1 mL chloroform ($CHCl_3$) but the latter in the dark at 4°C. The chloroform sinks and the solutions can be decanted off.

2.2.2.2. Propagation of spores

The fermentations described here were made using freshly grown spores which were propagated from a sub-master culture stored at 5°C, onto agar plates, incubated for 3 days at 37°C and then harvested on day 3 using sterile 0.1 % "Tween 80" (BDH Chemicals Ltd) and a glass "scraper" to remove the spores. All procedures were done aseptically. The spores were counted with a counting chamber (as for *A. niger* sect. 2.1.2.2) to determine its concentration.

For storage as freeze-dried culture, the master culture plate was propagated. Several agar plates were grown with the culture and harvested as above with Tween 80, counted, and resuspended in a 20 % (w/v) skimmed milk suspension (in sterile deionised water). The milk powder cannot be sterilised by heat because of denaturation of protein. Therefore only unopened (sealed) cans were used, which did not result in contamination. The spores suspension containing 5 x 10^7 mL^{-1} for one batch called batch A, and 1.1 x 10^8 mL^{-1} for batch B were pipetted into sterile glass ampoules (0.75 mL each) and freeze dried. These ampoules sealed by heating and fusing the glass at the mouth, and stored at 4°C. Spores can be used by resuspension in sterile water before re-propagating on agar plates.

2.2.3 Submerged growth medium

The follwing recipe for the growth medium was provided by Allelix Inc. (Mississauga, Canada):

Constituents	g L^{-1}	Supplier
Glucose	15	Fisons plc
Yeast extract	5	As for sporulation media
Ammonium nitrate, NH_4NO_3	8	Fisons plc
Potassium dihydrogen phosphate	4.5	Fisons plc
Magnesium sulphate, $MgSO_4$	0.5	Fisons plc
Trace elements solution	1.0 mL	As for sporulation media

The medium was made up with deionised water and pH adjusted to 5.0.

2.2.4 Growth in fermenters

2.2.4.1 Fermenters

All the fermentations were done in 12.5 L stainless steel fermenters (MBR Bioreactor AG, Switzerland) in a batch mode with 10 L working volume. The configuration of these fermenters were: Height = 0.5 m; diameter = 0.2 m; 3 x 12 bladed Rushton turbine impellers of 0.1 m diameter, equispaced; a ring sparger; and 4 baffles at 90°. Instrumentation was as described for the 42 L LH fermenters (section 2.1.4.1) except that the mass spectrometer was not used and foam control was through the use of silicone antifoam - "Dow Corning 1520 Antifoam" (20 % active food grade, Dow Corning, Reading, Berks., U.K.). This antifoam was diluted by x2 for use. The media was sterilised by steam in situ at 121°C, 0.1 MPa for 20 minutes; the glucose solution was sterilised separately.

2.2.4.2 Growth conditions

The following physical parameters were set: temperature 37°C; aeration rate 0.5 vvm (5 L min^{-1}); impeller tip speed 4.2 m s^{-1} (800 rpm), reduced during induction stage to 2.1 m s^{-1} (400 rpm). pH was controlled at 5 by 2.5 M ammonia solution (Fisons plc) and 2.5 M sulphuric acid. Inoculum size was 10^5 spores per mL of fermentation medium.

When the glucose is exhausted, as detected by a glucose analyser based on an immobilised glucose oxidase membrane (Model 27, Yellow Springs Instrument, U.S.A.), the inducer to cause expression of ADH is added : 100 mM of methyl ethyl ketone (Sigma Ltd) and 1.0 g fructose (Fisons plc) per L fermentation medium. The fructose keeps the cells alive without significant catabolite repression.

2.2.5 Dry Weight Measurement : the method for the *A. niger* strain was used (2.1.5).

2.3 Cell Recovery

This will deal with the harvesting and recovery of cells. The basket centrifuge was used for the *A. niger* strain grown in the 42 and 1500 L fermenters, whilst the polypropylene mesh was used for *A. nidulans* grown on a 10 L scale. Washing of the cells will also be mentioned.

2.3.1 *A. niger* - Basket centrifuge

2.1.3.1 42 L fermentation

The fermenter was pressurised to between 0.2 and 0.3 barg to force out the fermentation broth via the harvest valve to a basket centrifuge operating at 500 g (1800 rpm) (Model 3000 from MSE Ltd., Crawley, U.K.). The centrifuge contains a removable nylon basket (which retains the cells) housed in a perforated stainless steel bowl which allows the filtrate to escape. "Cake" capacity is 3 litres. If at any stage, cells appeared in the filtrate, then the centrifuge was stopped, the cells were removed and centrifugation resumed. After the first run to remove the growth medium, the cells were resuspended in 30 L of deionised water at 4°C and then re-centrifuged. This wash step was repeated once more.

2.1.3.2 1500 L fermentation

In this case the broth was fed through a mono pump at a flowrate of 720 L h^{-1} to a basket centrifuge operating at 500 g (1500 rpm) (Model RC 40 VPxR from Rousselet & Compagnie S.A., Annonay, France). This centrifuge also has a removable polypropylene filtration bag in a perforated bowl with a facility for simultaneous spray washing of cells during recovery (not used in this case). The bowl is 0.4 m in diameter, 0.25 m height and operates up to 3000 rpm (2000 g), handling a solids capacity of 18 kg. Similarly, the cells were washed the with deionised water by resuspension and re-centrifugation, except that only one wash was made. This still provided a more or less colourless filtrate indicating good removal of the molasses in the first stage.

2.3.2 *A. nidulans* - Polypropylene mesh

This simply involves filtration through a 50 µm pore size polypropylene sheet. The broth is poured onto the mesh and allowed to filter through on its own. Washing of cells was done twice with an equal sample volume of deionised water.

2.4 Cell Disruption

The materials and methods involved in the resuspension of the dewatered cells, high pressure homogenisation and bead milling will be covered here. Both *A. niger* and *A. nidulans* strains will be dealt in the same section.

2.4.1 Preparation of Cells : Resuspension and Storage

2.4.1.1 Resuspension

(i) ***A. niger***

Tris buffer : this was the standard buffer used for the re-suspension : 0.05 M Tris(hydroxymethyl)aminomethane (BDH Chemicals Ltd.) adjusted to pH 7.5 using hydrochloric acid. This buffer was used for compatibility with the G6PDH assay buffer. Normally, chloride ions should not be used in high pressure equipment, especially below pH 6, because of its corrosive nature, but in this case, since the pH was above 6, and the homogeniser rinsed thoroughly, it was considered safe.

Acetate buffer : this was made using 0.07 M sodium hydroxide (Fisons plc) and adjusting the pH to 5.2 with glacial acetic (ethanoic) acid (BDH Chemicals Ltd.) to form the sodium acetate buffer.

Both buffers were stored at 4°C before use and the cell suspension was kept likewise before disruption. As far as possible, all materials associated with the disruption step was kept cold, in order to minimise proteolytic and thermal degradation of proteins, as well as arresting cell activity. Resuspension simply means mixing in the cells with the buffer. The volume prepared will depend on the concentration of cells required and the minimal volume possible for the number of trials at various pressures and passes in the case of the homogeniser. For most cases there were six pressures for six passes each, disrupted at about 10 g L^{-1}. The minimal volume for six passes was 2.5 litres for the Lab 60 homogeniser and therefore about 15 L of buffer was prepared. This is provided the operator is very familiar with the piping network, because it is imperative that it is not run without liquid, especially under pressure. Resuspension should also take place within 30 minutes of cell recovery because there may be difficulties in achieving an homogeneous suspension if the cells are left dewatered, especially with a very dry cell paste.

(ii) ***A. nidulans***

All *A. nidulans* disruptions were done in 0.1 M sodium phosphate buffer at pH 8.3 stored at 4°C.

2.4.1.2 Storage of cells and homogenate

One experiment with *A. niger* using acetate buffer as the resuspension media, involved the storage of whole cells in various media for 24 hours before disruption. Subsequently, the homogenate samples from this experiment were stored as unclarified homogenate ie. cell debris and supernatant, at 4°C.

Whole cells storage - this experiment followed the procedure below:

At the peak of glucose oxidase productivity, the fermentation broth was harvested. 7.5 L out of the 30 L of broth was removed and stored. The remainder was dewatered in a basket centrifuge as described in section 2.1.3.1 and then stored in various media for 24 hours at 4°C before disruption in the homogeniser at 60 MPa for seven passes. These were:

(i) **Growth media** : cells harvested at 25 hours were stored as fermentation broth without any treatment - no dewatering; this storage mode is referred to as "broth".

(ii) **Water** : cells harvested, dewatered and washed were kept as a concentrated cell paste of 5% (w/w). It cannot be stored as it comes out from the centrifuge because at 15-20 % (w/w), it does not resuspend well - cells remain as 5 mm to 5 cm lumps. Therefore, some deionised water had to be added to make it more fluid. These are still referred to as "dewatered" cells.

(iii) **Acetate buffer** : dewatered cells from the centrifuge were resupended in acetate buffer as a concentrated cell paste, 5 % (w/w); This storage method is referred to as "buffer".

(iv) **Control** : this was simply cells harvested, dewatered, resuspended, disrupted and analysed within 2 hours of harvesting ie. no storage.

For the cells stored as "broth", these were dewatered in the basket centrifuge and resuspended in acetate buffer prior to disruption, whereas the other two were made up to the required concentration with acetate buffer. Therefore, the disruption itself still occurs in acetate buffer for all the cells.

Homogenate storage - the homogenate samples from the seventh pass (60 MPa) from cells stored in various media were stored as unclarified homogenate at 4°C for up to 12 weeks. Settling of cell debris occurred but on taking a sample for analysis, the homogenate was mixed thoroughly. After a period of 8 and 12 weeks, a sample of the homogenate was taken, centrifuged at 16000 g for 5 minutes and the supernatant assayed for glucose oxidase activity.

Another experiment used homogenate and the undisrupted cells from unstored cells in acetate buffer. Instead of only analysing for glucose oxidase in the seventh pass sample, protein and catalase were assayed for, in all the passes including the one at 0 pass ie. undisrupted. These were stored at 4°C as unclarified homogenate and analysed after 22 weeks.

One further experiment examined the protein content in stored homogenate from unstored cells using the Folin-Lowry protein assay instead of the Bio-Rad protein assay as used for all other experiments. Analysis was made after 2 and 5 weeks.

2.4.2 High Pressure Homogenisation

There will be two sub-sections : one dealing with the homogenisers used and another describing the procedures followed.

2.4.2.1 Equipment

Two different homogenisers were used both of which are from APV Gaulin Inc. (Everett, Mass., U.S.A.) and are models Lab 60 and 30CD. These are positive displacement pumps incorporating a single, adjustable, restricted orifice discharge assembly as described in section 1.3.3.2.

(i) Lab 60 homogeniser

This model has a double piston pump and was used for all pressures below 65 MPa. It is capable of operating up to 65 MPa and has a fixed feed flowrate of 60 L h^{-1}. A feature of this model is an aseptic sealing chamber around the pistons to allow sterilisation which is filled with running cold water as a coolant. It also serves as an indicator of product leakage through the pistons, if the clear water is clouded with homogenate.

To cool the homogenate quickly after disruption, the exit homogenate line is coupled to an APV Junior plate heat exchanger (APV Baker, Crawley, Sussex, U.K.) with a 70 % aqueous solution of ethylene glycol at -5°C as coolant. The cylinder block housing the suction and discharge valves can also be kept cold with ethylene glycol. Flow of coolant was regulated by a manual valve to ensure the cooled homogenate did not approach freezing point and was usually maintained at about 4°C. The temperature of fluid after the heat exchanger and on entry to the homogeniser was monitored by platinum resistance probes (model 3745 PT 100, Digitron Instrumentations Ltd., Hertford, U.K.) connected to a Digitron multi selector unit.

All three stainless steel vessels acting as feed and collecting vessels for the process batch had a capacity of 5 L. The pipework were all of stainless steel with stainless steel 3 way ball valves. The pressure of operation was indicated by a gauge mounted on top of the cylinder block. Fig. 2.4 shows a schematic diagram of the system.

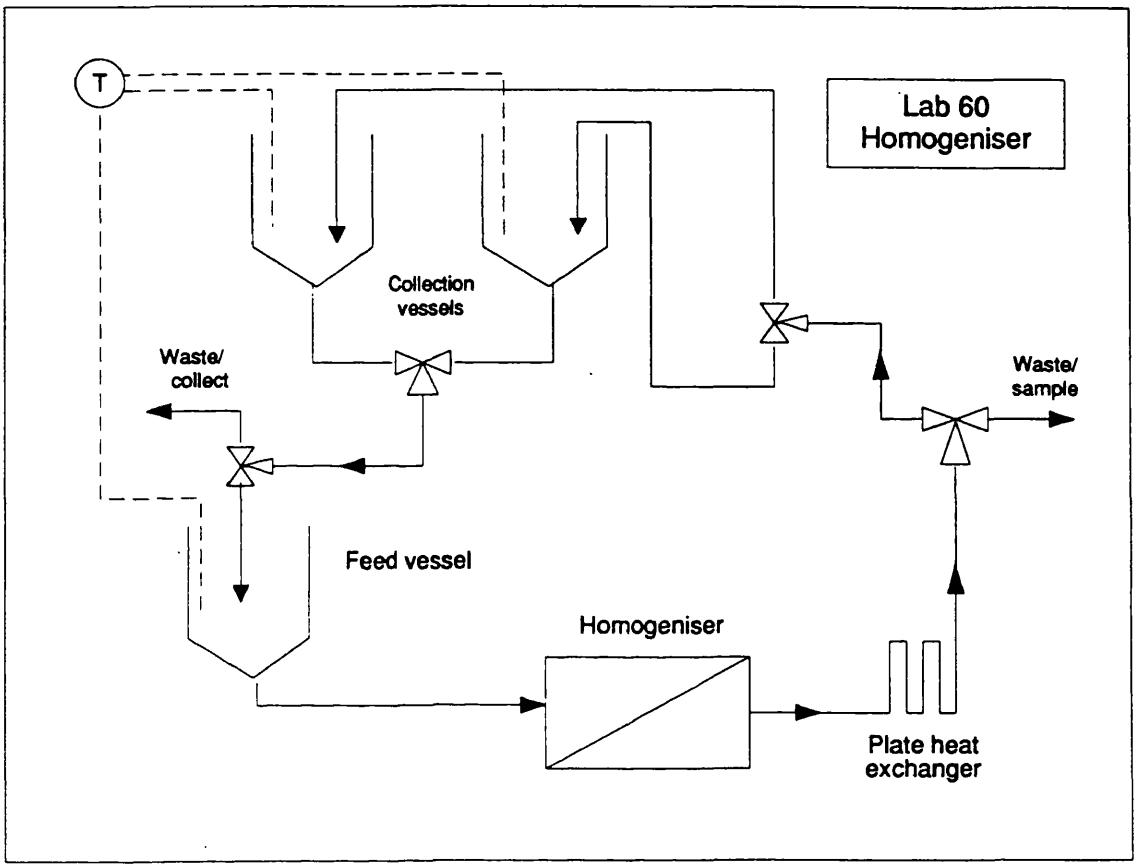


Fig. 2.4 : Schematic diagram of Lab 60 homogeniser system

(ii) 30CD homogeniser

Unlike the Lab 60, the homogeniser itself has a three piston pump and is a fully contained machine designed to operate up to a pressure of 104 MPa (15000 p.s.i.) routinely and at 124 MPa for periods up to 10 minutes. This machine is linked to a variable frequency drive which enables it to operate between 70 - 113 L h^{-1}. It has been designed with partially contained ancillary feed and collection vessels, which may be converted into pressure vessels for a completely contained system. There are a number of special features:

(1) The piping and vessels have been designed so that a very small volume of between 1.5-2 L of feed can be disrupted on a discrete pass basis by switching between the two feed hoppers.

(2) A back pressure relief valve on the exit line from the homogeniser which will operate if there is a build up of pressure above 4 bar in the system.

(3) A cooling system based on chilled water at 2°C, cooled by ethylene glycol at -5°C. This ensures that no freezing of homogenate is possible.

(4) All lines and vessels including the homogeniser can be sterilised by steam when fully contained.

(5) Aerosol release is minimised through double seals on the pistons and the homogeniser valve unit. Any leakage can be detected through a series of transparent silicon tubings.

Fig. 2.5 shows a line diagram of the 30CD homogeniser system.

(iii) Disruption valve

The disruption valve used in all experiments was the "cell disruption" type unit as mentioned in section 1.3.3.2 . A diagram (not to scale) of the valve is shown in fig. 2.6. In the Lab 60, the two valves used were identical but one was ceramic and the other tungsten carbide. There should be no difference in performance, except that the ceramic one is more hard wearing.

2.4.2.2 Homogenisation procedure

(i) Lab 60 homogeniser - operating procedure

Homogenisation was always done in a discrete pass basis to ensure that there is no mixing between passes. In order to do this, it was necessary to determine the time that it takes for the feed to travel from the homogeniser inlet to one of the collection vessels - this is called the hold up time. This time was found to vary at different pressures, due to an increased flowrate as the pressure decreases. the lag times are as follows:

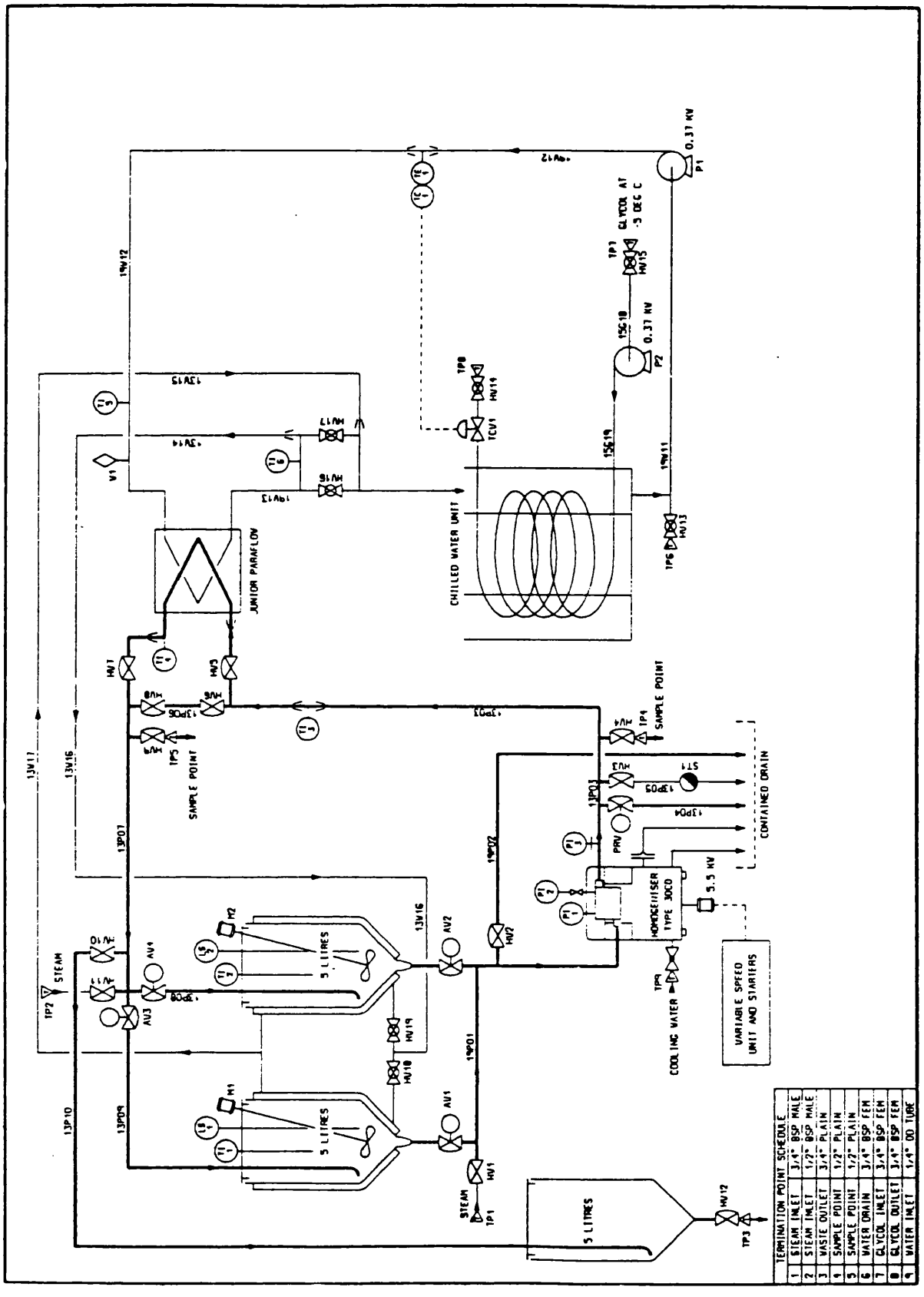


Fig. 2.5 : Line diagram of 30CD integrated homogeniser system

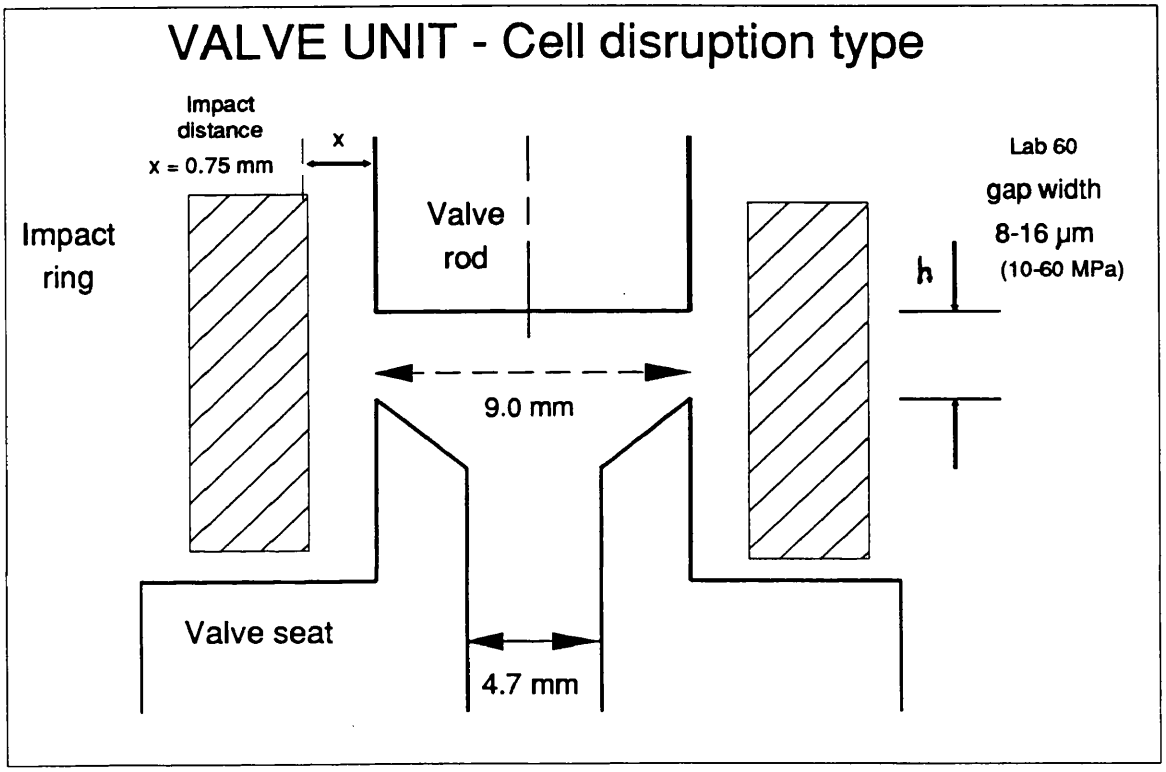


Fig. 2.6 : Schematic diagram of cell disruption type (CD) valve unit used (not to scale)

N.B.: Please note that the edges on the valve seat are not as sharp as drawn. A more accurate diagram of the CD valve unit is shown in Fig.1.10

Pressure (MPa)	Lag time (s)	Flowrate (L h^{-1})
10	47	74
20	49	71
30	50	70
40	52	67
50	55	64
60	58	60

The procedure for discrete pass operation

(a) Ensure that the lubrication water is flowing through the aseptic chamber at a steady but low flowrate; the handwheel on the valve assembly is on the open position (loose) and there is clean soft, deionised water or buffer in the feed vessel. Arrange valves on piping for recycle back into feed vessel.

(b) Switch on the pump and clear the system of any air by recycling liquid without any pressure for a few minutes. At the same time the cooling system can be turned on to start cooling the homogeniser. When the test liquid is recycling at about 4°C, turn the handwheel to raise the pressure to requirements, let it stabilise, check temperature of outlet and adjust cooling as necessary.

(c) Now that the system is set with the test liquid, switch the exit flow to discharge into waste. Once the feed vessel is almost empty, pour in the cell suspension quickly but without entraining too much air and keep it stirred. Simultaneously, record the hold up time plus 5 seconds for that pressure at which time switch one of the ball valves to collect homogenate in either collection vessel. The additional 5 seconds will ensure that any mixing between passes expelled and only pure homogenate from the first pass is collected.

(d) When the feed vessel is empty again (ie. 1st pass complete), empty the 1st pass homogenate into the feed vessel again to begin the second pass and after the hold up time, discard 5 seconds of homogenate (which is the mixed volume between 1st and 2nd pass), and then switch over to collect 2nd pass homogenate in the other collection vessel. The first one containing the 1st pass homogenate is still emptying into the feed vessel.

(e) Again when the feed vessel is empty, ie. 2nd pass complete, repeat step (d), switching between collection vessels for each pass, until the required number of passes is completed. When the last pass is completed, fill the feed vessel with water instead of homogenate, and wait for the hold up time before reducing the pressure and switching off pump.

(f) Samples of 7 mL were collected when about half the feed volume for the pass is homogenised ie. in the middle of the pass.

On no account must the pump be switched on when there is no liquid feeding in, and must not be switched off when the valve assembly is pressurised. At all times it is essential to observe the temperature of exit liquid. As the whole cells go through the first pass, there tends to be a knocking sound due to the tendency of the mycelial cells to separate from the suspending liquid just before entering the homogeniser and thus resulting in a heterogeneous mixture, which may lead to air pockets being formed. As the air passes through the compression and decompression causes the knocking sound. This is not a problem provided, it does not persist beyond the first pass.

(ii) 30CD homogeniser - operating procedure

As with the Lab 60, there is a hold up time but this is only 30 seconds for the flowrate of 84 L h^{-1} which was used for all pressures below 10 MPa. At 10 and 124 MPa it was necessary to use a higher flowrate of 94 L h^{-1} to avoid possible air pockets causing "knocking". The resulting hol up time was 26 seconds. Instead of manual valves, there are pneumatically controlled ones for changing the flow path, operated from the control panel. In addition, the level meter placed at the bottom of the feed vessels will sound an alarm if the liquid level is too low. The cooling system can be turned on before operation because there is no possibility of freezing. The operating procedure is also on a discrete pass basis, except that, the two feed vessels also act as collecting vessels. In this case, because of the small suction and discharge valves, it was anticipated that the mycelial cells may block these up. Therefore, the cells were preliminarily broken up in the Lab 60 at 50 MPa for 1 pass before feeding into the 30 CD.

(a) Once more, ensure lubricating water for pistons is flowing, cooling system turned on and there is some liquid in the feed. The procedure is then similar to steps (b) and (c) for the Lab 60 except that instead of pouring the cells into the feed vessel, it can be already in the other feed vessel so that once the pressure is set and the water/buffer emptied, the feeding vessel is simply switched over to the one with cells to start the 1st pass. Also, stirring is automatically carried out by the 2 bladed stirrers installed. The hold up time is now 30 seconds but the 5 second purge is maintained.

(b) For the other passes, the steps are similar to (d) and (e) for Lab 60 except that there is no need to empty from collection vessel to feed vessel. To move from one pass to the other the feeding vessels are simply switched over. Therefore, the two vessels alternate between acting as feed and collection vessels. Temperature control is not a problem since freezing is not a potential problem. Even at 124 MPa the 35°C exit homogenate is quickly cooled to 10°C. Similarly samples are taken at the "middle" of the pass.

2.4.3 Bead Mill

2.4.3.1 Equipment

The bead mill used was a horizontal agitator bead mill called "Dyno-mill" Type KDL (Willy A. Bachofen AG, Basel, Switzerland). The same bead mill was used for both *A. niger* and *A. nidulans* strains except that for the former the disruption chamber was a 0.6 L stainless steel vessel with a four impeller system whilst the latter used a 0.15 L lead free, glass disruption chamber with one impeller. The type of agitator discs used were made of 64 mm diameter "open" polyurethane type as shown in fig. 1. in section 1. . The machine was capable of operating at tip speeds of 6.7, 10, 15 and 20 m s^{-1} corresponding to agitator speeds of 2000, 3000, 4500 and 6000 rpm. Cooling of the suspension was made by 70 % aqueous ethylene glycol at -5°C in the case of the 0.6 L vessel and chilled water at 2°C for the 0.15 L chamber.

2.4.3.2 Operating procedure

All disruptions were batch mode operations with the chamber filled with 0.5 mm diameter ballotini glass beads. For the 0.15 L chamber, 225 g beads (70 % w/w) and 90 g of fungal cell suspension were mixed together and loaded into the chamber. For the 0.6 L chamber, 500 mL of beads were loaded (83 % v/v), and then the cell suspension pumped into the chamber with the agitator manually turned to mix in the suspension, until cells start to exude from the top. This is when the chamber was full. In both cases, any air pockets were expelled by gently mixing the suspension. The suspension was left to cool down to about 5°C if necessary, before disruption. For the *A. niger* cells, these were resuspended in acetate buffer.

Bead milling was done simply by switching on the motor driving the agitator shaft. At the appropriate times for sampling, the agitator was stopped 5 seconds before the actual time required to allow it to slow down. Samples of 1 mL and 5 mL were taken for the 0.15 and 0.6 L chambers respectively, after allowing the beads to settle. It was found that the temperature in the 0.6 L chamber rose to about 25°C after the first minute of disruption at 2000 rpm and so it was left to cool down to below 10°C before continuing. For the other chamber, this cooling period was omitted as the suspension was usually below 15°C.

2.5 Analytical Techniques

This section will cover the analytical work from the preparation of samples for assays through to the assay method itself. All absorbances were measured in a spectrophotometer (model PU 8800, Pye Unicam Ltd., Cambridge, U.K.) unless otherwise specified.

2.5.1 Sample preparation

All *A. niger* homogenate samples were processed in the same manner for the protein and enzyme assays. *A. nidulans* samples will be mentioned separately. One other preparation of *A. niger* samples using sodium dodecyl sulphate (SDS) for determination of total protein content will also be presented.

2.5.1.1 Protein and enzyme assays

(i) *A. niger*

All samples taken from the disruption trials were immediately placed in ice. These samples were then transferred to 1.5 mL micro test tubes (eppendorf vials), ensuring that the sample was well mixed (because of cell debris settling), and centrifuged at 16000 g for 5 minutes to separate the supernatant for assays. Once centrifuged, the samples were returned to ice but since the cell debris compacts well into a dense pellet it was unnecessary to decant or pipette off the supernatant except for the whole cells (0 pass sample). The supernatant was then used for assays. All samples were assayed as soon as possible and never more than 12 hours after disruption, unless otherwise specified.

(ii) *A. nidulans*

These samples which were already in 1.5 mL micro test tubes were centrifuged at 16000 g for 6 minutes and supernatant pipetted off. The supernatant was then frozen in "dry ice" at -70°C, because of the unstable ADH, and thawed at room temperature when ready to assay, and re-centrifuged for 5 minutes at 16000 g, after which the supernatant was used for assays. All samples were kept in ice.

2.5.1.2 SDS treatment of homogenate

Unclarified homogenate samples were added to 5 mL of 0.5 % (w/v) of sodium dodecyl sulphate (SDS) (Sigma Ltd.) solution in volumes of 50, 100, 200, 500 and 1000 µL to achieve various dilutions so as to be in the range of the protein standard curve (section 2.6.2.2). These mixtures were then mixed thoroughly by vortexing and then incubated in a boiling water bath (100°C) for 20 minutes, allowed to cool at room temperature, mixed again before using the whole mixture for protein assay by the method of *Ehresmann et al (1973).*

2.5.2 Protein assays

Three protein assays were used: the Bio-Rad protein assay, Folin-Lowry assay and the method by Ehresmann et al (1973) which will be called the "dual wavelength method". All assays were done in duplicates.

2.5.2.1 Bio-Rad Protein Assay

This was the method used for virtually all the protein assays including for the *A. nidulans* except that the technique was slightly modified. Unless otherwise specified all protein contents were determined by this method.

The Bio-Rad protein assay (Bio-Rad Labs. Ltd., Hemel Hempstead, Herts., U.K.) is based on the observation that the absorbance maximum for an acidic solution of Coomasie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. This is also the basis of the Bradford method *(Bradford 1976)*, the preparation being commercially prepared in this assay.

The standard assay method was followed :

1. A protein standard curve was prepared each time the assay was performed using bovine serum albumin (BSA) (lyophilised powder, Sigma Ltd.) made up to various known concentrations in deionised water from 0.1 to 1.0 mg ml^{-1} in 0.1 mg ml^{-1} intervals. The same procedure for the samples was applied to these standard BSA solutions.
2. Place 0.1 mL of standards or appropriately diluted samples (supernatant from homogenate) in plastic disposable 4.5 mL cuvettes. Place 0.1 mL of sample diluent (deionised water) in two cuvettes as the "blank". Assay performed at room temperature.
3. Add 3 mL of 5 x diluted dye reagent concentrate and mix well. (modified from 5 mL)
4. After a period of 15 minutes the absorbance at 595 nm was measured against the reagent blank.
5. The absorbance at 595 nm was plotted against the concentration of BSA standards and the unknown protein concentrations read from this standard curve.

For the *A. nidulans* samples, this assay was modified to use with a "Titertek Multiskan MCC/MCC340" spectrophotometer which only takes 300 µL volumes in its assay "wells" on specialised trays : 10 µL of sample was added to 200 µL of 5 x diluted dye concentrate reagent, incubated for 5 minutes and read at 595 nm. This was also the same for the BSA standards.

2.5.2.2 Folin-Lowry Protein Assay

This method *(Robyt & White 1987)* is based on the Lowry method *(Lowry et al 1951)*.

The following reagents were prepared : (All chemicals from BDH Chemicals Ltd)

1. **Alkaline Copper reagent** : Add 1.0 mL of a solution containing 0.5 % (w/v) copper sulphate ($CuSO_4.5H_2O$) and 1.0 % (w/v) sodium-potassium tartrate to 50 mL of 0.1 M sodium hydroxide containing 2 % sodium carbonate.
2. **Folin-Ciocalteu phenol reagent** : Dilute 1:1.

Method : (volumes modified - halved)

1. 0.25 mL of the appropriately diluted sample or BSA standard (Sigma Ltd) was added to 2.5 mL of the alkaline copper reagent, mixed vigorously and allowed to stand at room temperature for 10 minutes.
2. Then, 0.25 mL of the Folin-Ciocalteu reagent was added rapidly, mixed and allowed to stand for 30 minutes at room temperature.
3. The absorbance at 600 nm was measured against the reagent blank and a standard curve for BSA concentrations from 0.02-0.4 mg ml^{-1} obtained from which unknown protein concentrations determined.

2.5.2.3 "Two wavelength" method

This method is from Ehresmann et al (1973) who used it to determine protein concentrations in extracts containing tRNA's and rRNA's. In the presence of SDS, the Bio-Rad and Folin-Lowry method based on charged dye binding would be completely invalid. This method was therefore used, which simply involved measuring the absorbance of the BSA standards or samples at a wavelength of 228.5 nm and 234.5 nm. The difference in absorbance at these wavelengths represents the protein content. A standard curve was made using BSA (Sigma Ltd.) concentrations from 0.02 to 0.2 mg ml^{-1} dissolved in 0.5 % SDS, and a plot of the absorbance difference against BSA concentration used to determine unknown protein concentrations. The assay was performed at room temperature.

2.5.3 Enzyme assays

Four enzymes were assayed : glucose-6-phosphate dehydrogenase (G6PDH), glucose oxidase, alcohol dehydrogenase, and catalase.

2.5.3.1 Glucose-6-phosphate dehydrogenase (G6PDH)

This method was from *Bergmeyer (1983)*. The following reagents were prepared in deionised water:

1. β-Nictotinamide adenine dinucleotide phosphate (NADP), Sigma Grade 98-100 % sodium salt: 3.8 mM
2. Glucose-6-phosphate, G-6-P : 33 mM.
3. Magnesium chloride, $MgCl_2.6H_2O$: 0.63 M
4. Tris buffer, Tris(hydroxymethyl)aminomethane : 0.5 M adjusted to pH 7.5 with HCl.

All reagents were from Sigma Ltd. These were all stored at -20°C except for the Tris buffer.
For each assay the following working reagent or assay mixture of 1 mL per sample was prepared:

Reagent	Volume (mL)	Concentration in assay mixture (mM)
Deionised water	0.58	N/A
NADP	0.10	0.38
G-6-P	0.10	3.3
$MgCl_2$	0.10	6.3
Tris	0.10	50

In practice a stock solution of assay mixture was made fresh for the required amount of assays. No maleinimide was used because it did not make any difference to the readings. It is used to inhibit 6-phospho gluconate dehydrogenase (6-PGDH) which causes further oxidation of the reaction products of G6PDH and hence misleading results.

Method : Add 0.98 mL of assay mixture (at 25°C) to 0.05 mL of sample in a 1.5 mL cuvette (10 mm width) and mix thoroughly. Wait for about 30 seconds before reading the absorbance change over a period of 1 minute at 339 nm in a temperature controlled (25°C) spectrophotometer against a deionised water blank. The rate of change of absorbance is used to calculate the activity in international units per mL of sample.

One IU = The conversion of 1 micromole of NADP in one minute under the specified assay conditions.

2.5.3.2 Glucose oxidase

This is a colourimetric assay based on the reduction of glucose to gluconic acid and hydrogen peroxide, the latter product oxidising O-dianisidine to form an orange colour. The rate of accumulation of oxidised O-dianisidine is a measure of enzyme activity. The method is based on the method of Bergmeyer (1974).

The following reagents were prepared in deionised water (all from Sigma Ltd):

1. 0.1 M potassium dihydrogen orthophosphate (KH_2PO_4) adjusted to pH 6.00 with 0.1 M potassium hydroxide (KOH) solution.
2. O-dianisidine : 2.5 g L^{-1} (25 mg in 10 mL).
3. 18 % w/v β-D-glucose solution allowed to mutarotate for 24 hours before use.
4. Peroxidase solution (from horseradish) : 1.5 g L^{-1} (15 mg in 10 mL)

These were all stored at -20°C except for the KH_2PO_4 buffer.

For each assay the following working reagent or assay mixture of 3 mL per sample was prepared:

Reagent	Volume (mL)
Buffer	2.5
o-dianisidine	0.1
Glucose	0.3
Peroxidase	0.1

In practice a stock solution of assay mixture was prepared fresh for the amount of assays required.

Method : 3 mL of assay mixture was added to 0.05 mL of appropriately diluted sample (in 0.1 M phosphate buffer) in a 1 cm cuvette and mixed thoroughly. The absorbance change was followed at 460 nm against the reagent blank (all reagents but no sample, buffer 2.55 mL) for 2 minutes between 1 and 3 minutes after the reaction was started. The temperature was maintained at 25°C. Samples diluted in buffer should be assayed within one hour and should giev a reading between 0.15 - 0.30 IU mL^{-1}.

Activity definition: One International unit (IU) of enzyme activity is the amount of enzyme which liberates one micromole of hydrogen peroxide per minute at 25°C under the specified assay conditions.

2.5.3.3 Alcohol dehydrogenase (ADH)

This assay was based on the isopropanol oxidation by alcohol dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD) to cause an increase in absorbance at 340 nm (Bergmeyer 1984).

The following reagents were prepared in deionised water (All reagents from Sigma Ltd):

1. 0.1 M sodium phosphate buffer at pH 8.3 containing 2 mM $MgCl_2$ and with 1 µL of 1 M dithiothreitol added per 10 mL buffer prior to use.
2. NAD (grade III 98 % from yeast) : 0.05 M stored at -20°C.
3. Isopropanol (pure)

Method : 20 µL of sample was mixed with 200 µL of buffer, followed by 20 µL of NAD solution. The absorbance due to non-specific reduction of NAD is now read at 340 nm. Then 20 µL of isopropanol was added to initiate the reaction, and the absorbance change followed at 340 nm for 15 minutes. These reactions were monitored in a "Titertek Multiskan MCC/MCC340" spectrophotometer with triplicate readings made. The reactions were carried out at room temperature.

ADH activity expressed as IU : One IU means the enzyme activity which converts 1 micromole of NAD to NADH in one minute under the specified assay conditions.

2.5.3.4 Catalase

This assay method was based on one from *Sigma Chemical Co. Ltd. (1990)*. It monitors the decrease in hydrogen peroxide in the reaction mixture with time by following the decrease in absorbance at 240 nm. Since catalase catalyses a first order reaction in decomposing hydrogen peroxide to water and oxygen, the amount of peroxide decomposed is directly proportional to the concentration of substrate and enzyme. To compare activities of various preparations, the assay conditions must be identical: the H_2O_2 concentrations from the start to finish must be accurately defined.

The substrate solution: Add 0.1 mL 30 % H_2O_2 to 50 mL 0.05 M phosphate buffer pH 7.0. Observe the absorbance at 240 nm. For reproducible results, it was recommended that this absorbance was between 0.550 and 0.520. If higher than this range, buffer was added to decrease it, and if lower, more H_2O_2 was added.

Method
1. Add 0.1 mL of sample to 2.9 mL of H_2O_2 solution at 25°C. The initial absorbance will exceed 0.45 and start to decrease.
2. Note the time required for absorbance to decrease from 0.45 to 0.40. This corresponds to the decomposition of 3.45 µmoles of H_2O_2 in the 3 mL solution.

Total activity in 3 mL : 3.45/ minutes required = Sigma units (ΣU). Calculate ΣU per mL sample.

3.0 RESULTS & DISCUSSION

3.1 Fermentation and Cell Morphology

This section will cover a description of the fermentations and the associated cell morphologies. It will not involve a detailed analysis of the fermentation since it is essentially a "tool" for obtaining the cells. Table 3.1 shows a summary of the fermentations.

Fermentation type	Changes from control	Fermentation time (hr)	Morphology compared to control
Control	-	25	Filamentous Clumped, entangled
Small pellets 1	Reduce spore concentration 10 X	32	Pellets 0.5 mm diameter, "hairy" non-spherical
Small pellets 2	Reduce spore concentration 10 X	32	Pellets 0.5 mm diameter, "hairy" non-spherical
Large pellets 1	Reduce spore concentration 100 X	38	Pellets 0.9 mm diameter, spherical
Large pellets 2	Reduce spore concentration 100 X	38	Pellets 1.8 mm diameter, spherical
Filamentous	Add "Junlon" polyacrylic resin	25	Free filamentous no clumping
Filamentous	Reduce agitation from 500 to 300 rpm	25	Slightly more filamentous
Scale up 1500 L	Tip speed constant	26	No difference

Table 3.1 : Summary of fermentation types and morphologies

3.1.1 The "control" fermentation

To establish the control fermentation, (without altering the industrial media and conditions provided) the optimal time at which the highest productivity of glucose oxidase (G.O.) per unit weight of cells must first be determined. This will be the point of cell harvest and therefore the glucose oxidase content will be maximal for cell disruption.

A fermentation was conducted for 42 hours in the 42 litre fermenter system to determine the glucose oxidase profiles. The growth curve (fig. 3.1) shows a cell concentration of about 10 g L^{-1} was achieved by about 35 hours. This is half of the expected yield from a mass balance of glucose and fructose available from beet molasses and conversion to gluconic acid : about 50 % (w/v) is sucrose, which will be converted to equal parts of glucose and fructose, resulting in 25 g L^{-1} each. Since the cell yield on glucose and fructose are similar at about 0.5 g dcw/g substrate, and taking into account the glucose used for gluconic acid (4 g L^{-1}, from a stoichiometric relationship between NaOH added and glucose converted), the maximum possible yield would be about 23 g L^{-1}. However, in this case, the stationary phase was reached at 10 g L^{-1}, which suggests that there may be limitation of another nutrient, such as oxygen, as the high cell concentration and viscosity will reduce available oxygen transfer rates significantly *(Brierly and Steel 1959).* * Please see end of 3.1.1.

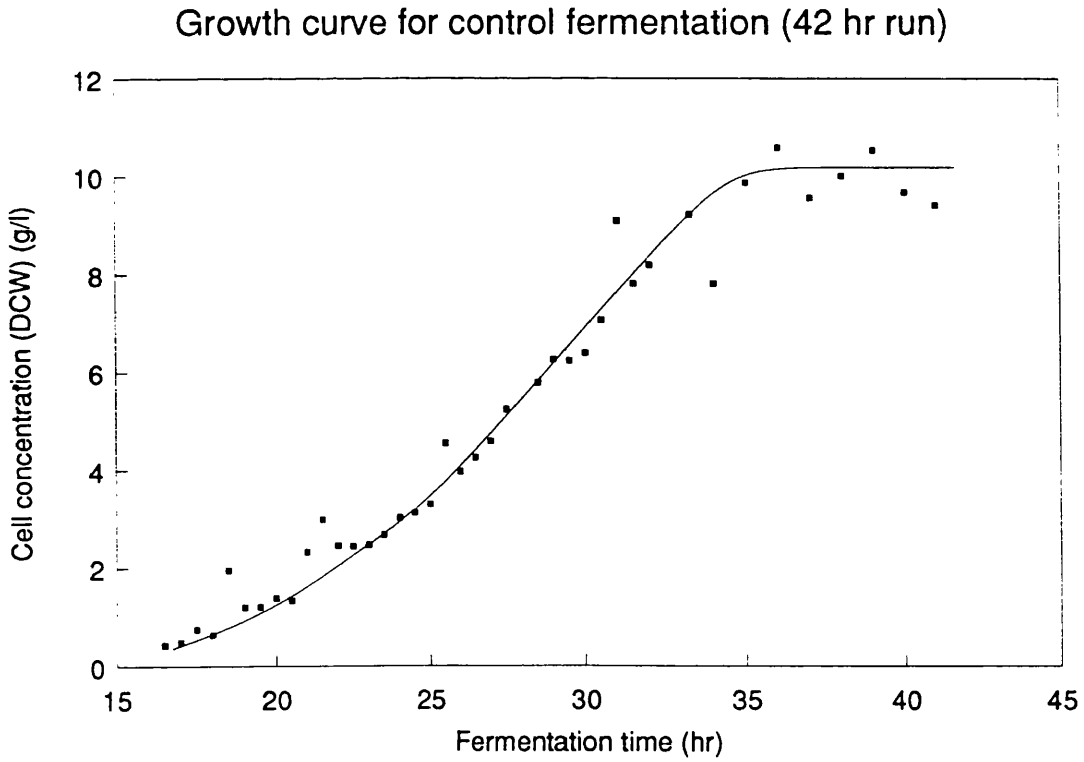


Fig. 3.1 : Growth curve of *A. niger* control fermentation in 42 litre fermenter

Fig. 3.2 shows the glucose oxidase profiles. These were obtained by ultrasonication of cell samples to release the glucose oxidase (see Section 2.1.7). The most important curve showing the "specific" intracellular glucose oxidase activity per gram of dry cell weight (dcw) clearly indicates a sharp peak of maximum productivity at about 23 hours after which it falls rapidly. Since, both glucose and oxygen are required to induce glucose oxidase production *(Mischak et al 1985, Rosenberg et al 1987, Reuss et al 1986)* the sudden decrease in "specific" G.O. activity may be due to diffusional limitation of these substrates, caused by the increasing viscosity and possibly increased clumping as cell concentration increases. *Traeger et al (1990)* found that the contribution of intracellular G.O. was half that of the extracellular G.O. in the production of gluconic acid from *A. niger* pellets of about 1 mm diameter and decreased further as pellet size increased to 1.5-2 mm diameter. They attributed this to oxygen transport limitation. The increasing viscosity may decrease the oxygen transfer coefficient *(Deindoefrer and Gaden 1955, Brierly and Steel 1959)* sufficiently to limit the mass transfer, so as to affect glucose oxidase production but not growth. The D.O.T. (at 25 hours) remains high at 50% (fig. 3.3), thus reflecting the possibiility that oxygen is not being transported from the liquid phase to the cells. * Please see end of 3.1.1.

More evidence for the dependence of glucose oxidase synthesis on the microorganism's environment is available : intracellular levels were increased 4 fold when the partial pressure of oxygen was increased by 3 fold in comparison with almost equal levels of intra- and extracellular enzyme when the partial pressure was the "normal" 0.21 bar *(Traeger at al 1990). Zetelaki and Vas (1968)* found that glucose oxidase production was doubled using pure oxygen for aeration; and that increased agitation also increased productivity. Further work by *Zetelaki (1970)* confirmed the agitation effects, and also showed that G.O. activity per g dcw decreased shortly after sucrose was depleted, the rate of decrease being faster for higher agitation rates and for increased overhead pressure in the fermenter. However, the growth in all cases continued to 2-3 % dcw (w/v), probably reflecting metabolism of the gluconate. Therefore, the G.O. and growth profile obtained here resembles the ones by *Zetelaki (1970)*, that is a rise and fall of G.O. per g dcw, and continuing growth, although in this case, sucrose was not likely to have been exhausted.

Intracellular glucose oxidase activity (IU/mL) also shows a similar trend - the activity decreasing after 26 hours which may mislead one to deducing that the enzyme is being turned over. However, since the extracellular enzyme increases gradually after 23 hours and total G.O. activity (obtained by the sum of intracellular and extracellular G.O.) is constant from 23 to 34 hours, it is more likely that there is leakage of the enzyme into the growth medium by either active secretion or cell wall damage. Total G.O. activity then falls after 34 hours, indicating that G.O. may be unstable. The most likely source of this loss is from the extracellular G.O., where shear associated damage or

proteolysis may occur. It is also interesting to note that total G.O. activity was constant between 23 and 34 hours, although "specific" G.O. activity was decreasing rapidly. This could mean that those cells which produced G.O. still retained the enzyme activity, but other cells (possibly ones experiencing oxygen limitation) did not synthesise any.

It is therefore clear that cell harvest should be performed at about 23 - 24 hours to obtain cells with maximum enzyme activity and prevent significant release of enzyme into the broth. At this time the cell concentration is about 2.5 - 3 g L^{-1} (dcw) (fig. 3.1), so that to obtain more cell mass for the homogenisation trials, without losing too much activity, it was decided that the harvesting be done between 25 - 26 hours to obtain about 4 - 4.5 g L^{-1}. This extended run was repeated and similar results obtained, but not shown here.

Fig. 3.3 shows the DOT, OUR & CER profiles for 25 hours only because the monitoring of parameters failed after this time. The DOT remains at 100 % until about 12 hours after which it starts to fall rapidly as the spores start to germinate, the OUR and CER also increased rapidly indicating vigorous growth. There is a long lag phase which is characteristic of a spore inoculum. Although a spore inoculum would not be practical on a large scale, the use of spores would result in better reproducibility of fermentation batches, especially if they were all grown from the same spore batch, and reduce the possibilities of contamination by reducing the number of transfer steps. The OUR is greater than the CER (ie. RQ < 1) as the production of gluconic acid (from glucose oxidation) needs oxygen.

The actual control fermentation is simply a shortened run of the 42 hour batch: it is exactly the same, except that after a period of 25 hours, the cells are harvested.

To characterise the morphology of the cells, photomicrographs were taken of random samples. This was the only feasible method since others such as one by *Metz et al (1981)* which characterises morphology by measuring various hyphal parameters (e.g. main hyphal length and number of tips) would not be able to distinguish individual hyphae in this case of highly entangled and clumped hyphae. Fortunately, the morphologies were so vastly different that a visual examination of the photomicrographs would suffice to distinguish them. Figs. 3.4 & 3.5 show the typical morphology of the control fermentations. These can be described as essentially filamentous, but with highly entangled and clumped hyphae and occasional large dense pellets of 0.1 - 0.2 mm diameter. This structure leads to a non-Newtonian pseudoplastic type broth (results not shown here).

* Alternatively, the fermentation could be nitrogen limited. With a supply of 0.86 g/L nitrogen and about a 10 % nitrogen content in the cell (w/w), the system will only give 8.6 g/L of biomass. This will also affect the protein synthesis in glucose oxidase production.

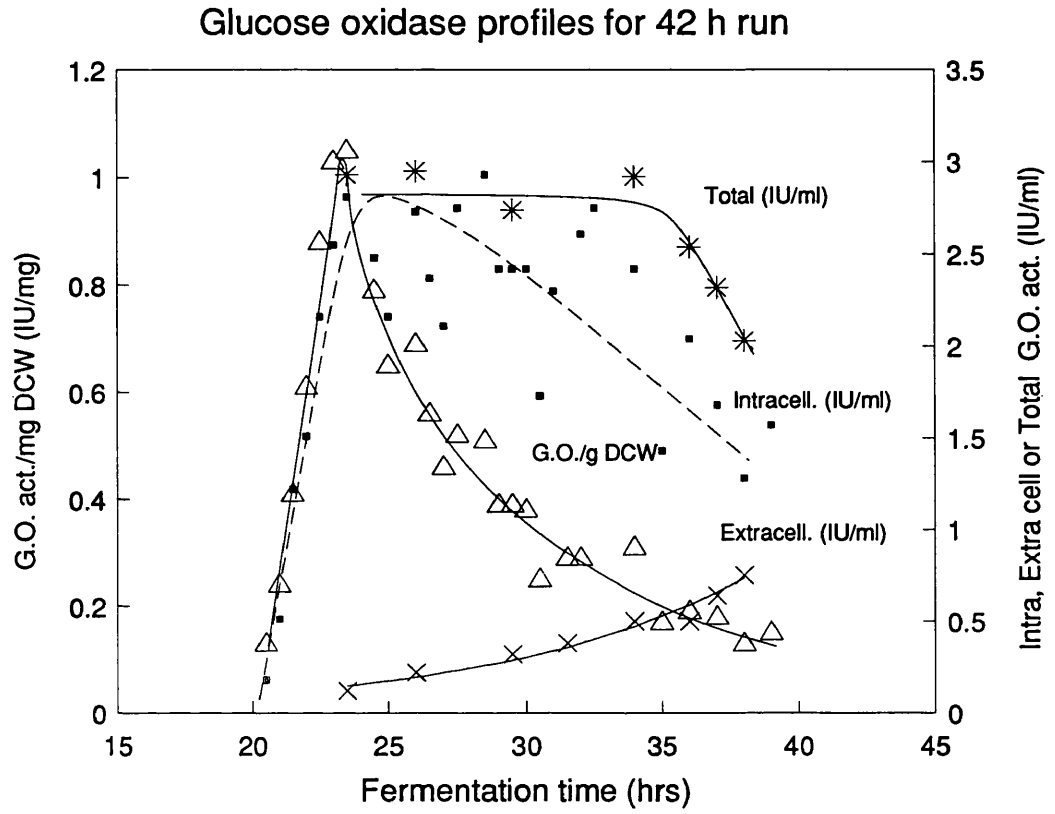


Fig. 3.2 : Glucose oxidase profiles : (△) G.O. activity per gm DCW; (◎) Intracellular, (X) Extracellular & (*) Total G.O. activity in IU/mL of fermentation broth.

