STUDIES ON THE GROWTH AND PRODUCTION OF AMYLOLYTIC ENZYMES BY *ASPERGILLUS* spp.

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ABSTRACT

The growth and production of α-amylase and amyloglucosidase by Aspergillus awamori NRRL 3112 and A. niger CBS 26265 were investigated, with a view to obtaining optimal levels of both these enzymes in shake flask culture, and subsequently on the large scale.

The effect of various parameters, such as carbon source, nitrogen source, presence/absence of buffering agents, carbon:nitrogen ratio, pH values, and time were examined.

Following the screening of ten strains of Aspergilli, A. awamori NRRL 3112 was found to be the superior amyloglucosidase producing strain, while A. niger CBS 26265, produced higher levels of α-amylase. The favoured nitrogen source for amylolytic enzyme production was corn steep powder, which had a strong buffering effect on the medium. Starch or its breakdown products stimulated α-amylase and amyloglucosidase expression as well as secretion. α-Amylase production was more sensitive to pH than amyloglucosidase. The former showed peak production after 24-48 hours while the rate of amyloglucosidase synthesis was linear for 6-7 days.

The fermentation was scaled up to 5 and 10 l laboratory scale fermenters. The effects of various agitation and aeration rates were examined. Oxygen transfer rate (OTR) or oxygen transfer coefficient ($K_L a$) was found to have a large effect on biomass and enzyme yields, with the conditions for maximal amylase production corresponding to suboptimal conditions for Aspergillus growth.

Controlling pH at 4.5 or 5.5 increased α-amylase production, but had little effect on amyloglucosidase yields. The average doubling time of A. awamori NRRL 3112 varied with the medium pH. Minimum values were recorded at pHi 4.1.

The specific growth rate of this filamentous organism reached a maximum at 12 - 16 hours, at 0.085-0.377 h^{-1}. This corresponded with the period of greatest change in pH.

It is concluded that A. awamori NRRL 3112 is a superior producer of amyloglucosidase, and is most suitable for large scale production of amylolytic enzymes.

SECTION 1 INTRODUCTION

1.1 The Genus Aspergillus

The Aspergilli have always been a factor in man's environment, but prior to the development of the microscope, they were brushed aside as white, yellow, green, red or black moulds without a serious attempt at interpretation. Micheli (1729) was the first to distinguish stalks and spore heads. He noted that the spore chains or columns radiated from a central structure to produce a pattern that suggested the aspergillum (mop for distributing holy water), with which he, as a priest, was familiar; hence he gave the name Aspergillus to the moulds he observed.

Aspergilli form a large proportion of all the moulds encountered in industrial processes. They are to be found almost everywhere and on every conceivable substratum. Their activities are not entirely destructive in nature, and several species have had their fermentative powers harnessed for commercial purposes.

In the overall classification of fungi, the Aspergilli fall together in a group called the Fungi Imperfecti. These fungi produce neither ascospores nor basidiospores, but reproduce solely by means of conidia, i.e. they only have an "imperfect" stage in their life cycle. Most of this grouping are probably Ascomycetes which produce the perfect stage (ascospores) only under special conditions which have not yet been discovered, or which have entirely lost the power of producing asci. (In a few cases, the perfect stage of Aspergillus spp. have been found, and are usually called Eurotium spp).

Since few Aspergilli form sexual (or perfect) stages, the basis for identifying the majority of isolates is found in the character of their colonies and in details of the morphology of their spore bearing structures. This is different from all other fungal groups in which classification is based on the morphology of the sexual stage.

In general, the submerged mycelia are septate, and colourless or lightly coloured. The fertile stalks arise perpendicularly from the mycelium and terminating in a

vesicle, which bears phialides or metulae; conidia successively cut off from the continuously elongating tips of the phialides. These spores have various shapes and colours depending on the species (Smith, 1969; Roper and Fennell, 1965).