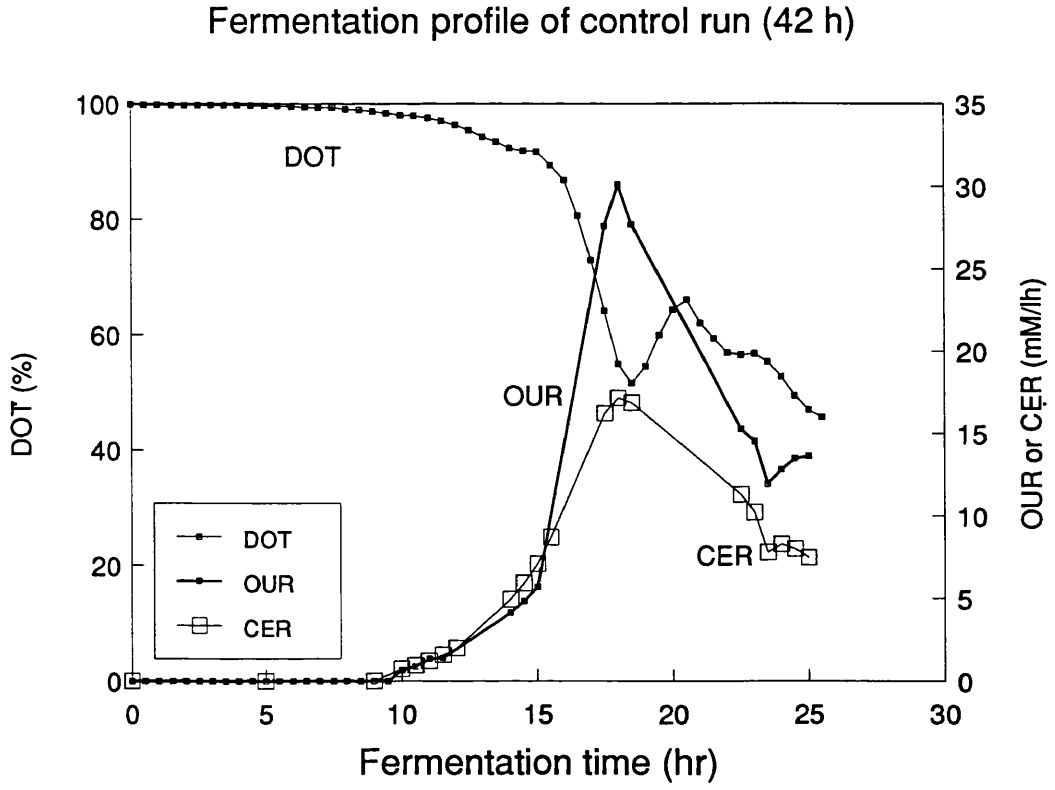


Fig. 3.3 : DOT, OUR & CER profiles for 42 hour fermentation (only up to 25 hours)

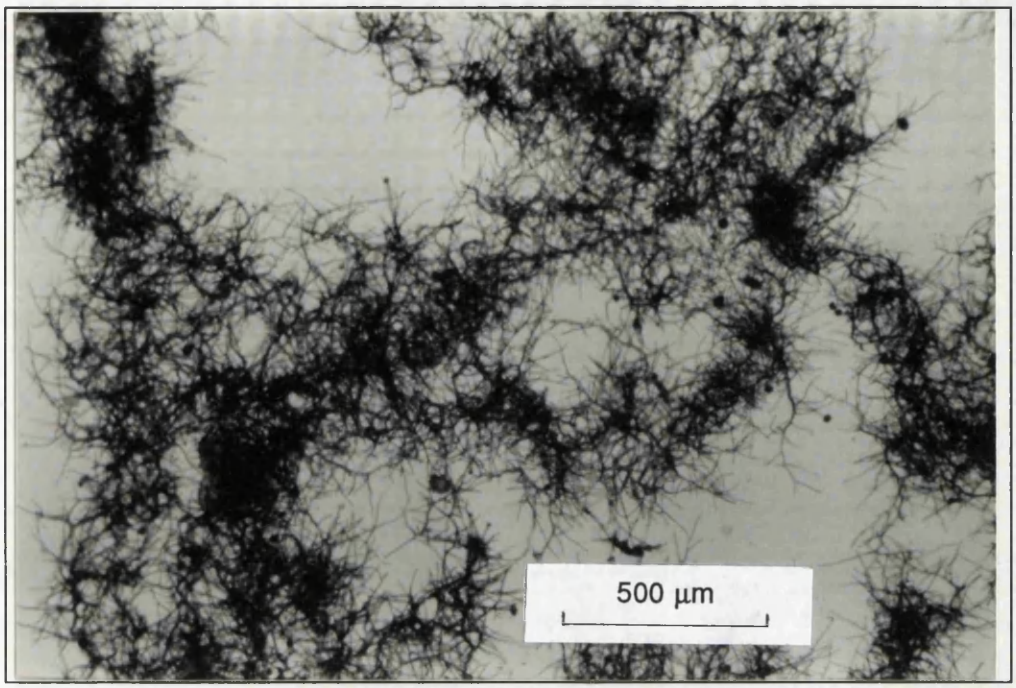


Fig. 3.4 : Photomicrograph of the typical morphology of control fermentations. Magnification 40 X

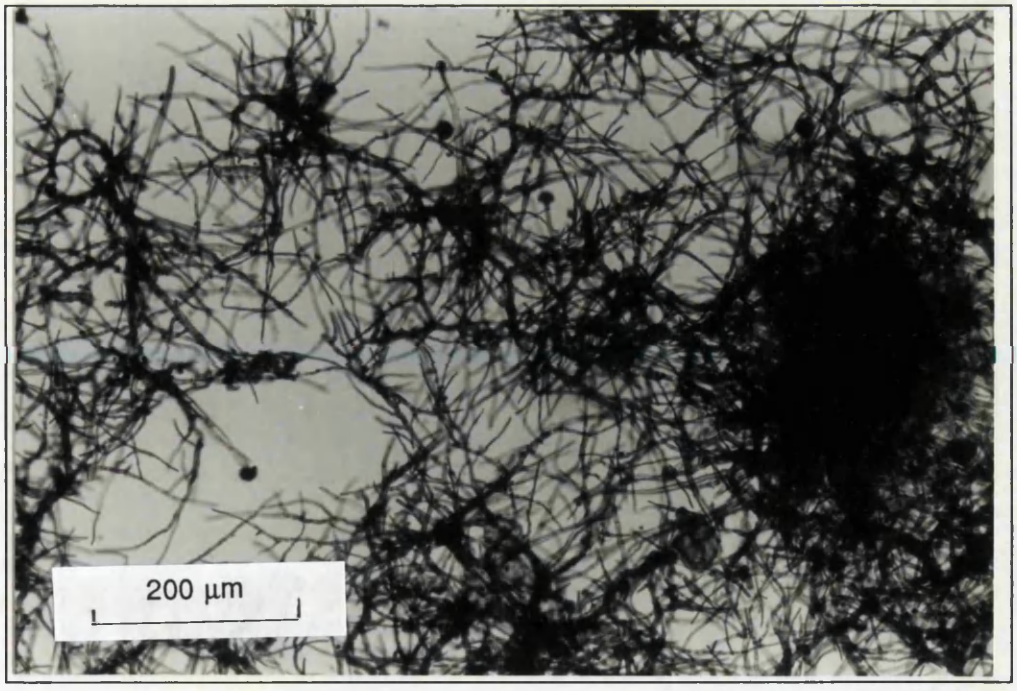


Fig. 3.5 : Photomicrograph of the typical morphology of control fermentation Magnification 100 X

3.1.2 Pelleted Growth

To determine the effects of morphology on cell disruption, various sizes of pellets were grown by decreasing the concentration of the spore inoculum from that of the control.

However it took a longer period of time to achieve the same level of biomass: the "small pellet" and "large pellet" batches were harvested at 32 and 38 hours respectively. Although a longer fermentation time was required to obtain a similar amount of biomass (5 g L^{-1} to have enough for homogenisation trials), the only period lengthened was the lag phase; the growth period was always from 13 -16 hours. Therefore the average age of the cells are similar in all the batches which means that the only significant change is the cell morphology. A lower spore inoculum therefore means a longer lag phase or a longer period to reach the "critical mass" before significant growth results, and does not affect the average age of the cells. Any change in the disruption results is therefore a result of the change in cell morphology.

From figs. 3.6 and 3.7, the fermentation profiles for the small and large types of pelleted morphologies are similar in trends but vary in the times at which changes occur. No OUR and CER data were available due to failure of the mass spectrometer. As for the control, the DOT starts to drop rapidly after the lag phase, (accompanied by a rise in OUR and CER in the control) but in this case this period is at 23 and 26 hours for the small and large pellets respectively compared to 12 hours for the control. So, it can be assumed that a similar pattern occurs for pelleted growth and in all cases the period of rapid growth (after lag phase) is for about 12 hours resulting in a similar average age. The pH also indicates the rapid growth phase (not shown for control) because when the pH rises a little, the DOT also falls rapidly. Therefore during occasions when OUR and CER data was unavailable, the DOT and pH can provide an estimation of the rapid growth phase.

These pellets were characterised by measuring the sizes of about 100 pellets by the image analysis used by *Adams et al (1988)*. Since the pellets were not fully spherical, the longest distance across the pellet was taken to be the "length" and the "breadth" was the distance perpendicular to the "length". Nevertheless, in all cases the lengths and breadths were not vastly different and so the length can be taken as the diameter of the pellet.

Small pellets (0.5 mm) : Reducing the spore concentration to $10^4 mL^{-1}$ from the control of $10^5 mL^{-1}$ of media, resulted in pellets of approximately 0.5 mm diameter (length 0.48 mm ± 4.7 % and breadth 0.38 mm ± 5 %) with "hairy" long branched hyphae on the outer surface around a loosely packed non-spherical pellet. Figs. 3.8 and 3.9-3.12 show the morphology and the size distributions respectively.

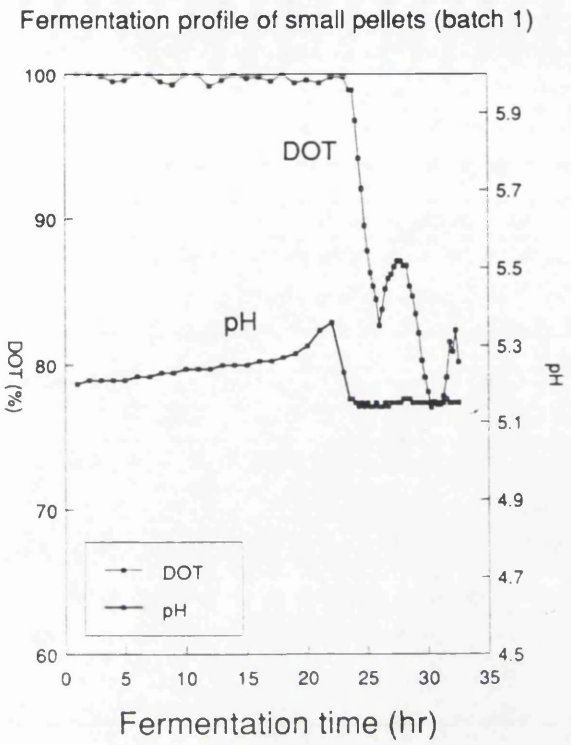


Fig. 3.6 : DOT & pH profiles for small pellets fermentation

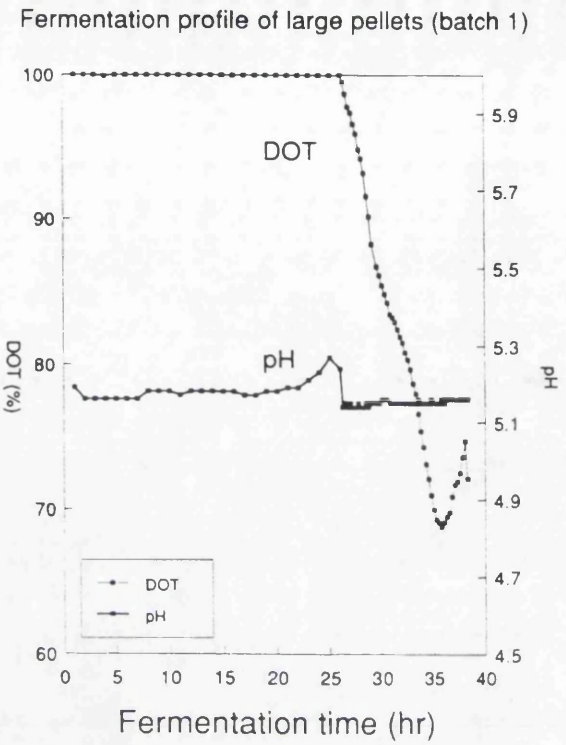


Fig. 3.7 : DOT & pH profiles for large pellets fermentation

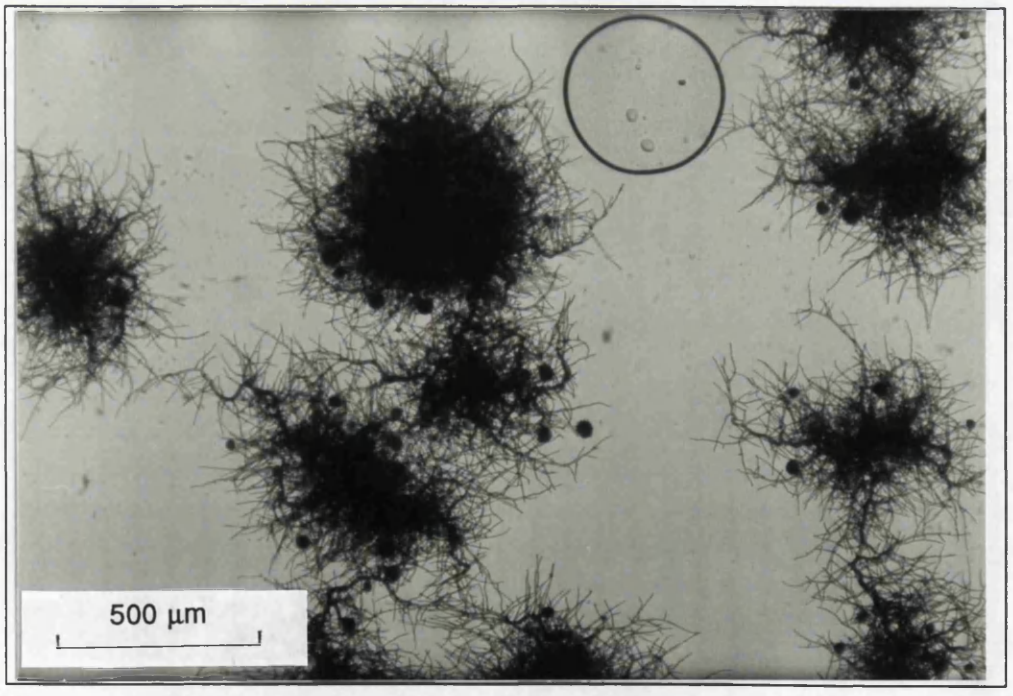


Fig. 3.8 : Typical morphology of the **small** pellets (0.5 mm diameter) batch no. **1**

Magnification 40 X

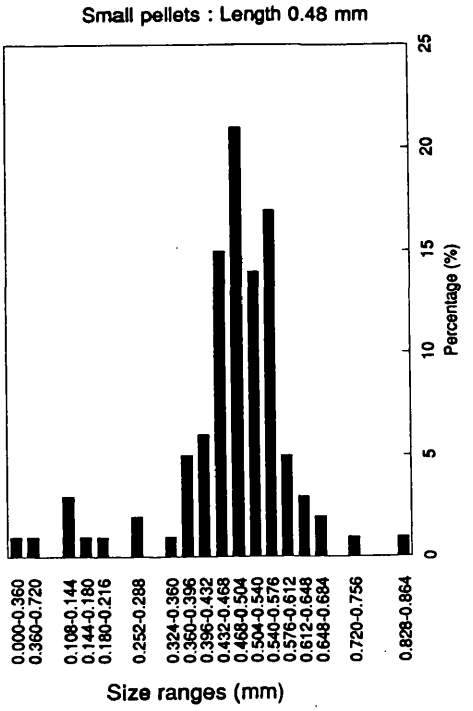


Fig. 3.9 : The "length" size distribution of **small** pellets batch no. **1**

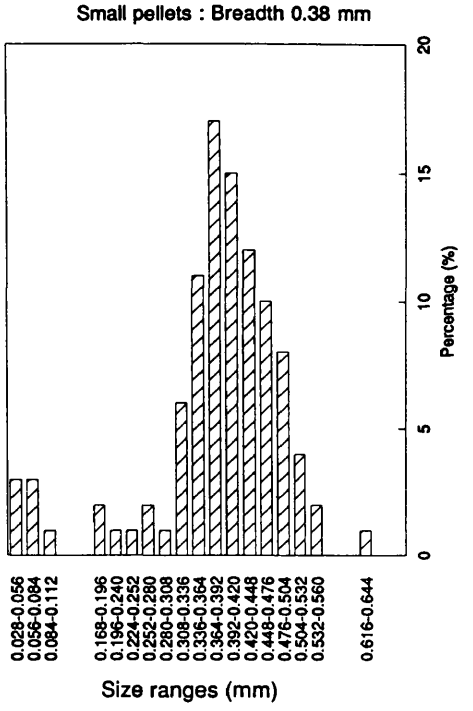


Fig. 3.10 : The "breadth" size distribution of **small** pellets batch no. **1**

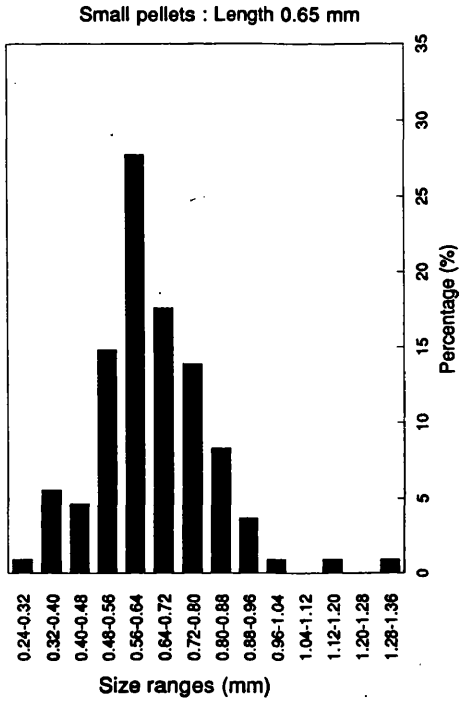


Fig. 3.11 : The "length" size distribution of **small** pellets batch no. **2**

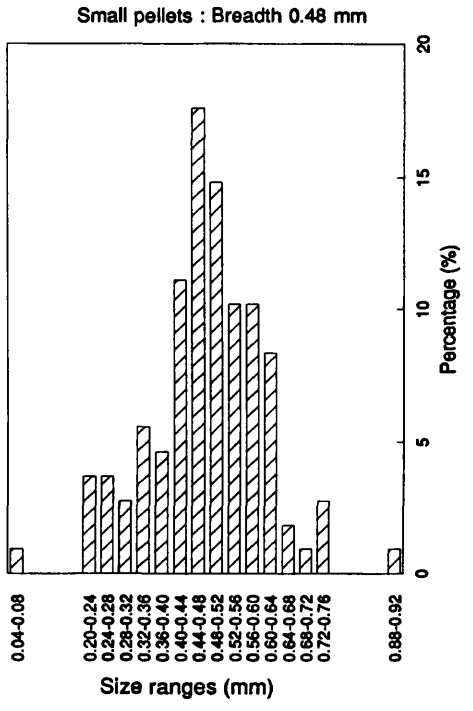


Fig. 3.12 : The "breadth" size distribution of **small** pellets batch no. **2**

The fermentation was repeated once and the size analysis and fermentation profiles proved to be reproducible : length 0.65 mm ± 5.3 % and breadth 0.48 mm ± 5.9 %.

Large pellets (0.9-1.8 mm) : A further reduction of the spore concentration by another factor of 10 (ie. a total reduction of 100) gave even larger pellets of about 0.9 mm diameter (length 0.93 mm ± 5.3 % and breadth 0.73 mm ± 5.9 %) and with a less "hairy" exterior of short, non-highly branched hyphae around a densely compacted spherical pellet. Another identical run was made, but the pellet sizes showed a much larger average size of 1.8 mm diameter (length 1.81 mm ± 4.7 % and breadth 1.4 mm ± 4.8 %). This could be due to a decreased viability of the spores, therefore effectively lowering the spore concentration further and hence the increased size, which may have been caused by the repeated freeze-thawing of the spore suspension. Nevertheless this increased size served as further evidence to the effects of pellet size on cell disruption. Figs. 3.13 and 3.14-3.15 show the typical morphologies and size distribution of these pellets respectively.

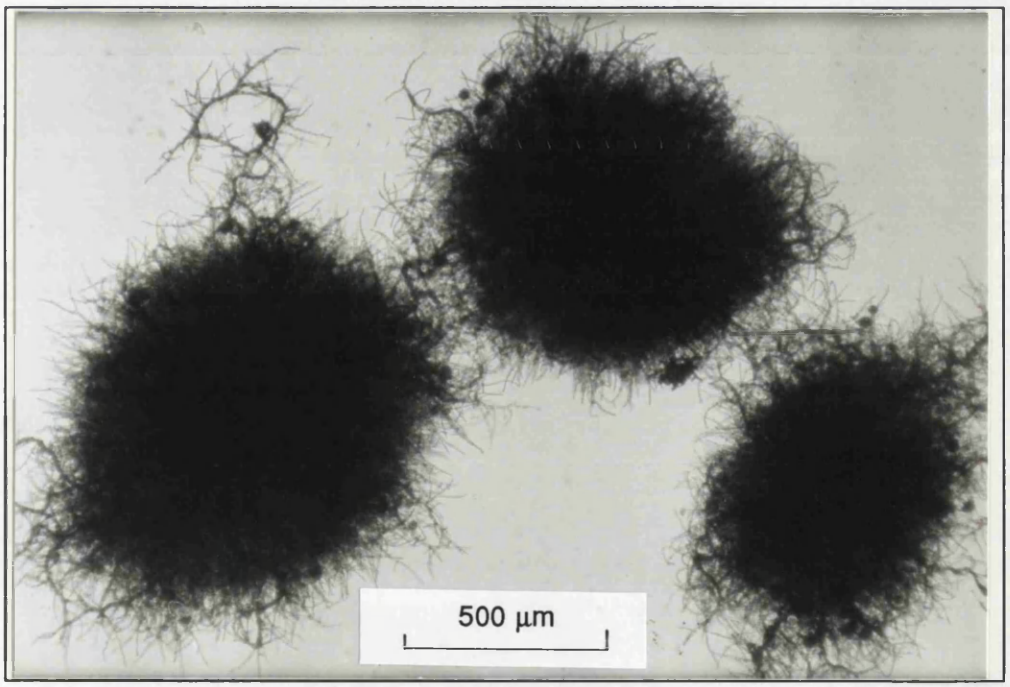


Fig. 3.13 : Typical morphology of the **large** pellets (0.9 mm diameter) batch no. **1**
Magnification 40 X

The effect of spore concentration on fungal morphology is well known : high concentrations giving filamentous growth and low concentrations producing pelleted growth *(Foster 1949)*. However, with *Aspergillus sp. Trinci (1970)* found that for all inoculum sizes, *A. nidulans* grew as pellets, and *Takahashi et al (1958)* reported an increase in the size of *A. niger* pellets but decrease in number,

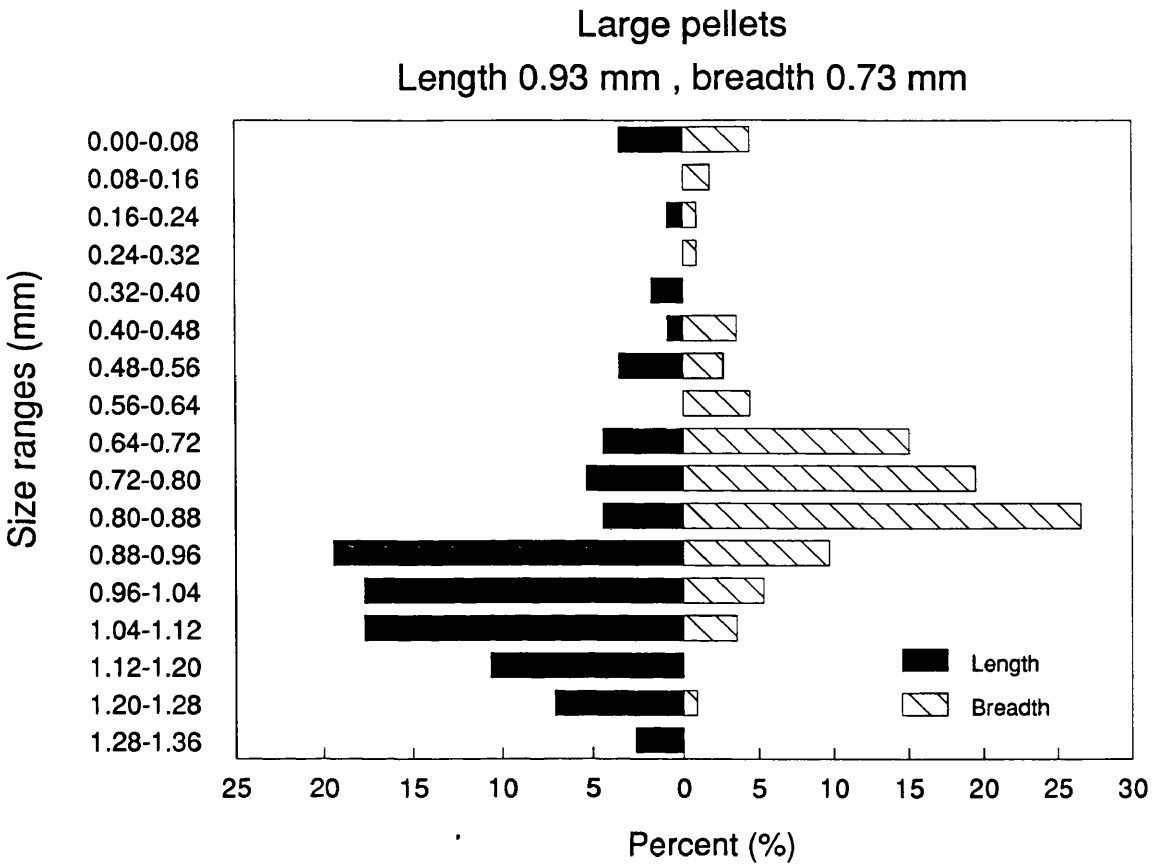


Fig. 3.14 : The "length" and "breadth" size distribution of **large** pellets batch no. **1**

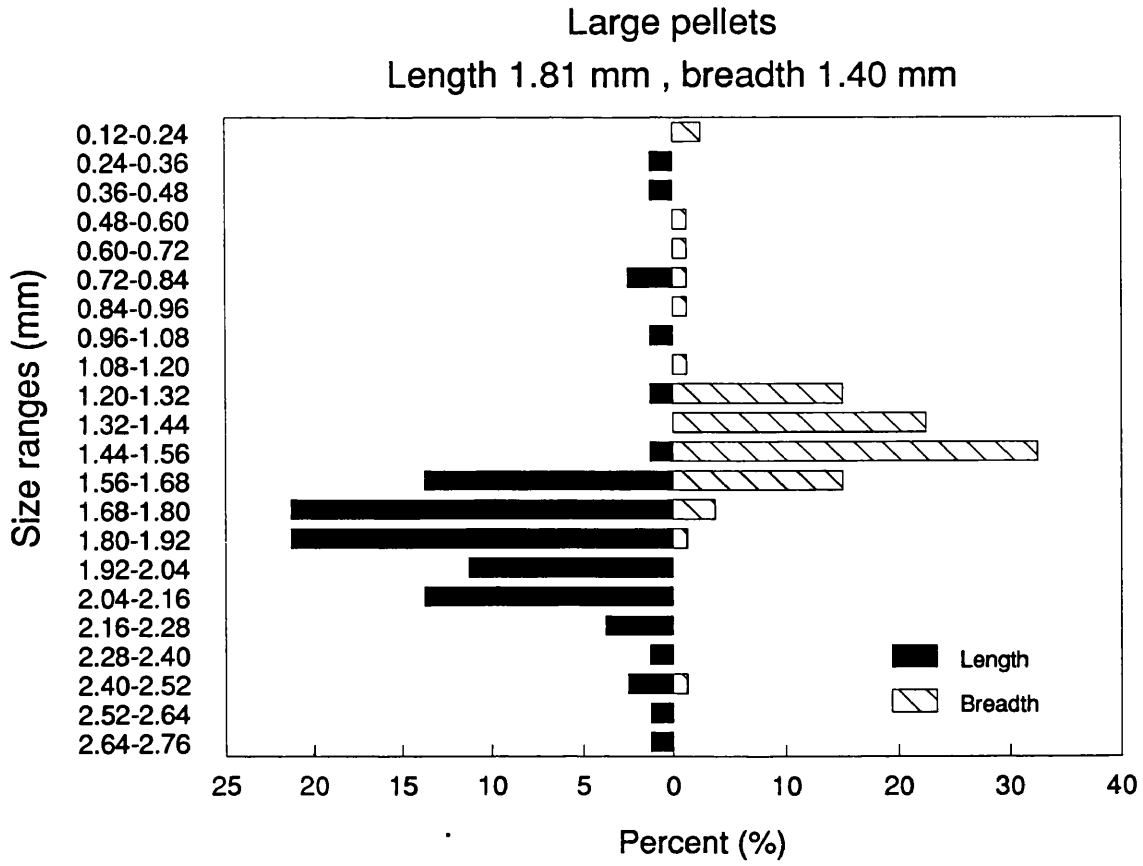


Fig. 3.15 : The "length" and "breadth" size distribution of **large** pellets batch no. **2**

when the inoculum size was reduced. Therefore, the results seen here are consistent with these studies. Nevertheless, it must be mentioned that other studies have found that pH had a more pronounced effect than inoculum size *(Galbraith and Smith, 1969; Steel et al, 1954)*. Hence, the morphology obtained for a particular system may not necessarily be applied to other systems.

As will be shown in section 3.4.1, the disruption of these pelleted cells gave lower glucose oxidase levels (IU/g dcw): for small pellets it was about 45 %, and for large pellets, 32 % of the control morphology (fig. 3.2). This is further evidence for the possibility of oxygen transfer limitations affecting glucose oxidase production. The decrease in glucose oxidase production would be expected due to the increased mass transfer resistance towards the centre of the pellet, resulting in reduced oxygen uptake rate for respiration and glucose oxidase production. According to *Kobayashi et al (1973)* the oxygen will be limiting for pellet sizes over 0.2 mm diameter, and so, in this case, both the small pellets of 0.5 mm diameter and large ones of 0.9 and 1.8 mm diameter will experience oxygen limitation. Therefore, the biomass in the centre of pellets will be "non-producing" cells. The formation of pellets should therefore be avoided when productivity is the prime objective. In this case, glucose oxidase production was not as important as pellet growth which was needed to examine its effect on cell disruption.

3.1.3 Filamentous growth

Although the control fermentation is on the whole filamentous, it is very much entangled and clumped. Preliminary studies with shake flask growth (not shown here) indicated that an increased spore concentration did not cause filamentous growth in the molasses medium. Changes in media composition was avoided because of the possibility of changing fermentation kinetics. A more "freely" filamentous, unclumped form where the individual hyphae can be distinguished, was obtained by the use of i) a polyacrylic resin called Junlon or ii) a reduction of the agitation speed.

3.1.3.1 The effect of a polyacrylic resin : Junlon PW-110

Junlon PW-110 is a cross-linked acrylic resin which is widely used as a thickening agent in the paint, paper, textile and cosmetic industry. Its use to promote the growth of *A.niger* as a filamentous form was first tested by *Trinci (1983)*, who used it on shake flask cultures.

In this case it was used in the 42 litre fermenter (30 L working volume) at 1 g L^{-1} and sterilised together with the media after making up an aqueous solution. The impact of sterilisation at 121°C and agitation was to completely destroyed the thickening effect of the resin as expected *(Nihon Junyaku Co., undated : commercial information)*. Hence there was no change in mixing conditions, and since Junlon was reported to be unmetabolised *(Trinci 1983)*, fermentation growth kinetics

would be unaffected . Nevertheless, the resin caused "free" filamentous, non-clumped morphology as shown in fig.3.16. Junlon is similar to "carbopol-934", an anionic polymer used by *Elmayergi and Moo Young (1973) and Elmayergi (1975)* to obtain filamentous growth : it also has anionic groups on its side chain radicals. Since pellet formation could be a result of spores forming flocs or aggregations, and subsequent entanglement of germinated spores *(Galbraith and Smith 1969)*, *Elmayergi (1975)* who found that only anionic polymers induced filamentous growth, proposed that there was electrostatic repulsion between the carboxyl groups and negative charged spores, resulting in unagglomerated spores. Therefore, Junlon's mechanism of causing filamentous growth may also be similar to that for carbopol.

The growth kinetics and fermentation time (fig.3.17) were unchanged except that DOT was lower (20 % ccf. 40-50 %) at about 24 hours. This is caused by the more pseudoplastic nature of the filamentous form which results in poorer mass transfer. No OUR and CER data were available, but the DOT and pH profile is very similar to the control fermentation.

The individual hyphae are distinctly separate and not clumped to form any pellets, although it is extensively entangled. In addition, there was no adhesion of media particles as observed in the control (cannot be seen in the low magnification photomicrographs), which showed itself in the very light brown colour of the biomass as opposed to the usual mid-brown colour imparted by the molasses. No characterisation of this form was made by image analysis because the individual organisms cannot be separated so that the hyphae are not crossed. As yet the image analysis system will discard any such cross-overs.

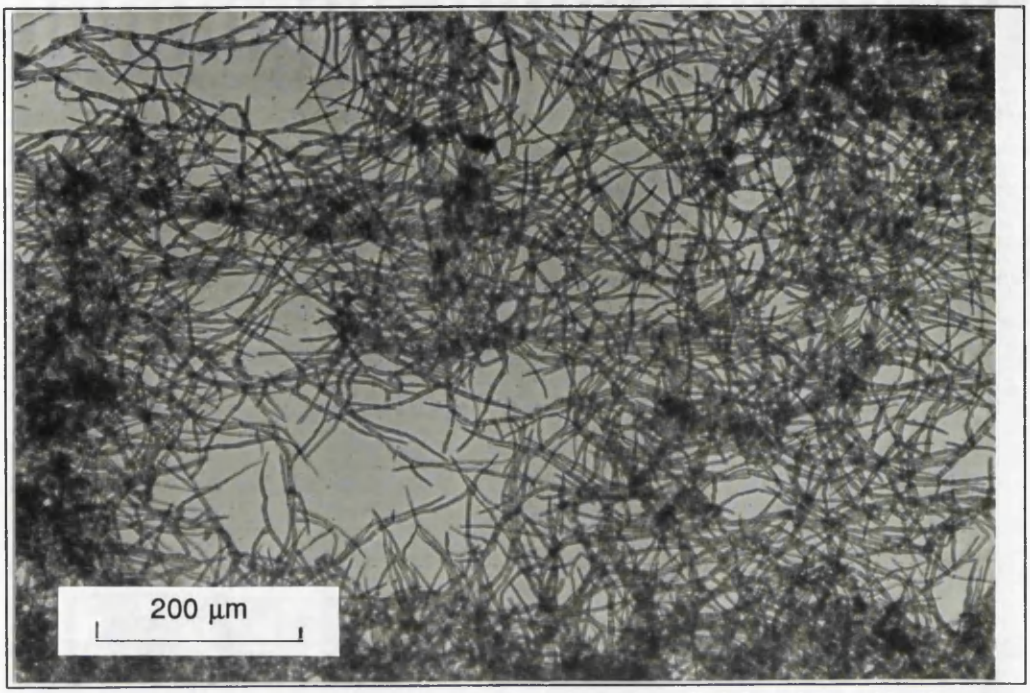


Fig. 3.16 : Typical morphology of **filamentous** type growth caused by Junlon. Magnification 100 X

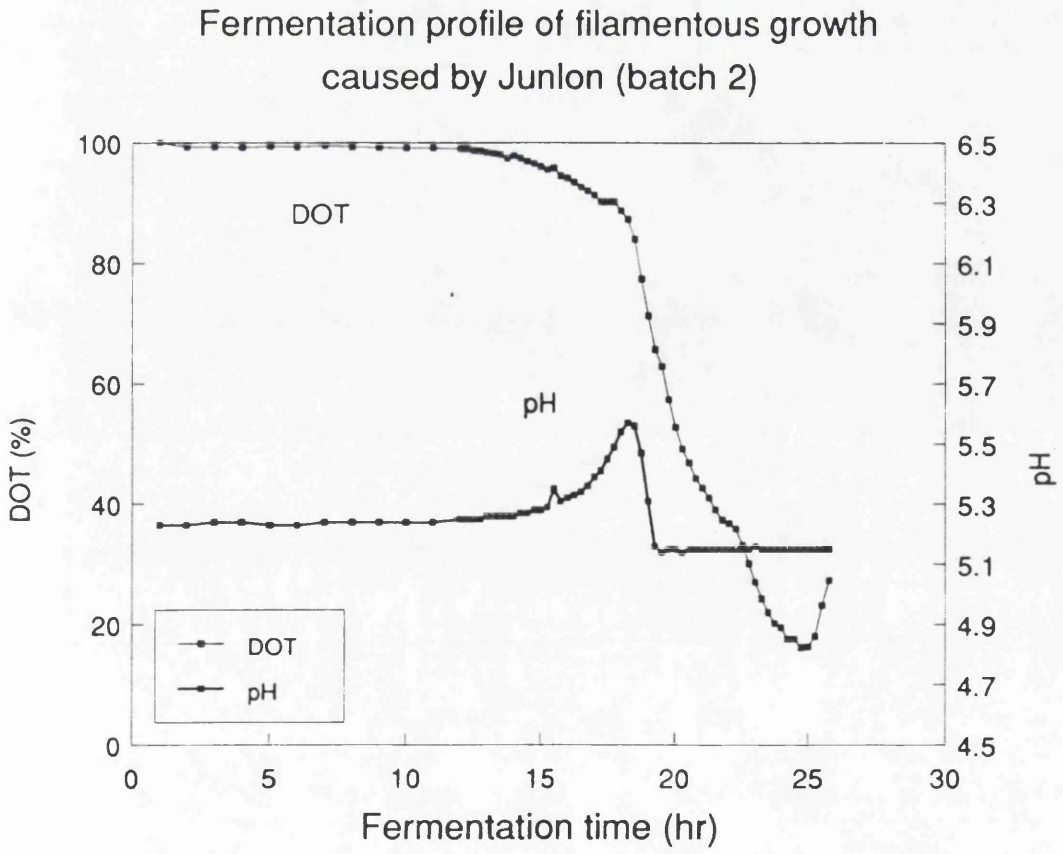


Fig. 3.17 : DOT & pH profiles for filamentous fermentation using Junlon.

3.1.3.2 The effect of reduced agitation

The usual agitation speed of 500 rpm or tip speed of 2.6 m s^{-1} was reduced to 300 rpm or 1.57 m s^{-1} tip speed. Fermentation time was unchanged for the same biomass level. However the DOT was very low (almost 0 %) towards the time of harvest and OUR & CER were about halved (fig. 3.18) and the respiratory quotient (RQ) was always close to 1 (not shown, but OUR = CER) in comparison with the control where RQ was less than 1 due to uptake of oxygen for glucose oxidation. The decreased OUR would indicate that glucose oxidase production would be low. This was found to be true in the disruption trials (section 3.4.2.2) where it was found that it was less than 20 % of that from the control fermentation. This is in agreement with extensive studies carried out by *Zetelaki (1970)* who found that glucose oxidase production was increased with agitation and RQ was also close to 1 for the lowest speed of agitation where the enzyme production was 30 % of that from a fermentation operating at twice the agitation speed. Therefore, the increased oxygen supply increases the productivity in terms of glucose oxidase production. This evidence fits in with the decreased productivity obtained from pelleted forms, to suggest that any oxygen transfer problem, either from the gas phase into the liquid medium, or from the liquid phase into the cells, can affect glucose oxidase production severely.

Fig.3.19 shows that the mycelia has longer hyphae and more diffuse, less compact clumps but are not very much different from the control. Therefore the reduction of agitation to change morphology significantly was not considered a good variable to change for this particular system. In addition the agitation change can also affect the strength of the cell walls and may thus affect the cell disruption.

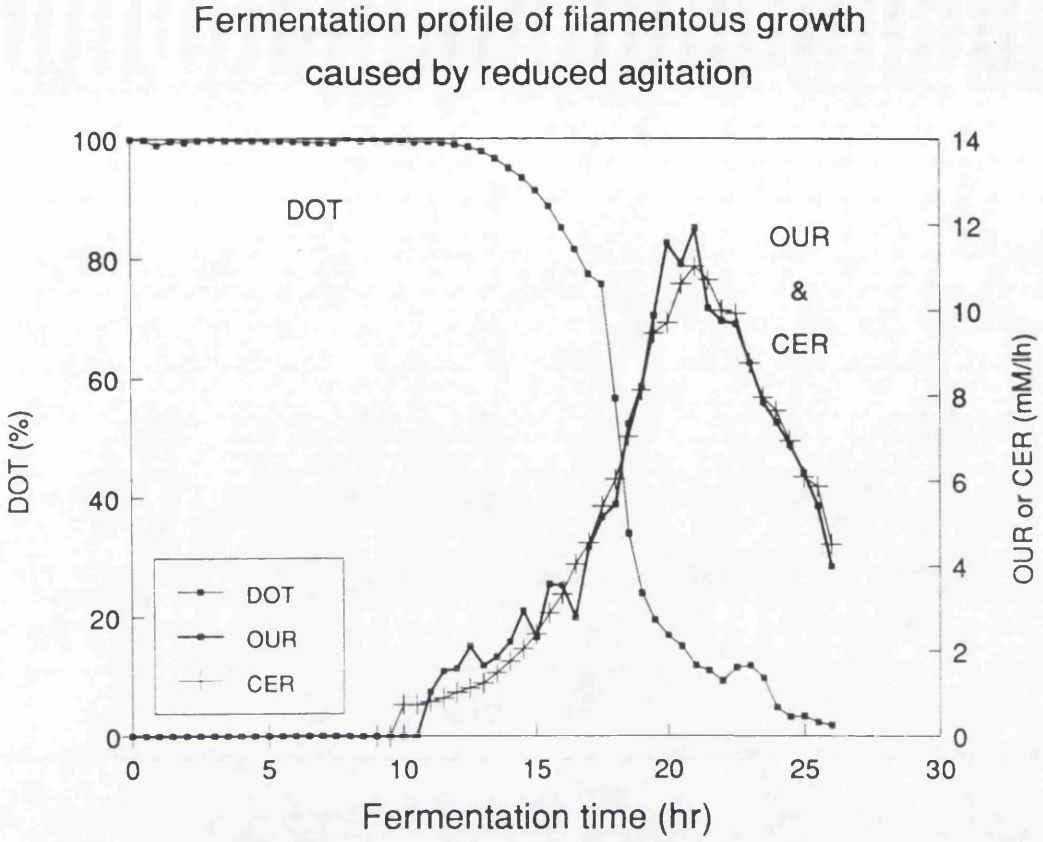


Fig. 3.18 : DOT & pH profiles for filamentous fermentation by reduced agitation.

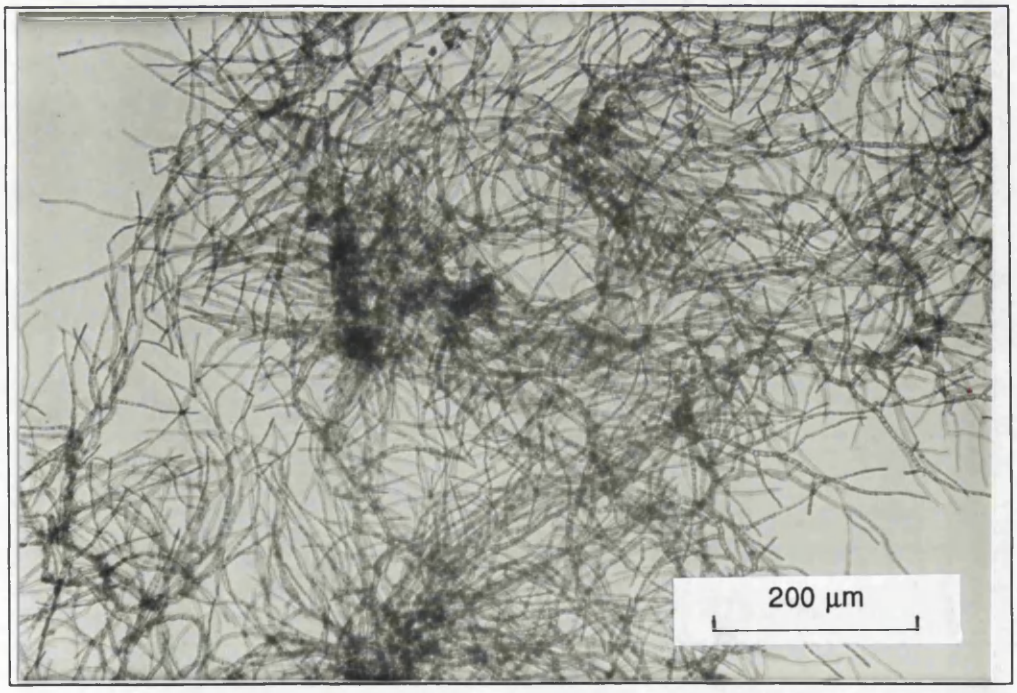


Fig. 3.19 : Morphology of slightly more filamentous mycelia grown under reduced agitation.

Magnified 100 X

3.1.4 Scale up

A study of the scale up of the fermentation is beyond the scope of this project. However one attempt was made by scaling up to a 1500 L fermentation with 1000 L working volume. All conditions were as before including the spore inoculum concentration, but the agitator speed was scaled up on constant tip speed of 2.6 m s^{-1} to give an operating speed of 200 rpm. A spore inoculum was maintained to avoid changing any conditions although this was a very laborious process in terms of producing and harvesting the spores. This was made easier by the use of large medical flats instead of the usual agar slants on universal bottles. At production scale, the use of spores may not be feasible and hence a vegetative inoculum will be used. However, this will also depend on the size of the plant, the capital costs of seed fermenters and the operating labour costs.

The growth curve (fig. 3.20) reproduced the 42 L control runs to give a similar cell concentration of 3.7 g L^{-1} compared to 4 g L^{-1} in the small scale. The DOT and pH profile were similar to the 42 L control fermentation (not shown here); no OUR and CER data were available.

In terms of glucose oxidase production, the profile (fig.3.20) was similar ie. a sharp rise to a peak activity and then a rapid fall, but the actual activity as determined by ultrasonication of cell samples only gave a peak activity of 575 IU/g dcw compared to about 1000 IU/g dcw in the 42 L. It is uncertain as to the reason, since oxygen supply was adequate. Nevertheless, the profile follows the one from the 42 L (fig. 3.2) scale in that it also decreases rapidly after 24 hours.

There was no noticeable change in the morphology : it remained essentially filamentous but highly entangled and clumped (Fig. 3.21).

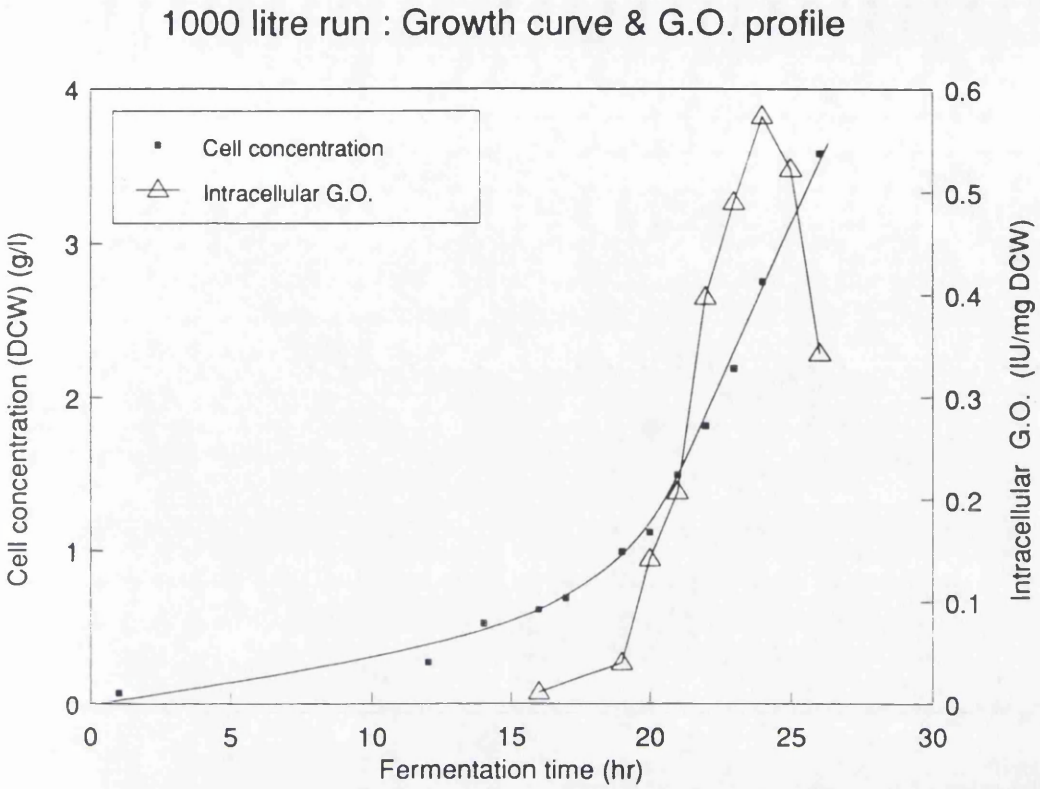


Fig. 3.20 : Growth curve & glucose oxidase profile for 1000 litre fermentation

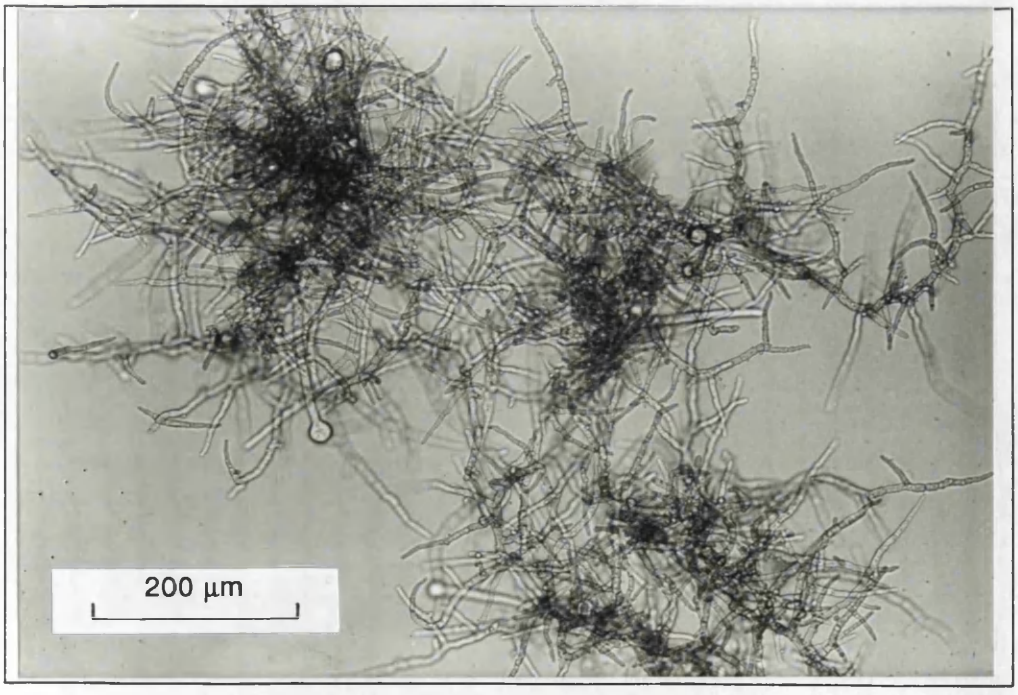


Fig. 3.21 : Morphology of cells grown in 1500 L fermenter. Magnification 100 X

3.2 Cell Harvesting and Resuspension.

Cell harvesting was performed in a basket centrifuge at about 2000 rpm (500 g) by forcing the fermentation broth out of the fermenter with positive pressure (2-3 x 10^5 Pa). A 30 L batch takes about 30 minutes. The cell paste is then removed manually from the nylon basket and washed by resuspension in cold deionised water and re-centrifuged to remove media. The supernatant of media or wash water was clear - the former was obviously dark brown molasses, whilst the latter showed a slight tinge of yellow from remaining media. Another wash step resulted in very clear, colourless supernatant. Therefore, this harvesting method was extremely suitable for the control type filamentous, entangled cells which formed a strong filtering network for the media.

In preparing for disruption, the very dry and flaky (20 % dry weight w/w) harvested cells caused difficulties in resuspension, if these cells were not resuspended in buffer within 0.5 - 1 hour after harvesting. The cells would not resuspend to give a homogeneous suspension of cells : instead there were large individual clumps of about 0.5 - 5 cm which had to be carefully loosened up. This situation means that a non-homogeneous (not due to cell structure) suspension of cells were being disrupted, which means keeping the conditions the same for reproducibility is invalid. It would seem that the cells have grown in the solid state and meshed together to form a more tangled mass.

In contrast wet cell pastes of 10-15 % dry weight (w/w) can be left to store at room temperature for a few hours or at 4°C over a few days without posing any resuspension problems. Of course, it is not good practice to store cells over a few days before disruption because of possible unknown changes in morphology, viscosity and physiology. Indeed it was found that overnight stored cell paste at 4°C had considerably reduced viscosity - the cells seemed to have "loosened up". This may affect the homogenisation.

During homogenisation itself, the cell suspension was constantly stirred to ensure homogeneity, because filamentous cells tend to separate from the liquid especially as it is being sucked into the homogeniser. At the first pass, a knocking sound occured, probably due to trapped air or clumps of mycelia being forced through. However, due to the viscous nature of the suspension, some air tends to get trapped in the pipe leading into the suction and discharge block from hopper. This is also where stirring cannot be done and so, aggregation to form highly dense clumps can happen. Fortunately, this only occurs at the first pass for about 5 mins, thus minimising the wear on the valve assembly. For further passes, operation is normal as the viscosity is much less. With very concentrated suspensions ($\approx$ 30 g L^{-1}) the "knocking sound" can get quite loud and so such high concentrations should be avoided.

3.3 Cell Disruption Studies on the Control Morphology

This section deals with the release of various products from disruption of the "control morphology" cells in a high pressure homogeniser and also (briefly) a bead mill. The control type cells are essentially filamentous, but highly clumped and entangled as shown in figs. 3.4 & 3.5 in section 3.1.1. These cells were harvested in a basket centrifuge, washed to remove media and resuspended in Tris HCl buffer for disruption in two APV-Gaulin high pressure homogenisers: models "Lab 60" & "30CD". The former was used for the lower range pressures of 60 MPa or below, whilst the latter for higher pressures than 60 MPa because the Lab 60 does not operate above 65 MPa. Most of the studies were done with the Lab 60 model because of the possibility of blocking the smaller ball valves in the 30CD by the filamentous cells. Therefore, for all operations in the 30CD the cells were homogenised at 60 MPa for 1 pass before passing through the 30CD. As yet it is still unknown if the 30CD will be able to handle these types of cells at a relatively high concentration (≈ 10 g L^{-1}).

In order to determine the extent of cell disruption or breakage, it was necessary to decide which cytoplasmic materials to monitor. Initially, the analysis of the protein and glucose oxidase release was chosen, the enzyme being used as an independent measure of disruption. This is because materials giving a positive result with the protein assay can be released from micronisation of cell debris (*Limon-Lason et al 1979, Keshavarz et al 1990a*).

Some preliminary studies assessed the effects of storage of whole cells and homogenate on the protein and glucose oxidase levels. The following results were found (details in Appendix 1):

1. There was an apparent drop in protein level after storage of cells for 24 hours with the assay based on the Bradford method but not with the Folin-Lowry method. Since it was decided that the Bradford method was better suited due to its rapid and simple procedure, all disruptions and assays were done within 12 hours of harvesting.
2. At a pressure of 60 MPa, although protein release was almost complete, only about 33 % of the glucose oxidase was released. This was demonstrated by the increased activity of the homogenate stored for several weeks at 5°C. Therefore a much higher pressure was required, as well as a different enzyme marker which was released at the same rate as protein. Therefore, another intracellular enzyme was chosen : glucose-6-phosphate dehydrogenase (G6PDH).

Three experiments were conducted for the control type cells. They are not identical and therefore show complementary information. Nevertheless, they are reproducible for those identical pressures of operation and most importantly for the complete release of each product as will be shown in the composite diagrams expressing product release as a percentage. They will be referred to as control 1, 2 & 3. Control 1 was operated between pressures of 10-60 MPa, control 2 at 20-80 MPa, and

control 3 at 20-124 MPa. They varied in their range of pressures due to the failure to achieve complete release of glucose oxidase in the first two controls and also because insufficient cells were available from one batch to conduct studies from 10-124 MPa at 10 MPa intervals. Each control run will be dealt with individually and then a composite diagram representing the control disruption kinetics will be shown. Due to the inevitable slight variations in protein and enzyme levels between batches, it is not possible to directly compare the kinetics with absolute values. Therefore for comparisons between control batches and between different morphological forms the value have been converted to percentage release. The definition of complete or 100 % release is when there is no further increase in the protein content or enzyme activity with increased pressure or number of passes. This is further supported by the analysis of protein after boiling the homogenate in 5 % sodium dodecyl sulphate (SDS) solution to break up all the cell walls, which will give the maximum possible protein content.

3.3.1 Control 1 : 10-60 MPa

This control run was operated at 5 pressures from 10-60 MPa to determine the disruption kinetics at the lower pressure range. The cells were disrupted at a concentration of 10.3 g L^{-1} (dcw).

Fig. 3.22 shows the disruption kinetics for the protein, G6PDH & glucose oxidase release up to 8 passes through the homogeniser. For all three products, the characteristics are :

1) a rapid release of product during the first one or two passes and then a considerable slowdown in the rate of release for subsequent passes. The release of product during the 1st pass is highly dependent on pressure and product release in subsequent passes is a weak function of the number of passes. The majority of product is therefore released at the first two passes.
2) there is no evidence of complete release since all show that the amount released is still increasing with both pressure and number of passes. This is especially so for glucose oxidase at 60 MPa where the rate of change of activity is higher than the other two products.

Therefore further work is necessary to determine the pressure required to release the products fully. It was anticipated that 80 MPa would be sufficient, as photomicrographs of the homogenate at 60 MPa showed very much fully broken cells consisting of empty cells broken perpendicularly to its length.

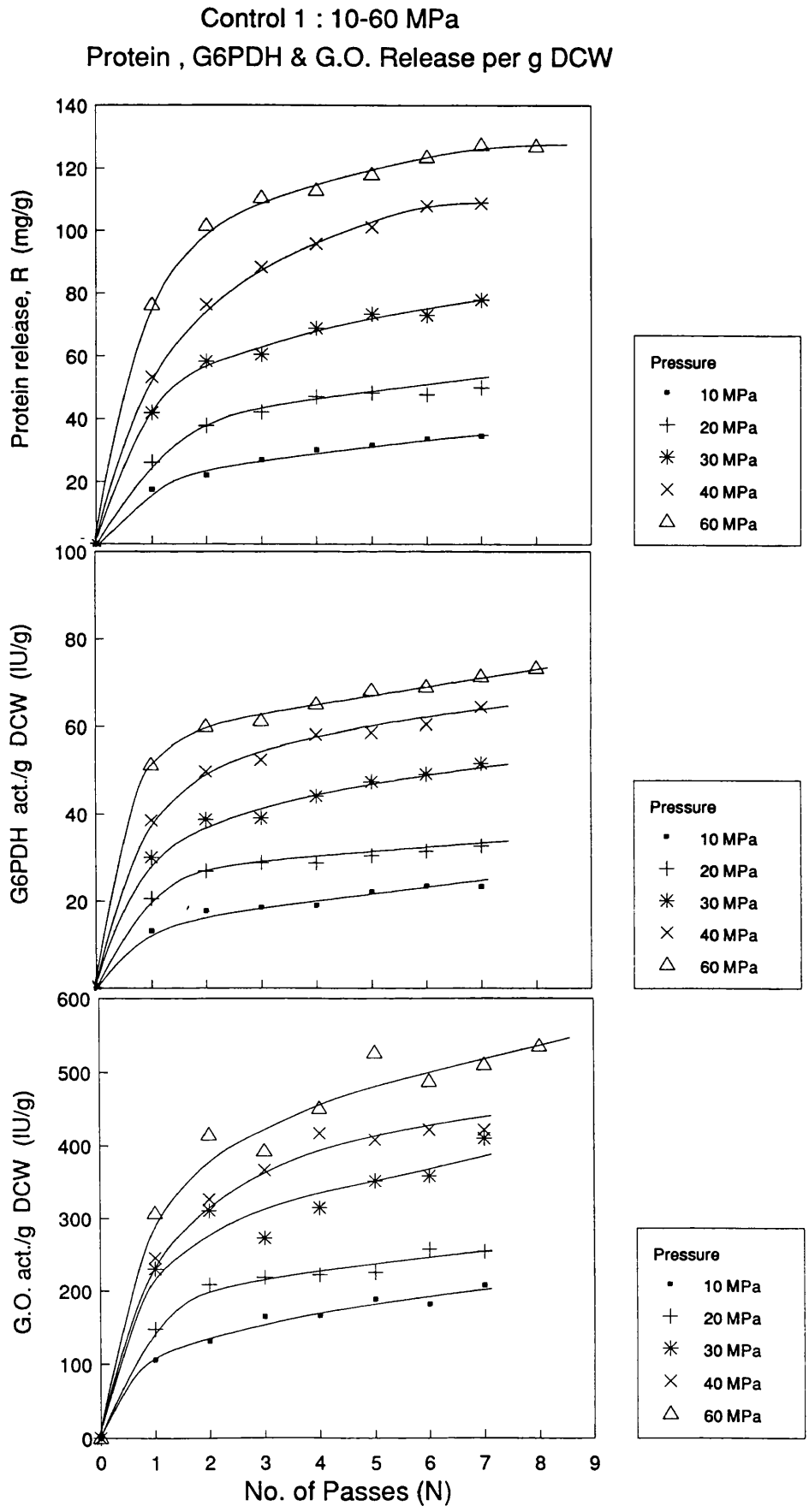


Fig. 3.22 : Protein, G6PDH & Glucose Oxidase release from **control no. 1 (10-60 MPa)** using Lab 60 homogeniser (expressed as absolute values).

3.3.2 Control 2 : 20-80 MPa

The pressure range was increased to 80 MPa to try to ascertain the pressure for complete product release. The concentration of cells was 10 g L^{-1}.

From fig. 3.23 the characteristics are :

1) again, a rapid release of product at the first two passes and then a very much slower rate of release for continuing passes.
2) maximum product release was achieved for protein and G6PDH since there is no further increase in the levels at 80 MPa. This maximum level of about 165 mg/g and 80 IU/g respectively, can be achieved after two passes at 80 MPa or in the case of G6PDH, after several passes (7) at 60 MPa. Therefore 100 % release of protein and G6PDH can be achieved by 80 MPa in 1 or 2 passes.
3) The difference in activity between 60 and 80 MPa for glucose oxidase is about 150 IU/g which is a very large jump. Although the level at 80 MPa is not increasing significantly, it is inconclusive as to whether this is the complete release point, because the level at 60 MPa does not even approach the one at 80 MPa. Therefore it was necessary to increase the pressure to determine the pressure needed for complete glucose oxidase release.

3.3.3 Control 3 : 20-124 MPa

In this control the maximum possible pressure in the 30CD homogeniser was used : 124 MPa (18000 p.s.i.). Disruption was conducted at a slightly lower concentration of 7.5 g L^{-1}.

Fig. 3.24 shows that :

1) once again the same trend of strong dependence of 1st pass release on pressure and weak dependence of further release on number of passes.
2) protein and G6PDH release is complete by about 80 MPa and is further confirmed here by no further increases in the levels at higher pressures.
3) glucose oxidase release is complete only by 100 MPa. At this pressure there is no further increase in the activity with number of passes as well as a further increase of pressure to 124 MPa. The maximum activity that can be achieved is about 950 IU/g which compares well with the level of about 1000 IU/g obtained by the ultrasonication of cell samples (fig. 3.2) during characterisation of the fermentation.

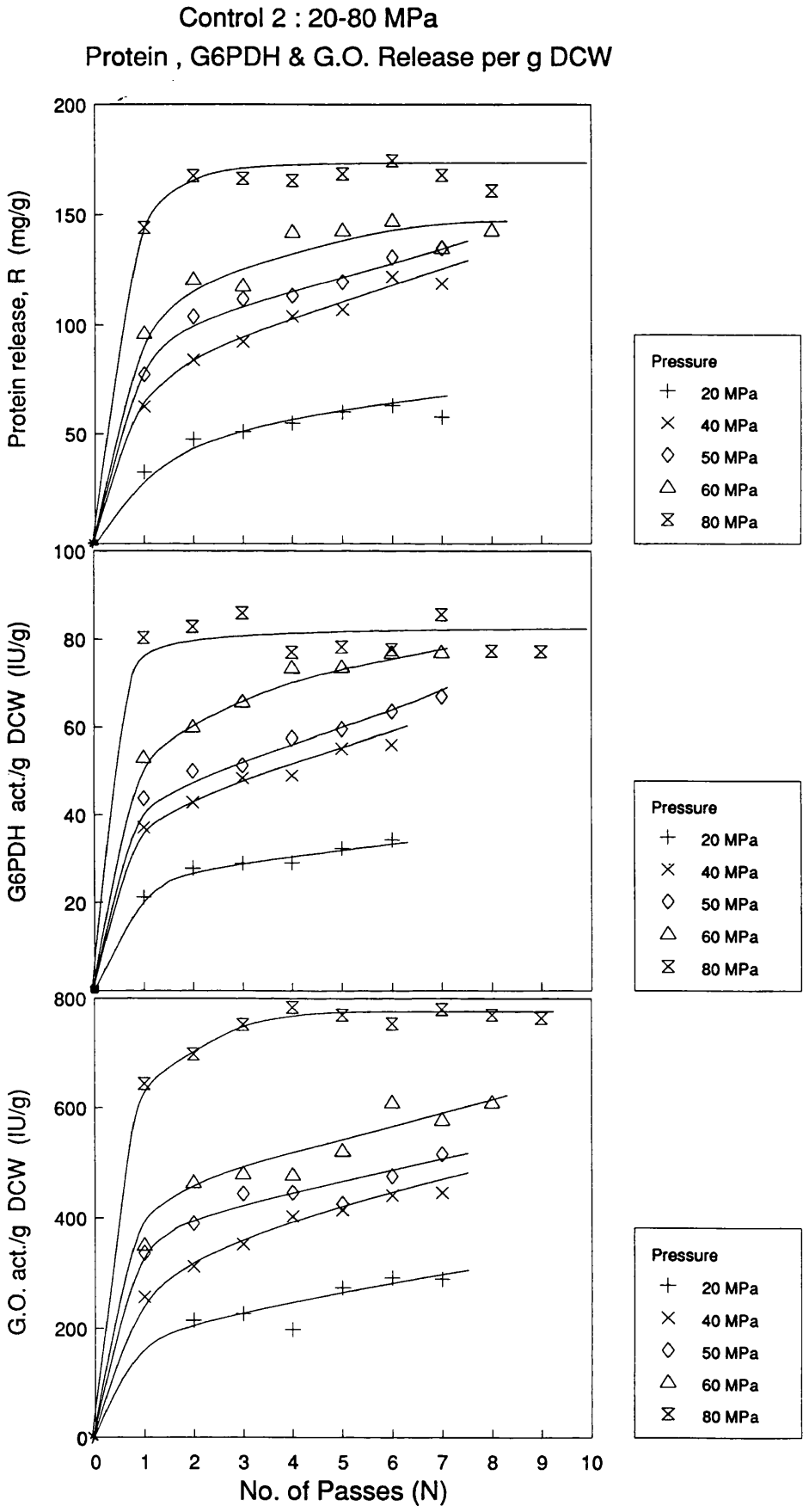


Fig. 3.23 : Protein, G6PDH & Glucose Oxidase release from **control no. 2 (20-80 MPa)** expressed as absolute values.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

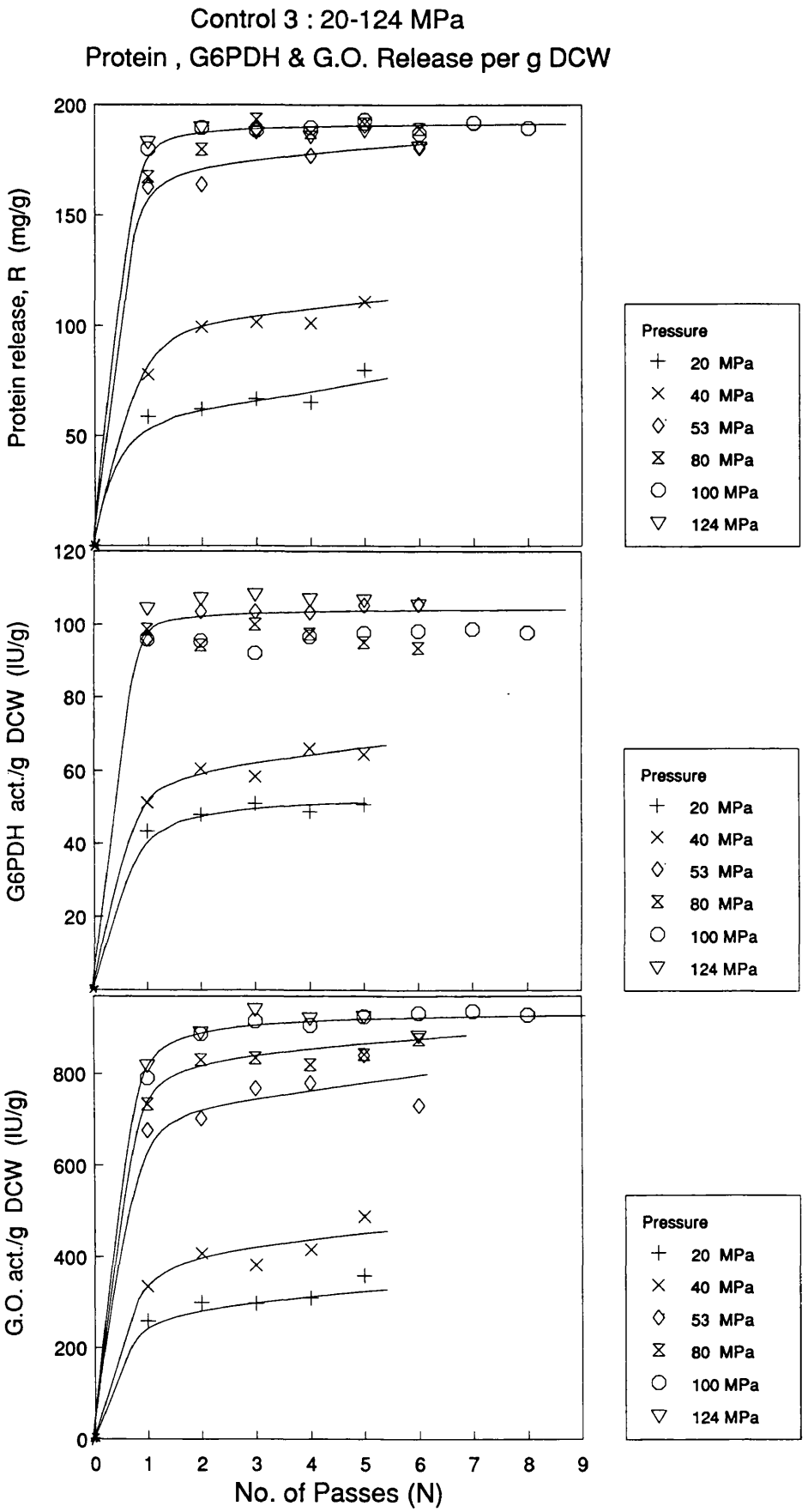


Fig. 3.24 : Protein, G6PDH & Glucose Oxidase release from **control no. 3 (20-124 MPa)** expressed as absolute values.
Homogenisers: Lab 60 - below 53 MPa; 30 CD above 53 MPa.

3.3.4 Generalised Disruption Kinetics of the Control

From figs. 3.22 - 3.24 it is evident from the actual amounts or activity that there is reproducibility in the disruption kinetics. For protein the maximum level is between 130 - 165 mg/g; G6PDH is 80-105 IU/g and glucose oxidase 1000 IU/g.

Nevertheless, they are not directly comparable because of the slight variations. Therefore, by normalising the values and representing them as percentage release composite diagrams covering the whole range of pressures can be compiled. Figs. 3.25 - 3.30 show the composite diagrams for protein, G6PDH and glucose oxidase release. By combining the three control runs to cover the entire pressure range, two sets of composite diagrams are obtained : set A and set B. Set A gives the low pressure (up to 60 MPa) data from control 1 (fig. 3.22), whilst set B data (up to 60 MPa) is from control 2 (fig. 3.23). The data for higher pressures (80 -124 MPa) for both sets A and B are from control 3 (fig.3.24).

3.3.5 Differential Rates of Product Release

From the above sections 3.3.1 - 3.3.2, there is clear evidence that glucose oxidase requires a much higher pressure (at least 100 MPa) than G6PDH and protein (80 MPa 2 pass) for its complete release. This difference in disruption kinetics can be attributed to the intracellular location of the two enzymes.

Further illustration of the slower rate of release of glucose oxidase relative to protein is shown in fig. 3.31. All three controls have been combined; the symbols distinguish each pressure range for a particular batch. A line of equal rate of enzyme and protein release is drawn in to show the extent of increased or decreased rates. It shows that G6PDH was released at a slightly faster rate than protein. In all cases the glucose oxidase release lagged behind protein and therefore also G6PDH.

Follows et al (1971) found that the relative rates of release of enzymes located in the periplasmic space was fastest followed by ones in the cytoplasm and lastly mitochodrion associated ones; the cytoplasmic ones being at the same rate as total protein. However these locations did not result in any difference in the pressure for complete release, as is the case here. Cytochemical staining studies by *Dijken & Veenhuis (1980)* on a similar glucose oxidase producing strain from the same industrial source found that glucose oxidase was located in microbodies or organelles called peroxisomes in the cytoplasm. These peroxisomes were associated with mitochondria and possessed an unit membrane which separated glucose oxidase from the cytoplasm as well as its coupled enzyme, catalase. This is a logical site because hydrogen peroxide is toxic, and since glucose oxidase produces hydrogen peroxide from the oxidation of glucose (which is reduced by catalase), they are

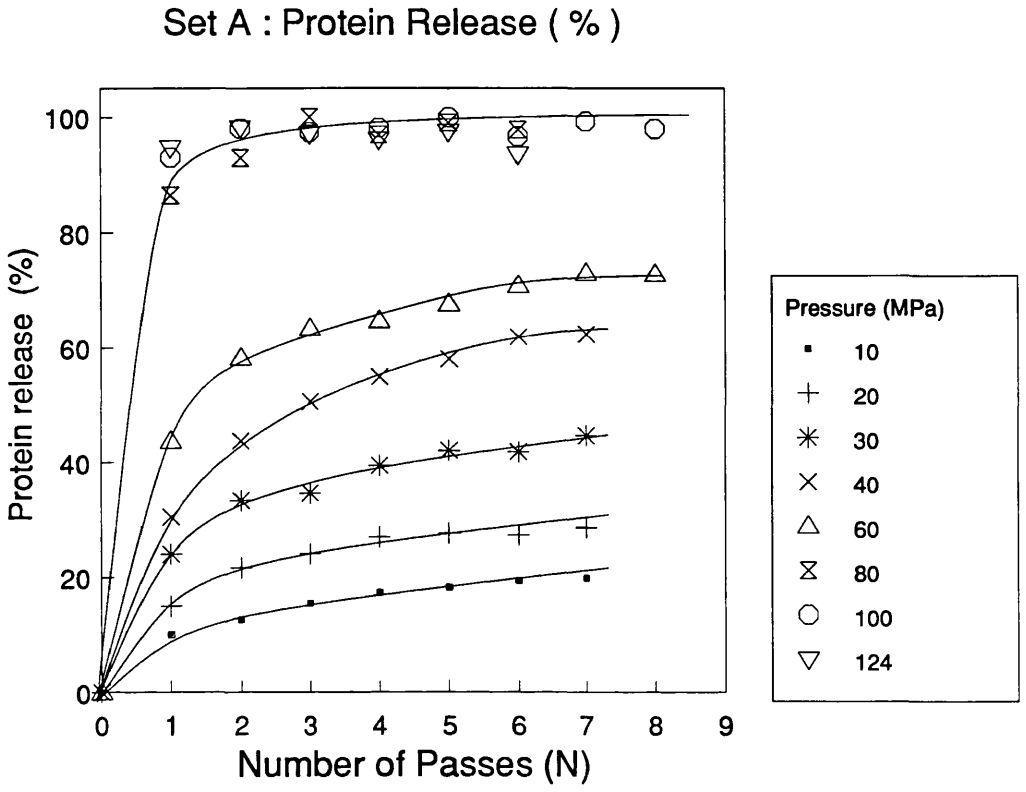


Fig. 3.25 : Protein release from "set A" expressed as percentage release.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.22 & 3.24)

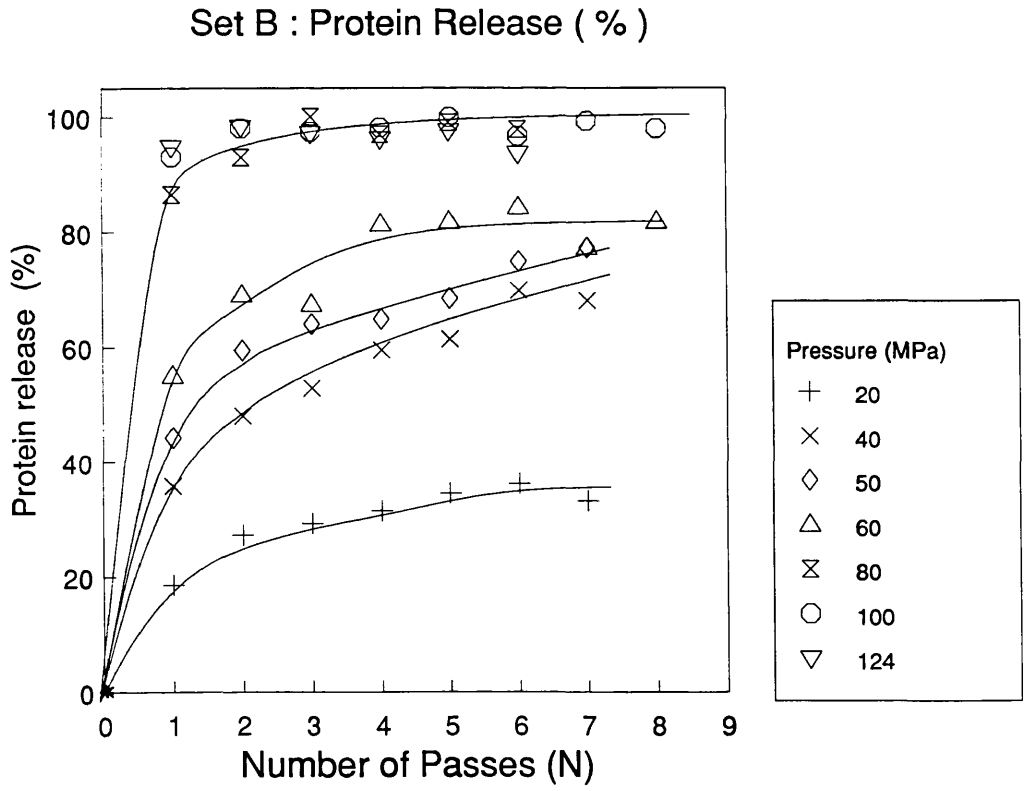


Fig. 3.26 : Protein release from "set B" expressed as percentage release
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.23 & 3.24)

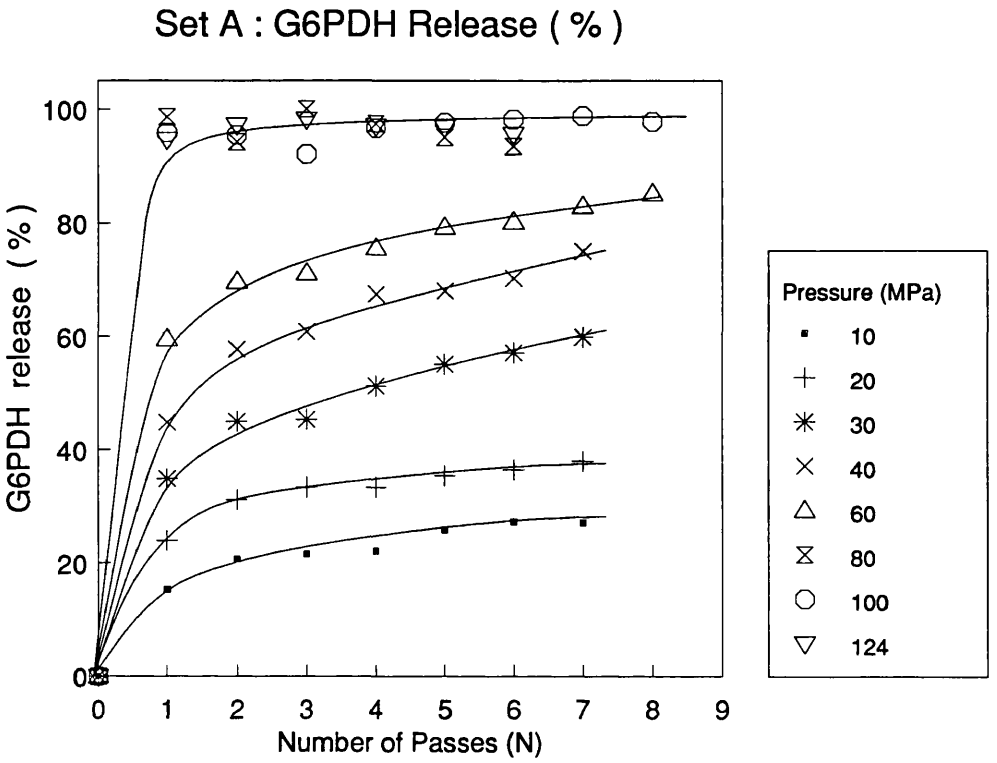


Fig. 3.27 : G6PDH release from "set A" expressed as percentage release
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.22 & 3.24)

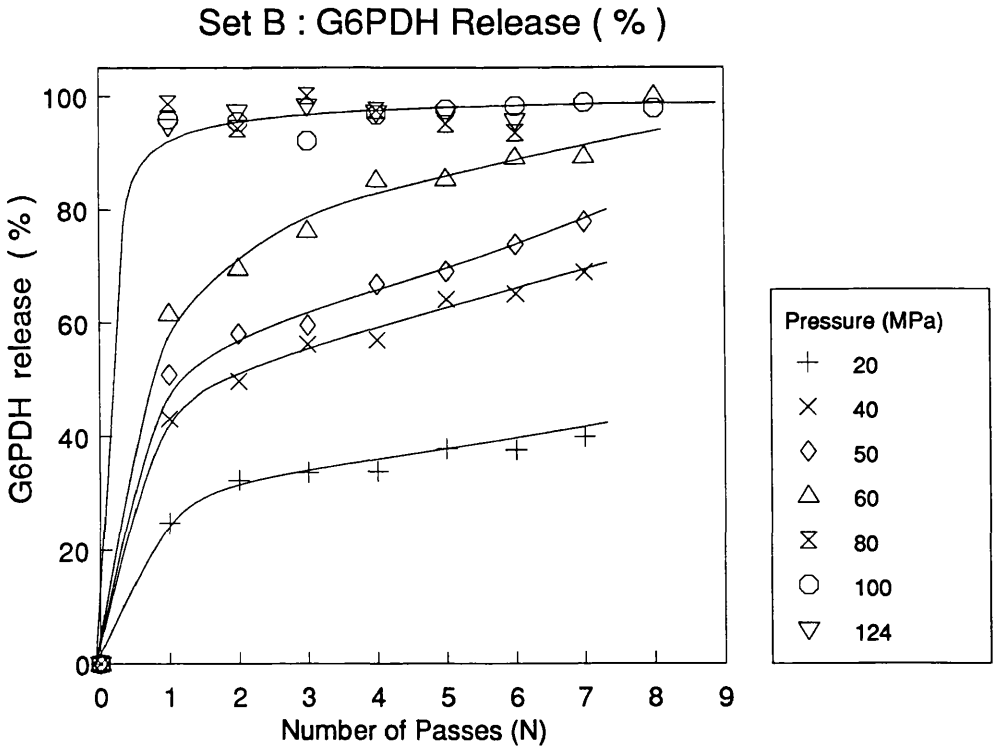


Fig. 3.28 : G6PDH release from "set B" expressed as percentage release
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.23 & 3.24)

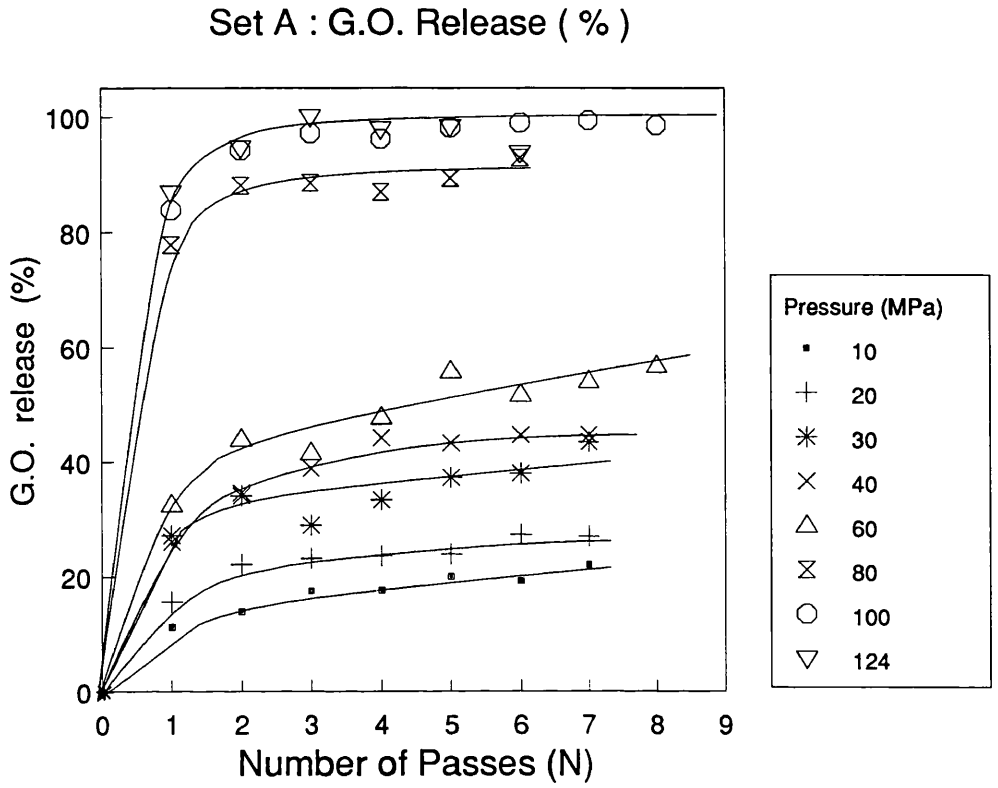


Fig. 3.29 : Glucose oxidase release from "set A" expressed as (%) release
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.22 & 3.24)

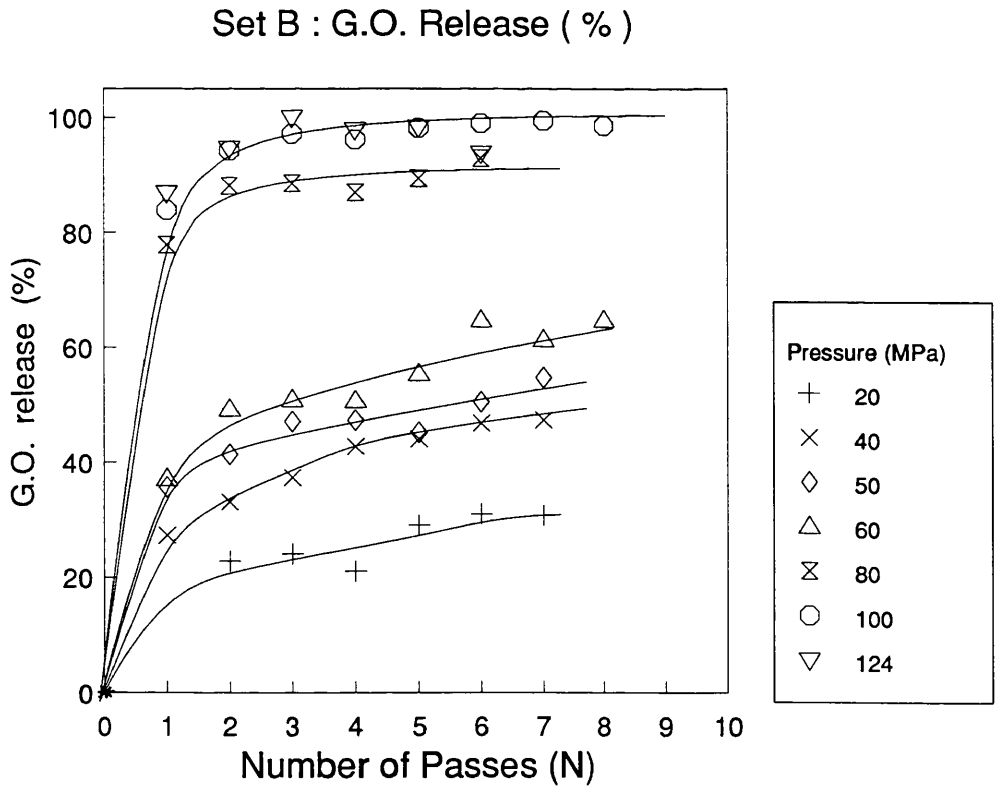


Fig. 3.30 : Glucose oxidase release from "set B" expressed as (%) release
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.
(data from fig. 3.23 & 3.24)

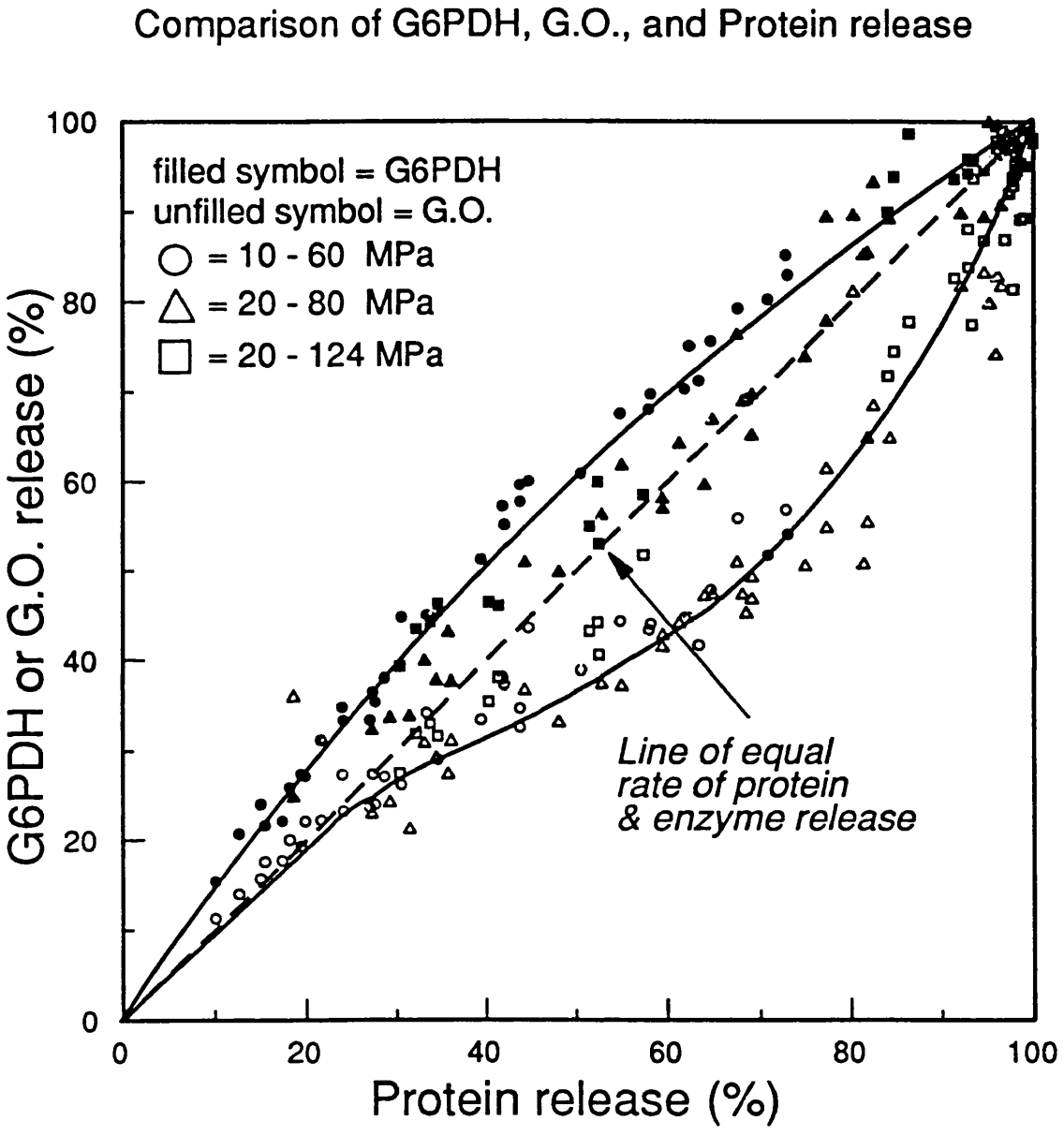


Fig. 3.31: A comparison of the rates of release of enzyme and protein (data from fig. 3.25 - 3.30)

located together. Therefore the disruption process has to breach another barrier after the cell membrane to release glucose oxidase. Hence, the need for higher pressure to break up these small (0.2 - 0.6 μm) structures and the slower rate of release.

Although G6PDH is an enzyme which is not located in any microbodies (it is freely soluble in the cytoplasm), its rate of release was slightly faster than overall protein release. This may be simply due to overall protein including glucose oxidase and other "difficult to release" proteins. This difference decreases with pressure, and at 80 MPa, when about 90 % glucose oxidase is released, the 10 % of unreleased G.O. does not represent a significant fraction of protein released leading to 100 % protein and G6PDH release.

3.3.6 Confirmation of Maximum Protein Content

As defined above, the point of complete release of product is where there is no further increase in the amount or activity with increase in pressure or number of passes, which is also the maximum amount. To confirm further the complete release, sodium dodecyl sulphate (SDS), an ionic detergent was used to dissociate all the proteins into their individual polypeptide subunits. The homogenate was heated at 100°C in a SDS solution to denature the proteins, during which the polypeptides were bound to the SDS. Following this, the proteins were analysed by the technique of measuring the absorbances at 228.5 and 234.5 nm devised by *Ehresmann et al (1973)*, since the Bradford method would be invalid due to the change in charges on the protein. This method will solubilise all the proteins and therefore gives the maximum possible protein that can be released through cell disruption.

Fig. 3.32 shows the protein content of various samples. Spun homogenate means that the cell debris has been removed by centrifugation leaving the supernatant to be used for the test, whereas unspun involves the whole homogenate being treated. The terms "in water" and "in SDS" simply means the suspending solution. The control sample is therefore UH H_2O and SH H_2O (unspun and spun homogenate in water) which contains homogenate taken from the final pass (8) at 80 MPa. Tests of unspun and spun homogenate in SDS (UH SDS & SH SDS) were used to determine if unheated SDS makes any difference. The most important sample is the treated unspun homogenate which was in SDS and heated.

Clearly, there is no significant difference in the protein content between the control unspun homogenate in water (UH H_2O) and both the heated and unheated SDS samples (TU H_2O & UH H_2O). All three show a level of about 400 mg/g which is equivalent to protein constituting 40 % of total dry weight. Unheated samples also showed no difference, which indicates that all barriers

to protein release have been breached by homogenisation and so the heating step is not necessary to break up any intact membranes, as the case may be with incomplete disruption. This is therefore conclusive evidence that high pressure homogenisation at 80 MPa for at least 2 passes will release all the protein, since the protein level at the second pass is the same as at the eighth pass.

However, for the spun homogenate (SH H_2O & SH SDS) the supernatant gave a 25 % lower protein content (300 mg/g). This can be attributed to the entrapment of protein by cell debris as it is spun down. Fortunately in these studies the absolute levels are not as important as the relative changes. This cell debris separation step was always carried out at the same centrifugal force of 16000 g in to ensure that the extent of protein entrapment is the same in all cases. In addition this protein assay technique has given a much higher protein level of 400 mg/g as opposed to almost 200 mg/g by the Bradford method. This is due to the different methods of detection: the Bradford depends on dye binding onto positive charged groups whilst the UV method is a measure of the difference in absorbance at those two wavelengths. Since the concern is the relative changes in protein as a measure of cell disruption, the Bradford method was chosen because of its simplicity.

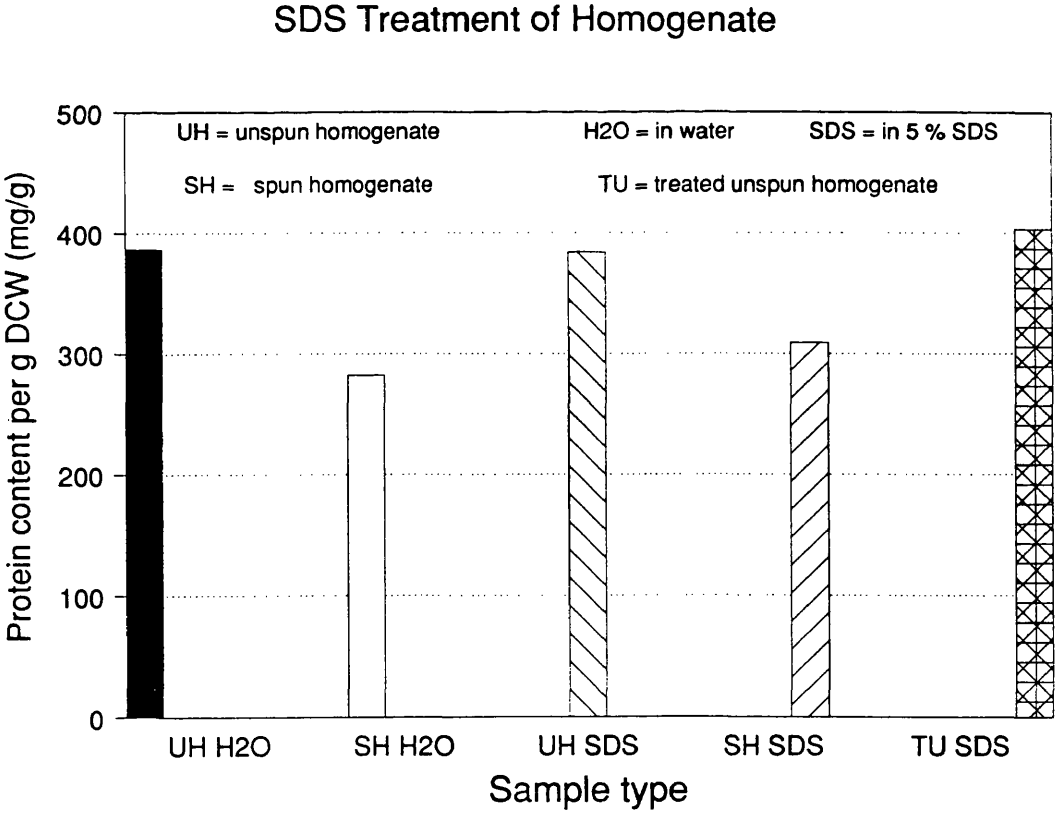


Fig. 3.32 : Comparison of protein content of SDS treated, untreated, spun and unspun homogenate from sample at 80 MPa after 8 passes

3.3.7 Direct Observation of the Disruption Process

3.3.7.1 Photomicrographs of homogenate

The following photomicrographs (figs. 3.33-3.38) of homogenised cells at various pressures and stages, show that there is good correspondence between the physical appearance and the enzymatic and protein analyses. By combining these two methods of detecting cell disruption, it is possible to formulate a qualitative description of the disruption process.

10 MPa : At the 1st pass (fig.3.33) the homogeniser appears just to disentangle the highly viscous and clumped mycelia mat with only a minimal number of cells disrupted. Further passes (fig.3.34) have negligible effect. These physical changes are reflected by the low release of products : 24 % for G6PDH, 17 % for protein and 13 % for glucose oxidase.

60 MPa : A large proportion of cells have been disrupted at the 1st pass (fig.3.35), which results in about 60 % protein and G6PDH release and 40 % glucose oxidase. The mycelia has been violently disentangled with the resultant high shear forces tearing and disrupting the cells. Fragments are now about from 40-100 µm. Continuing for 7 passes (fig. 3.36) causes reduction in the particle size to about 30 µm and the range of sizes seems to have narrowed considerably. At this high pressure there is distinct change in appearance with increased passes: fragments are now 2-3 cell lengths, and some whole cells remain. However, protein release was 77 %, G6PDH 92 % and glucose oxidase 60 %.

124 MPa : This extremely high pressure causes almost instantaneous and 100 % cell disruption at the 1st pass, as shown in fig. 3.37 by the 10 µm or one cell length particles and no whole cells. Of course this results in 100 % release of all products. After 5 passes (fig. 3.38) the cell debris is reduced to less than 10 µm or of the same size as media particles from the molasses. This debris size is still larger than whole yeast cells of 5 µm diameter and therefore separation is not a problem. In fact the debris settle very well to leave a relatively clear supernatant which can be decanted. Centrifugation at 16000 g (in a microcentrifuge) resulted in a dense pellet.

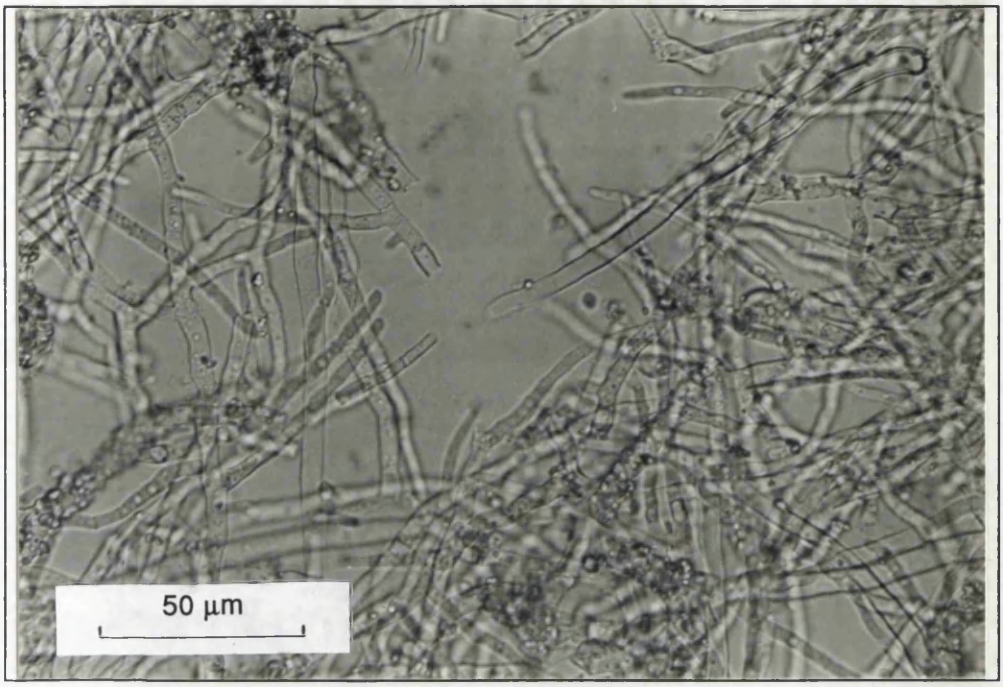


Fig. 3.33 : Photomicrograph of homogenised cells at **10 MPa after 2 passes.** Magnified 400 X Mostly intact cells. Minimal number are broken: hollow cell wall shell with no cytoplasm. Mycelial clumps disentangled. This corresponds to 13-24 % of product release.

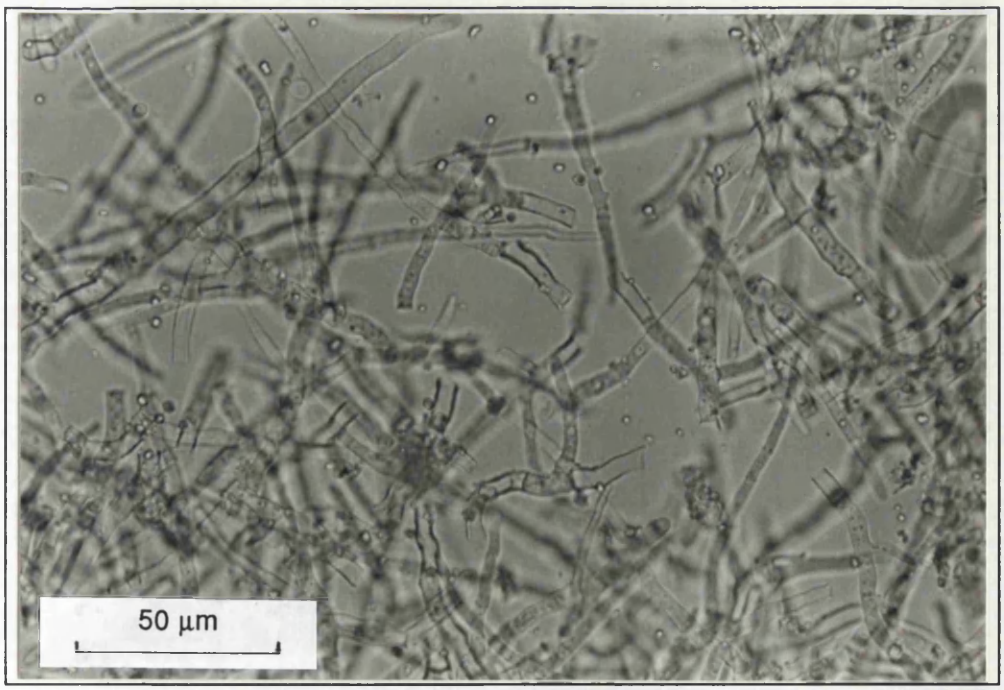


Fig. 3.34 : Photomicrograph of homogenised cells at **10 MPa after 7 pass.** Magnified 400 X. No significant difference from 2nd pass; still minimal cells disrupted.