Black Aspergilli are of very common occurrence. Their colonies spread very rapidly; mycelia are white initially, but frequently develop areas of bright yellow. Conidia are usually dark brown or black, which gives the name to this group - Aspergillus niger (Lat. niger, black). The two organisms used for most of this study, A. niger CBS 26265 and A. awamori NRRL 3112, are part of this group.

1.1.1 Diversity of Extracellular Products of Aspergilli

Filamentous fungi are very diligent producers of a wide range of biochemical products. These can roughly be divided into three groups - primary and secondary metabolites, and enzymes. Primary metabolites are generally simple chemicals, involved in normal cell metabolism e.g. organic acids, simple sugars, amino acids. Industrially, the most important primary products of Aspergilli are organic acids, principally citric (A. niger), itaconic (A. terreus), oxalic and gluconic (Rehm and Reid, 1983; Abou-Zeid and Ashy, 1984).

Filamentous fungi produce numerous extracellular enzymes, many of which are produced on a commercial basis by Aspergilli e.g. α-amylase, amyloglucosidase, pectinases and proteases (Godfrey and Reichelt, 1983). Aspergilli have proved particularly successful due to their ability to secrete proteins. Van Brunt (1986) suggested that A. niger could produce up to 20g of amyloglucosidase per 1 of medium. High levels of extracellular proteins which are biologically active mean that recovery and eventual purification are relatively simple processes.

When one views the remarkably wide range of synthetic capabilities of Aspergilli, it would seem almost inevitable that some poisonous compounds would be formed. Aflatoxins are a closely related group of secondary metabolites, produced by organisms in the A. flavus/oryzae group, which produce greenish-yellow spores. These toxins can be fatal in animals and man in quantities as small as parts per billion (ppb) (Wyllie and Morehouse, 1977,1978). For this reason,

members of the A. niger family, which do not produce these compounds, are the favoured source of enzymes, particularly those used in food processing. (Only A. niger and closely related species have been used in this study).

1.2 Basic Biochemical Pathways of Aspergilli

There seems little doubt that the Embden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate sequence, and the tricarboxylic acid cycle pathways occur in many fungi (Burnett, 1976). Exogenously supplied carbohydrates are utilised by many fungi, but they are usually broken down to monosaccharides by extracellular enzymes before being transported into the hyphae. For example, Aspergilli can produce α-amylase, β-amylase, α-glucosidase, amyloglucosidase, cellulose, βglucosidase, laminarinase, α-1.6 glucan hydrolase, invertase, β -1.4 xylanase, endo-polymethylgalacturonase, exo-polygalacturonase and many more, therefore many types of polymers can be attacked.

Much of the carbohydrate transferred into the mycelium is in the form of glucose, but the presence of free glucose is difficult to demonstrate in the hyphae. This is because it is phosphorylated by hexokinase, an intracellular enzyme operating near the membrane, to glucose-6-phosphate. This compound is the starting point for the EMP pathway of glycolysis. Alternative pathways for glucose-6-phosphate include the pentose phosphate pathway (common in many fungi including A. niger, Penicillium chrysogenum and Rhizopus sp.) and the Entner-Doudoroff sequence (mainly found in bacteria and perhaps a few fungi including A. flavus-oryzae) (Blumenthal, 1965). Various authors (Ng et al, 1972, Carter and Bull, 1969) have shown that the pentose phosphate pathway replaces the EMP pathway during conidiation. This explains why primary products such as citric acid are not produced initially, but only when the spores have germinated and the mycelium is growing. In the final degradation, pyruvate is dissimilated, usually aerobically, via the TCA or glyoxylate cycles, to CO_2. Synthesis and accumulation of acids, for which Aspergilli are well known, can readily be explained through basic biochemical pathways.

1.3 Nature of Amylolytic Enzymes

Starch is a high molecular weight polymer of D-glucose, which occurs in the form of water insoluble granules, as the major reserve carbohydrate in all higher plants. It consists of two polymers: amylose, an unbranched chain-like molecule containing only α-1.4 glucosidic links, and amylopectin which has a backbone of α-1.4 links, but contains 3-5% branching linkages (α-1.6 glucosidic bonds).