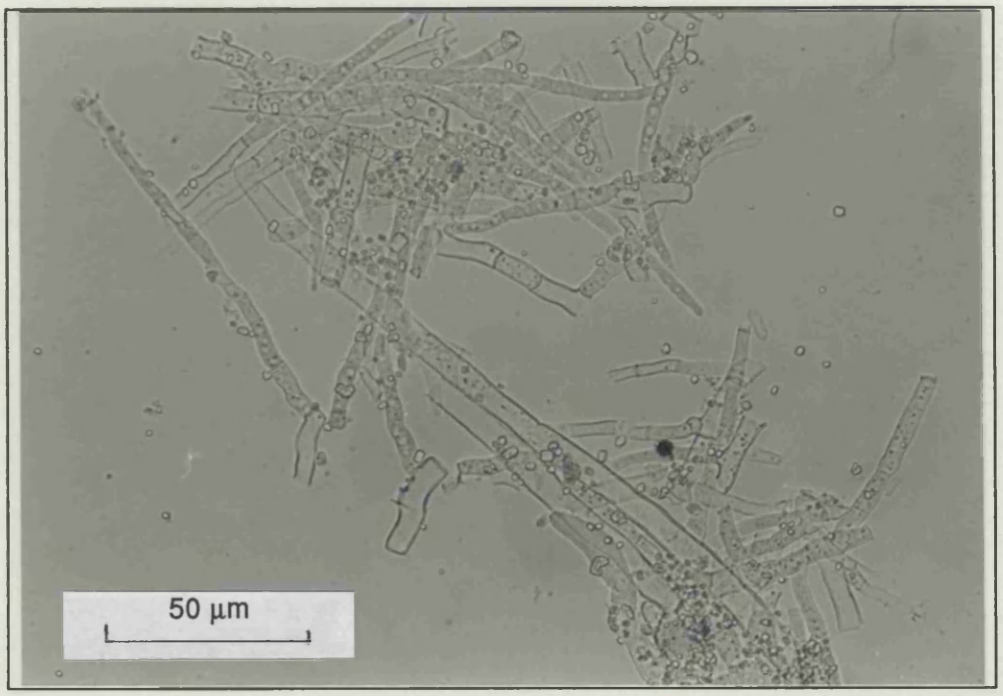


Fig. 3.35 : Photomicrograph of homogenised cells at **60 MPa after 1 pass**. Magnified 400 X. Mycelia fully disentangled and many cells broken but not all. This corresponds to release of 49% protein, 60% G6PDH and 40% glucose oxidase.

Fig. 3.36 : Photomicrograph of homogenised cells at **60 MPa, 6 pass.** Magnified 400 x. Most cells disrupted: fragments of 10-30 µm. 77% protein, 92% G6PDH and 60 % glucose oxidase released.

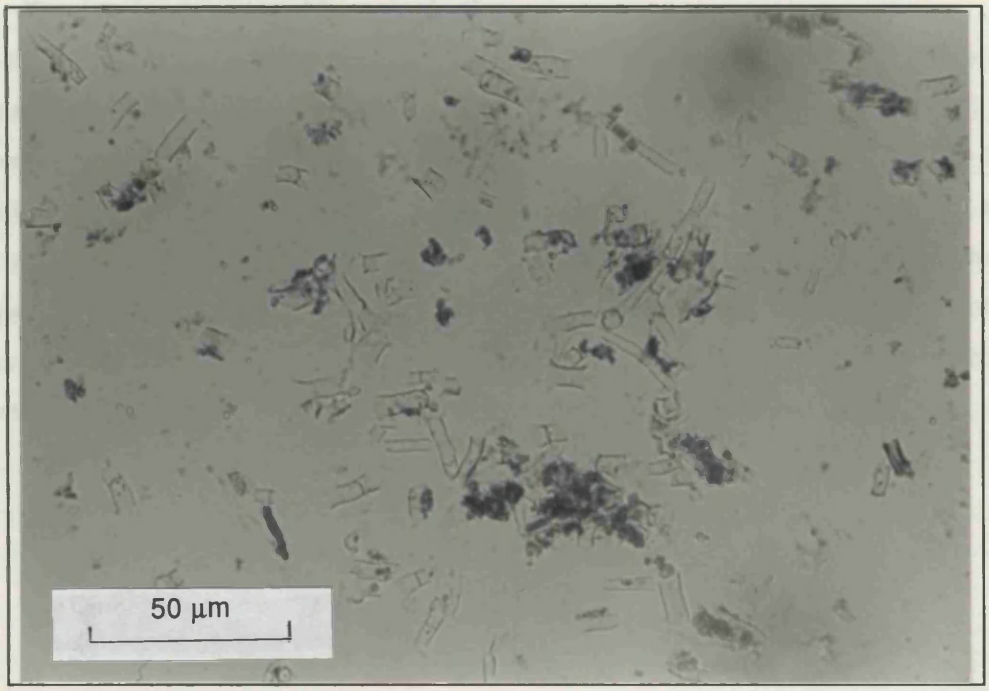


Fig. 3.37 : Photomicrograph of homogenised cells at **124 MPa after 1 pass.** Magnified 400 X. Further reduction of particle size to less than one cell length - all disrupted. Corresponds to 100 % release of all products.

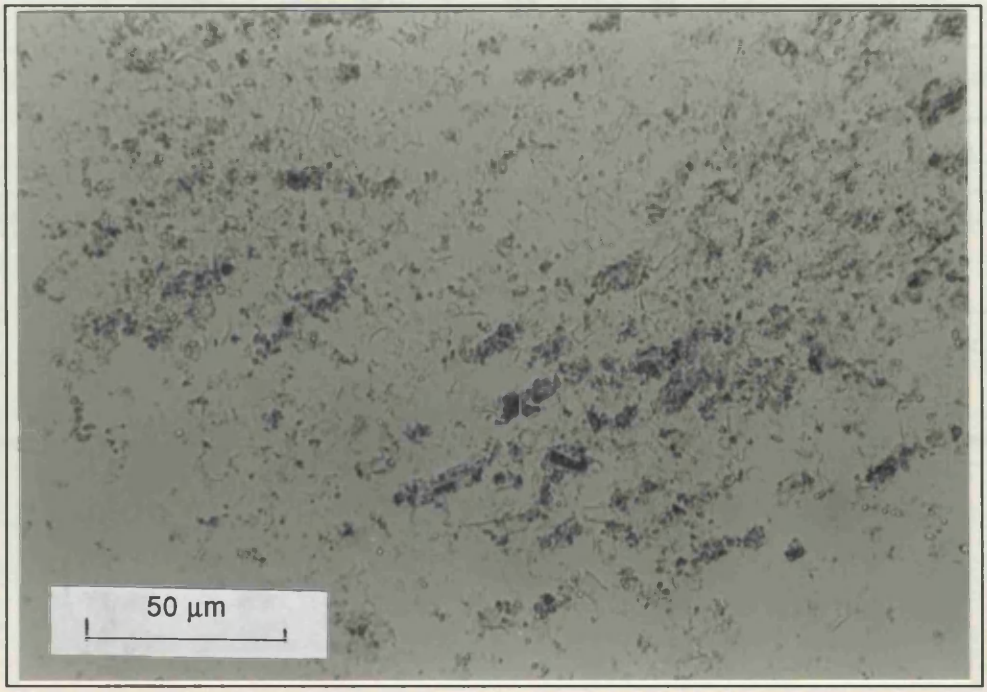


Fig. 3.38 : Photomicrograph of homogenised cells at **124 MPa, 5 pass.** Magnified 400 X. Cell fragments now indistinct - same size as media particles (darker) less than 10 µm.

3.3.7.2 Temperature control of homogenisation

In both the "Lab 60" & "30CD" homogenisers, the homogenate was cooled immediatedly after disruption to below 10°C by ethylene glycol at 5°C (Lab 60) or chilled water at 3°C (30CD). Exit homogenate temperature from the 30CD at 124 MPa at any pass was about 35°C which is an extremely high temperature rise from about 6°C cell suspension. Nevertheless, this did not affect enzyme activity or protein content, as shown by figs. 3.22-3.24. No loss of activity was seen even after 8 passes at 100 MPa. Therefore for these products, the temperature increase is not a problem. However, for thermolabile products, it may cause denaturation but since the residence time through the valve is likely to be less than a second, deanaturation may be minimal.

3.3.8 Summary of the "Control" Disruption Kinetics

Pressure (MPa)	Number of Passes	Product Release (%)			Observations of homogenate
		Protein	G6PDH	G.O.	
10	1	10	18	11	Disentanglement, minimal disruption
	7	20	32	22	
60	1	49	60	40	Particles 40-100 μm
	6	77	92	60	Particles 30 μm
124	1	100	100	100	Particles 10 μm ≈ 1 cell length
	5	100	100	100	Particles less than 10 μm

Table 3.2 : Summary of Control Disruption Kinetics

3.3.9 Discussion

From the disruption kinetics of protein, G6PDH and glucose oxidase, shown above (fig. 3.22-3.24), the characteristics are:

1) a high level of product release is achieved in the first pass and the extent of this release is a strong function of pressure.
2) product release after the first pass proceeds quite slowly.

In some cases, the increase in product release after several passes at a given pressure is almost negligible. This may mislead one to assume that complete release has been achieved, especially at a relatively high pressure of 60 MPa, as is the case for protein release in control 1 (fig. 3.22). A higher pressure must therefore be used, and if no further increase in product concentration is seen, then complete release can be assumed.

For the three products examined there were different minimum pressures required for its complete release within a few passes: G6PDH and protein required 80 MPa, although the former can be fully released after 6 or 7 passes at 60 MPa; glucose oxidase required 100 MPa. The differences have been attributed to their location inside the cell (sect. 3.3.5). The optimal operation of an homogeniser will therefore depend on the product required.

Since *A. niger* is a septate fungi, disruption may be expected to be more difficult than for aseptate fungi, as each cell would need to be broken, whereas for aseptate fungi, once the cell wall is broken anywhere in the whole mycelia, then the contents of all cells will be released. *Keshavarz et al (1990a)* found that the complete release of alcohol dehydrogenase (ADH) from *Rhizopus nigricans* was achieved at 10 MPa in a high pressure homogeniser and that photomicrographs showed that at this pressure, although the filamentous structure was retained with only a few points of cell wall breakage, all the cell contents were emptied. As expected from its aseptate nature, the disruption kinetics of *R. nigricans* was a weak function of pressure, which contrasts the high dependency on pressure for *A. niger*. Despite the differences due to the septate/aseptate structure, the kinetics of release are similar in that a majority of product is released at the first pass. This is in contrast to the first order disruption kinetics exhibited by unicellular microorganisms such as baker's yeast *(Hetherington et al 1971)* and *E. coli (Gray et al 1972)*. Therefore, filamentous fungi would appear to have an entirely different mechanism of disruption from unicellular microorganisms.

An attempt to postulate the mechanism of disruption of *A. niger* will be made here, based on a qualitative analysis of its disruption kinetics and photomicrographs of homogenate (fig. 3.33-3.38):

First pass: At a low pressure (10 or 20 MPa), there is a small release of products and it appears that the highly entangled cells are disentangled with only some cells broken. At higher pressures, (> 30 MPa) the disentanglement is more complete and also results in considerable breakage. The cells are highly entangled in clumps or pellets of the order of several hundred microns. This compares with estimates of the gap width between the valve rod and valve seat of the order of 8-16 μm (calculation in Appendix 3). Consequently, as the mycelia is passed through the orifice of the valve seat, and then is forced through the very small gap width, the cells will initially be torn apart with very high shear forces generated, thus disentangling and rupturing the cells. As the gap width decreases, it becomes more difficult to force the cells through, and so, the shear forces will be higher, thus causing not only disentanglement but also extensive breakage of cells.

After the first pass: the cells are now disentangled, whether it is at 10 MPa or 60 MPa. The only difference is the degree of disruption. At a low pressure, the disentanglement only "loosens up" the mycelia, and then in subsequent passes, the cells do not undergo much change in structure. This could be due to the gap width being about 15 μm at 10 MPa, which compared to the hyphal width of about 3 μm, is much bigger. It may be possible that these long hyphae just "slip" through the gap width, resulting in minimal disruption. However, at high pressures, at say 60 MPa, the fragments of hyphae which were reduced to 40-100 μm at the first pass are further reduced to 30 μm lengths (2-3 cell lengths) with significant increase in product release. Therefore, the cause may be through impingement of the fungal particles on the impact ring, since the forces of impact at 60 MPa would be much higher than at 10 MPa. The mechanism of impingement was considered to cause 80 % of disruption for yeast cells *(Keshavarz et al 1990b)*. However, in the case of filamentous cells, this may not be so significant especially at the first pass, and low pressures. Once the hyphae lengths are reduced to sizes of about 40 μm, which is not much bigger than yeast cells, then the mechanism may be similar to the disruption of unicellular microorganisms.

3.4 The Effect of Morphology on Disruption

The pelleted and filamentous type cells as described in sect. 3.1.2 and 3.1.3 were disrupted, as for the control type morphology. For filamentous cells and pelleted cells, the disruption kinetics cannot be compared directly with each other and have to be treated separately. As far as possible the cell concentration for disruption was maintained at about 10 g L^{-1} to avoid introducing another variable.

3.4.1 Disruption of Pelleted Cells

Pellet sizes can be loosely classified as "small" (about 0.5 mm diameter) and "large" (0.9 & 1.8 mm) pellets. The latter group includes two different sizes because the disruption kinetics do not appear to vary above the 0.9 mm size.

3.4.1.1 "Small" pellets (0.5 mm)

There were two identical batches resulting from a reduction in spore inoculum concentration by a factor of 10, which gave mean sizes of 0.48 mm "length" and 0.38 mm "breadth" : batch 1; and 0.65 mm "length" and 0.48 mm "breadth" : batch 2. Disruptions were done at 11 and 9.4 g/L (dcw) respectively. The following figures 3.39 and 3.40 show the disruption kinetics for both batches, expressing product concentrations as absolute values. By comparing the two batches, the kinetics are very similar. Normalising the values to a percentage release basis gives fig. 3.41, which is an average of both batches. These percentage values can then be compared to the control.

Both batches show similar characteristics to the control morphology (fig. 3.25-3.30):

* Rapid rate of product release at the 1st pass.
* Much slower rate of release after the 1st pass.
* Maximum release of G6PDH was achieved at 60 MPa after 6 passes.
* Glucose oxidase was not fully released at 60 MPa 6 passes; complete release was achieved by further disrupting the homogenate for 6 passes at 124 MPa.

A difference to the control morphology is :

* Protein release was fully released after 6 passes at 60 MPa. It is not achieved until 80 MPa for the control.

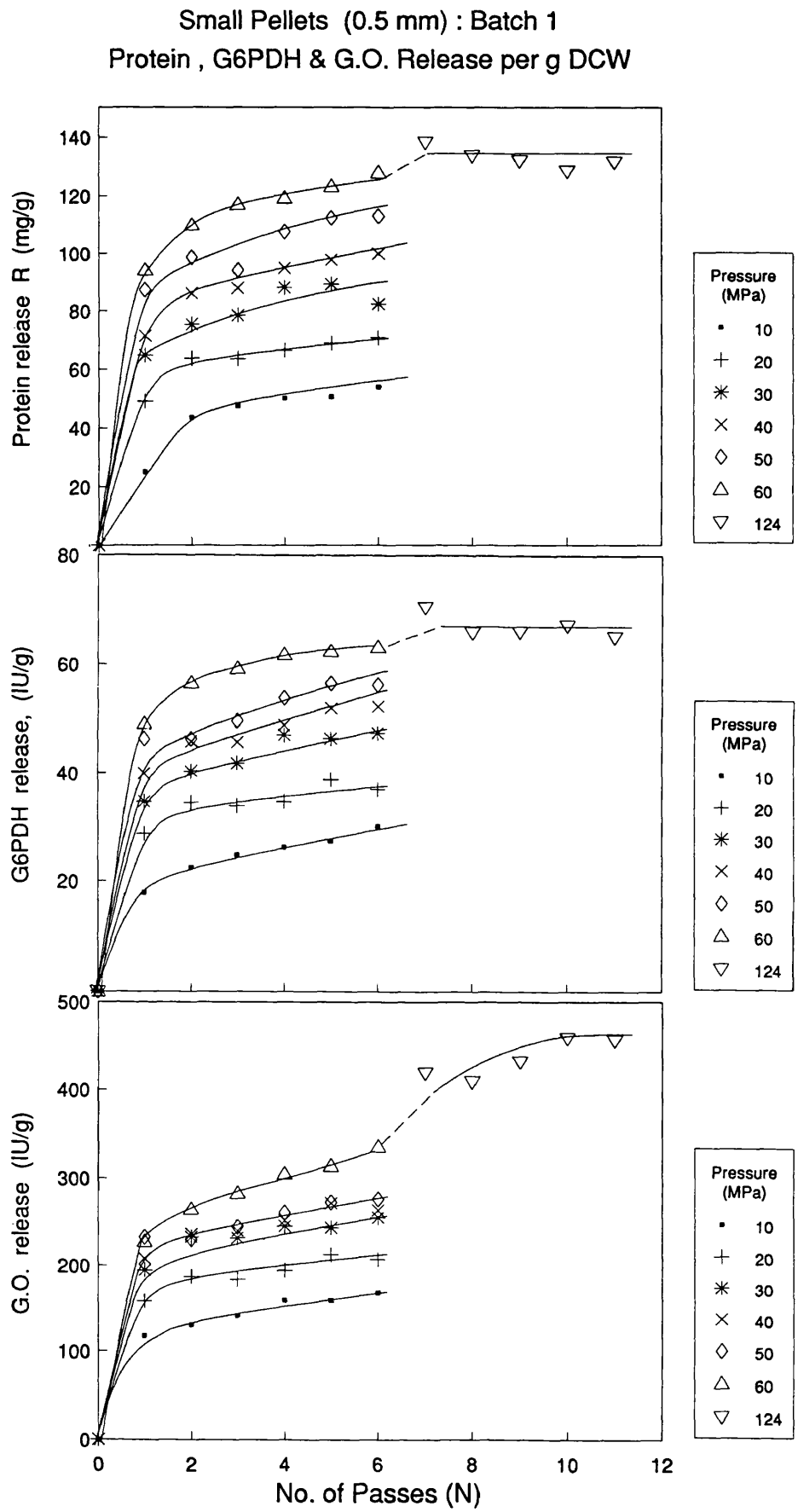


Fig. 3.39 : Protein, G6PDH & Glucose Oxidase release from **small pellets batch 1** expressed as absolute value.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

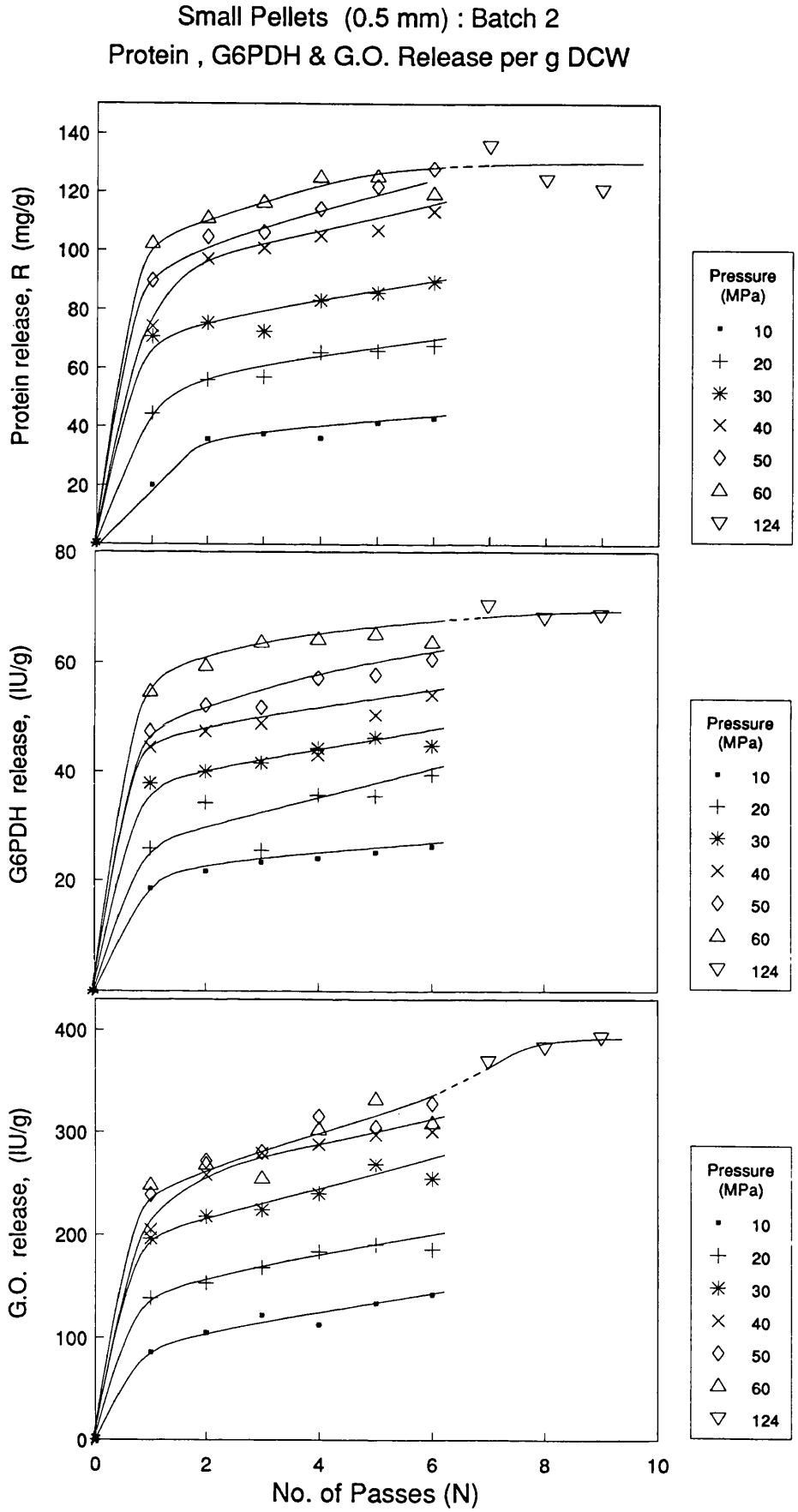


Fig. 3.40 : Protein, G6PDH & Glucose Oxidase release from **small pellets batch 2** expressed as absolute value.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

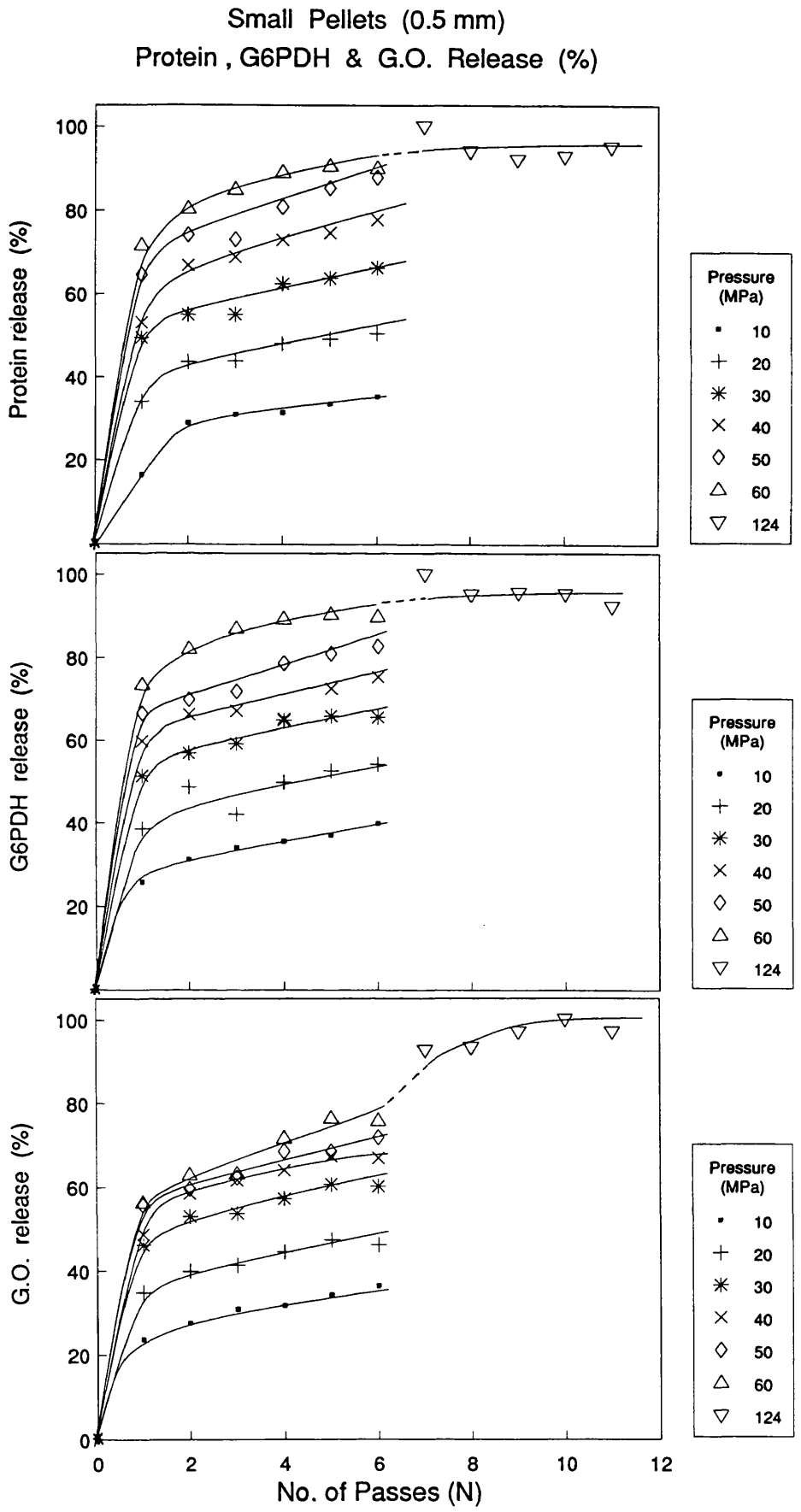


Fig. 3.41 : Protein, G6PDH & Glucose Oxidase release from **small pellets** expressed as an average percentage release calculated from batch 1 & 2. Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

3.4.1.2 "Large pellets" (0.9 & 1.8 mm)

Two batches of different pellet sizes are classed in this group :

Batch 1 : length 0.93 mm , breadth 0.73 mm; disruption concentration : 9.9 g/L (dcw)

Batch 2 : length 1.81 mm , breadth 1.40 mm; disruption concentration : 9 g/L (dcw)

The disruption kinetics, represented both as absolute values and percentage release are shown in figs. 3.42 and 3.43.

Again, the characteristics of the kinetics were similar to the control and small pellets. However, for both large pellet batches, there was an unexplained 20 % loss of protein at 124 MPa but not for G6PDH activity which did not change, and glucose oxidase activity which as expected increased to its maximum release on disrupting the 60 MPa homogenate again at the higher pressure.

3.4.1.3 Discussion

Figs 3.39-3.43 show that the form of disruption kinetics did not change for pelleted type growth. This means that they show the characteristic rapid rate of release during the first pass, after which the rate decreases significantly. Since there is a change in shape and overall size of the mycelial entity ie. the mycelia is accumulated as a pelleted mass as opposed to the dispersed structure in the control morphology, it may be anticipated that the disruption kinetics would be completely different, but it still retains a similar pattern to the control, which means that the mechanism of disruption may be similar to the control as postulated in section 3.3.9. The difference occurs in the extent of disruption at a given condition. It is recognised here that the pressure required for protein release has decreased to 60 MPa, as compared to 80 MPa for the control morphology. This indicates that pellets may be easier to disrupt and this will be illustrated more clearly in section 3.4.3, where a comparison between the different morphologies are made.

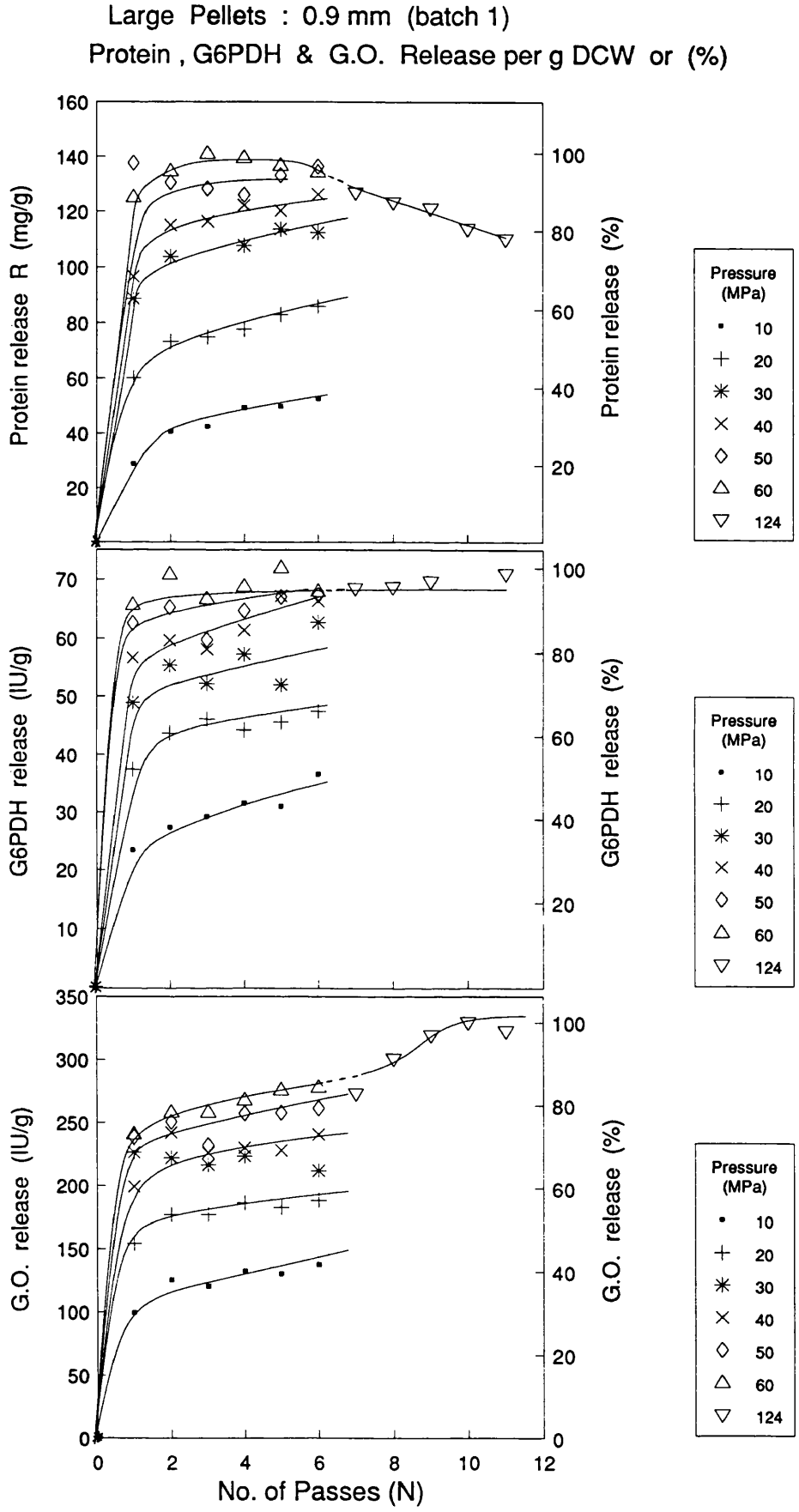


Fig. 3.42 : Protein, G6PDH & Glucose Oxidase release from **large pellets batch 1** expressed as absolute and percentage value.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

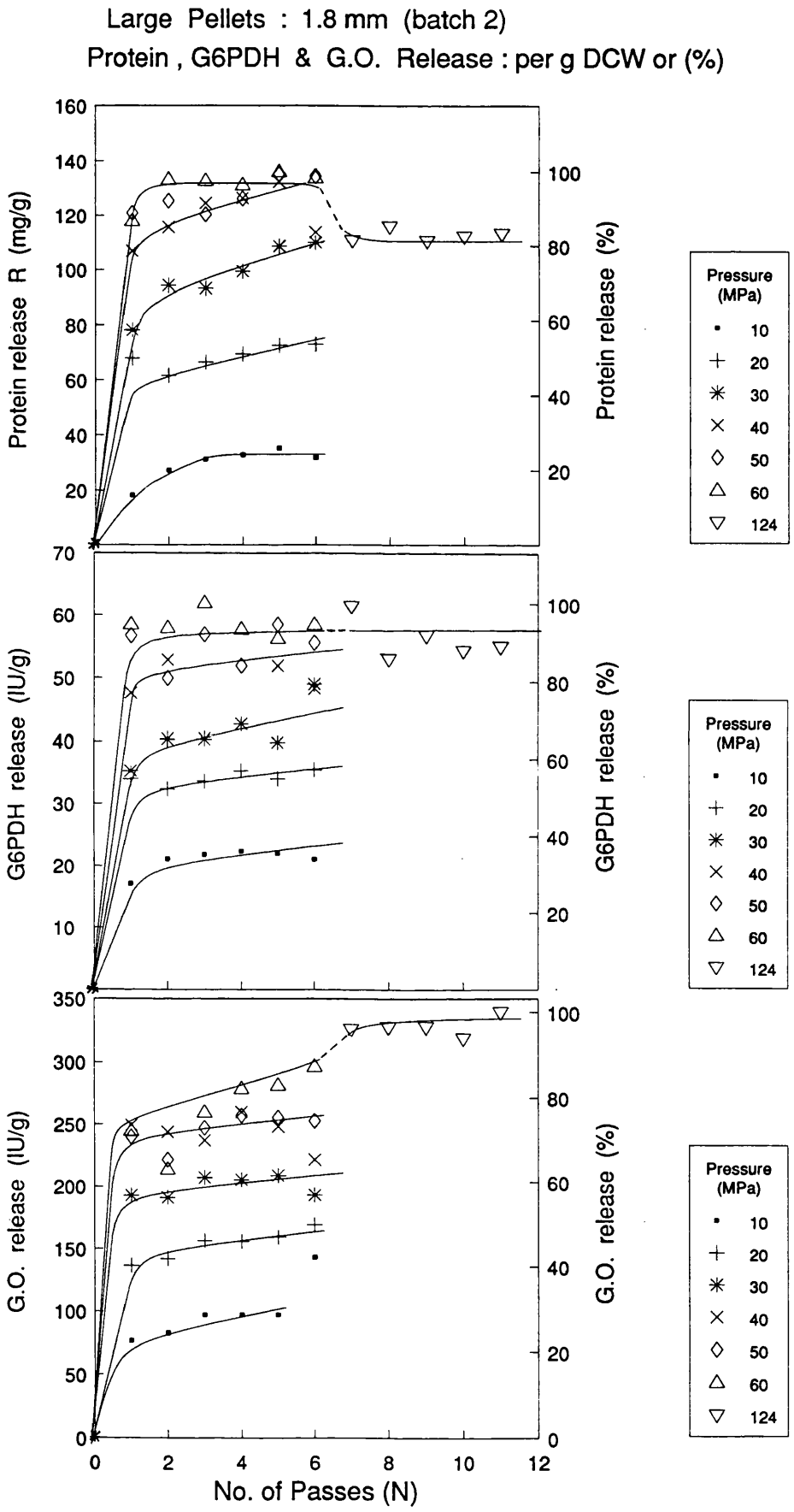


Fig. 3.43 : Protein, G6PDH & Glucose Oxidase release from **large pellets batch 2** expressed as absolute and percentage value.
Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

3.4.2 Disruption of Filamentous Cells

Filamentous growth was produced by the addition of "Junlon" (section 3.1.3.1) or by reducing the agitation (3.1.3.2). Although both changes resulted in filamentous cells, these disruption kinetics were quite different from each other. Therefore it is necessary to treat them separately.

3.4.2.1 Filamentous growth caused by "Junlon"

The disruption kinetics of two identical batches (disrupted at 10.6 and 8.6 g/L (dcw) for batch 1 and 2) are shown in figs. 3.44 & 3.45. Again, as with the pelleted cells, this representation of the kinetics does not show immediately any differences from the control morphology (fig. 3.25-3.30). They have the similar characteristics of high rate of release for the first two passes, slow rate of release for further passes.

The most striking difference is that glucose oxidase release at 60 MPa 6 pass has reached almost 90 - 99 %, compared to 85 % for large pellets, 80 % for small pellets and 60 % for the control (for the same pressure and pass). Complete release of protein and G6PDH was achieved by 60 MPa, 6 pass.

3.4.2.2 Filamentous growth caused by reduced agitation

As mentioned in 3.1.3.2, the glucose oxidase production was reduced to 20 %. Although filamentous growth was obtained by this method, it was possible this parameter can affect the cell wall strength. Therefore, this method was superseded by the use of "Junlon". Disruption was done at 6.7 g/L (dcw). The results are shown in fig. 3.46.

The kinetics were again similar to those of the control morphology (3.25-3.30). There was irregularity in some of the data in that only one line can be drawn through the 50 and 60 MPa data points, and also for 30 and 40 MPa for protein release.

3.4.2.3 Discussion

Again the form of disruption kinetics for filamentous cells have not changed from the control morphology and is also similar to the pelleted cells. Therefore, despite an apparent change in the morphology the basic process of disruption seems to be similar for all the morphologies, since the basic shape of *A. niger* consists of several cells forming filamentous hyphae, whether these hyphae are "packaged" in a pellet form or dispersed form. Where they differ due to the morphology is the extent of disruption.

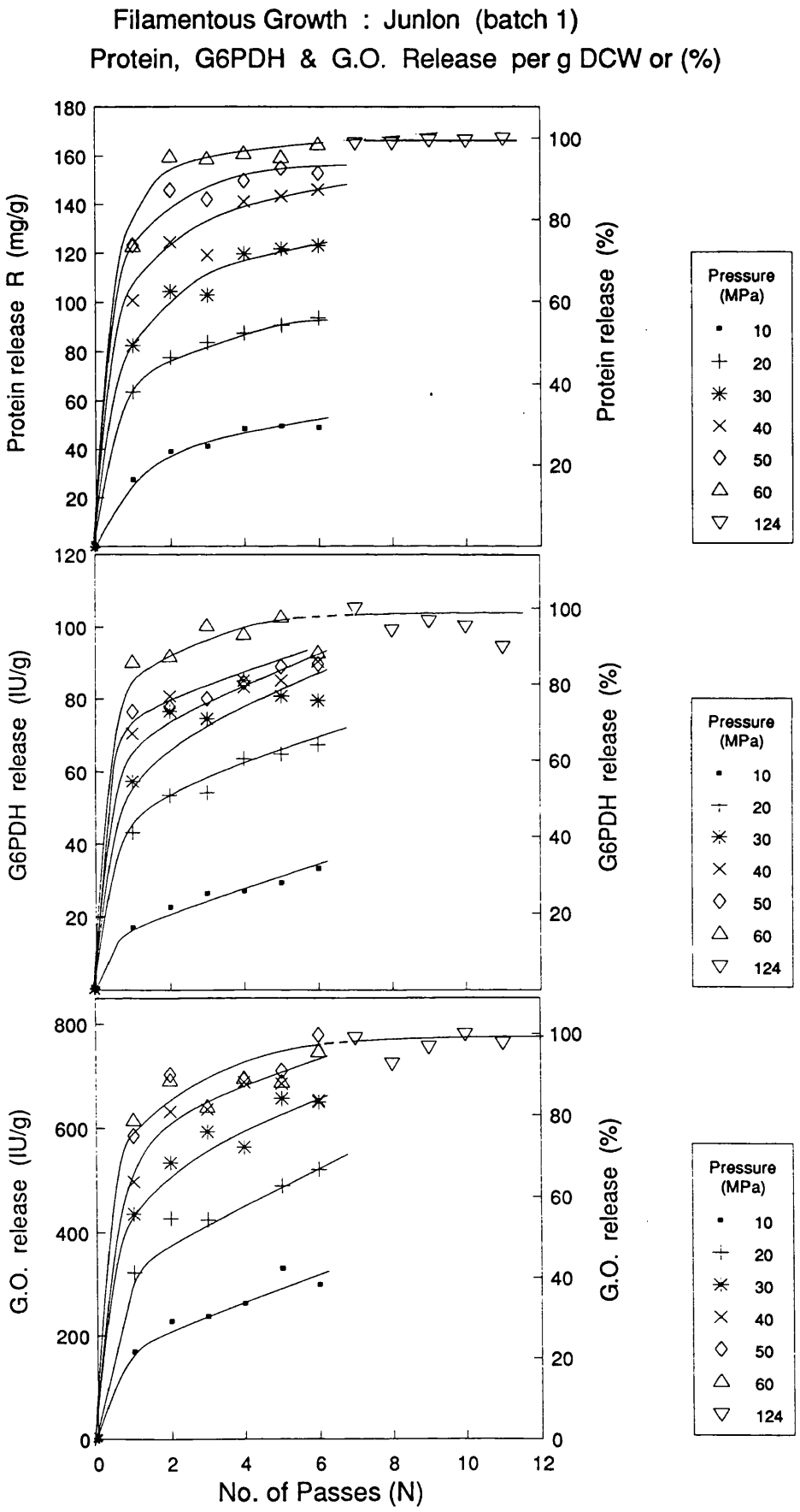


Fig. 3.44 : Protein, G6PDH & Glucose Oxidase release from **filamentous cells (Junlon) batch 1** expressed as absolute and percentage value. Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

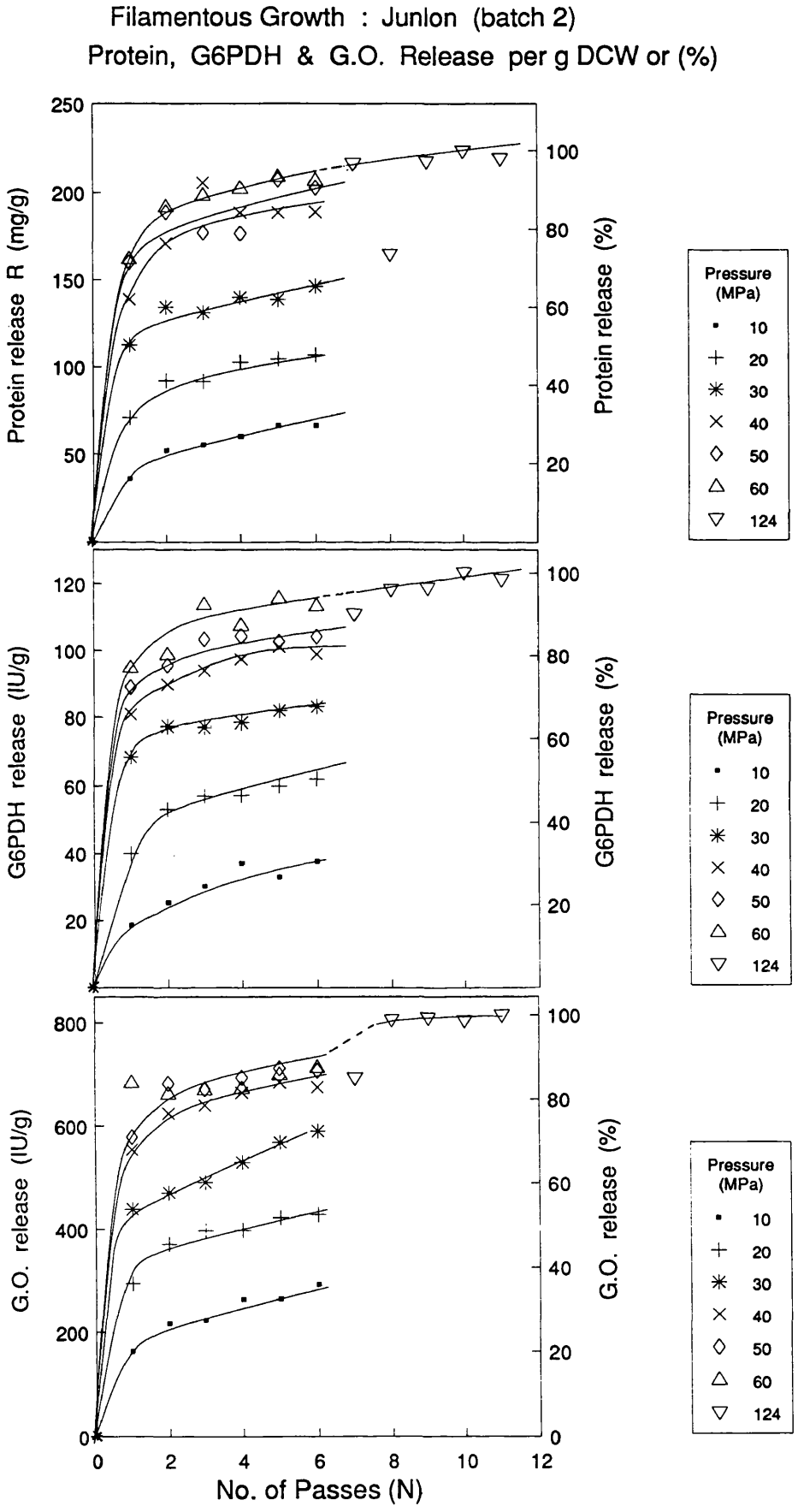


Fig. 3.45 : Protein, G6PDH & Glucose Oxidase release from **filamentous cells (Junlon) batch 2** expressed as absolute and percentage value. Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

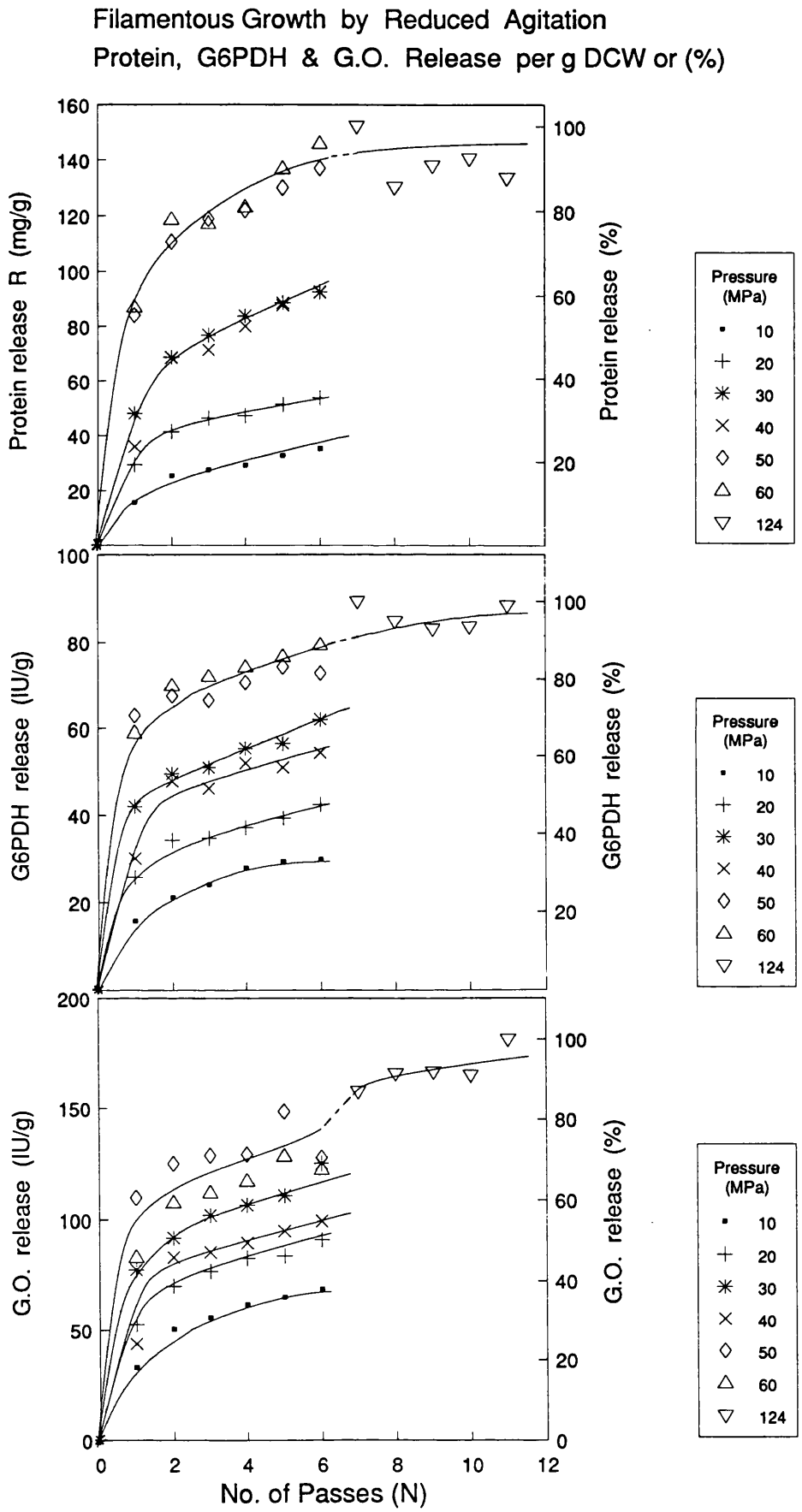


Fig. 3.46 : Protein, G6PDH & Glucose Oxidase release from **filamentous cells (reduced agitation)** expressed as absolute and percentage value. Homogenisers: Lab 60 - below 60 MPa; 30CD - above 60 MPa.

3.4.3 Pelleted and Filamentous Cells vs. Control

The disruption kinetics of pelleted and filamentous cells will be compared with the control in order to determine if morphology affects disruption. It must be emphasised that it would be incorrect to draw any trends from a direct comparison of filamentous and pelleted disruption kinetics because these morphologies were achieved through different means and so may actually affect the kinetics together with the morphology. Therefore they will be compared separately in relation to the control. Since several pressures were tested against each morphology, only one representative figure will be shown for a pressure of 40 MPa. Other pressures also gave similar trends (results not shown here). As it is also evident from photomicrographs of homogenate from the control (fig. 3.33 - 3.38), that the morphology of the cell is completely changed at the first pass, ie. filamentous or pelleted cells are reduced to similar sized and shaped particles, then it is likely that any interaction of morphology with disruption occurs at the first pass. Hence, figures comparing the extent of disruption at the first pass for all the pressures could provide a greater insight into the comparison.

3.4.3.1 Pelleted cells vs. control

Figs. 3.47 show the disruption kinetics of pellet sizes of 0.5, 0.9 and 1.8 mm diameter in comparison with the control, for protein, G6PDH and glucose oxidase at 40 MPa, which clearly indicates that there is an increase in percentage release for all three products with increasing pellet size, but there also seems to be a limiting size of 0.9 mm, above which there is no further increase, as shown by the 1.8 mm pellets (for G6PDH & G.O.). At the sixth pass the release for the control, 0.5 mm and 0.9 & 1.8 mm pellets respectively was : 65, 70-80 and 90-95 % for protein, 60, 70-80, and 95 % for G6PDH, and 45, 60 and 70 % for glucose oxidase. Although there is a small degree of scatter especially for the 0.5 mm pellets (for protein and G6PDH), there is definitely a trend that larger pellets tend to release more product at the same pressure.

Similarly, fig. 3.48 showing the release at the first pass for all pressures show the same trend of increased product release with increased pellet size. The most significant difference occurs at 60 MPa where there is a 30 % increase in protein and G6PDH release, and 35 % increase in glucose oxidase for the 0.9 & 1.8 mm pellets in comparison with the control at 60 MPa. Both the protein and G6PDH kinetics are similar, but the glucose oxidase differs. Again this can be attributed to the peroxisomal location of glucose oxidase, as mentioned in section 3.3.5.

Therefore pelleted morphologies (achieved through a reduction in spore inoculum size) gave increased rates of product release compared with the filamentous, clumped morphology of the control. The possible cause of this may be due to the initial interaction of the pellets with the

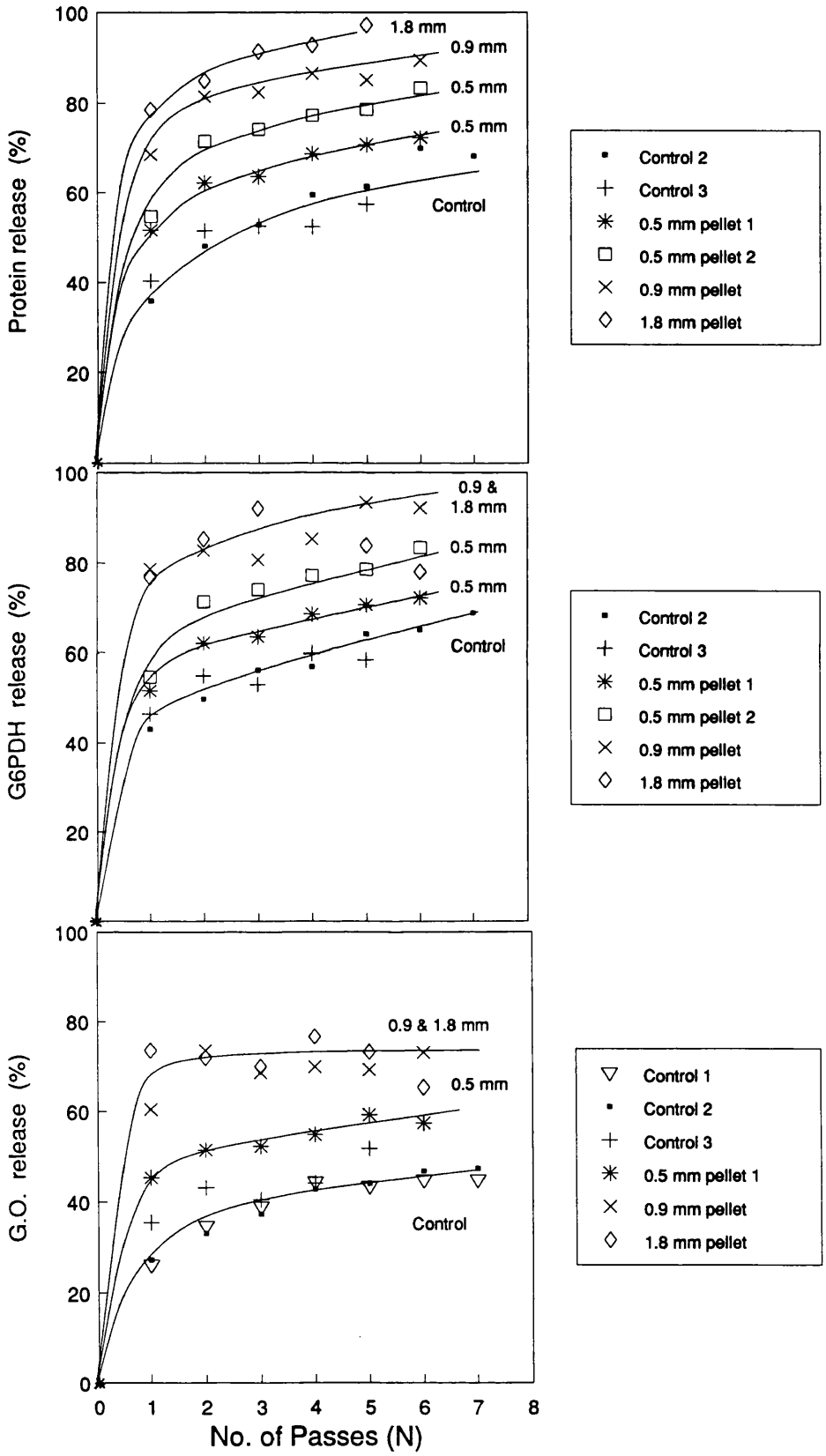


Fig. 3.47 : Protein, G6PDH & Glucose Oxidase release from **all pelleted cells compared to control at 40 MPa**

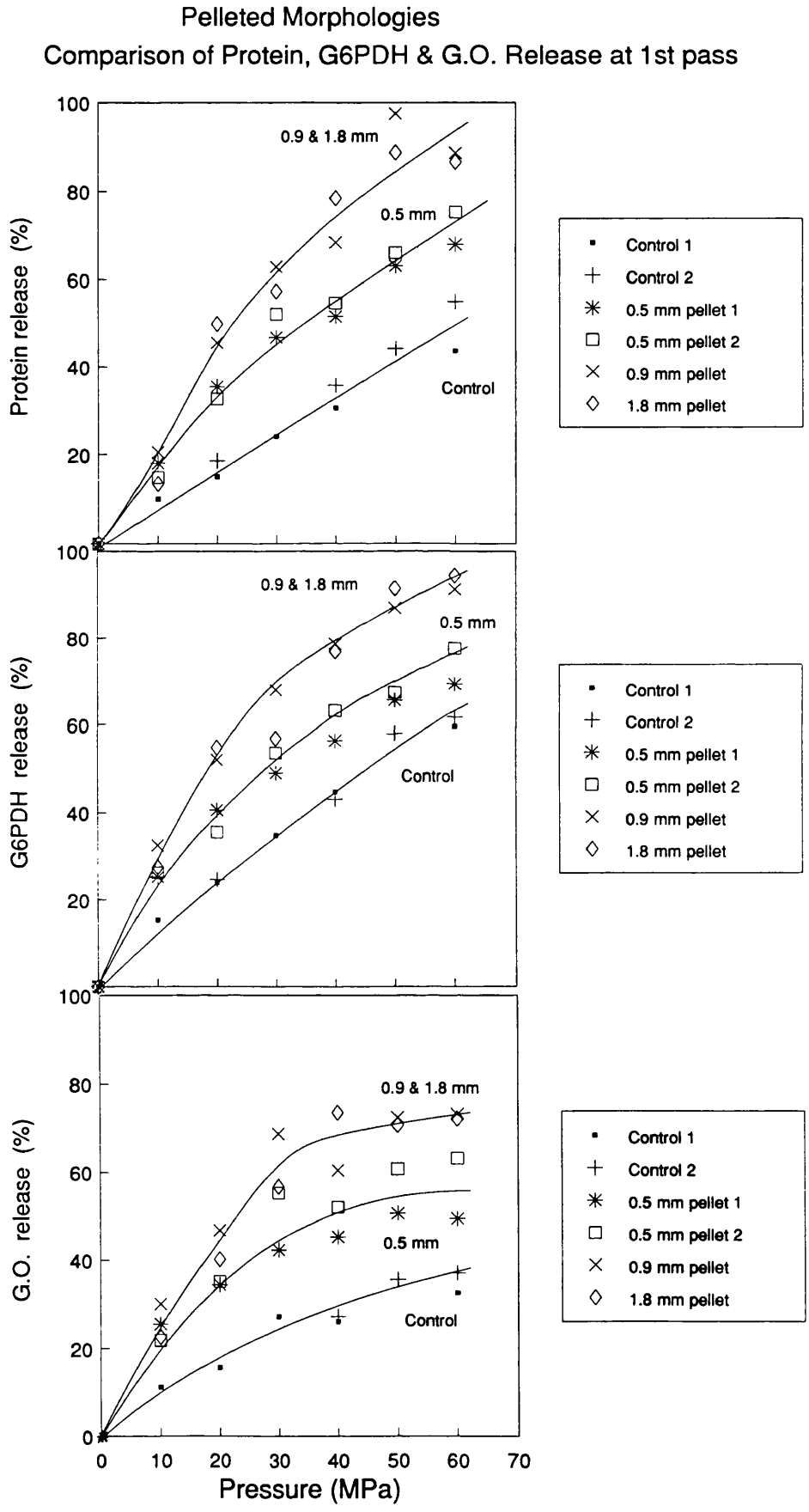


Fig. 3.48 : Protein, G6PDH & Glucose Oxidase release from **all pelleted cells compared to control at 1st pass**

homogeniser during the first pass. As postulated in section 3.3.9, the mycelia is forced through the gap width between the valve seat and valve rod, which decreases as the pressure is raised. This gap width could be hundreds of times smaller than the entangled mycelia and so very high shear forces could be generated as the cells are forced through, which will disentangle and tear apart the hyphae, resulting in disruption. The smaller width at high pressures would result in greater shear forces, which will cause more disruption, instead of mainly disentanglement as observed for a low pressure of 10 MPa. in the case of pellets, these could be visualised as relatively dense, rigid spheres in comparison with a more malleable form of the filamentous structure. Whilst the filamentous form would still undergo severe stress being forced through the gap width, a certain degree of flexibility may be possible because the structure may be able to "squeeze through". However, if a dense, rigid pellet is forced through, the shear stress resulting can be imagined to be much larger than for a malleable filamentous structure, and thus resulting in greater disruption. This postulation, would seem plausible since the larger pellets would undergo greater stress than the small pellets thus causing more disruption, as evident in the results obtained.

The interesting point is that the rates of release for pellets of 1.8 mm were similar to the 0.9 mm pellets ie. no further enhancement of the product release with pellet size. It is uncertain why this is the case. The extent of packing of organisms within a pellet would also contribute to the extent of breakage due to the almost violent disentanglement process during the first pass.

3.4.3.2 Filamentous cells vs. control

With the untangled, unclumped filamentous type growth obtained through the use of Junlon, (fig. 3.49) there is an increased release of product compared to the control : 25, 20 and 40 % increase for protein, G6PDH and glucose oxidase respectively at the sixth pass. This represents a significant increase, especially for glucose oxidase. However, for the more filamentous growth achieved through a reduction in agitation, there is virtually no difference with respect to the control for protein and G6PDH, but glucose oxidase release was increased by about 10 %.

Again product release at the first pass for all the pressures (fig. 3.50), shows that filamentous cells grown by Junlon gave increased product release: at 60 MPa a 15-20 % increase for protein and G6PDH, but a 50 % increase for glucose oxidase. The other filamentous growth by reducing the agitation showed no difference for protein and G6PDH, but an increased release for glucose oxidase (although the data is scattered). The cause for this anomaly is uncertain.

Therefore, the use of Junlon for obtaining filamentous growth has increased the rate of product release, whilst reduced agitation to obtain a similar morphology did not affect the disruption kinetics. This exemplifies the possible impact of a change in growth media on product extraction. In obtaining filamentous growth, the medium or growth conditions have been changed. This introduces another variable to the system. The results obtained must therefore be attributed to the change made; in this case either the use of Junlon or reduced agitation, and not necessarily because it is filamentous. The consequences can therefore be interpreted in three ways:

1. Junlon or reduced agitation has a direct and sole effect on the cell physiology which results in a weaker or unchanged cell wall strength respectively. The filamentous growth does not affect disruption : it is merely a "side effect".
2. Since Junlon is not metabolised, it can be assumed to have no effect on cell physiology and so the filamentous form is the cause of the increased product release. Similarly with the reduced agitation, although it is less likely that agitation will not affect cell wall strength.
3. A combination of the effects of Junlon or agitation and the filamentous structure.

A possibility for explaining the enhancement of product release by the use of Junlon, is that Junlon acts a protective film around the hyphae, since *Trinci (1983)* found that the resin was sometimes difficult to remove from the mycelia by washing (although not observed in this case), and no media particles were attached to the hyphae as mentioned in 3.1.3.1. In forming a film around the hyphae, it may shield the hyphae from the shear effects of agitation and thus form a less strong cell wall, because it is known that high shear rates can cause *A. niger* cells to

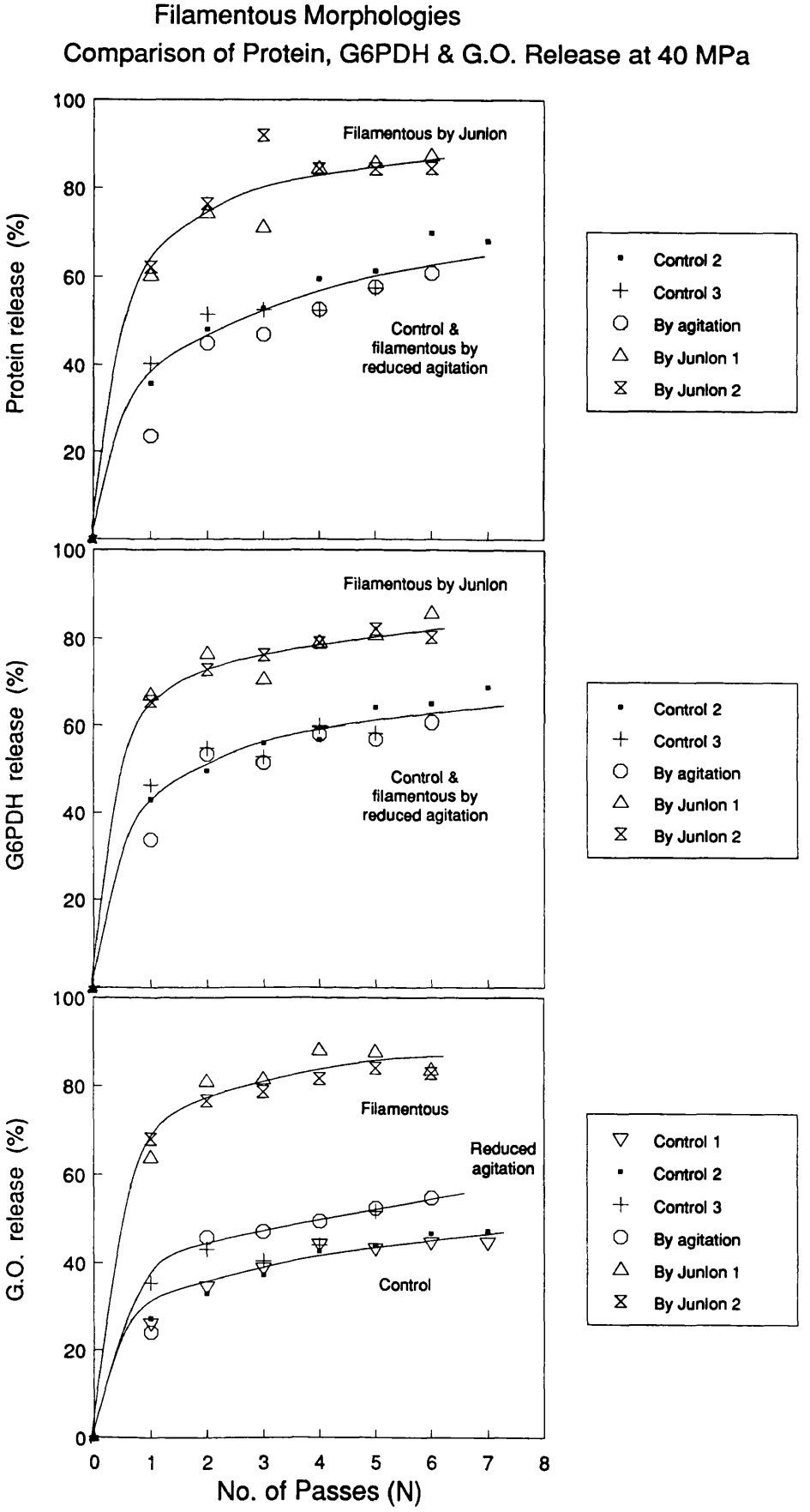


Fig. 3.49 : Protein, G6PDH & Glucose Oxidase release from **all filamentous cells compared to control at 40 MPa**

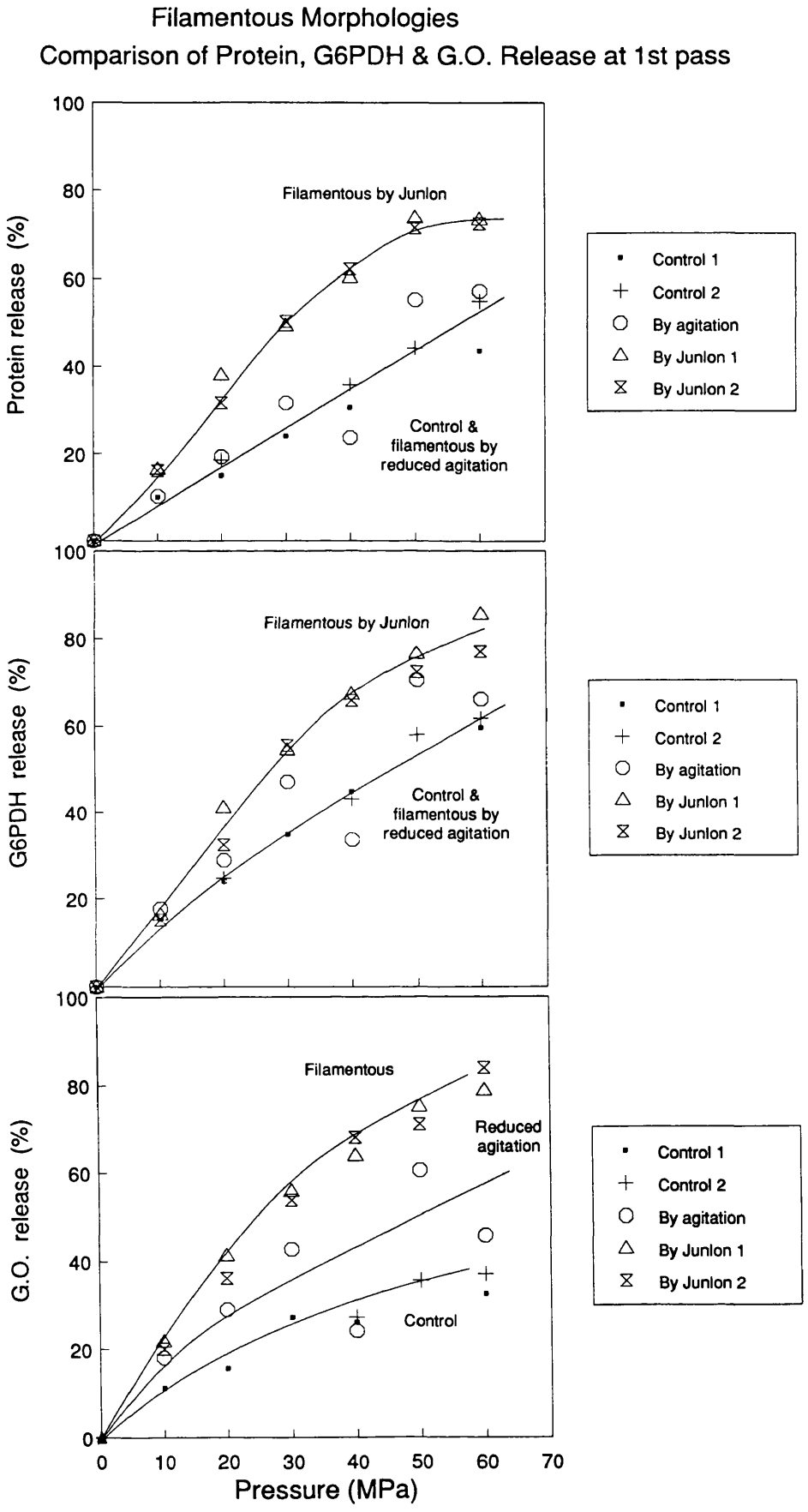


Fig. 3.50 : Protein, G6PDH & Glucose Oxidase release from all filamentous cells compared to control at 1st pass

assume a more resistant form *(Mitard et al 1988)*. With this explanation, the filamentous growth may not be contributory to the disruption kinetics.

In the case where agitation was reduced to achieve the filamentous morphology, the rate of release of protein and G6PDH did not seem to change from the control. This may be because the filamentous morphology although less clumped and entangled than the control, is not vastly different, so as to affect the disruption kinetics.

3.5 Disruption Studies Using a Different Cell Resuspension Buffer : Acetate buffer pH 5.2

The use of this buffer as a resuspension media was recommended by the industrial source of this fungal strain, which at this pH serves to optimise the activity of the glucose oxidase. As will be evident, this buffer lead to apparently very different disruption kinetics between the glucose oxidase and protein release in that the rate of release of protein was much faster than for glucose oxidase. The protein content was unusually low at 2 % of dry cell weight, which is too low compared to 15-20 % obtained from using the Tris buffer. Therefore it is reasonable to deduce that the acetate buffer at pH 5.2 was precipitating up to 90 % of the protein which was then removed with the cell debris and undetected. However, the glucose oxidase activity and thus amount was unchanged with this buffer. The significance of this is that unwanted proteins have been almost completely removed by this very simple step. This means a recovery step yielding a very high purification factor and thus simplifying further purification steps.

The fermentations here were exactly the same as for the control type; the only difference is the resuspension buffer.

3.5.1 High Pressure Homogenisation

Two identical batches of control type cells were disrupted at 9.7 and 9.2 g/L (dcw) for batch 1 and 2 respectively. From figs. 3.51 & 3.52 the homogenisation in acetate buffer at pH 5.2 gives very similar disruption kinetics for the protein release in comparison with the use of Tris buffer (fig. 3.22-3.24):

1) Rapid rate of release of protein at the first two passes and then a considerably slower rate of release for subsequent passes.
2) Complete or 100 % release of protein is achieved quickly at 80 MPa after 2 passes but can also be attained at 60 MPa after 6 or 7 passes.

However, on examining the actual value of protein released, for both batches only 28-35 mg protein per g dry cell weight compared to 130-190 mg/g for Tris buffer, which represents only 15-27 %. This discrepancy was due to the buffer used, as will be shown below.

The kinetics were substantially different for glucose oxidase release (fig. 3.51 & 3.52) which shows that there is negligible or no release of glucose oxidase at pressures from 10-40 MPa and even at 50 MPa there is only a minimal amount. Only at 60 MPa there is a significant release, but the activity is still increasing after 6 or 7 passes. Moving onto the higher pressures of 80 and 100 MPa, there is a considerable difference in the amount released, reaching a maximum activity of about 850 IU/g dcw, which is comparable to 940 IU/g dcw obtained with the Tris buffer. The

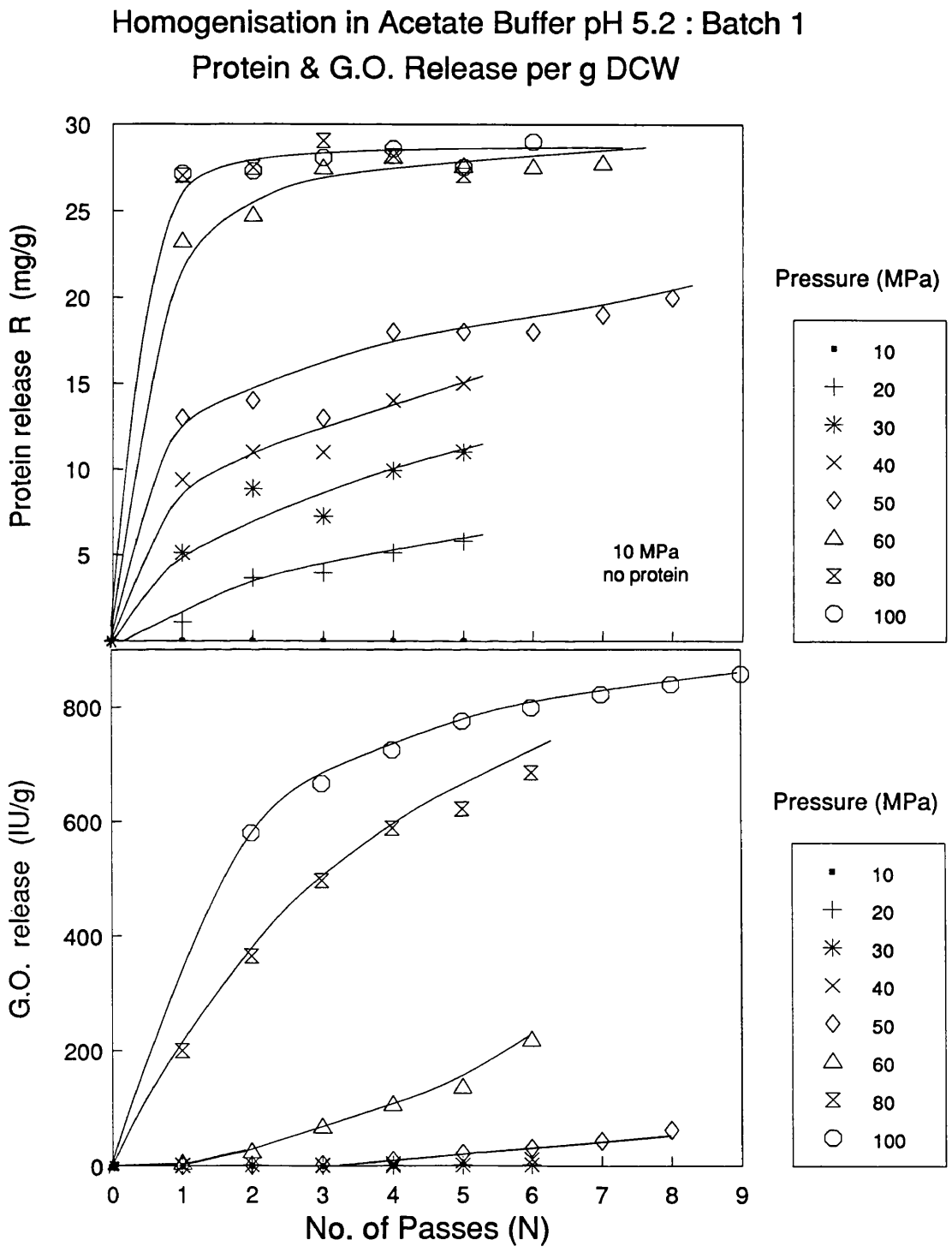


Fig. 3.51 : Protein & Glucose Oxidase release from control type cells in acetate buffer pH 5.2 - **batch 1**
Homogenisers : Lab 60 - below 60 MPa; 30CD - above 60 MPa

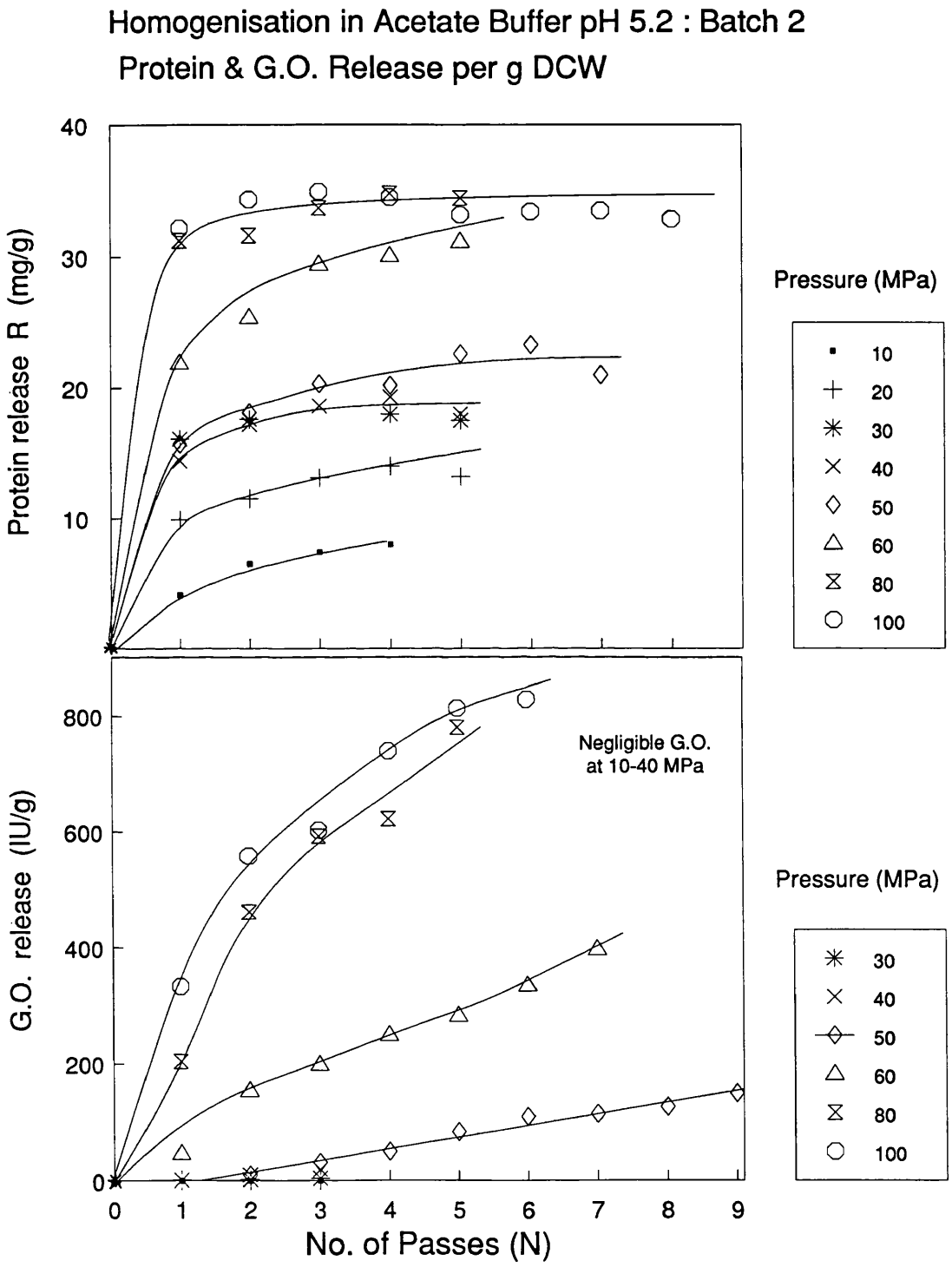


Fig. 3.52 : Protein & Glucose Oxidase release from control type cells in acetate buffer pH 5.2 - **batch 2**
Homogenisers : Lab 60 - below 60 MPa; 30CD - above 60 MPa

kinetics differed with the protein release (and so also with the Tris buffer work):
The release is almost linear for glucose oxidase; there is no sudden and rapid release at the first two passes. No enzyme was released between 10-40 MPa. The only similarity with the Tris buffer results is that the pressure required for complete release is about 100 MPa, but it takes several passes (9) before there is no further increase in activity. This is in contrast to the Tris buffer where almost all the enzyme was released after 2 passes at 100 MPa. Therefore, although the removal of 90 % of the protein by the use of this buffer enhances the purification, the need for several passes to extract the enzyme is necessary, but it is likely that benefits of easier purification will outweigh the minor disadvantage in homogenisation.

The absence of enzyme release at the lower pressures and the need for several passes to achieve high release suggests that the acetate buffer may have made the release of glucose oxidase more difficult. However since there was no change to the protein release trends compared to the Tris buffer, it could mean that the cell structure or strength may be unchanged, but somehow, the peroxisomes containing the glucose oxidase have been affected in a way that made release more difficult. No plausible cause can be suggested at present, and further work will need to be done.

A comparison of the differential rates of release of the protein and glucose oxidase is shown in fig. 3.53. Clearly, there is a considerable lag in the release of glucose oxidase with respect to the protein, since the curve is well below the "equal rate of release line". In contrast with the Tris buffer, the release of glucose oxidase is far slower than protein. There would appear to be a threshold pressure of 50 MPa before there is any significant release of the enzyme.

Fig. 3.54 shows an experiment to determine if it is the buffer which is affecting the product release. The disruption was done at 40 MPa using cells from the same batch but resuspended in different buffers. From this, protein release with the acetate buffer at pH 5.2 was only 24 % of the one with Tris buffer at pH 7.5; G6PDH was undetected and minimal glucose oxidase released using the acetate buffer. This very substantial "loss" of protein can be attributed to the precipitation of protein by the buffer which due to its low pH of 5.2 compared to Tris at 7.5 will cause denaturation of some proteins followed by precipitation and separation together with the cell debris, when the samples are prepared for soluble protein analysis.

It may be argued that because of the different pH buffers, the Bio-Rad protein assay *(Bio-Rad Laboratories 1990)* which is based on the Bradford method *(Bradford 1976)* will bind differently, because the positive charges onto which the dye binds will change with pH. This is not the case because by using the Folin-Lowry method *(Lowry et al 1951)* which binds on specific amino acid

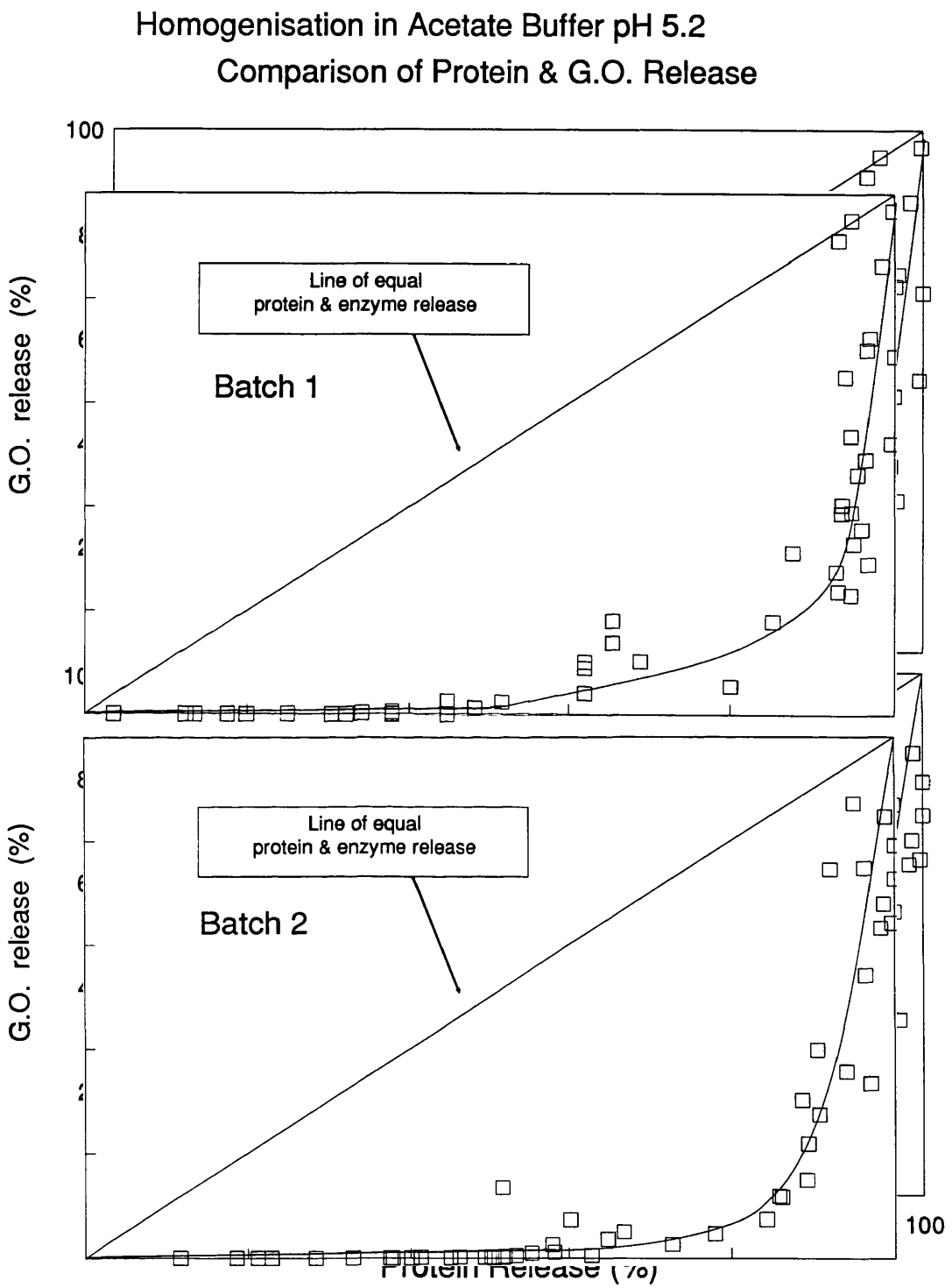


Fig. 3.53 : Comparison of protein and glucose oxidase release from control type cells in acetate buffer pH 5.2 - batch 1 & 2.

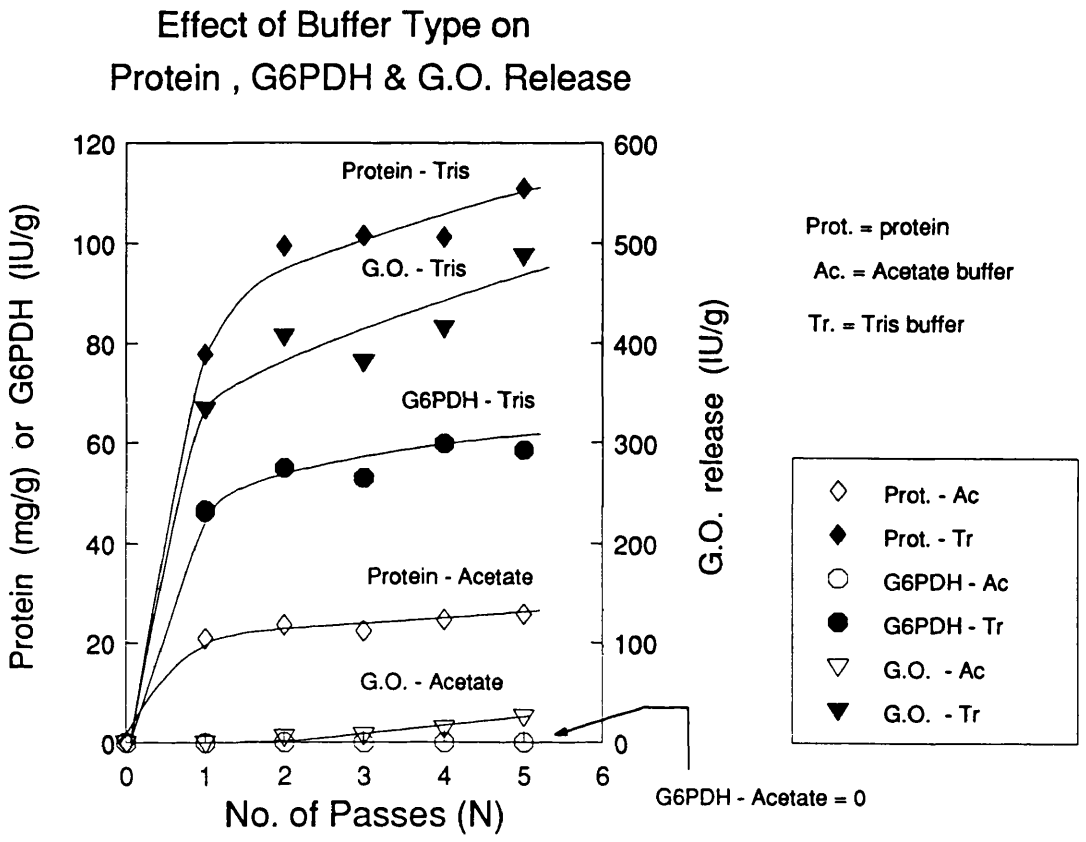


Fig. 3.54 : Protein, G6PDH & Glucose Oxidase release at 40 MPa from control type cells resuspended in Tris HCl or acetate buffer

groups, it only detected 1.7 times more protein, which is still low compared to the protein levels assayed when using the Tris buffer during release. Different protein assays will give varied results *(Jernejc et al 1986)*. The Folin-Lowry method detected more protein, but the Bradford method was used as only relative values were needed and this was a fast and simple technique.

In addition, it is worthwhile noting that the acetate buffer also gave a much clearer "transparent" supernatant with a densely packed cell debris pellet when the homogenate was centrifuged at 16000 g for 5 minutes, whereas the Tris buffer gave a less clear supernatant and the cell debris is not so densely packed so that any turbulence may re-mix the debris with the supernatant. Further observation also showed that the homogenate in Tris buffer was far more susceptible to microbial spoilage than the acetate buffered homogenate (even at 4°C), as evident from the odour of decomposition. The latter would keep clear and glucose oxidase activity retained for about six months, whereas the former could only keep for one or two weeks. This could be due to the lack of protein in the acetate buffered homogenate as well as the pH value.

3.5.2 Bead Mill

The bead mill was used as an alternative method of disruption, since homogenisation would require a very high pressure of 100 MPa to completely release all the enzyme. This was not intended to be an extensive study of the use of a bead mill for *A. niger* disruption. Bead milling was done using 0.4-0.5 mm ballotini beads and polyurethane impellers in a 0.6 L bead mill (Dyno-mill) operating in a batch mode. Two identical batches of control type cells were disrupted, the first being disrupted for up 2 minutes because this batch did not give full release of glucose oxidase and the second up to 5 minutes. The disruption concentrations were 9.7 and 9.2 g/L (dcw) for batch 1 and 2 respectively.

Batch 1 : Fig. 3.55 shows that the protein levels decrease with increasing disruption time and tip speed. Protein level decreases by 20 % for a tip speed of 10 m/s and 33 % for 15 m/s from the disruption time of 0.5 to 2.5 minutes. The protein levels at 15 m/s were 30 % less than at 10 m/s. This "loss" of protein can be caused by shear associated damage of proteins at air-interfaces present during the agitation due to difficulty filling the mill completely and extraction of samples leading to entrainment of air as the volume is decreased. Such a cause of protein dentauration was suggested by *Narendranathan and Dunnill (1982)*. The higher tip speed obviously causes more intensive agitation and higher turbulence resulting in more protein damage. One sample was extracted from the 20 m/s run before it failed, possibly due to too much cooling on the rotor causing freezing of condensate and straining the motor which cut off the power. Nevertheless, the only sample gave a protein value close to the 15 m/s sample.

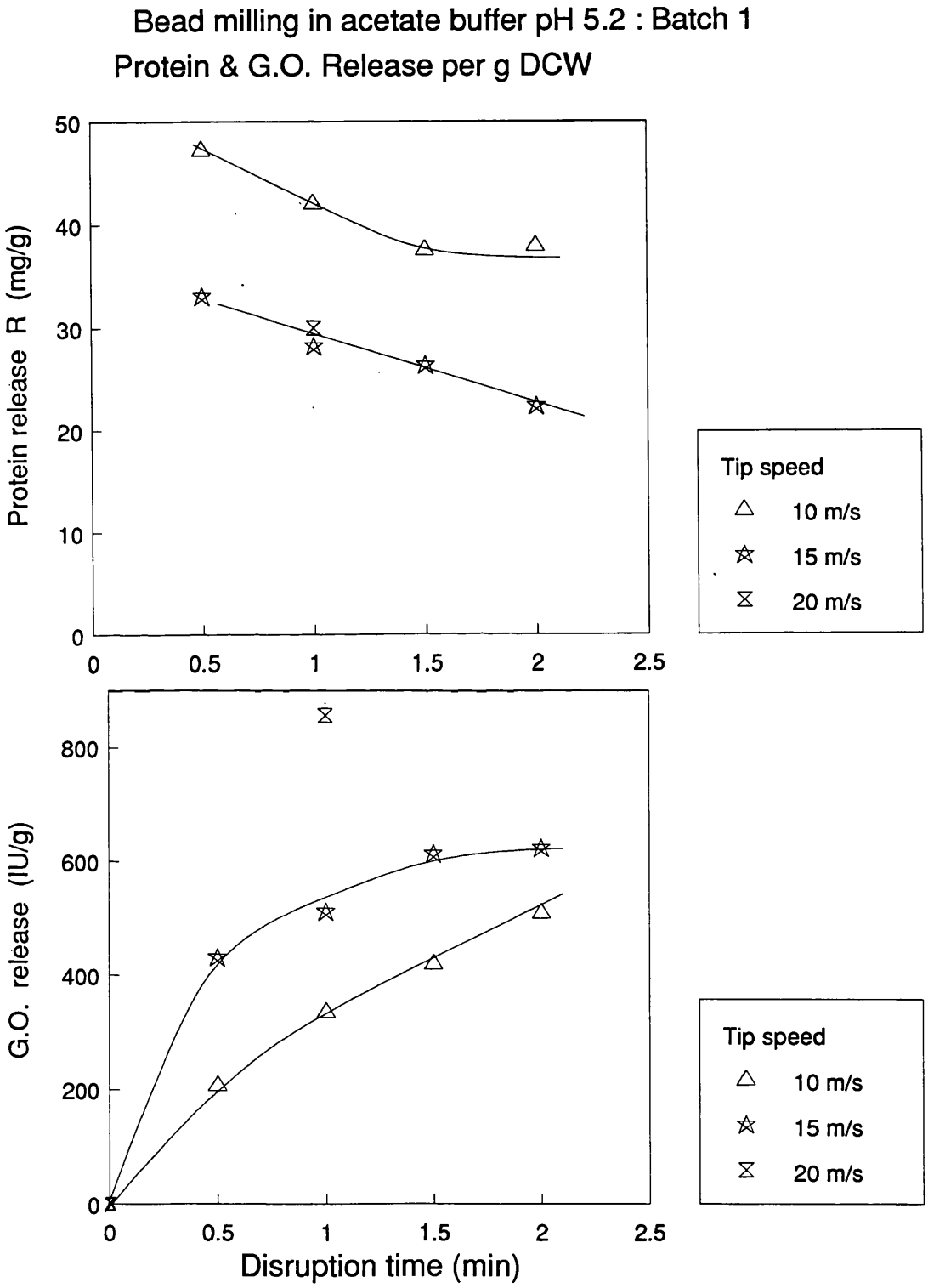


Fig. 3.55 : Protein & Glucose Oxidase release from control type cells in acetate buffer using a bead mill - **batch 1**

With glucose oxidase, (fig. 3.55) no denaturation was experienced. In fact the activity increased with increasing disruption time and tip speed. The one sample at 20 m/s gave the highest activity of 856 IU/g dcw which was comparable to the maximum from homogenisation studies. As it is still unclear as whether full release has been achieved it was necessary to repeat the run with extended disruption times.

Batch 2 : Fig. 3.56 shows that the protein release decreased with increasing disruption time but the kinetics was the same for all tip speeds. The protein level decreased by 50 % from 0.5 to 3 minutes and stayed constant after 3 minutes. This batch had less than 50 % protein of the first batch after 0.5 min at 10 m/s. This discrepancy could be due to uneven loading of the disruption chamber leading to varying amounts of cell suspension relative to beads since the filling was done by pumping the cells into the already bead filled vessel, mixing it slowly by rotating the impeller by hand and once the cell suspension starts to exude out from the outlet, it was considered full.

The release of glucose oxidase (fig. 3.56) was successful in that the maximum release was achieved in 3 minutes at 20 m/s and confirmed by the unchanging activity up to 5 minutes. It would also seem that the 15 m/s speed would have caused maximum release because the last sample at 1.5 minutes was as high. These results did not reproduce the ones from batch one especially for the 15 m/s run which gave 70 % release after 2 mins in batch 1 but 100 % release in batch 2.

Discussion: The objective to determine the maximum release of glucose oxidase and confirm if homogenisation in acetate buffer achieved complete release was successful, as both values are comparable: about 900 IU/g dcw for bead milling and 850 IU/g dcw for homogenisation. This is also comparable to the known level of 1000 IU/g dcw from sonication of the fermentation sample (fig. 3.2). Since only two trials using the bead mill were made, it would not be expected to give reproducible results, without some optimisation for the system. In particular the loading of the disruption chamber was probably not consistent, and would result in differences in performance *(Limon-Lason et al 1979)*. It would probably be better if continuous operation is used. In comparison with the disruption of yeast which released 90 % of protein after 1 minute disruption at 10 m/s *(Limon-Lason et al 1979)*, protein release would seem to be complete at 10 m/s in 0.5 minute (although this is obscured by the decreasing protein content), but glucose oxidase release requires 3 minutes at 15 m/s. Therefore, cell rupture (based on protein release) *A. niger* appears to be easier than yeast. Glucose oxidase must be treated differently because of its location in the cell, and so its complete release does not correlate with cell wall rupture.

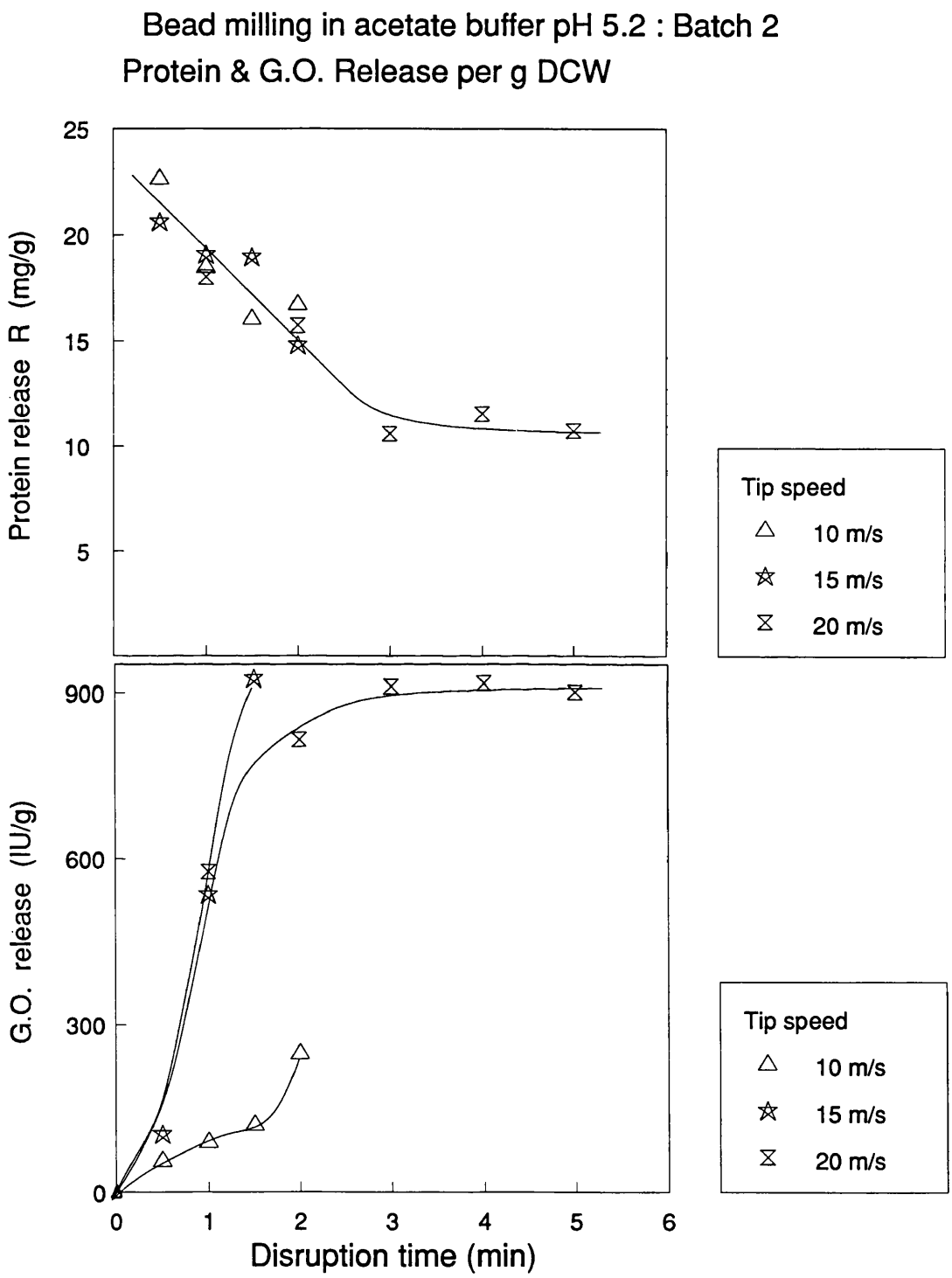


Fig. 3.56 : Protein & Glucose Oxidase release from control type cells in acetate buffer using a bead mill - **batch 2**

Comments on operation : The bead mill is inconvenient to use with regards to the beads. Attrition of beads resulting in fine particles contaminating the homogenate will require an additional separation step. Besides this, a very important factor is the cooling capability. In the batch mode of operation, the temperature of suspension was cooled to 5°C by ethylene glycol through the chamber jacket before operating, but over the period of one minute the temperature would rise to about 25°C and so it was necessary to re-cool the suspension before continuing. By operating under a continuous mode this problem can be overcome as well as the aforementioned problem of air entrainment and consequent loss of protein. However, the bead mill is better contained than the high pressure homogeniser, as it is unpressurised.

3.5.3 Effect of Cell Concentration

Two identical batches of control type cells were homogenised at 60 MPa from a cell concentration of 7 to 17 g/L to determine any effect of this variable on the disruption kinetics of cells resuspended in acetate buffer.

These kinetics are shown in figs. 3.57, where the release of glucose oxidase is shown as a specific release per g protein, instead of release per g dcw, because of the possibility of some uncertainties in the cell concentration determination. The protein release per g dcw showed a slight dependence on cell concentration whereas glucose oxidase per g dcw was highly dependent on cell concentration (results not shown here). Fig. 3.57 comprises of data from the two batches : the ones at 7.3, 11.4, and 17.3 g/L are from one batch and 8.3, 13.1 and 15.9 g/L from the other. There is clearly a strong dependence on the release of glucose oxidase as the cell concentration increases, and this dependence would appear to weaken between 11 and 17 g/L.

This study on the effect of cell concentration has only been performed on cells resuspended in acetate buffer. Therefore, this cannot necessarily be applied to the cells resuspended in Tris buffer.

It is intriguing that there is a strong dependence of glucose oxidase release on cell concentration but not so for protein release. As shown in 3.5.1, glucose oxidase release was released much slower than protein, thereby indicating that enzyme release was more difficult. The disruption curves for shown in fig. 3.57, resemble the glucose oxidase ones in fig. 3.51 & 3.52 in the general shape. Strictly speaking these should not be compared directly as the units are different, but since the protein release is almost constant after the first pass, the trend for specific G.O. release tends to be similar to G.O. release per g dcw. The curve for the highest concentration (17.3 g/L) is similar to the one for highest pressure (100 MPa) and the one for lowest

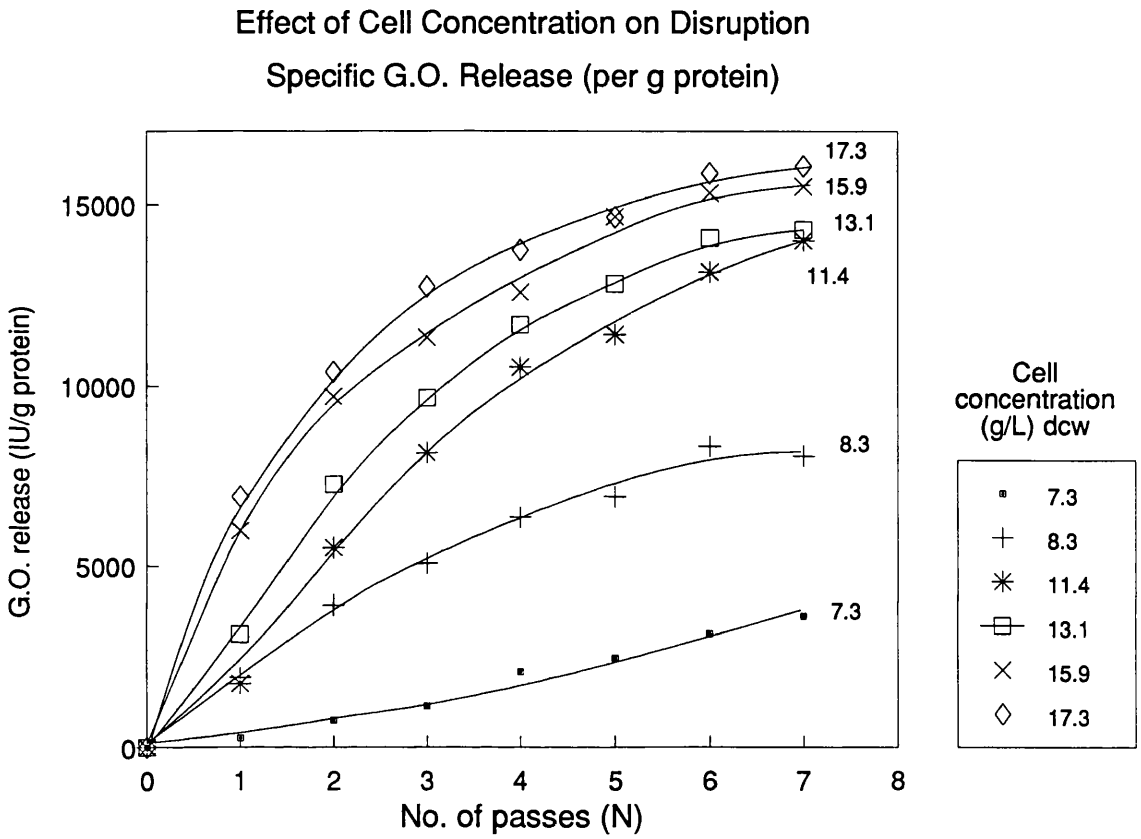


Fig. 3.57 : Specific glucose oxidase release (per g protein) from control type cells at various cell concentrations in aectate buffer at 60 MPa.

concentration (7.3 g/L) similar to the one for 60 MPa. As the higher pressure involves a greater energy input into the homogeniser resulting in greater release, a similar analogy may be applied to the higher concentration. With a higher concentration, the pump will need to exert a greater force to maintain the same pressure and flowrate with a more viscous suspension. By putting in more energy, the disentanglement process may be more violent. Consequently, disruption of the cells is also much greater. This effect was not observed with protein release, because at this pressure most of it will be released anyway, and therefore any increases cannot be significant. However, glucose oxidase is not fully released at 60 MPa, so that if there is an increase in the rate of disruption, it will be manifested in an increased enzyme release. Similarly, as seen here the dependence on concentration decreases at the higher concentrations because the amount released is approaching the maximum and not much more enzyme can be released.

This postulation ties in with the one suggested for pelleted cells where the rate of release is greater than for the filamentous control morphology. It is probably more difficult to force a rigid pellet through the gap width than the more flexible filamentous form, and therefore more energy is expended to force the pellet. If this is true, then even for the cells resuspended in Tris buffer, a similar effect of concentration may be expected. However, it may be less pronounced, because these cells seem to release the glucose oxidase more easily than the ones in acetate buffer. Fortunately, all the work done in comparing control morphology with pelleted and filamentous ones have been at similar concentrations of about 10 g/L. Clearly further work will need to be done to assess this variable with different presssures and buffers.

3.5.4 Effect of Scale Up - 1000 litre Fermentation.

The control fermentation was scaled up to a 1000 litre fermentation as described in section 3.1.4., and was a successful attempt. Some of these cells were homogenised in acetate buffer at 12.5 g/L (dcw) to determine any difference in kinetics. However, it must be noted that these cells had been in 5°C storage as a dewatered but moist paste for 7 days before disruption.