Various amylases have been found to use starch as a substrate. Table 1.1 reviews the properties of these amylolytic enzymes is given under the headings of type of reaction, physical changes brought about by the enzyme and products of the reaction; this summarises the work of a number of authors.

1.3.1 α-Amylase - the Nature of α-Amylase

α-Amylase (α- 1.4 - D - glucanohydrolase, E.C. 3.2.1.1) belongs to the class of amylolytic enzymes known as dextrinogenic amylase or endoamylases. It is widely distributed in animals, plants and microorganisms. It hydrolyses the α-1.4 - glucosidic bonds in amylase and amylopectin in an endo fashion (i.e. randomly) but the α-1.6 links are resistant to attack. The α-1.4 bonds situated terminally in the molecule are less susceptible to cleavage than those situated centrally.

α-Amylases are unable to hydrolyse maltose, but can attack higher molecular weight oligosaccharides of the same homologous series. Thus the action of this enzyme on amylose gives maltose and small amounts of glucose and maltotriose, while action on amylopectin yields maltose and α-limit dextrins (glucose polymers which contain at least one α-1.6 glucosidic link). Radio tracer studies have shown that the point of cleavage in the hydrolysis of α-1.4 glucosidic bonds by α-amylase is the carbon - 1 - oxygen bridge.

The mode of action of fungal α-amylases on starch can be characterised by four changes in the substrate.

A. Increase in free reducing power - the liberation of reducing aldehyde groups due to hydrolytic cleavage results

Table 1.1 Summary of Starch Degrading Enzymes

	α-Amylase	β-Amylase	AG*	α-Glucosidase	Debranching Enzymes
Produced by	B,Y,M	B,P	M	B.M,Y	B
EC Number	3.2.1.1	3.2.1.2	3.2.1.3	3.2.1.2	3.2.1.68 3.2.1.41
Hydrolyse α-1,4	Yes	Yes	Yes	Yes	No
Hydrolyse α-1,3	No	No	Yes	Yes	
Hydrolyse α-1,6	No	No	Yes	Yes	Yes
Bypass α-1,6	Yes	No	Weakly Bond Cleaved	Weakly Bond Cleaved	Bond Cleaved
Mode of attack	Endo	Exo alternate bonds	Exo successive bonds	-	Endo (Mainly)
Configuration of end product	α	β	β	α	-
Viscosity reduction	Fast	Slow	Slow	-	-
Reducing sugar production rate	Slow	Fast	Fast	-	-
Decrease in Iodine staining power	Fast	Slow	Slow	-	(Increases)
Side reactions	No	No	Reversion (also TG)	Substitution	-
Rate of Hydrolysis with Increasing mw	-	-	Increase (up to G_5)	Decrease	-
Hydrolysis of :					
glycogen	G_1 G_2 +limit dextrins	G_1	G_1	No	Yes
amylose	G_1 G_2	G_1	G_1	No	-
amylopectin	G_1 G_2 +limit dextrins	G_2+limit dextrins	G_1	No	Yes
maltose	No	No	No	G_1	-

* Amyloglucosidase

1. B = bacteria, M = moulds, Y = yeasts, P = plants.
2. Viscosity reduction with respect to number of bonds hydrolysed.
3. mw = molecular weight
4. G = glucose, G_1 = maltose, G_2 = Maltotriose, G_3 = Maltopentose.
5. Officially α-glucosidase is not a "starch degrading" enzyme but it is useful to compare its properties with those of amyloglucosidase
6. Enzyme acts at the non-reducing end of the substrate.
7. Transglucosidase = TG.

in increased reducing power, which can be measured using the Somogyi-Nelson or dinitrosalicylic acid methods.

B. Changes in iodine staining properties - the maximum absorption of light of the iodine complex of amylose or amylopectin moves rapidly to shorter wavelengths as α-amylase acts on starch, until all iodine-staining properties disappear (Bhella and Altosaar, 1984).