The protein and glucose oxidase release kinetics are shown in fig. 3.58. In comparison with the homogenisation of the 42 L scale cells in acetate buffer (section 3.5.1), the general trend remains the same : 1) for protein release, there is a rapid release at the first two passes and then little changes for the subsequent passes. Maximum protein release also seems to be achieved by 60 MPa after 6 passes, although this needs confirmation with higher operation pressures.

2) glucose oxidase shows a more linear increase in its release and does not achieve 100 % release at 60 MPa. There is a small difference in that for the low pressures between 10-40 MPa, a significant release is experienced, in contrast with none being detected for the 42 L scale one. This could be due to the storage period which may result in some breakdown or weakening of cell walls and membranes, resulting in greater disruption.

Large scale grown cells therefore do not differ significantly in its disruption kinetics from small/pilot scale grown cells disrupted in acetate buffer (fig. 3.51 & 3.52). It would not be expected to differ very much because all the conditions were maintained as for the 42 L scale, including the tip speed of the impeller during fermentation. Consequently, the morphology and cell wall strength in the 1000 L cells were unchanged and so disruption kinetics would also not be expected to vary.

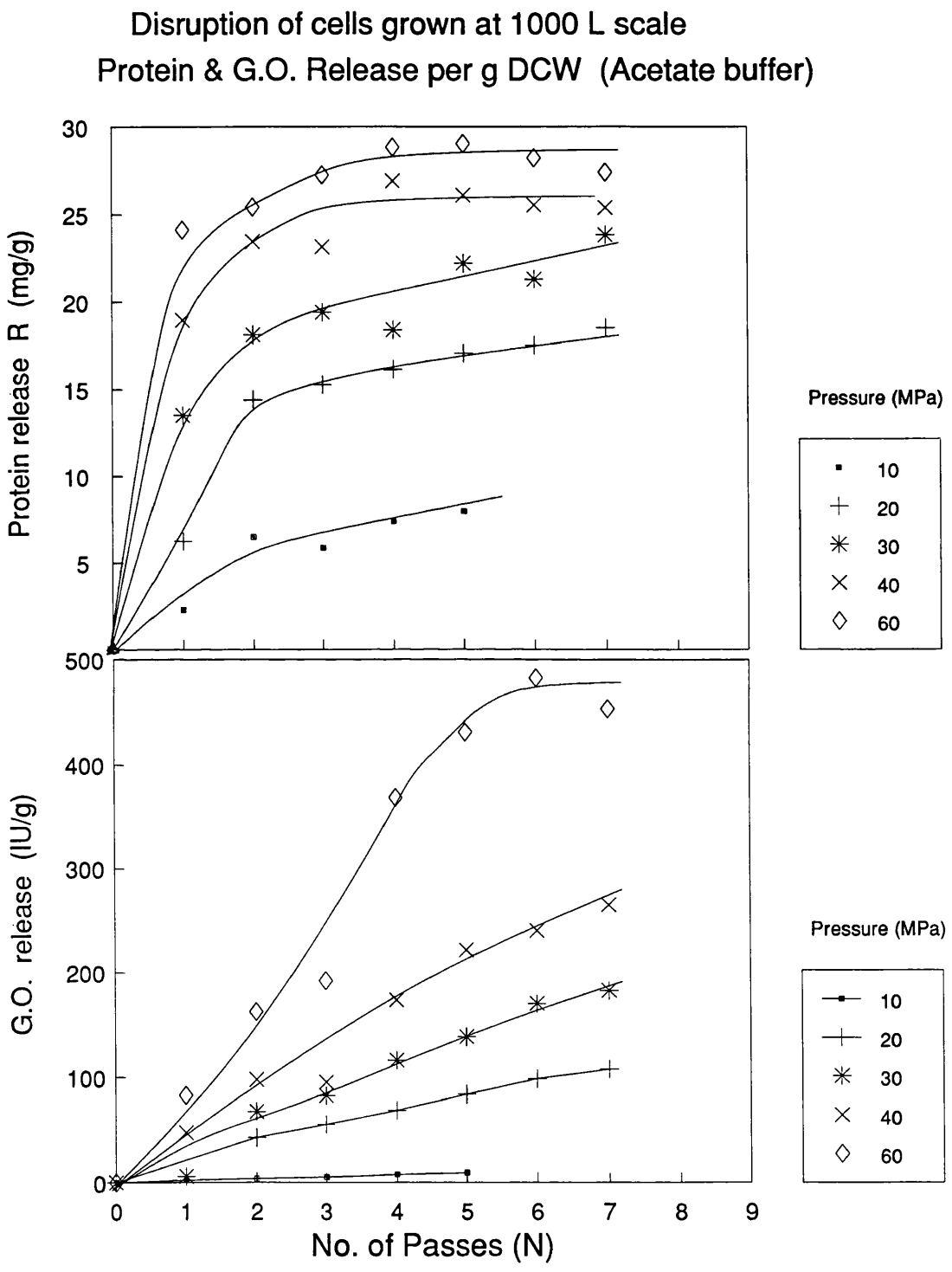


Fig. 3.58 : Protein & Glucose Oxidase release from cells grown at 1000 L scale. (resuspended in acetate buffer)

3.6 Disruption of a Recombinant Strain of *Aspergillus nidulans*

The recombinant strain of *Aspergillus nidulans* producing high levels of intracellular alcohol dehydrogenase (ADH) as described in 2.2 was disrupted in a 0.15 L bead mill. The effects of tip speed and cell concentration were investigated. To examine if there is a period after induction where the cells are more susceptible to disruption, the effect of post-induction time was also studied. Due to the short time available for the study of this strain, the results obtained are not as detailed as found in the *A. niger* strain. The results can be found in Appendix 2. Nevertheless, a few points can be drawn from this work. These will be discussed briefly here.

1. In most cases, complete release of protein or ADH was achieved within the first minute, if not in the first 30 seconds of disruption.

2. After the maximum release was reached, the protein content and ADH activity would decline rapidly. This can be attributed to shear associated denaturation of proteins, when air is entrained into the disruption chamber, and together with the high velocity gradients, results in oxidation of proteins at the liquid-air interface. The entrainment of air resulted from the suspension volume decreasing as samples were removed. *Narendranathan and Dunnill (1982)* suggested that this may be the cause of protein loss in high shear fields experienced in ultrafiltration processing. Therefore, in batch disruption it is necessary either to minimise the sampling or to replace the sample volume with buffer.

3. There was no significant effect of tip speed, cell concentration or post-induction time on the disruption kinetics: complete release was always achieved within one minute of disruption.

Since disruption was completed within one minute of disruption regardless of the tip speed, it would appear that these are very easy cells to break. In comparison to the *A. niger* strain (3.5.2), which also required only half a minute to fully release the protein, the recombinant *A. nidulans* strain does not seem to differ in its susceptibility to disruption. However, in comparison to yeast cells, where protein release showed some dependence on tip speed, *A. nidulans* cells are easier to break, because there is no dependence on tip speed. At 10 m/s after 1 minute, 90 % protein is released *(Limon-Lason 1979)*.

3.7 Overall Discussion

This will discuss the general strategy in operating a high pressure homogeniser to disrupt filamentous microorganisms, and whether this strain of *A. niger* has been successful as a model system; the interactions between fermentation conditions and disruption; and briefly, the impact of the acetate buffer on further downstream processing. Lastly, the implications of this work on process development will be mentioned. The interactions of fermentation and downstream processing in these studies is illustrated in fig. 3.59.

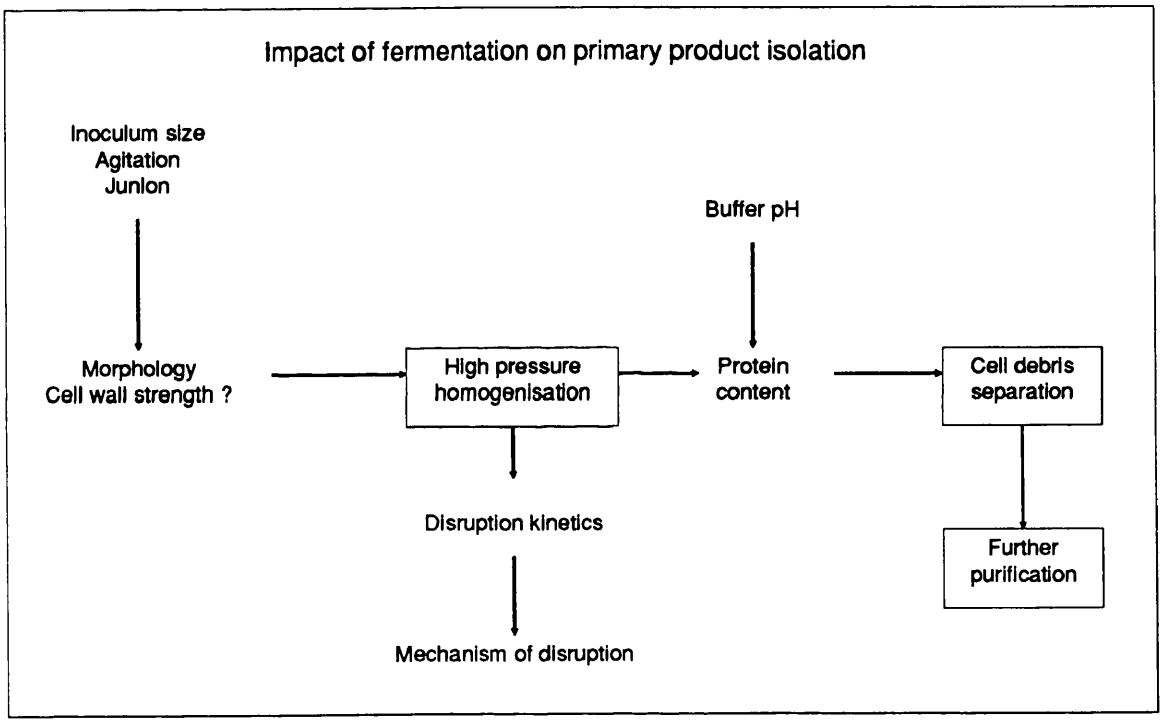


Fig. 3.59 : The interaction between the process variations made and its effects.

3.7.1 High pressure homogenisation.

3.7.1.1 General observations

From all the above studies on the disruption kinetics of *A. niger* using the high pressure homogeniser, a common characteristic exists : the release of any product (except for glucose oxidase from cells in acetate buffer) is highly dependent on pressure and is largely determined by th operation of the first pass. This is demonstrated in the rapid release of product during the first pass and a much slower rate of release for subsequent passes.

The general strategy for optimising the use of the homogeniser is to operate at the minimum pressure which releases almost 100 % of the product after one or two passes. This minimum pressure varies slightly with morphology and fermentation conditions. In the case of the control morphology (filamentous, entangled), the minimum pressure for the release of G6PDH and protein in one or two passes is 80 MPa, and for glucose oxidase is 100 MPa. Since there was an increase in product release for pelleted and filamentous cells, the pressures to achieve 100 % release in two passes changed for the large pellets (0.9 and 1.8 mm) and the filamentous growth by Junlon to 60 MPa for protein or G6PDH. However, with glucose oxidase, this pressure has not changed : 100 MPa is still required, but it is possible to achieve about 90 % release after 6 passes at 60 MPa with the large pellets or filamentous cells grown using Junlon.

The homogenisation should be operated for the minimum number of passes to avoid further disintegrating the cell debris, minimise exposure of possibly delicate proteins to high shear and temperature, and clearly to minimise processing time and power. Temperature rise during homogenisation has not affected the activity of the two enzymes monitored, but in other cases, it may be expected that temperatures of about 35°C will denature some proteins. Since the residence time through the valve assembly may be less than a second, the exposure time to high temperatures will be minimal. With the homogeniser and bead mill, this potential problem must be assessed for the particular system.

The glucose oxidase system here has served as a good example of the impact of intracellular product location on the operation of the homogeniser. Due to its location in microbodies called peroxisomes, and thus presenting another barrier to disruption, the pressure required for its complete release was raised to 100 MPa from 80 MPa which is necessary to release freely soluble cytoplasmic enzymes such as G6PDH. This implies that even if the cell can be broken easily, the product, depending on its location may not necessarily be released easily.

Protein release as a marker for cell disruption was considered not so appropriate because of other materials causing a positive result with the protein assay, such as from micronisation of cell debris (sect. 3.3). There seems to be more evidence that this is the case with this glucose oxidase system. For the control morphology, the release of G6PDH was apparently faster than protein although both are in the cytoplasm as "free" substances, unlike glucose oxidase. This was attributed to the fact that since glucose oxidase is also a protein, it was not possible to achieve 100 % protein release without 100 % G.O. release. So when a significant proportion of G.O. is unreleased, as at 60 MPa after 6 passes, when 40 % of G.O. is unreleased, then it is a significant enough fraction of total protein, that only 77 % protein is released, compared to 92 % G6PDH.

However at 80 MPa, when only 10 % G.O. is unreleased, then almost 100 % protein and G6PDH was released. Therefore, as glucose oxidase is released more slowly than G6PDH, it also tends to lower the overall protein release. Further evidence to support this is shown in the different morphology cells. Since, the pelleted and filamentous cells (by Junlon) were found to be easier to disrupt, and thus G.O. release in comparison with protein and G6PDH will be less slow than for the control morphology, then the protein release will represent the release of "free" cytoplasmic proteins more closely. In addition, the total glucose oxidase in the pelleted cells have decreased, and so will be a smaller fraction of total protein. This means that protein release can then be a suitable marker. Therefore, the use of protein to monitor release of cytoplasmic material can be misleading if there is a particular protein constituting a large fraction which is released at a different rate.

All the work done with Tris buffer as the resuspending solution, showed similar disruption kinetics (rapid release during first pass and much slower rate of release at subsequent passes) but the acetate buffer gave a linear increase with number of passes for glucose oxidase although protein release kinetics remained similar. Glucose oxidase release in acetate buffer was considerably more difficult: no release was obtained between a pressure of 10-40 MPa, and even at 100 MPa, complete release was not achieved until 7 or 8 passes. Consequently, G.O. release lagged behind protein release to a much greater extent than for cells in Tris buffer. The reason is still unknown. The acetate buffer at pH 5.2 had denatured and precipitated 90 % of the protein, but this should not affect the release of glucose oxidase.

A speculative attempt was made in section 3.3.9, as to the mechanism of filamentous cell disruption in the high pressure homogeniser. The process can be split into two phases : first pass and subsequent passes. At the first pass, the mycelia undergo violent disentanglement as it is forced through the gap width in the valve assembly. Depending on the pressure and hence the gap width, the disentanglement may result in almost 100 % disruption as at 100 MPa or minimal disruption as found at 10 MPa. At subsequent passes, at a low pressure of 10 MPa, the cells appear to remain loosely intertwined with minimal cell breakage. It may be possible that these cells "slip through" the estimated gap width of about 16 μm (calculations of gap width in Appendix 3 - these are speculative, but give an estimate for comparison with cell fragments). However at a high pressure of 60 MPa, the first pass has reduced the mycelia to about 40-100 μm fragments, which are further broken up to 30 μm fragments, probably through impingement on the impact ring. The mechanism of disruption after the first pass may then be similar to the first order rate kinetics as found in yeast or unicellular microorganisms, since the cell fragments will not be much larger than yeast cells. This mechanism involving firstly disentanglement,

which may be unique for filamentous cells, and secondly a yeast type mechanism of mainly impingement, has been used to model the disruption kinetics. This will be discussed later.

The characteristic shape of the disruption curves for the control morphology, pelleted and filamentous cells would suggest that the mechanism is similar for all morphologies because the final shape and size of the organism is the same : filamentous hyphae. The difference is in the extent of disruption during the first pass. In the case of the pellets, it was speculated that the energy input used in forcing a "rigid" pellet through the gap width is greater than for the more "flexible" filamentous (control) cells. An increased cell concentration also resulted in increased glucose oxidase release. A similar reason may be applied: the higher cell concentration involves a greater energy input to maintain the same pressure and flowrate.

However, in the case of filamentous growth using Junlon, the cell wall strength may be weaker than the control, and so the disruption was easier due to a change in cell wall strength, rather than the morphology. The change in its susceptibility to disruption should be attributed to the effect of Junlon, instead of effect of morphology. This can be supported by the filamentous growth obtained by reduced agitation where no change in disruption rate was found.

Since there have been no extensive studies on high pressure homogenisation of septate filamentous fungi, it is difficult to assess this *A. niger* disruption as a model system. The only work has been with *Rhizopus nigricans* where the process is highly dependent on the first pass release *(Keshavarz 1990a)*. However, due to this fungus being non-septate, any large enough break anywhere in the organism will release the entire contents. Consequently, the disruption kinetics is also a weak function of pressure: a very low pressure of 10 MPa and 1 pass will result in 100% release. Therefore the only similarity is the extent of product release in the first pass.

3.7.1.2 Quantitative description : a model

A relatively simple model is presented here to describe the high pressure homogenisation of *A. niger* cells. It has been modelled in two parts: the first, applicable to the first pass (N=1) disruption based on a power law equation, and the second, for subsequent passes (N>1) based on Hetherington's model *(Hetherington et al 1971)* of a first order disruption process (eqn. 1.1).

The Hetherington model *(Hetherington et al 1971)* which describes the rate of release of protein to be proportional to the amount of unreleased protein, cannot be applied to the disruption of filamentous microorganisms. This can be shown by applying the model to experimental data from the control morphology (fig. 3.22-3.24). An example of this is shown in fig. 3.60, for

G6PDH release from control 1 (fig. 3.22), where $\log_{10} (R_m / R_m - R)$ is plotted against number of passes (N). If the model fits the experimental data, then the transformation should result in a linear relationship between $\log_{10} (R_m / R_m - R)$ and N. This is clearly not the case here. However, it would appear that a straight line can be fitted onto data points from the first pass onwards but without going through the origin at (0,0).

In view of this, the model by Hetherington et al (1971) would seem to apply for the passes after the first one. Therefore, the disruption during the first pass was modelled separately from the disruption after the first pass. This two staged modelling method also fits in with the postulated mechanism of disruption in section 3.3.9: a process of disentanglement and disruption in the first pass, followed by a first order disruption in subsequent passes typical of that observed for many unicellular microorganisms. Data used to develop the model are from the Lab 60 homogeniser disruption only and thus covers the pressure range from 10-60 MPa. Data from the 30CD homogeniser (80-124 MPa) was not used because the starting material was always homogenate from the Lab 60, and not whole cells.

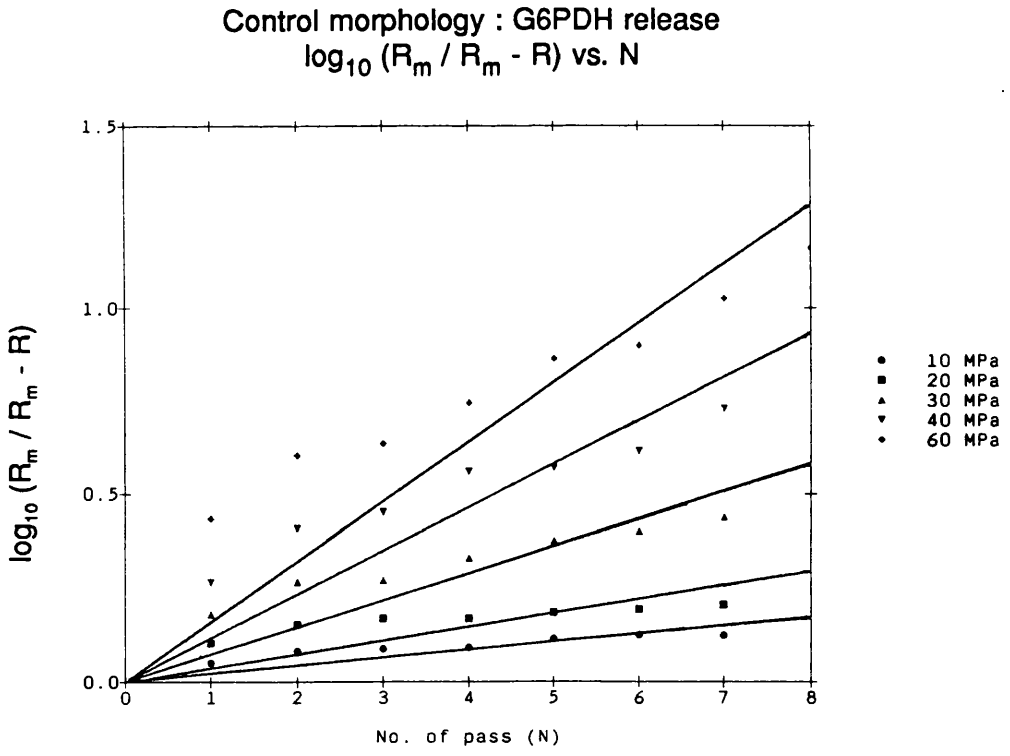


Fig. 3.60 : Application of Hetherington's first order disruption model to data of G6PDH release from control 1 (fig. 3.22)

First pass modelling (N=1)

The disruption during the first pass (R_1) is obviously independent of number of passes (N), but clearly a function of pressure (P) :

$$R_1 = f^n (P) \qquad \text{eqn. 3.1}$$

Converting R_1 to a fractional release and introducing a dimensional constant k_1 (MPa^{-a}) and an exponent "a", equation 3.1 is transformed to 3.2:

$$(R_1 / R_m) = k_1 . P^a \qquad \text{eqn. 3.2}$$

where R_m = maximum available product for release (protein, G6PDH or glucose oxidase)

P = pressure (MPa)

Depending on the product, the units for R_1 or R_m can be (mg/g dcw) for protein, or (IU/g dcw) for G6PDH or glucose oxidase. The maximum product released R_m, was determined experimentally. R_m was achieved when there was negligible increase in the product released on increase of pressure. For the purpose of modelling, an average R_m value was calculated, since there is a small degree of experimental error between the highest values.

In order to determine the constant k_1, and the exponent "a", equation 3.2 can be re-written as:

$$\log_{10} (R_1 / R_m) = \log_{10} k_1 + a.\log_{10} P \qquad \text{eqn. 3.3}$$

and $\log_{10} (R_1 / R_m)$ plotted against $\log_{10} P$.

This calculation was performed on all the first pass data for the various morphologies. Figs. 3.61 and 3.62 shows the plots of $\log_{10} (R_1 / R_m)$ vs. $\log_{10} P$, for pelleted and filamentous growth respectively. The control morphology data has been shown in both figures for comparison. From these figures, the model for the first pass fits relatively well. However, it must be noted that the model applies only to the Lab 60 disruptions in the range of 10-60 MPa. It obviously breaks down when the value of P is such that R_1 is greater than R_m.

Modelling for N > 1

The first order disruption kinetics by Hetherington et al (1971) must be adapted here, since the origin is not at N=0, and R=0, but at N=1 and R=R_1. Therefore, the modified form is:

$$\log_{10} [(R_m - R_1) / (R_m - R)] = K.(N - 1) \qquad \text{eqn. 3.4}$$

where R = product released at N. The R_1 value used for modelling is the one predicted from the

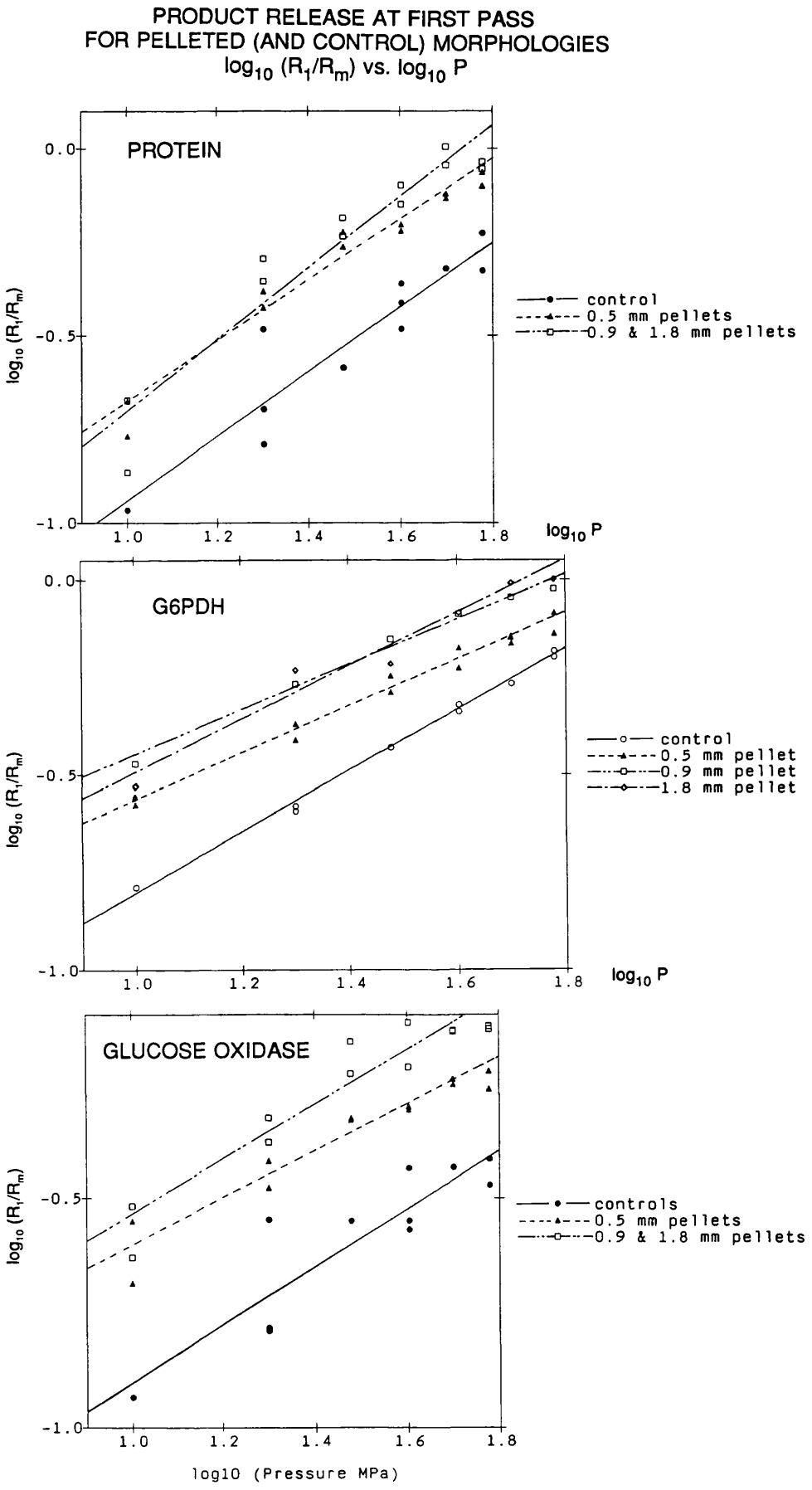


Fig. 3.61 : First pass model: plot of $\log_{10}$ (R_1/R_m) vs. $\log_{10}$ P for protein, G6PDH and glucose oxidase release from pelleted and control morphologies (data from fig. 3.22, 3.23, 3.39, 3.40, 3.42 & 3.43). Lines through points are best fit lines.

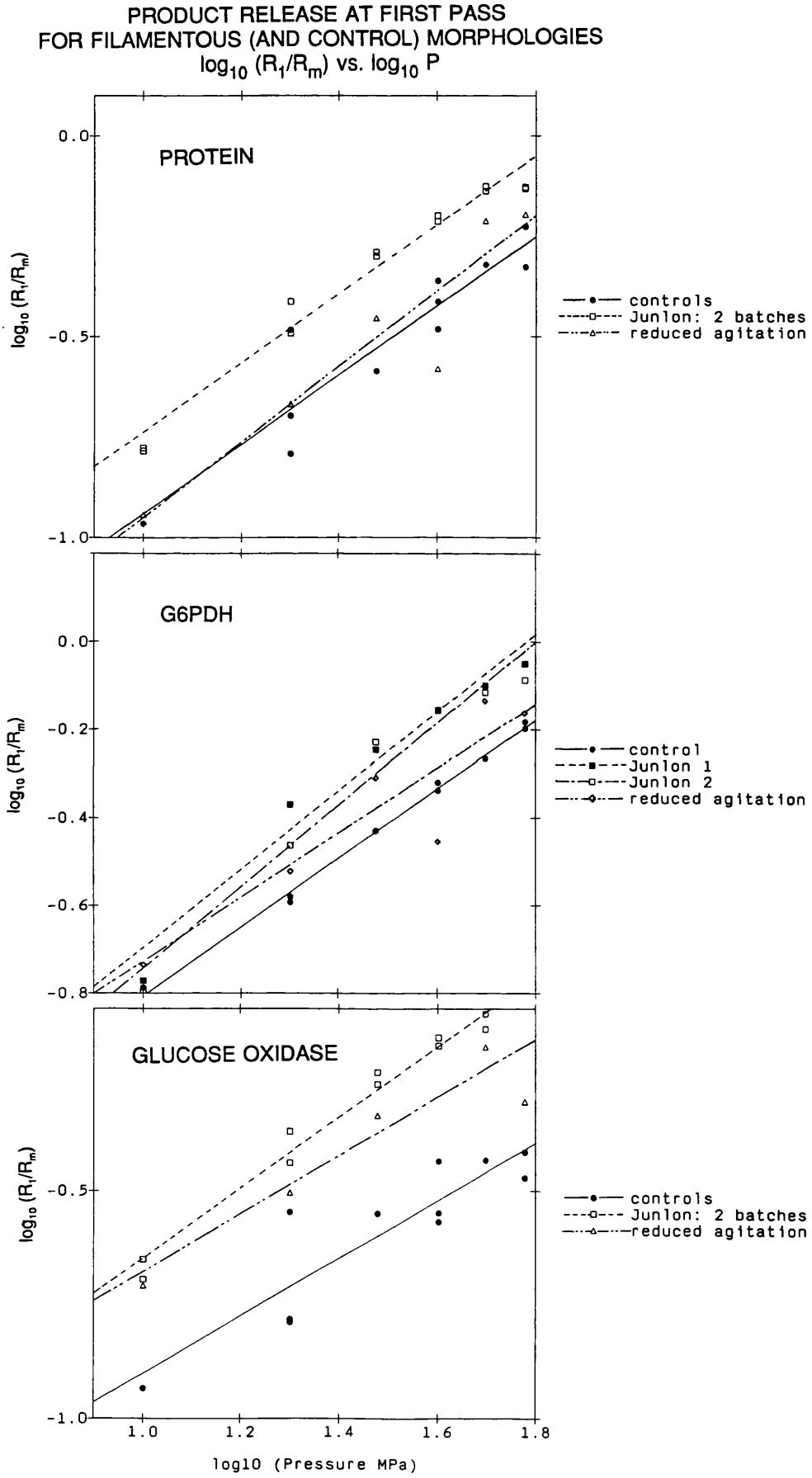


Fig. 3.62 : First pass model: plot of $\log_{10}(R_1/R_m)$ vs. $\log_{10}$ P for protein, G6PDH and glucose oxidase release from filamentous and control morphologies (data from 3.22,3.23 & 3.44-3.46). Lines through points are best fit lines.

model for N=1 ($(R_1/R_m) = k_1.P^a$), as described above. Plots of $\log_{10} [(R_m - R_1) / (R_m - R)]$ vs. N-1 were made for the different morphologies and the rate constant K (dimensionless) calculated for each pressure, by linear regression. An example of such a plot is shown in fig. 3.63 for protein G6PDH and glucose oxidase release from control 1.

Since the rate constant K, is a function of pressure, it can be expressed as:

$$K = k_2.\ P^b \qquad \text{eqn. 3.5}$$

where k_2 = dimensional constant (MPa^{-b}),

b = pressure exponent; pressure P in MPa.

Rewriting equation 3.5 by taking logs gives:

$$\log_{10} K = \log_{10} k_2 + b.\log_{10} P \qquad \text{eqn. 3.6}$$

and a plot of $\log_{10}$ K vs. $\log_{10}$ P will yield k_2 and b. Fig. 3.64 shows such a plot for the three products released from control 1 and 2. Values of k_2 and b are then obtained by linear regression.

Model predictions vs. experimental data

Having determined all the constants in the models, the product release R, can be calculated using the model, and these predictions compared with experimental data.

Fig. 3.65 shows the comparison of model predictions with experimental data for the control type morphology (data from control 1: fig. 3.22). The solid lines represent the model predictions whilst the non-solid lines (either dashed, or dot-dashed) are extrapolations of the model for pressures higher than 60 MPa. The dotted line shows the R_m value. The extrapolation for 80 MPa does not fit the experimental data where the first pass was at 60 MPa in the Lab 60 and the remaining passes at 80 MPa in the 30CD. For G6PDH and protein it was shown that a pressure of 80 MPa after 2 or 3 passes will cause 100 % release, but the model does not predict this. An extrapolation of the model underestimates glucose oxidase release at 100 MPa by a very large margin, because in practice, maximum release was achieved at this pressure in the 30CD. Therefore, the model is only valid for the Lab 60 homogeniser in the range of 10-60 MPa. Hypothetically, if the Lab 60 operated up to 100 MPa, it may be expected to perform as the model predicts. For the 30CD another model must be used. Evidently, there are differences in the performance of the two homogenisers, the 30CD being the more efficient.

To test the model further, an attempt was made to model the experimental situation where the first pass was at 60 MPa in the Lab 60, and the rest at 80 MPa in the 30CD. Using the model, the first pass prediction R_1, was made with P = 60 MPa, and then for subsequent passes, this R_1 value was used, but with P = 80 MPa. As shown in fig. 3.65, (for G6PDH, as dot-dot-dashed line

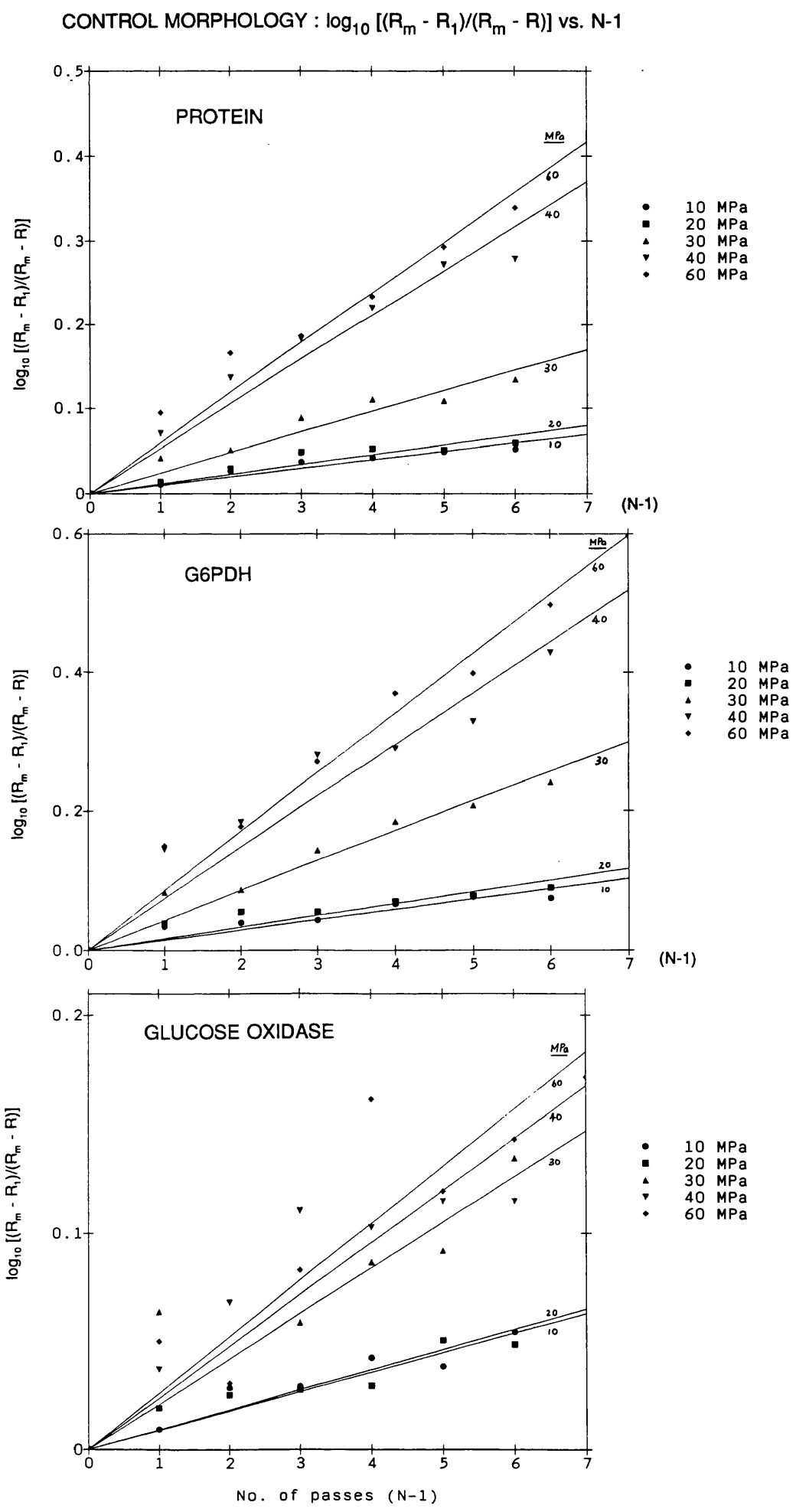


Fig. 3.63 : Plot of $\log_{10}[(R_m - R_1)/(R_m - R)]$ vs. N-1 for protein, G6PDH and glucose oxidase release from control 1 (data from fig. 3.22). Lines through points are best fit lines.

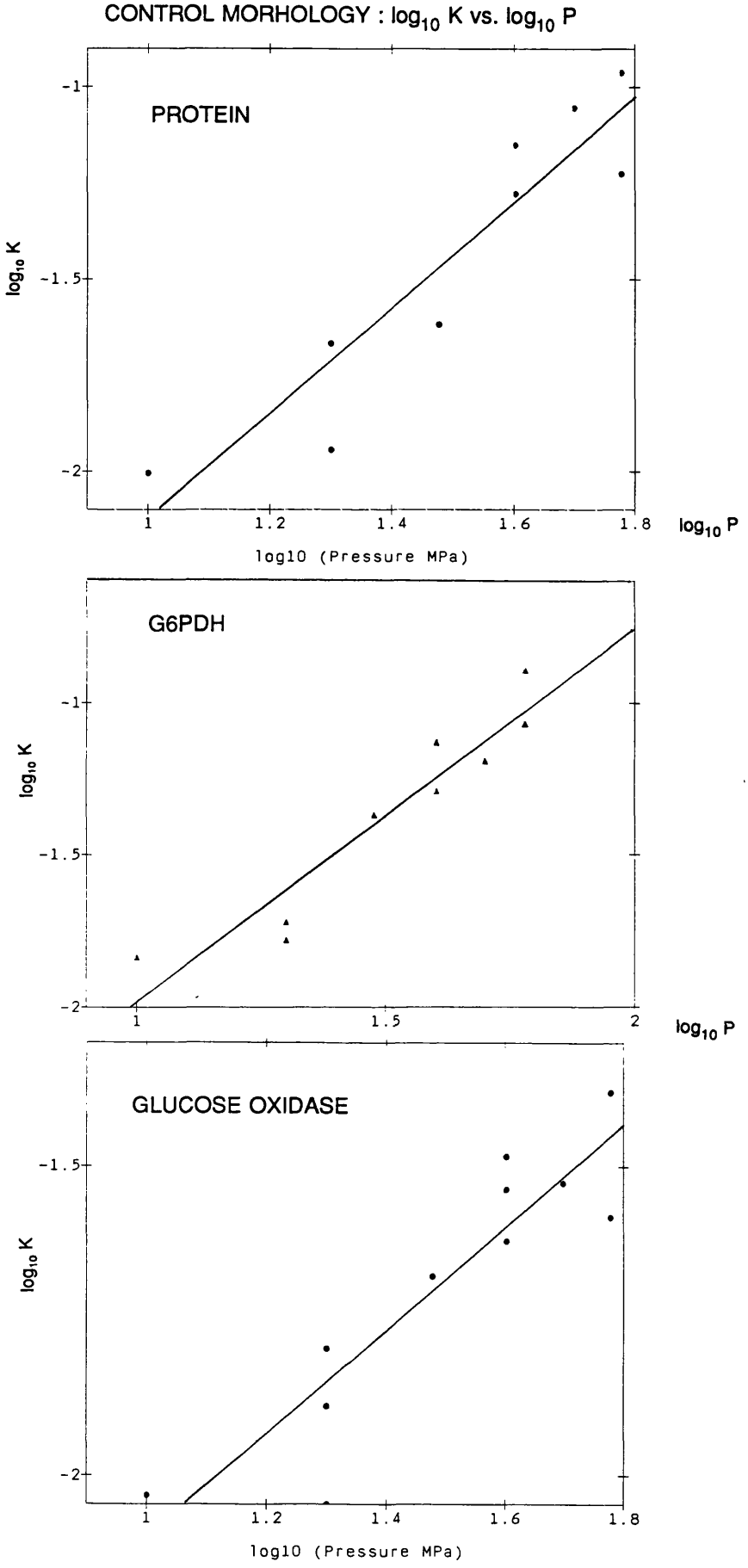


Fig. 3.64 : N > 1 model: Plot of $\log_{10}$ K vs. $\log_{10}$ P for protein, G6PDH and glucose oxidase release from control 1 (K values from fig. 3.63) to determine k_2 and b in $K = k_2 . P^b$. Lines through points are best fit lines.

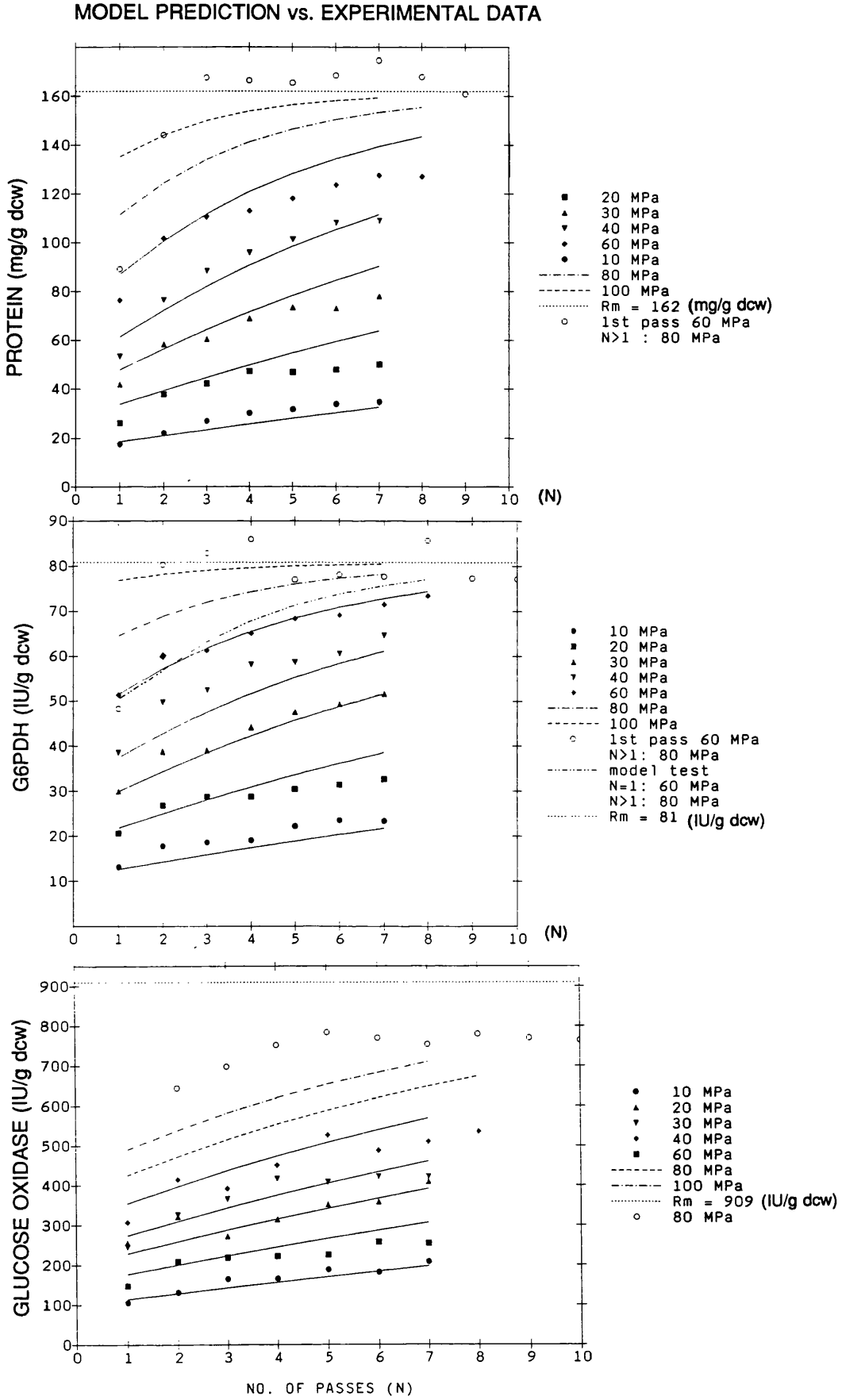


Fig. 3.65 : Comparison of model predictions and experimental data (from fig. 3.22) for protein, G6PDH and glucose oxidase from control morphology. Solid lines: model predictions; non-solid lines: extrapolation of model to P > 60 MPa.

- model test), the prediction falls short of the experimental value where the change in pressure from 60 to 80 MPa, from the first to the second pass resulted in a considerable rise in G6PDH release. It also falls short of the model prediction when P = 80 MPa is used to predict R_1 (dot-dash). This clearly shows the importance of R_1 prediction on the second part of the model for N>1. In practice, this was not the case. If cells are disrupted at 10 Mpa for 1 pass, and then at 80 MPa for subsequent passes, the product released at N>1 would be similar to the case where all passes are at 80 MPa.

Similarly, for the pelleted and filamentous morphologies, a comparison of the model predictions and experimental data can be made. These are presented in figs. 3.66-3.70. Clearly, the form of the model used fits the data for all the different morphologies, but the constants k_1, k_2, a and b will vary between them. Therefore, the basic mechanism of disruption for these *A. niger* cells in the Lab 60 homogeniser in the range of 10-60 MPa is the same for all the morphologies. This was thought to be the case, simply from the similar shapes of the disruption curves. The values of these constants are tabulated in table 3.3 and 3.4. Where the constants are identical for the 0.9 and 1.8 mm pellets, or the two "Junlon" batches, the one model was found to fit the two batches. From these values, it is difficult to draw any trends to the variation in disruption between the different morphologies, because the product released depends on both the rate constants (k_1 and k_2) and pressure exponents (a and b). Since the disruption is highly dependent on the first pass release, an analysis of the first pass model should provide a better insight into any trends.

From table 3.3, an average value of the exponent "a" is calculated for each product. Correspondingly, a new value of k_1 is calculated from:

$$k_1 . P^a = (R_1 / R_m) = k_{1,av} . P^a av \qquad \text{eqn. 3.7}$$

where a_{av} = average value of the exponent a for different morphologies

$k_{1,av}$ = "new" value of k_1 calculated from a_{av}.

$k_{1,av}$ is therefore calculated from:

$$k_{1,av} = k_1 . P^{(a - a_{av})} \qquad \text{eqn. 3.8}$$

for a pressure of 40 MPa, as an example.

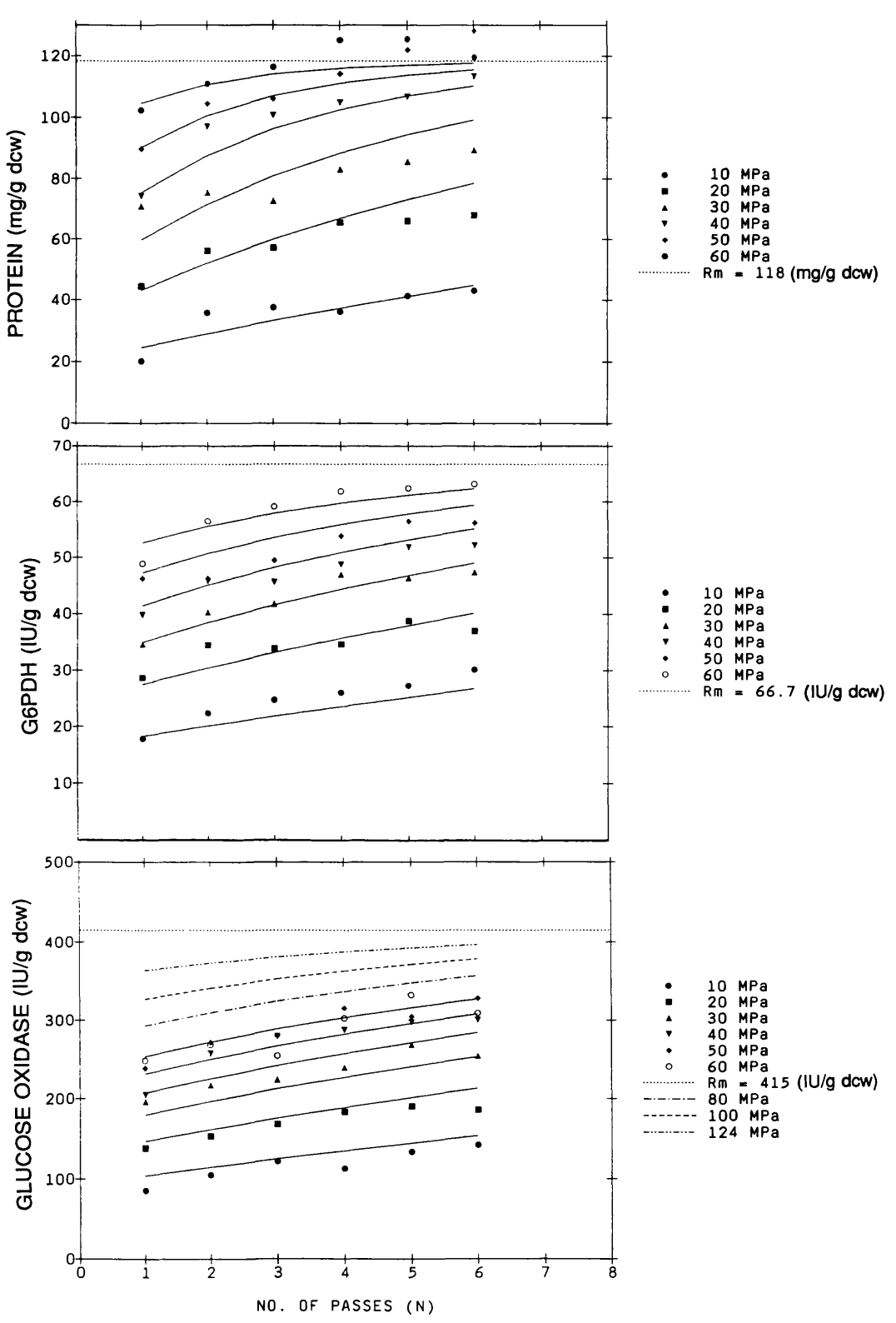


Fig. 3.66 : Comparison of model predictions and experimental data (from fig. 3.40) for protein, G6PDH and glucose oxidase from 0.5 mm pellets. Solid lines: model predictions; non-solid lines: extrapolation of model to P>60 MPa.

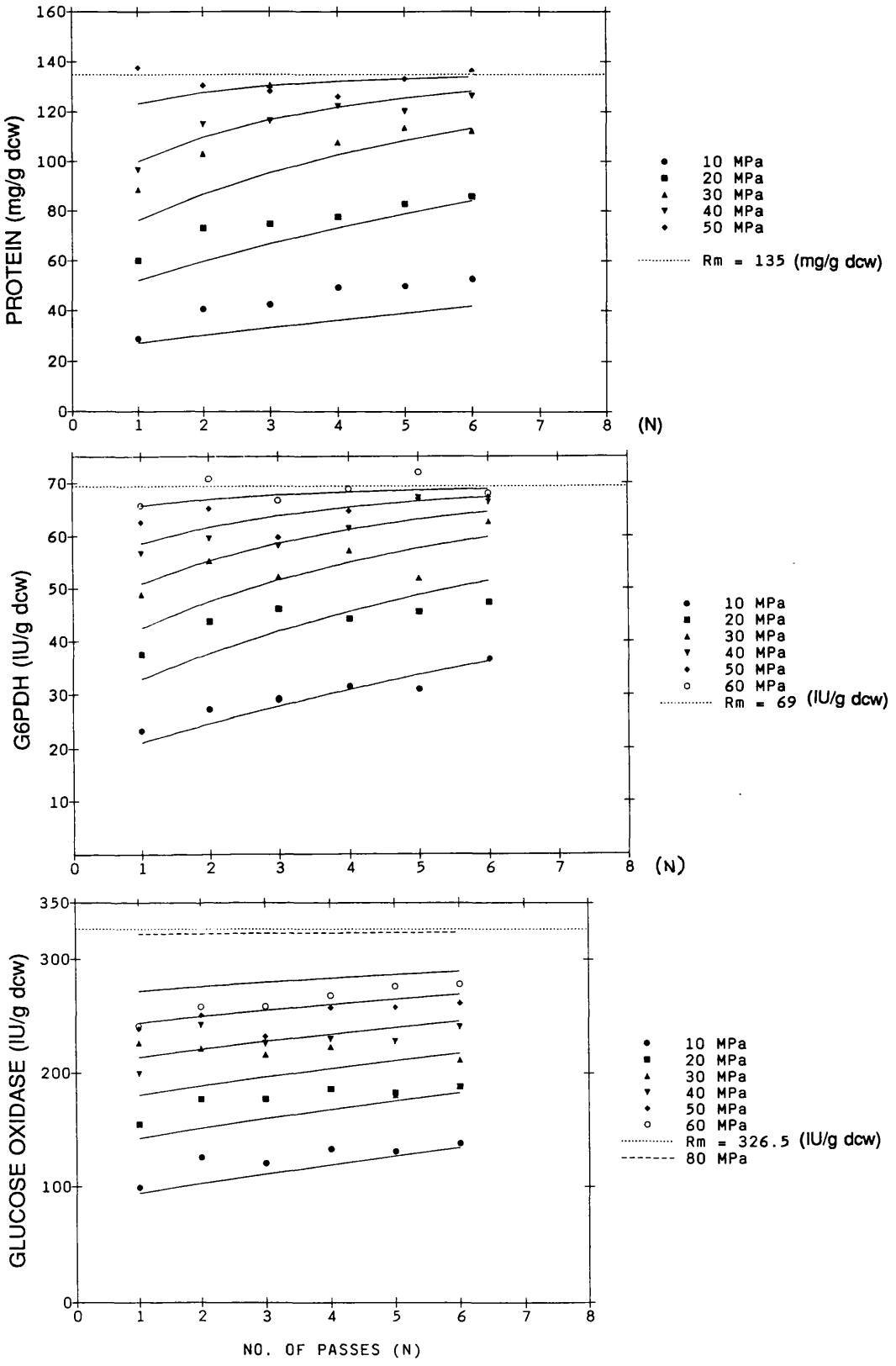


Fig. 3.67 : Comparison of model predictions and experimental data (from fig. 3.42) for protein, G6PDH and glucose oxidase from 0.9 mm pellets. Solid lines: model predictions; non-solid lines: extrapolation of model to P>60 MPa.

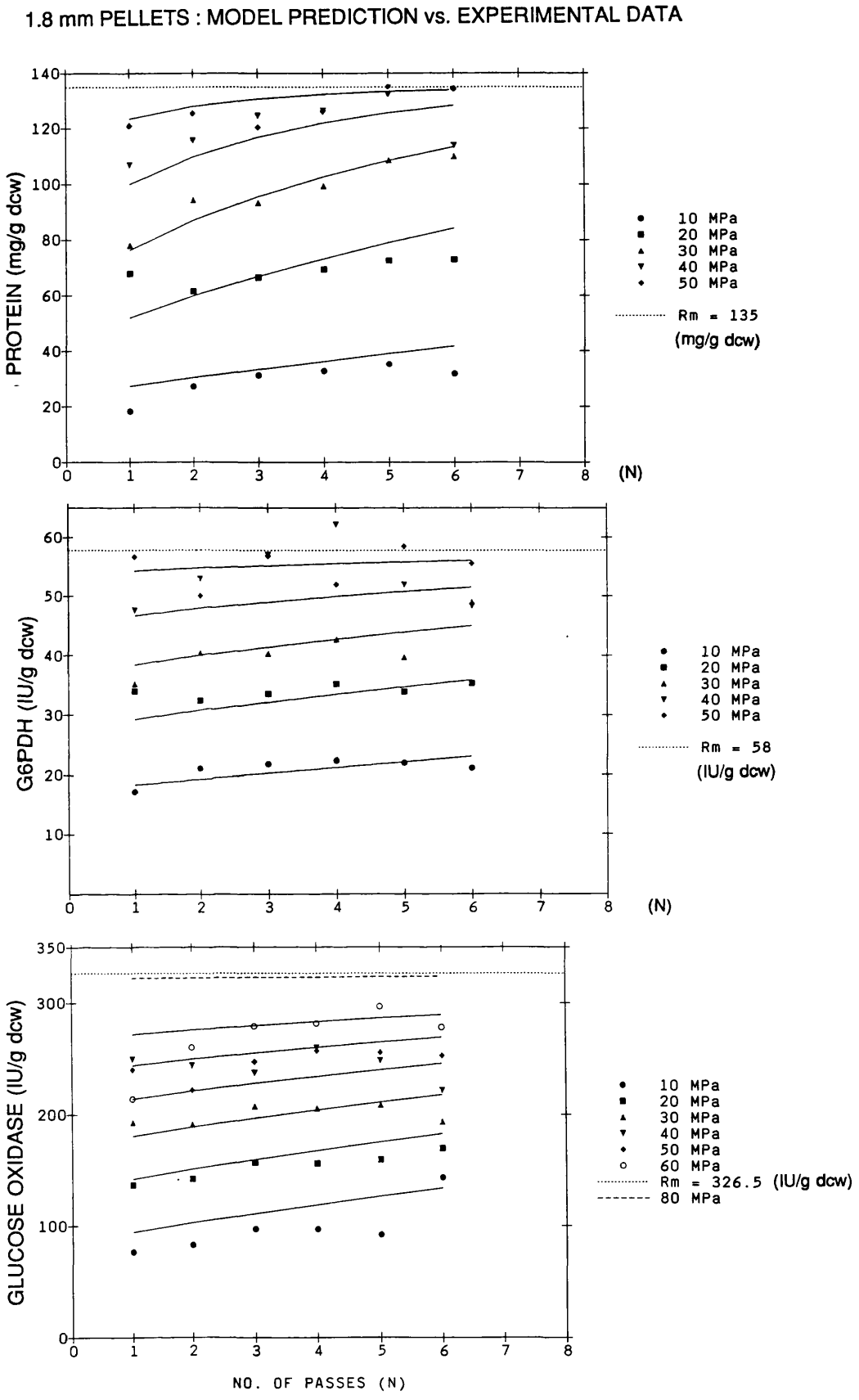


Fig. 3.68 : Comparison of model predictions and experimental data (from fig. 3.43) for protein, G6PDH and glucose oxidase from 1.8 mm pellets. Solid lines: model predictions; non-solid lines: extrapolation of model to P>60 MPa.

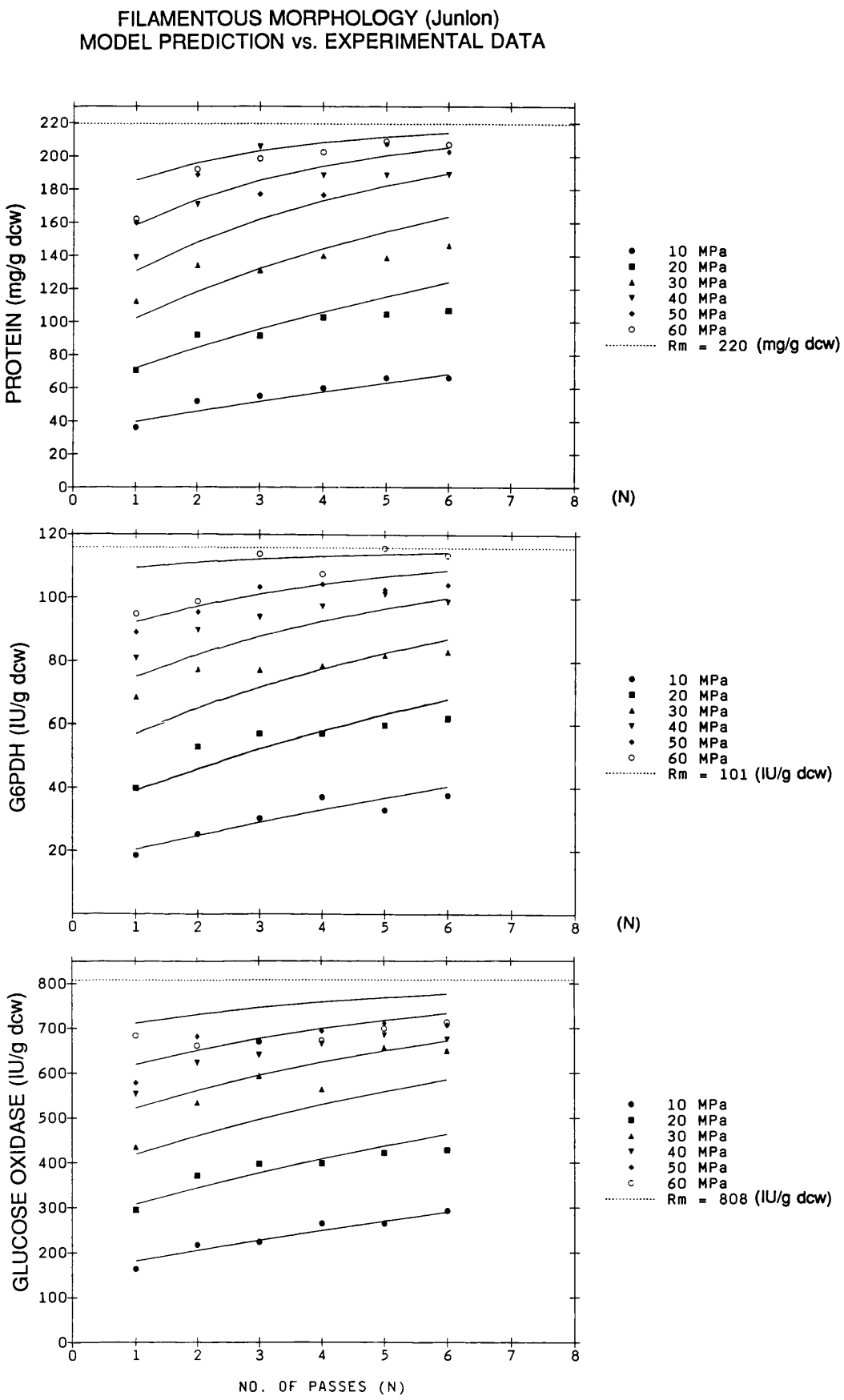


Fig. 3.69 : Comparison of model predictions and experimental data (from fig. 3.45) for protein, G6PDH and glucose oxidase from filamentous growth by Junlon. Solid lines: model predictions.

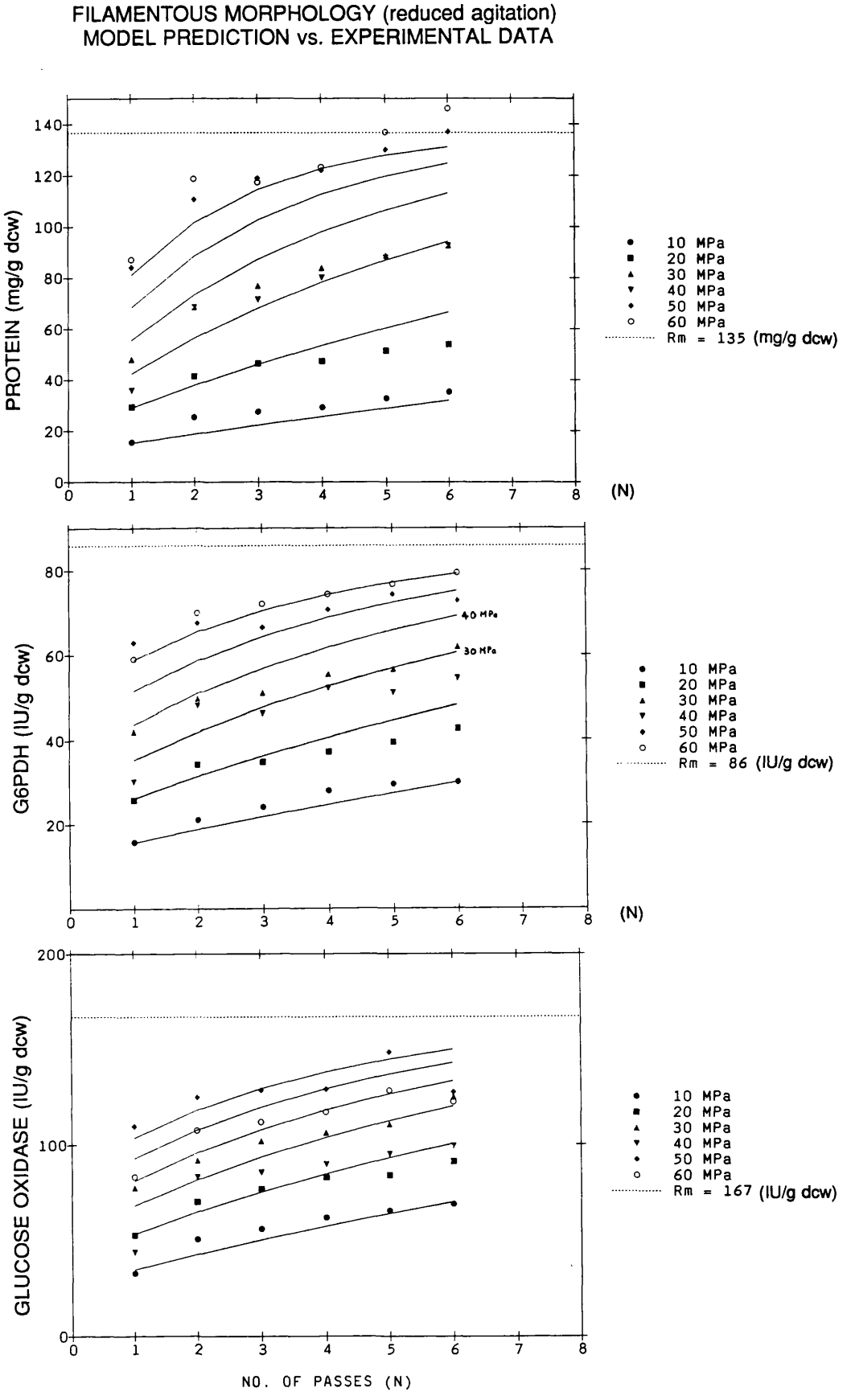


Fig. 3.70 : Comparison of model predictions and experimental data (from fig. 3.46) for protein, G6PDH and glucose oxidase from filamentous growth by reduced agitation. Solid lines: model predictions.

$(R_1 / R_m) = k_1 . P^a$						
Type	**G6PDH**		**PROTEIN**		**GLUCOSE OX.**	
	k_1 (MPa^{-a})	a	k_1 (MPa^{-a})	a	k_1 (MPa^{-a})	a
Control	0.026	0.784	0.016	0.863	0.029	0.632
0.5 mm pellets	0.071	0.589	0.032	0.809	0.080	0.497
0.9 mm pellets	0.091	0.587	0.023	0.940	0.074	0.591
1.8 mm pellets	0.067	0.676	0.023	0.940	0.074	0.591
Filamentous : Junlon 1	0.024	0.906	0.025	0.860	0.039	0.763
Filamentous : Junlon 2	0.021	0.935	0.025	0.860	0.039	0.763
Filamentous : reduced agitation	0.034	0.737	0.013	0.933	0.051	0.610

Table 3.3 : Values of the rate constant k_1 and pressure exponent "a" in the first pass model $(R_1 / R_m) = k_1 . P^a$

$\log10 [(R_m - R_1) / (R_m - R)] = k_2 . P^b . (N - 1)$						
Type	**G6PDH**		**PROTEIN**		**GLUC. OX.**	
	k_2 ($x10^{-3}$) (MPa^{-b})	b	k_2 ($x10^{-3}$) (MPa^{-b})	b	k_2 ($x10^{-3}$) (MPa^{-b})	b
Control	0.616	1.228	0.319	1.371	0.117	0.831
0.5 mm pellets	1.663	1.004	0.850	1.394	3.003	0.702
0.9 mm pellets	3.847	0.929	0.229	1.745	6.298	0.413
1.8 mm pellets	0.948	1.072	0.229	1.745	6.298	0.413
Filamentous : Junlon 1	1.993	1.009	0.718	1.332	2.399	1.100
Filamentous : Junlon 2	1.993	1.009	0.718	1.332	1.735	0.978
Filamentous : reduced agitation	1.856	1.025	0.385	1.526	4.186	0.807

Table 3.4 : Values of the rate constant k_2 and pressure exponent "b" in the N>1 model $\log10 [(R_m - R_1) / (R_m - R)] = k_2 . P^b . (N - 1)$

The values of $k_{1,av}$ is shown in table 3.5. For each product, the $k_{1,av}$ value increases with increasing pellet size, in comparison with the control. The value is also greater (than control) for the filamentous morphology obtained by Junlon, whereas for the filamentous form obtained by reduced agitation, for protein and G6PDH, the $k_{1,av}$ value is similar to the control. The increased $k_{1,av}$ would result in greater product release in the first pass and consequently in subsequent ones. This is as expected from the experimental results. By calculating an average value of "a". the k_1 values can be compared directly. Looking back at figs. 3.61 and 3.62, the gradient of the best fit lines are quite similar, that is "a" is similar between the various morphologies. Therefore, the dependency of disruption on pressure could be the same for the different morphologies. It is likely that the rate constant k_1 decides the extent of disruption.

$(R_1 / R_m) = k_{1,av} \cdot P^a av$ a_{av} = average "a" for all morphologies calculated from table 3.3 $k_{1,av}$ = "new" value of k_1 calculated using a_{av} and eqn.3.8, for P = 40 MPa			
Type	**G6PDH** $a_{av} = 0.745$	**PROTEIN** $a_{av} = 0.886$	**GLUCOSE OX.** $a_{av} = 0.635$
	$k_{1,av}$ (MPa^{-a})	$k_{1,av}$ (MPa^{-a})	$k_{1,av}$ (MPa^{-a})
Control	0.030	0.014	0.029
0.5 mm pellets	0.040	0.024	0.048
0.9 mm pellets	0.051	0.028	0.063
1.8 mm pellets	0.052	0.028	0.063
Filamentous : Junlon 1	0.044	0.023	0.062
Filamentous : Junlon 2	0.041	0.023	0.062
Filamentous : reduced agitation	0.033	0.015	0.047

Table 3.5 : Values of the rate constant $k_{1,av}$ and an average pressure exponent a_{av} in the first pass model. $k_{1,av}$ values are now directly comparable between morphologies.

The two stage model described above would seem to fit the experimental data. Therefore, the mechanism of disruption suggested may be plausible: at the first pass disentanglement is the likely cause of disruption, followed by a first order disruption process for subsequent passes. The first order disruption process is similar to the yeast disruption process, and hence, the mechanism could be by impingement.

3.7.2 Impact of fermentation conditions on cell disruption

From the changes made in inoculum size, media (Junlon) and agitation, it is quite clear that fermentation conditions can have a considerable effect on the disruption and release of intracellular products. These effects are summarised below in table 3.6. In addition the effect of buffer type and pH used for disruption is included for comparison.

Reducing the inoculum size resulted in pelleted growth which proved to be easier to disrupt. Product release rates also tend to increase with pellet size but there appears to be no further enhancement of the rate of release beyond the 0.9 mm diameter pellets. The reduction in inoculum size does not change the media or fermenter operating conditions such as agitation. Thus, it is reasonable to assume that the cells are identical to the ones from the control except that they grew as pellets which means that the cause of the increased rate of release can be attributed to the change in morphology, with a degree of certainty. However, this is not the case with the filamentous growth.

Using the two methods of Junlon and reducing the agitation to obtain filamentous growth, the results were different. The ones grown in Junlon were easier to disrupt than the control morphology whereas the ones grown with reduced agitation showed little difference. If it was the filamentous structure which caused the change in disruption, then both types of filamentous growth should have similar results. Therefore the cause may be due to the effect of Junlon on the cell wall strength as discussed in section 3.4.3.2. and not due to its filamentous structure. Similarly, agitation can have an effect on cell wall strength, but does not seem to have affected the cells so as to make any noticeable change in disruption. This illustrates the difficulty in changing fermentation conditions and expecting only one parameter (for example, morphology) to change. The complexity of the interactions between fermentation conditions and microbial physiology is such that any one change can affect several aspects of the fermentation. A clear example is the decreased production of glucose oxidase with pelleted cells or reduced agitation. In practice, the decrease in productivity of G.O. cannot be sacrificed for the increased ease in cell disruption.

This work has demonstrated that it is possible to manipulate fermentation conditions to improve the performance of cell disruption. However, it must be mentioned that the manipulations made with this system may not necessarily have the same effects on another system.

Changes from control	Morphology	No. of pass	Product Release (%) * 60 MPa		
			Soluble Protein	G6PDH	G.O.
None	Filamentous, clumped, entangled	1	49	60	40
		6	77	92	60
Reduce spore conc. 10 x	Pellets 0.5 mm diameter	1	70	70	55
		6	95	95	78
Reduce spore conc. 100 x	Pellets 0.9 mm diameter	1	90	93	73
		6	100	100	85
Reduce spore conc. 100 x	Pellets 1.8 mm diameter	1	90	100	70
		6	100	100	90
Add "Junlon" polyacrylic resin	Free filamentous no clumping	1	73	78	80
		6	100	100	93
Reduce agitation from 500 to 300 rpm	Filamentous, little clumps	1	57	67	45
		6	92	90	72
Change buffer: Tris pH 7.5 to acetate pH 5.2	Filametous, clumped, entangled	1	63	0	12
		6	100	0	47

* The percentage of product released is calculated based on 100% being the maximum product available for release on complete disruption under the specified environmental conditions. Therefore the 100 % value is different for each case. Where the Tris buffer was changed to acetate buffer pH 5.2, the soluble protein assayed was considerably less than the other cases.

Table 3.6 : Effects of fermentation and disruption conditions on product release

3.7.3 Impact of cell resuspension buffer on further purification.

The selection of a cell resuspension buffer for disruption is usually based on one which will maintain the activity of the enzyme product. A widely used buffer such as Tris HCl at pH 7.5 would be able to maintain solubility and activity of most proteins. However, the use of acetate buffer at pH 5.2 does offer an alternative. The resultant precipitation of 90 % of the protein without affecting the required product (glucose oxidase) is a potentially valuable tool in reducing the problems in purification of intracellular proteins.

The immediate benefit of this "conditioning" technique is in the separation of cell debris. As mentioned on section 3.5.1, the clarity of the acetate buffer was very good compared to the Tris buffer. In fact a quick measurement of turbidity of centrifuged supernatant from the homogenate at a 650 nm gave an absorbance of 0.007 and 0.11 for acetate and Tris buffered cells, respectively. This indicates that the supernatant from the acetate buffer does not contain much proteins or lipids which tend to cloud the homogenate. The separation of cell debris is not a problem as found with yeast or *E. coli* : the particle sizes are about 10 µm which is larger than whole yeast cells, and can therefore be easily centrifuged to form a dense sediment. In fact separation of debris is so simple that even by gravitational force, the debris would settle within a few hours to leave a clear supernatant in the case of the acetate buffer.

Purification of an enzyme solution with 90 % of initial protein impurities removed will be made much easier. The initial purification made by this buffer means that there will be a smaller load of impurities to remove in subsequent purification steps.

3.7.4 Implications for process development

The studies have shown that it is possible to manipulate fermentation conditions to improve the cell disruption stage. Simple conditioning techniques in the primary steps of downstream processing can also make a significant impact on further purification with expensive methods such as chromatography. In these studies, the location of the product can affect the operation of the homogeniser. Therefore, in process development, it may be beneficial to genetically engineer the product to be located in the cytoplasm as a an unbound substance ie. not further bound in an organelle, which may make the disruption more difficult. Unless it is possible to selectively separate such organelles containing the product, and then disrupting these to liberate the product, it would probably be better located freely in the cytoplasm. Obviously, this would depend on the nature of the product. In the case of glucose oxidase, its location is due to the toxic hydrogen peroxide formed during glucose oxidation, and therefore must be isolated from the rest of the cell.

The tendency for pellets to be easier to disrupt than filamentous cells indicates that this could be a method to improve product release from high pressure homogenisers. In addition the use of pellet growth will change the fermentation broth from a non-Newtonian fluid which causes problems in mass and heat transfer, and mixing to a Newtonian fluid without these problems. However, pellets sizes must be reduced considerably because of the mass transfer limitations experienced with pellet sizes greater than 0.1 to 0.2 mm diameter. Of paramount importance is the productivity of the fermentation. This must not be adversely affected in the pellet growth form, as in glucose oxidase production. Therefore, the need for an integrated approach to process development in order to achieve a high overall productivity is very important.

4.0 CONCLUSIONS AND FUTURE WORK

High pressure homogenisation of filamentous microorganisms to release intracellular products has proven to be a reliable method. The use of *Aspergillus niger* cells as a model system revealed a characteristic feature which may be unique for filamentous microorganisms: a high level of product release is achieved during the first pass, after which, the rate of release decreases considerably. This characteristic was prevalent for the three products monitored as markers of cell disruption: intracellular soluble protein, G6PDH and glucose oxidase.

In the course of this study, a number of factors were found to affect the rate of release of the products:

1) Intracellular location of product

   Protein and G6PDH which are both freely soluble in the cytoplasm, were released at a faster rate than glucose oxidase which is located in a membrane bound organelle called a peroxisome (in the cytoplasm). This difference was reflected in the minimum pressure required to achieve complete release within a few passes. Protein and G6PDH required a pressure of 80 MPa, whereas glucose oxidase required a pressure of 100 MPa.

2) Fermentation conditions

   The control morphology was filamentous and clumped. A decrease in the spore inoculum size resulted in pelleted morphology which showed a trend towards increased rates of release with increasing pellet size (in comparison to control). Similarly, the incorporation of "Junlon", (a polyacrylic resin) in the growth medium also improved the rates of release, and produced a loose filamentous morphology. This was important, especially for the release of glucose oxidase. At a pressure of 60 MPa after a single pass, cells grown with Junlon released 80% of its glucose oxidase compared to only 40% for the control. However, a reduction in agitation speed which also gave a more filamentous form did not result in any change to the disruption kinetics.

3) Resuspension buffer

   The pH of the buffer used for resuspending the cells for disruption can affect the disruption kinetics. Tris buffer at pH 7.5 which is the "usual" buffer retains all the proteins in solution, whereas an acetate buffer at pH 5.2 precipitated 90 % of the released protein but without affecting the glucose oxidase activity. However, the disruption kinetics of glucose oxidase was almost a linear function of number of passes, and negligible release was obtained below

40 MPa. Protein release still followed the characteristic disruption kinetics as mentioned above.

As the disruption kinetics for the various morphologies were similar, a common mechanism of disruption was postulated. During the first pass, the mycelia undergo disentanglement, resulting in disruption. At subsequent passes, the disruption process is similar to a yeast type first order kinetics, where impingement may be the major cause of disruption. This two stage mechanism was then used as the basis for the development of a model. It was shown that, the form of the model was applicable to the various morphologies, and it was the rate constants and pressure exponents which varied between them. Therefore, for the first pass, the model is:

$$(R_1 / R_m) = k_1 \cdot P^a \qquad \text{model for N=1}$$

and for subsequent passes (N>1):

$$\log10 [(R_m - R_1) / (R_m - R)] = k_2 \cdot P^b \cdot (N - 1) \qquad \text{model for N>1}$$

In this study, the work has centred on the disruption kinetics and the interaction between fermentation and disruption. The results have provided evidence to postulate a mechanism for the disruption of filamentous microorganisms. However, further work is necessary to obtain more direct evidence that it is a process of disentanglement during the first pass. Due to the complex nature of disentanglement, such evidence may not be easy to discover.

The model obtained has pointed the way forward to developing a relationship between the fermentation conditions and cell disruption. Further experimental work involving other changes to the fermentation will provide a more complete picture to the complex interactions between fermentation and downstream processing.

Further work can also be performed on the 30CD homogeniser. To date, whole filamentous cells have not been used as the feed, due to the possibility of blockage, as the dimensions are generally smaller than the Lab 60. Clearly, it would be useful to determine if it can handle highly viscous and concentrated filamentous cell suspensions. Following that, the disruption kinetics can be determined and compared to the Lab 60, because, it would seem that the 30CD is more efficient.

Another area of work which requires attention is the dependence of disruption kinetics on cell concentration. So far, it would appear that a product which is difficult to release, such as glucose oxidase, has a strong dependence on cell concentration whereas protein release does not. More extensive studies have to be made to clarify this issue.

Lastly, the disruption of a recombinant strain of *Aspergillus* in a high pressure homogeniser could be of interest, since the physiology of such cells may affect disruption kinetics. However, it may be advisable to ensure that the cells have not been "weakened" substantially prior to disruption. In the case of the *A. nidulans* strain used, the inducer was toxic to the cells, which may explain its high susceptibility to mechanical disruption in a bead mill.

BIBLIOGRAPHY

Adams H.L. and C.R. Thomas, "The Use of Image Analysis for Morphological Measurements on Filamentous Microorganisms" Biotech. Bioeng. **32** 707-712 (1988)

Andrews B.A., Huang R.-B., Asenjo J.A. "Differential product release from yeast cells by selective enzymatic lysis" in "Separations for Biotechnology 2" ed. D.L. Pyle, Elsevier Applied Science, London and New York, 21-28 (1990).

APV Gaulin "Technical Bulletin 74" (1985)

Asenjo J.A. and Dunnill P. "The Isolation of lytic enzymes from *Cytophaga* and their application to the rupture of yeast cells" Biotech. Bioeng. **23** 1045-1056 (1981)

Augenstein D.C., Thrasher K., Sinskey A.J., Wang D.I.C. "Optimization in the recovery of a labile intracellular enzyme" Biotech. Bioeng. **26** 1433-1447 (1974)

Baldwin C. and Robinson C.W. "Disruption of *S. cerevisiae* using enzymatic lysis combined with high-pressure homogenisation" Biotech. Techniques **4**, (5) 329-334 (1990)

Barbesgaard P. "Industrial Enzymes produced by members of the genus *Aspergillus*" in Genetics and Physiology of *Aspergillus* ed. J.E. Smith & J.A. Pateman Br. Myco. Soc. Symp. Series No.1 Academic Press

Bergmeyer H.U. (editor) "Methods of enzymatic analysis" 3rd edn. Vol. 3 (1983) Verlag Chemie, Weinheim, Florida, Basel.

Bergmeyer H.U. (editor) "Methods of enzymatic analysis" 2nd edn. Vol. 2 457-458 (1974) Verlag Chemie, Weinheim, Florida, Basel.

Belmar Campero M.T. and Thomas C.R. "The Effect of Stirrer Speed on the Morphology and Clavulanic Acid Production of *Streptomyces clavuligerus*"
2nd International Conference on Bioreactor Fluid Dynamics 21-23 Sept 1988 Cambridge U.K. BHRA 215-227 (1988)

Bio-Rad Laboratories, "Instructions for Bio-Rad Protein assay" (1989)
Bradford M.M. "A rapid and sensitive method for the quantitation of microgram quantities quantities of protein dye binding" Anal. Biochem. **72** 248-254 (1976)

Brierly M.R., Steel R. "Agitation-aeration in submerged fermentation. II. Effect of solid dispersed phase on oxygen absorption in a fermentor" Appl. Microbiol. **7** 57-61 (1959)

Brocklebank M.P. "Large Scale Separation and Isolation of Proteins" in Food Biotechnology ed. R.D. King and P.S.J. Cheetham 139-185 Elsevier Applied Sciences (1987)

Brookman J.S.G. "Mechanism of Disintegration in a High Pressure Homogeniser Biotech. Bioeng. **16** 371-383 (1974)

Buckland B.C., Richmond W., Dunnill P., Lilly M.D. "The large scale isolation of intracellular microbial enzymes: cholesterol oxidase from *Nocardia*" in Industrial Aspects of Biochemistry **30** part 1, ed. Spencer B., 65-77 (1973)

Byrne G.S., Ward O.P. "Effect of nutrition on pellet formation by *Rhizopus nigricans*" Biotech. Bioeng. **33** 912-914 (1989)

Carter B.L.A. , Bull A.T. "The Effect of oxygen tension in the medium on the morphology and growth kinetics of *A.nidulans*" J. Gen. Microbiol. **65** 265 (1971)

Clark D.A. "Submerged citric acid fermentation of ferrocyanide treated beet molasses: morphology of pellets of *Aspergillus niger*" Can. J. Microbiol. **8** 133-136 (1962)

Coakley W.T., Baxter A.J., Lloyd D. "Disruption of Microorganisms" in Advances in Microbial Physiology ed. A.H.Rose, D.W.Tempest **16** 279-341 Academic Press (1971)

Cocker R., Greenshields R.N. Pap.Pres.2nd Ann. Res. Meeting IChemE Bradford

Currie J.A., Dunnill P., Lilly M.D. "Release of protein from bakers' yeast (*Saccharomyces cerevisiae*) by disruption in an industrial agitator mill" Biotech. Bioeng. **14** 725-736 (1972)

Deindoerfer F.H., Gaden E.L. "Effect of liquid physical properties on oxygen transfer in penicillin fermentation" Appl. Microbiol. **3** 253-257 (1955)

Dion W.M., Carilli A., Sermonti G., Chain E.B. "The effect of mechanical agitation on the morphology of *Penicillium chrysogenum Thom* in stirred fermenters." Rend. Ist. Super. Sanita **17** 187-205 (1954) English edn.

Doulah M.S., Hammond T.H., Brookman J.S.G. "A hydrodynamic mechanism for the disintegration of *Saccharomyces cerevisiae* in an industrial homogeniser"
Biotech. Bioeng. **17** 845-858 (1975)

Dunnill P., Lilly M.D. "Protein extraction and recovery from microbial cells" in Single Cell Protein 2, eds. Tannenbaum S.R. & Wang D.I.C. MIT Press Cambridge MA USA and London U.K. 179-207 (1975)

Edebo L. "Disintegration of Cells" in Fermentation Advances Ed. D.Perlman Academic Press New York 249-271 (1969)

Ehresmann B., Imbault P., Weil J.H., "Spectrophotometric determination of protein concentration in cell extracts containing tRNA's and rRNA's"
Anal. Biochem. **54** 454-463 (1973)

Elmayergi H., Scharer J.M., Moo-Young M. "Effect of polymer additives on fermentation parameters in a culture of *Aspergillus niger*" Biotech. Bioeng. **15** 845 (1973)

Elmayergi H. "Mechanism of pellet formation of *Aspergillus niger* with an additive"
J. Ferment. Technol. **53** no.10, 722-729 (1975)

Engler C.R., Robinson C.W. "Disruption of *Candida utilis* cells in high pressure flow devices" Biotech. Bioeng **23** 765-780 (1981a)

Engler C.R., Robinson C.W. "Effects of organism type and growth conditions on cell disruption by impingement" Biotech. Lett. **3** (2) 83-88 (1981b)

Fatile I.A. "Rheological characteristics of suspensions of *Aspergillus niger* : Correlation of rheological parameters with microbial concentration and shape of the mycelial aggregate" Appl. Microbiol. Biotechnol. **21** 60-64 (1985)

Fish N.M., Lilly M.D., "The interaction between fermentation and protein recovery" Bio/technology **2** (7) 623-627 (1984)

Follows M., Hetherington P.J., Dunnill P. Lilly M.D., "Release of enzymes from Bakers' yeast by disruption in an industrial homogeniser" Biotech. Bioeng. **17** 549-560 (1971)

Foster J.W. "Chemical activities of fungi" pp. 62-64 Academic Press, New York (1949)
Galbraith J.C. and Smith J.E. "Filamentous growth of *Aspergillus niger* in submerged shake flask culture" Trans.Br.Mycol.Soc. **52** 237 (1969)

Gomez R., Schnabel I., Garrido J. "Pellet growth and citric acid yield of *A.niger*" Enzyme Microb.Technol. **10** 188-191 (1988)

Gray P.P., Dunnill P., Lilly M.D. "The continuous flow isolation of enzymes" in Fermentation Technology Today ed. G. Terui 347-351 Soc. Ferm. Tech. Japan, Proc. 4th International Fermentation Symp.

Harrison S.T., Dennis J.S., Chase H.A. "The effect of culture history on the disruption of *Alcaligenes eutrophus* by high pressure homogenisation" in "Separations for Biotechnology 2" ed. D.L. Pyle, Elsevier Applied Science, London and New York, 38 -47 (1990).

Hermersdorfer H., Leuchtenberger A., Wardsack C.H., Ruttloff H. "Influence of culture conditions on mycelial structure and polygalacturonase synthesis of *Aspergillus niger*"
J. Basic Microb. **27** (6) 309-315 (1987)

Hetherington P.J., Follows M., Dunnill P., Lilly M.D. "Release of protein from bakers' yeast (*Saccharomyces cerevisiae*) by disruption in an industrial homogeniser"
Trans. IChemE **49** 142-148 (1971)

Hettwer D. and Wang H. "Protein release from *Escherichia coli* cells permeabilized with guanidine-HCl and Triton X-100" Biotech. Bioeng. **33** 886-895 (1989)

Higgins J.J., Lewis D.J., Daly W.H., Mosqueira F.G., Dunnill P., Lilly M.D. "investigation of the unit operations involved in the continuous flow isolation of β-galactosidase from *E.coli*" Biotechnol. Bioeng. **20** 159-182

Hoare M. and Dunnill P. "Biochemical engineering challenges of purifying useful proteins" Phil. Trans. R. Soc. Lond., B **324**, 497-507 (1989)

Ishimori Y., Karube I., Suzuki S. " Continuous production of glucose oxidase with *Aspergillus sp.* under ultrasound waves"
Enzyme Microb. Technol. **4** 85-88 (March 1982)

Jernejc K., Cimerman A., Perdih A., "Comparison of different methods of protein determination in *Aspergillus niger* mycelium"
Appl. Microbiol. Biotechnol. **23** 445-448 (1986)

Kelemen M.V., Sharpe J.E. "Controlled cell disruption : a comparison of the forces required to disrupt different micro-organisms." J. Cell. Sci. **35** 431-441 (1979)

Keshavarz E., Hoare M., Dunnill P. "Biochemical engineering aspects of cell disruption" in Separations for Biotechnology ed. Verall M.S., Hudson M.J. 62-79 Soc. Chem, Ind. Ellis Horwood, Chichester, U.K.

Keshavarz E., Bonnerjea J., Hoare M., Dunnill P. "Disruption of a fungal organism *Rhizopus nigricans*, in a high-pressure homogenizer" Enzyme Microb.Technol. **12** 494-498 (1990a)

Keshavarz E., Hoare M., Dunnill P. "Disruption of baker's yeast in a high-pressure homogenizer : new evidence on mechanism." Enzyme Microb.Technol. **12** 764-770 (1990b)

Kim J.H., Lebeault J.M., Reuss M. "Comparative study of rheological properties of mycelial broth in filamentous and pelleted forms"
Eur.J.Appl.Microbiol Biotechnol. **18** 11-16 (1983)

Kisser M., Kubicek C.P., Rohr M., "Inluence of manganese on morphology and cell wall composition of *Aspergillus niger* during citric acid fermentation"
Archives of Microbiol. **128** 26-33 (1980)

Kloostermann J., Van Wassenaar P.D., Slater N.K.H. "An integrated approach to the recovery of intracellular products from yeast by bead milling and precoat filtration"
The Chem Eng J. **37** B47-B54 (1988)

Kobayashi T., Van Dedem G., Moo-Young M. "Oxygen transfer into mycelial pellets" Biotech. Bioeng. **15** 27-45 (1973)

Lilly M.D., Dunnill P. "Isolation of intracellular enzymes from microorganisms - the development of a continuous process" in Fermentation Advances Ed. D.Perlman Academic Press New York 225-247 (1969) Proc. 3rd Int.Ferm.Symp.

Limon-Lason J., Hoare M., Orsborn C.B., Doyle D.J., Dunnill P. "Reactor properties of a high speed bead mill for microbial cell rupture"
Biotechnol. Bioeng. **26** 745-774 (1979)

Lowry O.H., Resebrough N.J., Farr A.L., Randall R.J., "Protein measurement"
J. Biol. Chem. **193** 265-275 (1951)

Marffy T., Kula M.R., "Enzyme yields from cells of brwers' yeast disrupted by treatment in a horizontal disintegrator" Biotechnol. Bioeng. **26** 623-634 (1974)

Mao H.H., Moo-Young Murray "Disruption of baker's yeast by a new bead mill"
Biotech. Techniques **4**, (5) 335-340 (1990)

Metz. B., DeBruijn E.W., Van Suijdam J.C. "Method for quantitative representation of the morphology of molds" Biotechnol.Bioeng. **23** 149-162 (1981)

Metz B., Kossen N.W.F. "Biotechnology review - The growth of moulds in the form of pellets - a literature review" Biotechnol. Bioeng. **19** 781-799 (1977)

Metz B., Kossen N.W.F., Van Suijdam J.C. "The rheology of mould suspensions" Adv. in Biochem. Eng. **11** 104-156 (1979)

Microfluidics Corporation, USA "Technical Bulletin M-120E Reverse Flow Microfludizer" (1989)

Miles E.A., Trinci A.P.J. "Effect of pH and temperature on the morphology of batch and chemostat cultures of *P.chrysogenum*" Trans. Br. Mycol.Soc. **81** 193-200 (1983)

Mischak H., Kubicek C.P., Röhr M. "Formation and location of glucose oxidase in citric acid producing mycelia of *Aspergillus niger*" Appl. Microbiol. Biotechnol. **21** 27-31 (1985)

Mitard A., Riba J.P. "Rheological properties of *Aspergillus niger* pellet suspensions" Appl.Microb.Biotechnol. **25** 245-249 (1986)

Mitard A., Riba J.P. "Morphology and growth of *Aspergillus niger* ATCC 26036 cultivated at several shear rates" Biotechnol. Bioeng. **32** 835-840 (1988)

Mogren H., Linblom M., Hedenskog G. "Mechanical disintegration of microorganisms in an industrial homogeniser" Biotech.Bioeng. **16** 261-274 (1974)

Morrin M., Ward O.P. "Biotransformation of progesterone to 11-α-hydroxprogesterone by different morphological forms of *Rhizopus arrhizus*" Biotech. Lett. **11** (5) 319-324 (1989)

Morrison K.B., Righelato R.C. "The relationship between hyphal branching, specific growth rate and colony radial growth rate in *P.chrysogenum*" J.Gen Microbiol, **81** 517-520 (1974)

Mosqueira F.G., Higgins J.J., Dunnill P., Lilly M.D. "Characteristics of mechanically disrupted bakers' yeast in relation to its separation in industrial centrifuges" Biotechnol. Bioeng. **23** 335-343 (1981)

Naglak T.J. and Wang H.Y. "Recovery of a foreign protein from the periplasm of *Escherichia coli* by chemical permeabilization." Enzyme Microb. Technol. **12** 603-611 (August 1990)

Naglak T.J. and Wang H.Y. "Protein release from the yeast *Pichia pastoris* by chemical permeabilization : comparison to mechanical disruption and enzymatic lysis." in "Separations for Biotechnology 2" ed. D.L. Pyle, Elsevier Applied Science, London and New York, 55-64 (1990).

Narendranathan T.J. and Dunnill P. "The effect of shear on globular proteins during ultrafiltration: studies of alcohol dehydrogenase" Biotech. Bioeng. **24** 2103-2107 (1982)

Nihon Junyaku Co., Ltd. Tokyo. "Rheologic®, Junlon" undated technical document.

Packer H.L., Belmar Campero M.T., Thomas C.R. "Morphological measurements of filamentous microorganisms by image analysis" in 4th Int. Congr. on Computer Applications in Ferm. Tech. 25-29 Sept 1988 Cambridge U.K. for Soc.Chem.Ind.

Pirt S.J., Callow D.S. "Continuous flow culture of the filamentous mould *P.chrysogenum* and the control of its morphology" Nature **184** 307 (1959)

Rehacek J., Beran K., Bicik V. "Disintegration of microorganisms and preparation of yeast cell walls in a new type of disintegrator" Appl.Microbiol. **17** 462-466 (1969)

Rehacek J., Schaefer J. "Disintegration of microorganisms in an industrial horizontal mill of novel design" Biotechnol. Bioeng. **19** 1523-1534 (1977)

Reuss M., Fröhlich S., Kramer B., Messerschmidt K., Pommerening G., "Coupling of microbial kinetics and oxygen transfer for analysis and optimization of gluconic acid production with *Aspergillus niger*" Bioprocess Engineering **1** 79-91 (1986)

Rosenberg M., Balaz S., Sturdik E., Skarka B. "Modelling cellular oxidation of D-glucose to D-gluconic acid" in Interbiotech '87 Enzyme Technologies, Proc. Int. Symp. Biotech. (Progress in Biotech. 4) edited by Blazej A., Zemek J. (1987) 523-535

Righelato R.C., Trinci A.P.J., Pirt S.J., Peat A. "The influence of maintenance energy and growth rate on the microbial activity, morphology and conidation of *P.chrysogenum*" J. Gen Microbiol **50** 399-412 (1968)

Robyt J.F., White B.J. "Biochemical Techniques : Theory and Practice" Brooks/Cole Publications Co., Monterrey, Calif. USA (1987)

Roels J.A., Van Der Berg J, Voncken R M "The rheology of mycelial broths" Biotechnol Bioeng **26** 181-208 (1974)

Russell A.J. PhD Thesis University of London "The production and purification of salicylate hydroxylase from *Pseudomonad*" (1980)

Sauer T., Robinson C.W., Glick B.R. "Disruption of native and recombinant *Escherichia coli* in a high-pressure homogenizer" Biotech. Bioeng. **33** 1330-1342 (1989)

Sanchez-Ruiz S.A. "Studies on cell disruption and cell debris removal in downstream processing" MSc. Thesis, Massachusetts University of Technology (1989)

Scawen M D, Atkinson A, Darbyshire J "Large Scale enzyme purification" in J Applied Protein Chemistry 281-325 (Grant R.A. - ed) Applied Science Publishers London (1980)

Schutte H, Kroner K H, Hustedt H, Kula M R, "Experience with a 20 litre industrial bead mill for the disruption of microorganisms" Enzyme Microb Technol **5** 143-149 (1983)

Sodeck G, Modl J, Kominek J, Salzbrunn W "Production of citric acid according to the submerged fermentation process" Proc. Biochem **16** (6) (1981)

Steel R., Lenz C., Martin S.M. "A standard inoculum for citric acid production in submerged culture" Can. J. Microbiol. **1** 150-157 (1954)

Su Z G, Kleizen H H, Van Brakel J, Wesselingh J A "Influence of cell disruption on filtration of yeast homogenate" in Proc 4th Eur. Congr on Biotech **2** 554-557 (1987) (O M Neijssel, R R Van der Meer & K.Ch.A.M. Lubven - eds) Elsevier Science Publishers

Takahashi J., et al J. Agr. Chem. Soc. (Japan) **32** 501 (1958)

Talboys B.L., Dunnnil P. "Effect of shear on membrane-associated enzymes: studies of the release of intracellular protein and of the progesterone 11-α-hydroxylase complex from *Rhizopus nigricans*" Biotech. Bioeng. **27** 1730-1734 (1985)

Thomas V. "Disruption of a filamentous fungi sing a high pressure homogenisation : release of a membrane bound hydroxylation complex" PhD Thesis, University of London, (1988)

Traeger M., Qazi G.N., Onken U., Chopra C.L. "Contribution of endo- and exocellular glucose oxidase to gluconic acid production at increased dissolved oxygen concentrations" J. Chem. Tech. Biotechnol. **50** 1-11 (1991)

Trinci A.P.J., "Effect of Junlon on morphology of *Aspergillus niger* and its use in making turbidity measurements of fungal growth" Trans. Br. mycol. Soc. **81**, (2) 408-412 (1983)

Trinci A.P.J. "Kinetics of the growth of mycelial pellets of *Aspergillus nidulans*" Arch. Mikrobiol. **73**, 353-367 (1970)

Van Brunt J., "Fungi: the perfect hosts?" Bio/technology **4** (12) (1986)

Van Dijken J.P., Veenhuis M., "Cytochemical localization of glucose oxidase in peroxisomes of *Aspergillus niger*" Appl. Microbiol. Biotechnol. **9** 275-283 (1980)

Van Suijdam J C, Metz B "Influence of engineering variables upon the morphology of filamentous moulds" Biotechnol. Bioeng. **23** 111-148 (1981)

Wang D.I.C., Cooney C.L., Dunnill P., Humphrey A.E., Lilly M.D., "Fermentation and Enzyme Technology" J. Wiley & Sons, New York (1979)

Whitworth D.A. "Hydrocarbon fermentation: protein and enzyme solubilization from *C. lipolytica* using an industrial homogenizer" Biotech. Bioeng. **16** 1399-1406 (1974)

Wimpenny J W T Proc Biochem **2** (7) 41 (1967)

Woodrow J R, Quirk A V "Evaluation of the potential of a bead mill for the release of intracellular bacterial enzymes" Enzyme Microb Technol **4** 385-389 (1982)

Zetelaki K, Vas K. "The role of aeration and agitation in the production of glucose oxidase in submerged culture I" Biotechnol. Bioeng. **10** 45-49 (1968)

Zetelaki K. "The role of aeration and agitation in the production of glucose oxidase in submerged culture II" Biotechnol. Bioeng. **12** 379-397 (1970)

NOMENCLATURE

a	pressure exponent	-
b	pressure exponent	-
c	exponent on number of pass	-
C	solids concentration in centrifuge effluent	g/L
C_o	solids concentration in feed stream	g/L
d	pressure exponent	-
h	gap width in valve unit	m
K	first order disruption rate constant	-
k	disruption rate constant	homogeniser Pa^{-c}, bead mill s^{-1}
k_1	disruption rate constant	MPa^{-a}
k_2	disruption rate constant	MPa^{-b}
L_b	mean length of hyphal branches	m
L_e	length of main hypha or effective length	m
L_e^*	dimensionless effective length	-
L_{hgu}	hyphal growth unit	m
L_s	mean length of segments	m
L_t	total length of all hyphae	m
n	number of branches in a mycelial particle	-
N	number of pass	-
P	pressure	bar or Pa
Q	volumetric flowrate	m^3/h
R	product release at N number of pass	(mg/g dcw) or (IU/g dcw)
R_1	product release at first pass (N=1)	(mg/g dcw) or (IU/g dcw)
R_h	cells disrupted by homogeniser alone	-
R_m	maximum product released	(mg/g dcw) or (IU/g dcw)
R_x	cells disrupted by enzymatic treatment alone	-
R_o	entry zone radius in valve unit	m
R_e	exit zone radius in valve unit	m
S_{eff}	separation efficiency of centrifuge	-
t	time	s or h
x	impact distance in disruption valve unit	m

Greek letters

ρ	density	kg/m^3
ν	kinematic viscosity	m^2/s
μ	dynamic viscosity	Pa s
γ	shear rate	s^{-1}
τ	shear strain/stress	Pa

Abbreviations

ADH	alcohol dehydrogenase	
CMC	carboxymethyl cellulose	
CD	cell disruption valve unit	
CER	carbon dioxide evolution rate	mM/Lh
CR	cell rupture valve unit with coned valve rod	
CRF	cell rupture valve unit with flat valve rod	
dcw	dry cell weight	g or kg
DOT	dissolved oxygen tension	%
DPR	differential product release	
FV	flat valve unit	
G.O.	glucose oxidase	
G6PDH	glucose-6-phosphate dehydrogenase	
Gu-HCl	guanidine hydrochloride	
IU	international unit of enzyme activity	
KE	knife edge valve unit	
OUR	oxygen uptake rate	mM/Lh
RQ	respiratory quotient	
SDS	sodium dodecyl sulphate	
SU	Sarette unit; a measure of glucose oxidase activity as it oxidises glucose. One SU = 10 mm^3 oxygen consumed per minute in the presence of excess oxygen in a phosphate buffer of pH 5.9 containing 3.3% glucose monohydrate, as determined in a Warburg manometer at a temperature of 30°C.	

APPENDIX 1 Effect of Storage of Whole Cells and Homogenate

Preliminary studies of the storage of cells and homogenate and its effects on the protein content and glucose oxidase were made, in order to determine the time schedule for the disruption and subsequent analyses ie. if it was possible to delay the disruption after harvesting or the assays for protein and enzyme after disruption without affecting the results obtained. Wherever mentioned, "buffer" means acetate buffer at pH 5.2.

A 1.1 Whole cell storage

The experiment performed here was to assess any changes in glucose oxidase levels and any effects on homogenisation by storage of cells as fermentation broth (cells and media), as dewatered and washed (by deionised water) cell paste at about 5 % dcw (w/w), and as a concentrated cell paste in acetate buffer pH 5.2 also at 5 % dcw (w/w). It is not possible to just store cells immmediately from the basket centrifuge because the dry weight is usually about 20 % (w/w) leading to difficulties in resuspending again for disruption. Hence the dewatered cells are made moist by addition of water. So, there are essentially three types of storage medium:

1) **Growth media** : cells still in spent media at about 0.6 % dry weight (w/w) ie. straight from fermenter.
2) **Water** : concentrated cell paste 5 % (w/w) (referred to as dewatered cells)
3) **Acetate buffer** : concentrated cell paste 5 % (w/w)

These suspensions were stored in the cold room at 5°C for 24 hours in storage vessels, after which the cells in media were dewatered, washed and resuspended in acetate buffer for disruption; the other two were just resuspended in buffer to the appropriate concentration for disruption. The control batch was cells dewatered, washed and disrupted within 2 hours of harvesting ie. no storage. Cells were grown as control type morphology in the 42 L fermenter.

The disruption suspensions were at the following concentrations (g/L) dcw :
Control = 12.3; Media = 11.1; Dewatered = 12.3; Buffer = 13.0
Homogenisation was carried out at 60 MPa.

Protein release : These results are shown in fig. A1.1. The protein levels have decreased by about 50 % from the control. Substantially the same protein release was obtained after 24 h storage in either buffer, media or as dewatered cells. The drop in available protein over 24 h storage is due to the nature of the Biorad assay and later results (fig. A1.4) using the Folin Lowry assay *(Lowry et al 1951)* show that no such drop exists.

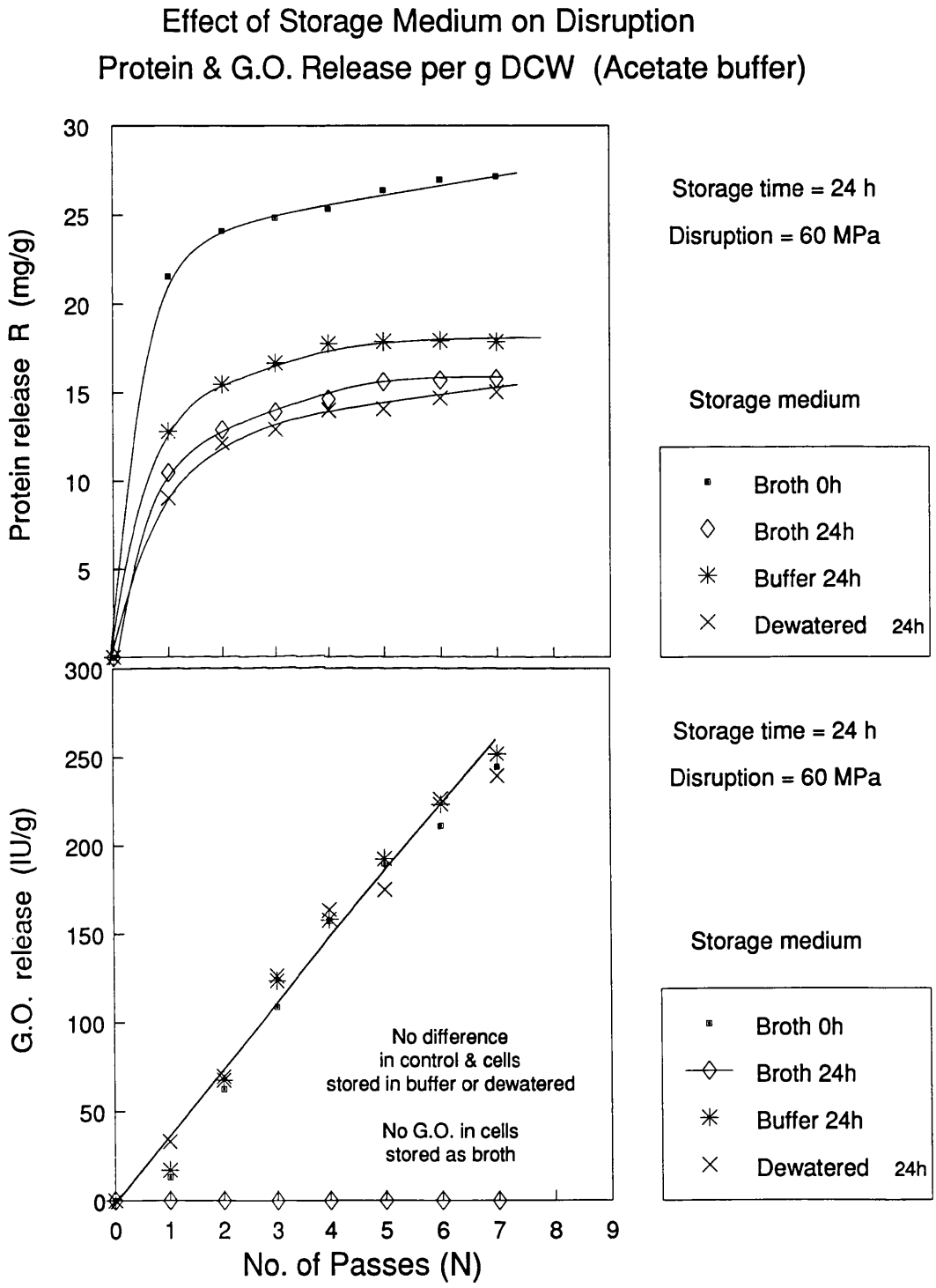


Fig. A1.1 : Protein & Glucose Oxidase release from cells stored in various conditions for 24 hours before disruption (resuspended in acetate buffer)

Glucose oxidase release : Fig. A1.1 shows that there is no difference in release of glucose oxidase between the control and cells stored as concentrated suspensions in water or buffer, but for the cells in media, no activity was detected when the homogenate was assayed on the same day as the disruption. The disruption curve is similar to previous ones in acetate buffer ie. almost a linear release. The strangest anomaly is the nil detection of any glucose oxidase even when protein is being released and the cells are clearly broken up. No glucose oxidase was detected in the media which means that it was not secreted or released through autolysis. Further analysis of this homogenate stored at 5°C resulted in a rise of activity up to the expected maximum within 12 weeks. This will be dealt in the next section.

There was no evident change in cell morphology during storage: they remained filamentous but very much entangled.

A 1.2 Homogenate storage

Before the use of the 30CD homogeniser which operated at pressures higher than 60 MPa, the maximum level of glucose oxidase was never attained at 60 MPa. It was only through the analysis of homogenate stored for several weeks at 5°C which gave activities over double as much as the unstored homogenate, that revealed the enzyme was not being fully released during homogenisation. Two such experiments were carried out :

1) **Homogenate from cells stored in various media :** From the previous section, it was found that no glucose oxidase activity was detected in the homogenate (assayed promptly) from cells stored in fermentation broth. In this experiment the unclarified homogenate (cell debris unseparated) of the seventh pass at 60 MPa from these differently stored cells were kept at 5°C and then assayed for glucose oxidase after 8 and 12 weeks. Fig. A1.2 shows that the enzyme activity in all samples, whether the cells were stored as broth, in water, in buffer or unstored (control), rose to a common value of around 780 IU/g dcw. This is especially surprising for the cells stored as fermentation broth. For the other samples it is plausible to suggest that the enzyme was not fully released at 60 MPa and that on storage, the cell debris gradually decomposed and released the enzyme. The increase from 0 to 860 IU/g dcw for the broth stored cells is difficult to explain. This shows that the glucose oxidase was still present and not denatured. Attempts to determine why the storage of cells as broth resulted in an increased G.O. levels, failed to provide any suggestions. Experiments such as one which spiked the homogenate with a known activity of pure glucose oxidase to test if there was any inhibitory agent proved negative. Therefore, to date this phenomenon remains unexplained.

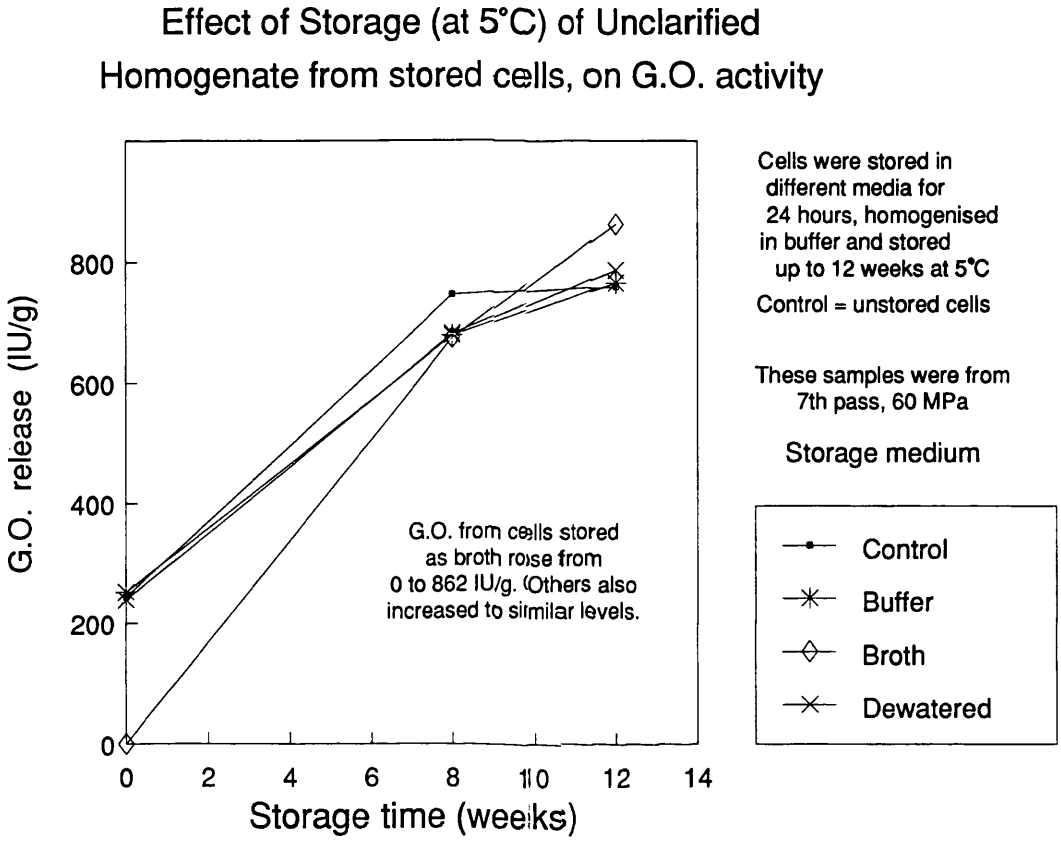
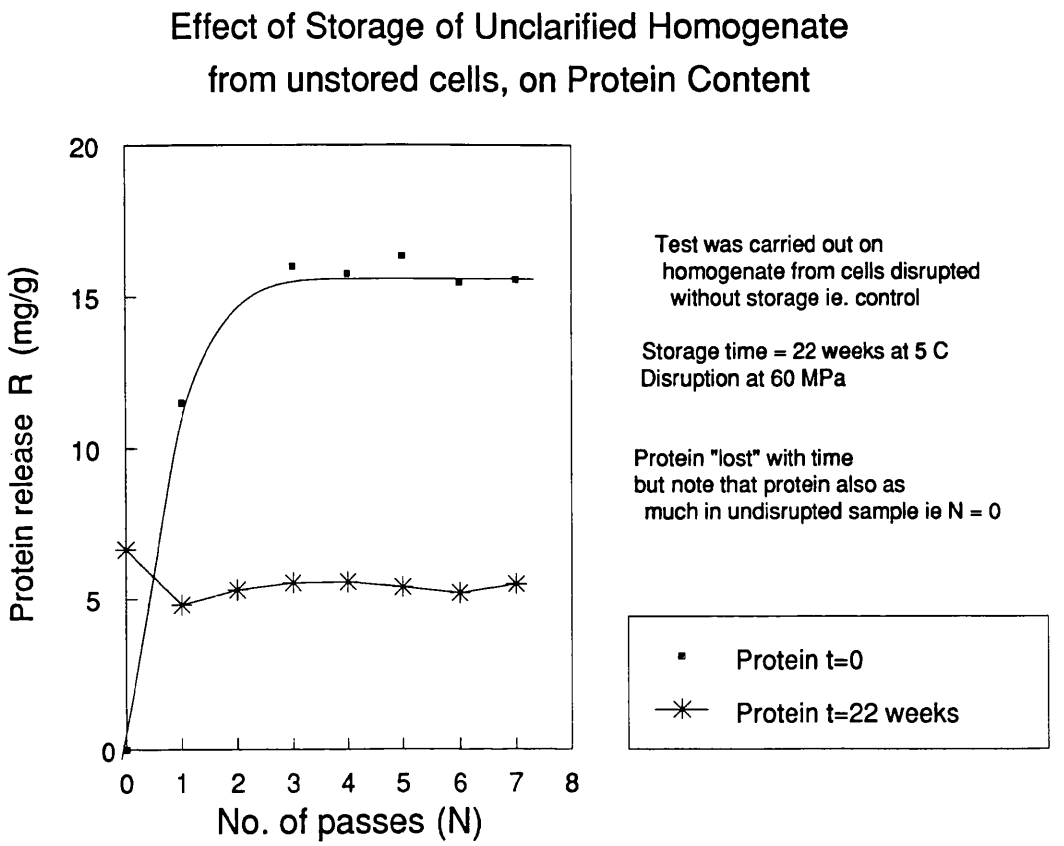


Fig. A1.2 : Changes in glucose oxidase levels upon storage of unclarified homogenate at 5°C (from cells stored under various conditions)

2) **Homogenate of 0-7 pass 60 MPa :** This experiment served to determine if the increased level of glucose oxidase was due to some form of cell decomposition by analysing the enzyme content in all the passes including the undisrupted sample at 60 MPa from stored homogenate of the control ie. cells which were disrupted immediately. Since at each pass there will be varying degrees of disruption, if the enzyme level for all passes (including the undisrupted one) were similar, then it would be fair to deduce that it was being released as the cell structure decomposed. In addition the catalase activity was also analysed, because it is coupled to glucose oxidase as it is necessary to remove the hydrogen peroxide from glucose oxidation and so is also located in the peroxisomes *(Dijken & Veenhuis 1980)*. This other enzyme would serve to support the glucose oxidase results.

From fig. A1.3 the glucose oxidase level at all passes including the undisrupted one has risen to a common level of around 800 IU/g dcw after 22 weeks at 5°C. The highest level with unstored homogenate was 380 IU/g dcw. To support this, the catalase level also increased correspondingly by about 3 fold at the seventh pass and the undisrupted cells also gave similar values. Only the seventh pass sample before storage was assayed for catalase activity but it is sufficient for comparison. Therefore, it is likely that cell decomposition has resulted in release of the enzymes from initially unbroken peroxisomes (assuming from a complete protein release that the cells were all disrupted).

However, the protein content has decreased by 3 fold as detected by the Bio-Rad protein assay *((Bio-Rad Labarotories 1990)* a commercial preparation based on the Bradford method). This protein assay was misleading because in a separate experiment, the protein content stayed almost constant using the Folin-Lowry *(Lowry et al 1951)* method (fig. A1.4). Protein level should be unchanged since all cytoplasmic proteins have been fully released at disruption. If any change should occur, it should be an increase due to the glucose oxidase released from the peroxisomes and not decrease unless protease degradation occurred. In this buffer which precipitates 90 % of the protein, it is unlikely that proteases will be excluded.



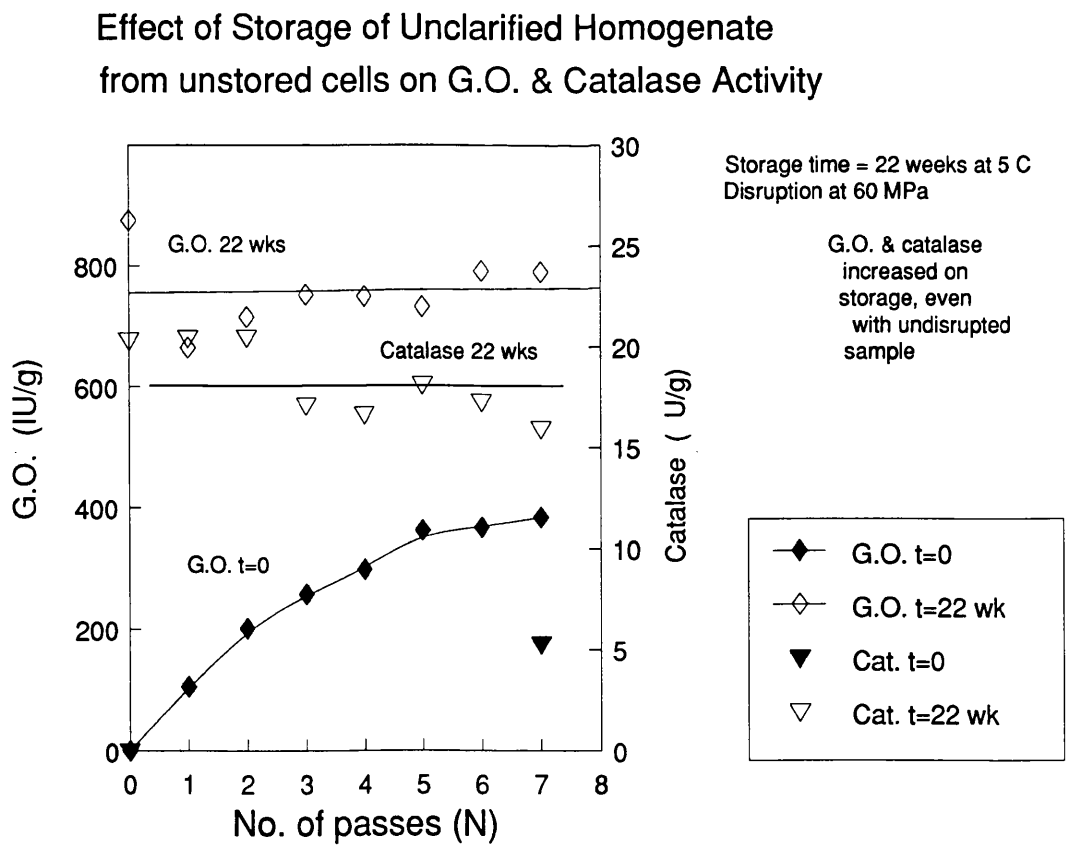


Fig. A1.3 : Changes in protein, glucose oxidase & catalase levels upon storage of unclarified homogenate from all passes at 60 MPa, at 5°C (from unstored cells)

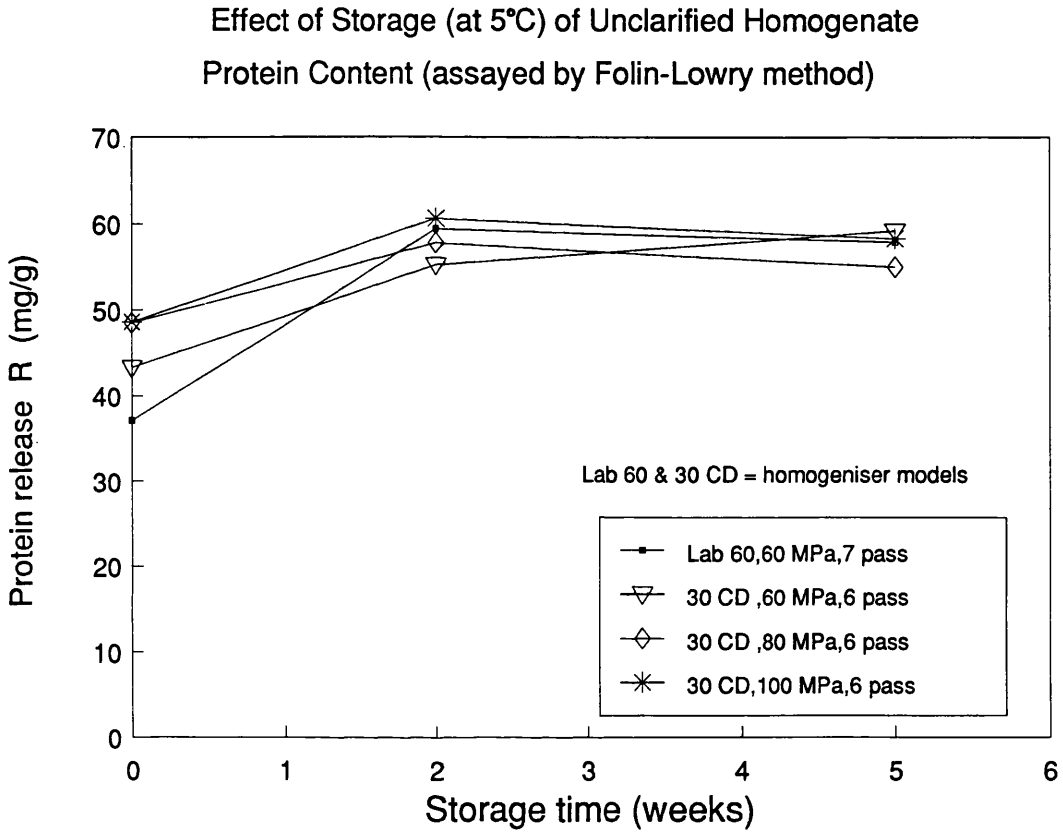


Fig. A1.4 : Changes in protein levels upon storage of unclarified homogenate at 5°C as detected by a different protein assay method (from unstored cells)

APPENDIX 2 A Recombinant Strain of *Aspergillus nidulans* - Fermentation and Bead Mill Disruption

This recombinant strain of *A. nidulans* has been self-cloned to express intracellular alcohol dehydrogenase (ADH) at a higher level by having more copy numbers of the ADH gene. The expression system was induced by the addition of methyl ethyl ketone when glucose is exhausted after the growth period. Work carried out on this strain involved the bead mill disruption and the effects of post-induction time, tip speed of impellers and biomass concentration on the disruption.

Due to the short period of time available for these experiments, it was not possible to repeat experiments to consolidate the results obtained. Consequently, the results may not be consistent. Nevertheless, it provides preliminary information from which further work can be based upon.

A2.1 Fermentation

All fermentation batches were grown according to the standard protocol as described in section 2.2. A typical profile of the fermentation is shown in fig. A2.1. The cells grow to about 4.5 g/L (dcw) in about 25 hours, using up the glucose in this period. Methyl ethyl ketone (MEK) was then added on glucose exhaustion to induce the expression of ADH (32 hours in this case). After induction, growth as expected slows down considerably with cell concentration creeping up to about 6 g/L by 50 hours which may be caused by growth on the fructose added with the MEK. The cell concentration starts to drop after 50 hours due to cell death and autolysis thereby resulting in decreasing dry weight since any small cell particles will be washed away through the glass fibre filter used for dry weight determination.

The morphology of the cells were similar to the control type for the *A. niger* strain : filamentous but entangled and clumped.

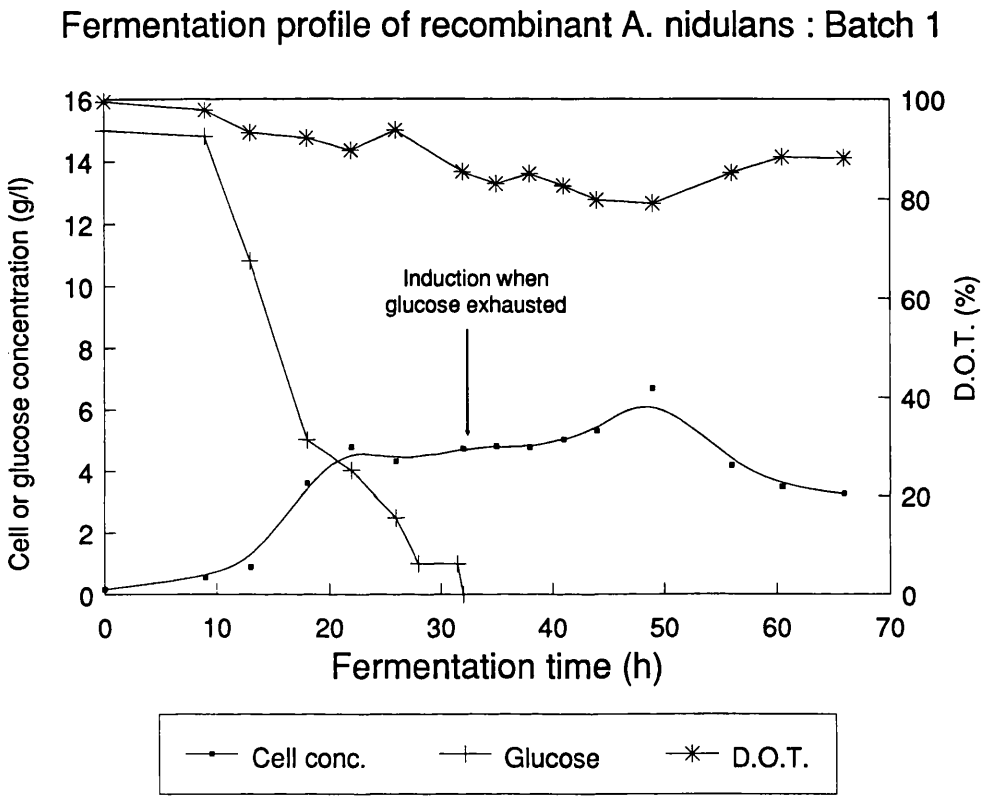


Fig. A2.1 : Fermentation profile of a recombinant *A. nidulans* - batch 1 & 2

A2.2 Bead Mill Disruption

The bead mill used was a 0.6 L glass disruption chamber fitted with one polyurethane impeller capable of operating at a tip speed of 6.7, 10, 15 and 20 m/s. The beads used were 0.5 mm diameter ballotini beads. Operation was on a batch mode, and cooling was by chilled water at 2.5°C. A few variables were examined :

1) To determine if there is any difference in disrupting cells at various post-induction times. This may show up a particular period where cells are most susceptible to breakage.
2) The dependence of disruption on tip speed.
3) The dependence of disruption on cell concentration.

A2.2.1 The effect of post-induction time

Samples of cells were removed at various post-induction times and disrupted for up to 10 minutes at a tip speed of 10 m/s. Two identical batches were grown. The two batches resulted in some variation of the protein and ADH levels between batches and also throughout the post-induction time. The latter is likely to be caused by the changing levels of protein and ADH during the induction period.

Batch 1

Protein release : Fig. A2.2 shows the general trend of a very rapid release of protein between 0.5 and 1 min disruption time rising from 0 to 50-70 mg/g dcw and then a loss of protein with increasing disruption time. This means that the cells are broken completely by 0.5-1 min as indicated by the maximum protein release. The loss of protein during disruption is likely to be caused by shear associated damage because there is considerable entrainment of air during the operation, especially as the volume decreases with sampling allowing more air space in the chamber. This possible cause was suggested by *Narendranathan and Dunnill (1982).* Denatured protein is then precipitated and separated with cell debris during the soluble protein assay.

With respect to post-induction time, there is an increase of protein release from 40 to 65 mg/g during the time from 3 to 12 hours and then is relatively constant at 50-60 mg/g between 17-28 hours, but a sudden drop to 10 mg/g was experienced at 34 hours. Since the cells are fully disrupted in 1 minute, suggesting that they are very easily disrupted, even if there is an effect of post induction time ie a possible variation in cell strength, it may not show up. Therefore, it is more likely that the changes in protein release were due to the rise and fall of protein in the cell. However, the very low level of protein at 34 hours could be caused by autolysis during cell death, thereby losing most of the cell contents.

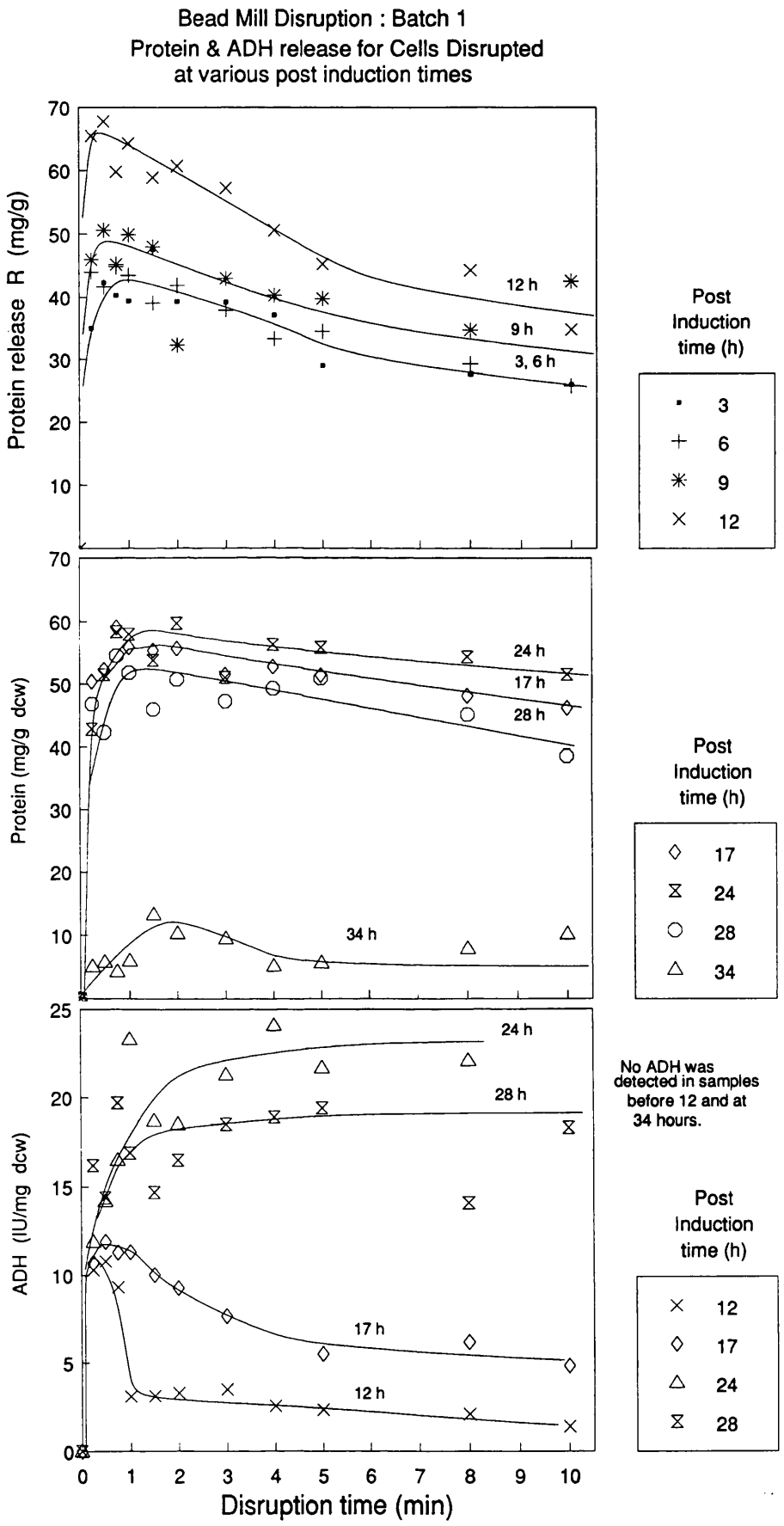


Fig. A2.2 : Protein and Alcohol dehydrogenase release from *A. nidulans* disrupted in a bead mill at various post induction times - **batch 1**

ADH release : No ADH was detected for the 3, 6, 9 and 34 hour samples. ADH release is also shown in fig. A2.2. These are entirely different from the protein release : there is a considerable difference in ADH values. Nevertheless it still appears that the peak is achieved by 1 minute. Only in the 17 and 12 hour run, the ADH activity falls after 1 minute of disruption; the 24 and 28 hour ones remain relatively stable. Again, the considerable variations may be due to changing ADH levels during fermentation, rather than any effect of stronger cell walls.

Batch 2

Protein release : The protein levels do not resemble batch 1 (fig. A2.3). There was much greater variation in protein levels over the post-induction time studied : it increased from 15 mg/g to 65 mg/g from 6 to 34 hours; only between 18 and 28 hours the values were similar (40 mg/g). Nevertheless, maximum release of protein was achieved by 1 minute of disruption. In this case, the loss of protein over the disruption period was less.

ADH release : ADH was not detected in the 6, 9, and 12 hour samples. The profiles (fig. A2.4) do not resemble the one from batch 1 : the samples before 34 hours are much lower and the one at 34 hours was about 3 times that of the maximum in batch 1, but once again disruption was completed after 1 minute and there was a decrease in activity after this.

A2.2.2 Effect of tip speed and cell concentration

Two batches from the same fermentation were disrupted at tip speeds from 6.7 - 20 m/s at a concentration of about 7 and 11 g/L cell concentration. Another one was done at 10 m/s with cell concentrations from 7.4 to 32 g/L. All 3 batches were harvested at 17 hours after induction, where the ADH productivity is at its maximum (as recommended by Allelix Inc.). The results will show that there was negligible dependence of disruption on tip speed nor cell concentration: the cells were so fragile that in all case 100 % disruption was achieved after one minute of disruption.

Tip speed change : 7 g/L dcw

Fig. A2.5 shows that the protein release reaches a maximum of about 90 mg/g dcw in less than 1 minute for all tip speeds, but the amount of protein loss for longer periods increase with increasing tip speed. This would seem logical as the higher tip speed would create more turbulence and higher shear, resulting in greater loss. The ADH release (fig. A2.6) is similar : maximum release (45 - 50 mg/g) in about 1.5 minutes and then loss of activity, but the decrease did not increase with tip speed. However, there is more scatter of data. The important point is that maximum release is obtained at the same time for all tip speeds.

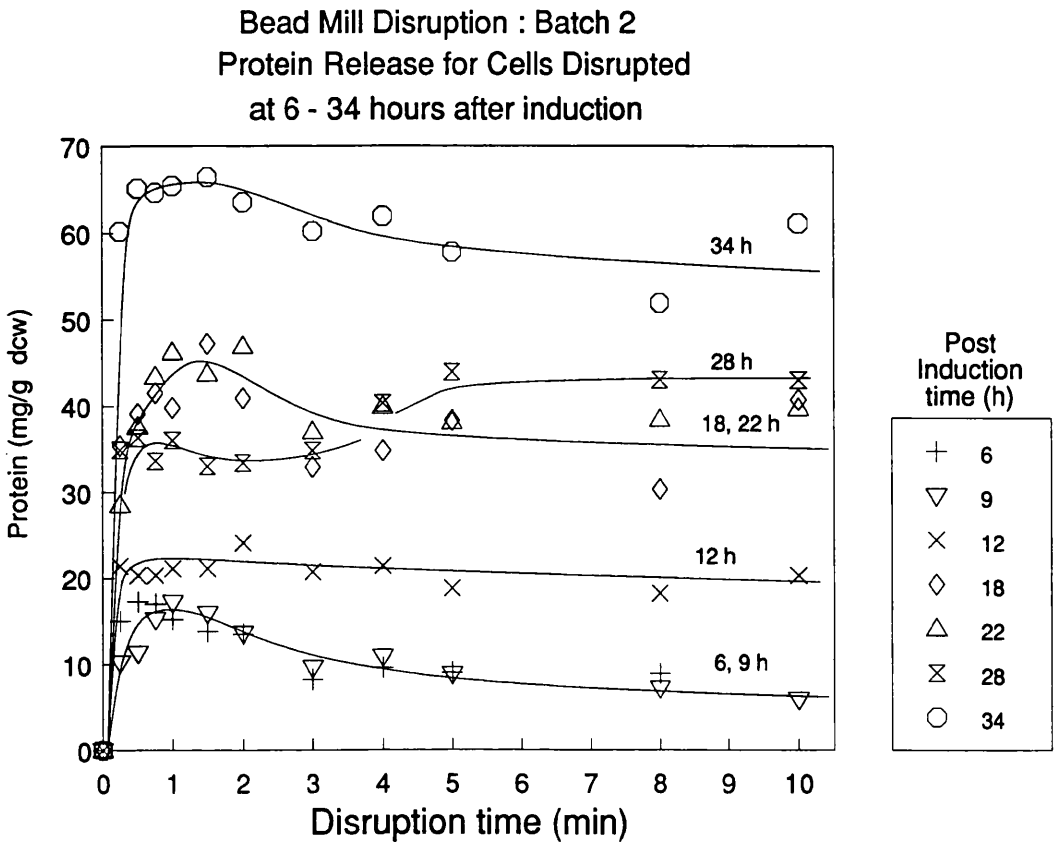


Fig. A2.3 : Protein release from *A. nidulans* disrupted in a bead mill at various post induction times - **batch 2**

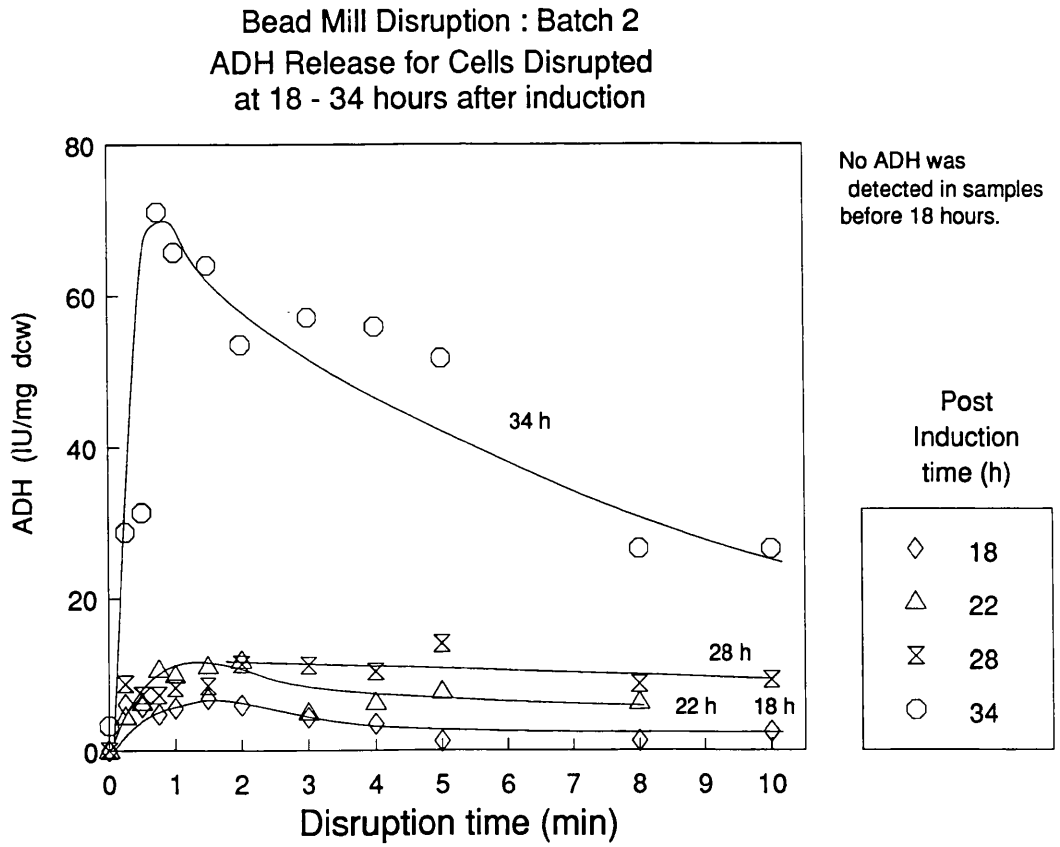


Fig. A2.4 : Alcohol dehydrogenase release from *A. nidulans* disrupted in a bead mill at various post induction times - **batch 2**

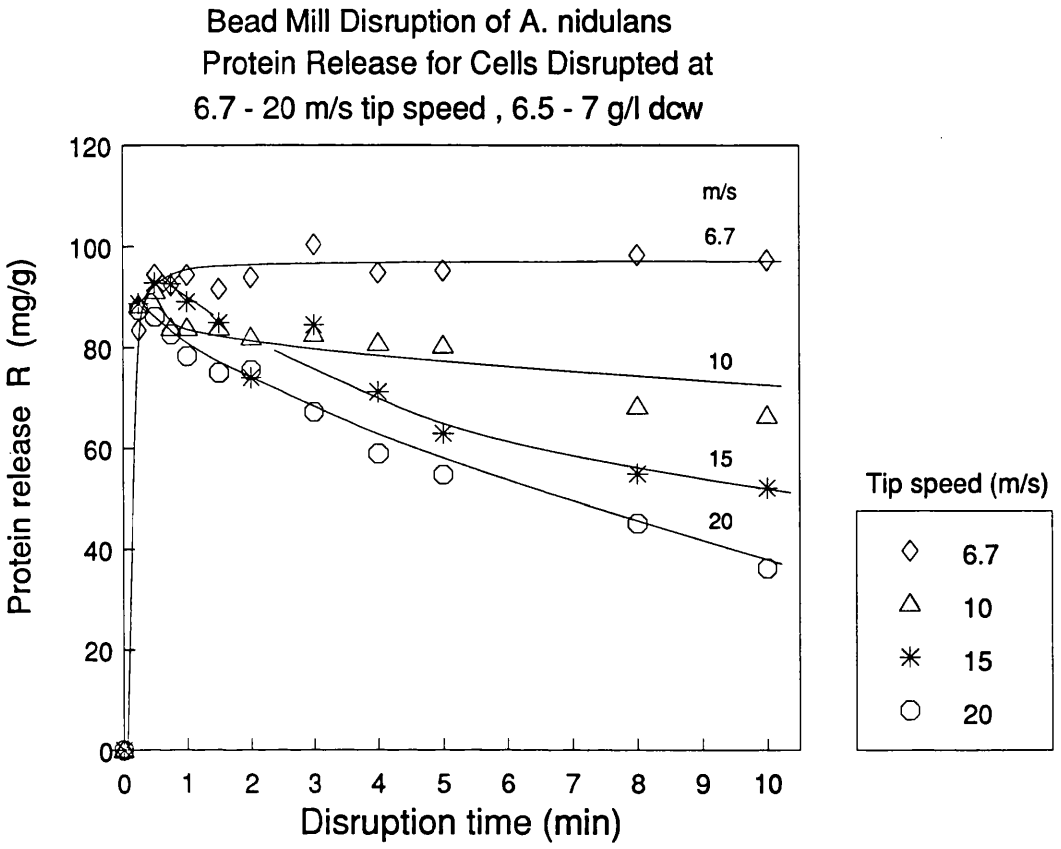


Fig. A2.5 : Protein release from *A. nidulans* disrupted in a bead mill at various tip speeds - **at 6-7 g/l dcw**

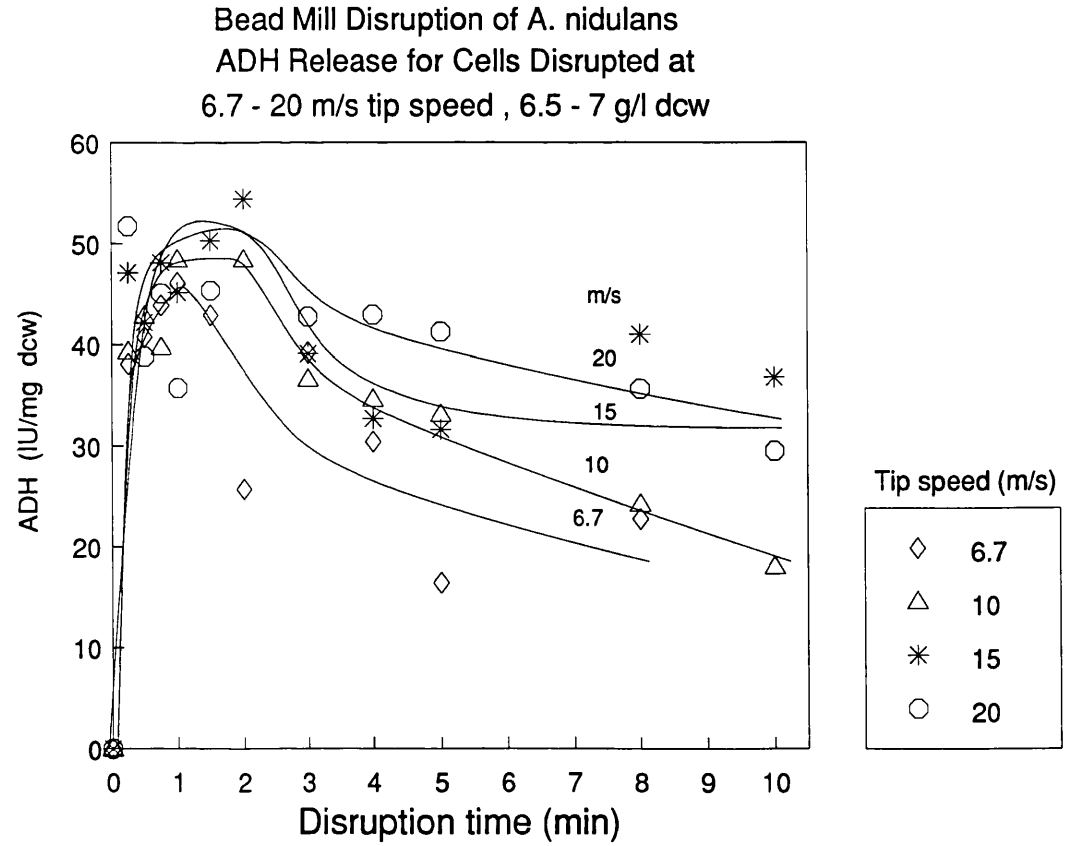


Fig. A2.6 : Alcohol dehydrogenase release from *A. nidulans* disrupted in a bead mill at various tip speeds - **at 6-7 g/l dcw**

Tip speed change : 11-12 g/L dcw

Protein and ADH results in fig. A2.7 & A2.8 show the same trend as observed for the less concentrated run. Protein values are similar, between 80 - 100 mg/g dcw but ADH values are higher at 80 IU/g dcw compared to 50 IU/g for the batch at 7 g/L. In this case the decline in both protein and ADH levels do not follow any trend with respect to the tip speed.

Cell concentration change : tip speed 10 m/s

In this fermentation no ADH was detected. Only protein release is shown in fig. A2.9. There appears to be a slight dependence of protein release on cell concentration : about 1.4 times more was released by the 35 g/L batch compared to the lowest at 7.4 g/L. Between 13.8 and 35 g/L there was only a small variation.

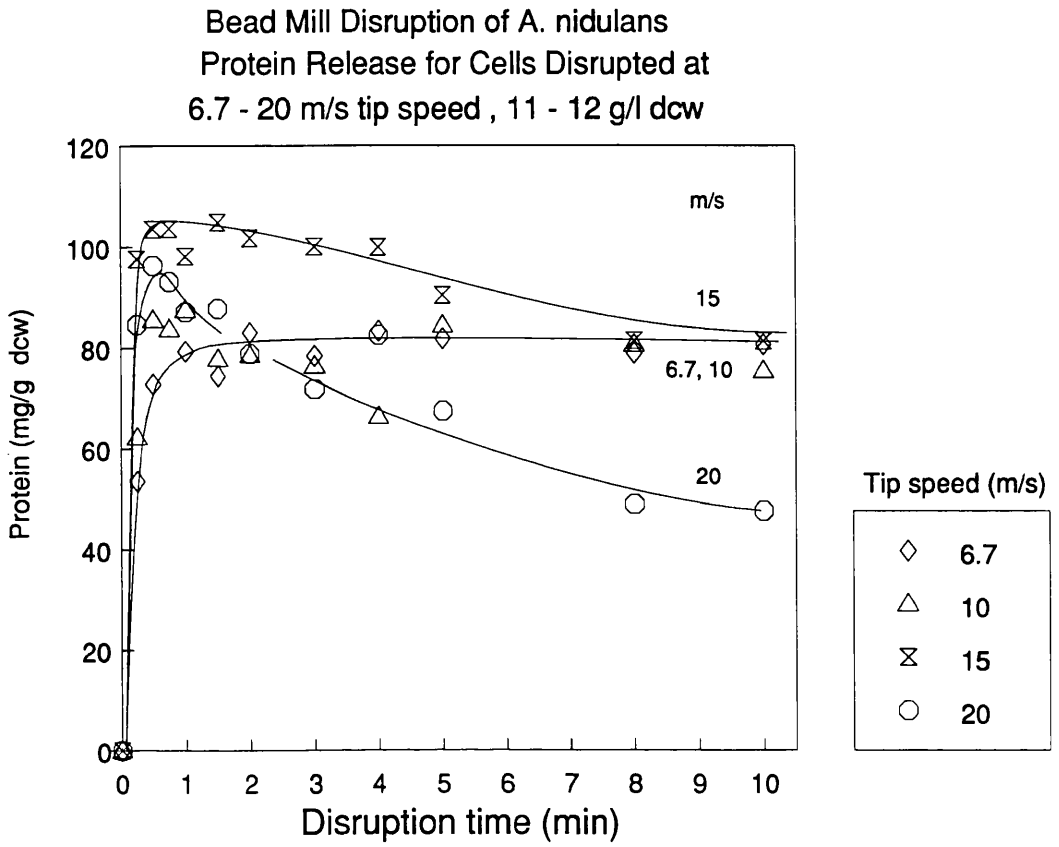


Fig. A2.7 : Protein release from *A. nidulans* disrupted in a bead mill at various tip speeds - **at 11-12 g/l dcw**

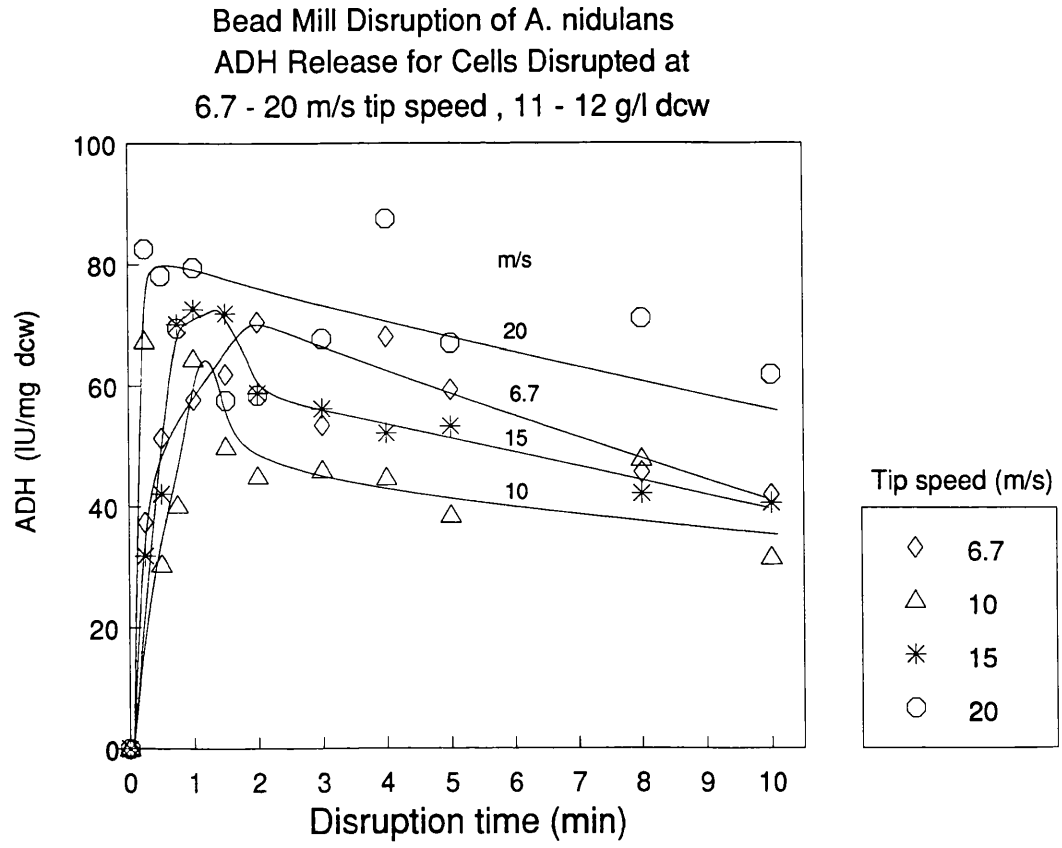


Fig. A2.8 : Alcohol dehydrogenase release from *A. nidulans* disrupted in a bead mill at various tip speeds - **at 11-12 g/l dcw**

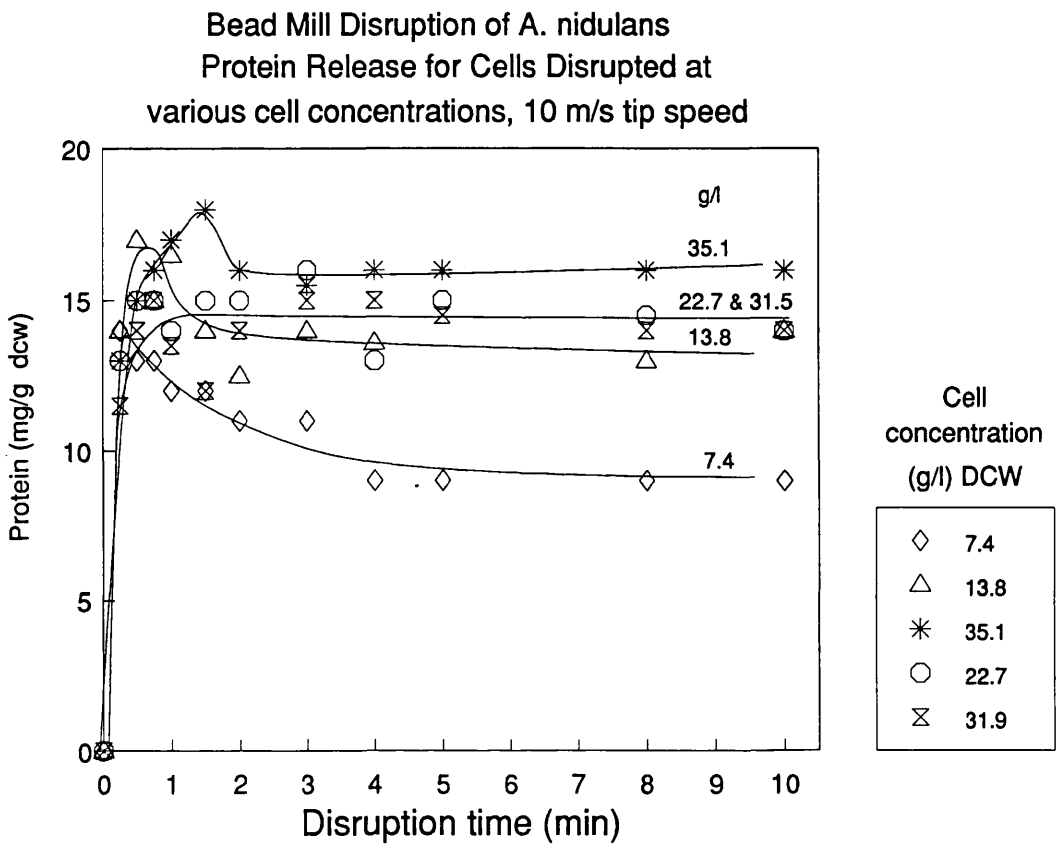


Fig. A2.9 : Protein release from *A. nidulans* disrupted in a bead mill at various cell concentrations

APPENDIX 3

Estimation of gap width between the valve rod and the valve seat in the Lab 60 homogeniser.

The gap width h, between the valve rod and the valve seat (as shown in fig. 2.6) decreases with increasing pressure. An estimation of this gap width would enable a comparison of its size with the microorganism. *Keshavarz et al (1990b)* developed a Bernoulli type expression and used it to calculate the gap width. This equation can be used to estimate the gap width h, for the Lab60 homogeniser:

$$P = \left[\frac{\rho}{4}\right]\left[\frac{Q}{2\pi.R_o.h}\right]^2 + \frac{5\,\rho\,\nu^{3/5}}{h^3}\cdot\left[\frac{Q}{2\pi}\right]^{7/5}\cdot\left[\frac{1}{R_o^{2/5}} - \frac{1}{R_e^{2/5}}\right] + \left[\frac{\rho}{2}\right]\left[\frac{Q}{2\pi.R_e.h}\right]^2$$

where	ρ	= density	(kg/m^3)
	Q	= flowrate	(m^3/s)
	ν	= kinematic viscosity = μ/ρ	(m^2/s)
	μ	= dynamic viscosity	(Pa.s)
	R_o	= entry zone radius (fig. A3.1)	(m)
	R_e	= exit zone radius (fig. A3.1)	(m)
	h	= gap width	(m)

In using this equation, some assumptions have been made:

1) $\mu = 5 \times 10^{-3}$

2) $\rho = 1000\ kg\ m^{-3}$

The dynamic viscosity μ, of a fungal suspension is obviously dependent on the shear stress applied (as it is non-Newtonian). In the case of a fungal pellet, the viscosity of the actual pellet is clearly unknown. Therefore no realistic value can be used. Instead the value of 5×10^{-3} Pa.s, as used by Keshavarz et al (1990b) is employed, and so, strictly speaking, the gap width estimated is for a 45% w/v suspension of baker's yeast. The entry and exit zone radii, R_o and R_e respectively, are also obtained from Keshavarz et al (1990b). R_e is calculated from R_o = 3.85 mm, and $R_e - R_o$ = 0.5 mm. In the Lab 60, the flowrate varies with pressure, as mentioned in section 2.4.2.2.. The estimated gap widths for the Lab 60 homogeniser are therefore speculative. These are tabulated in table A3.1.

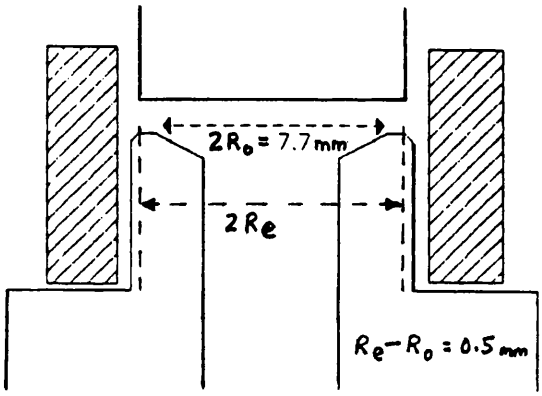


Fig. A3.1 : Cell disruption type valve unit showing entry and exit radii (R_o and R_e)

Pressure (MPa)	Gap width (μm)
10	15.5
20	12.0
30	10.4
40	9.1
50	8.3
60	7.5

Table A3.1 : Estimated gap widths for Lab 60 homogeniser.