C. Decrease in viscosity - the ability of α-amylase to hydrolyse centrally located α-1.4 glucosidic links enables it to reduce rapidly the viscosity of starch solutions.

D. Changes of optimal rotatory power.

Numerous methods for assaying amylase activity have been described in the literature, and are usually based on one of the phenomena listed above.

α-Amylases can be classified into two types - liquifying and saccharifying. Liquifying amylases, which decrease the viscosity without much hydrolysis of the substrate, are used for 'thinning' of starch solutions. Generally, they are produced by Bacillus spp. with temperature optima of 70-92 °C.

Saccharifying amylases are produced by fungi, including Aspergillus (Aski et al, 1971; Kundu and Das, 1970), Penicillium, Mucor (Adams and Deploey, 1976), Neurospora (Gratzner, 1972) and Trichoderma spp. (Nagai et al, 1976).

Purification of fungal α-amylase has been carried out using a number of techniques including ammonium sulphate precipitation, ion exchange chromatography, starch adsorption techniques, acetone and alcohol precipitation. Recently Bhella and Altosaar (1984) purified α-amylase from A. awamori NRRL 3112 by means of ethanol precipitation, Sephacryl-200 gel filtration and anion-exchange chromatography on Dowex (AGI-X4) resin. The properties of this enzyme were in good agreement with previous results. (Table 1.2).

Table 1.2 Properties of some Aspergillus ∝-amylases.

Organism	pH optimum	temp. optimum	pI	mw ($x10^3$)	Reference
A. awamori NRRL 3112	4.5-6.0	50	4.2	54	Bhella & Altosaar (1984)
A. niger					
acid unstable	5.0-6.0	35	3.75	61	Aria et al (1968)
acid stable	4.0-5.0	50	3.44	58	Ramachandran et al (1978)
A. oryzae	5.5-5.9	40	4.0	52.6	McKelvy & Lee (1969)
				51	Takagi (1981)
					Yakubi et al (1977)

There are no marked similarities in the amino-acid composition of ∝-amylases obtained from fungi as compared with those obtained from bacteria. A. oryzae ∝-amylase (Taka-amylase A) is poor in basic amino acid residues, which might contribute to its acidic nature. A. niger produces two ∝-amylase types: acid-stable and acid-unstable. The acid-unstable enzyme contains twice the number of lysine residues of the acid stable. The acid stable ∝-amylase is rich in free carboxyl groups and therefore has a lower isoelectric point of 3.44. The carbohydrate content of fungal ∝-amylases is low. (Table 1.3).

Table 1.3 Carbohydrate composition of some fungal α-amylases.

mole/mole of enzyme

Organism	Mannose	Xylose	Hexosamine	
A. oryzae (Taka amylase A)	8	1	2	Hanafusa et al (1955)
A. niger				
acid unstable	7	NR	1	Aria et al
acid stable	24	NR	4	(1968)

NR = Not Recorded

α -Amylases are calcium metalloenzymes with at least one atom of calcium per molecule of enzyme. The strength of the binding of the metal to the protein is dependent upon the source of the enzyme. Furthermore, α-amylases require this element for catalytic activity. In the presence of calcium they are quite resistant to extremes of temperature, pH, treatment with urea or exposure to proteases. The acid-stable and acid-unstable α-amylases of A. niger contain 1 gramme-atom of calcium which may be removed by chelating with EDTA (ethylenediaminetetra-acetic acid) (Aria et al, 1969). Partial restoration of activity occurs on readdition of calcium. Magnesium, strontium and barium can replace calcium in Taka amylase A without causing appreciable change in amylase activity.

Although α-amylase is expressed extracellularly, in some cases it has been detected intracellularly or bound to the cell membrane (Fogarty, 1983). Yabuki et al (1977) showed that between 5 and 19% of the total α-amylase produced by A. oryzae was detected intracellularly, and that intra and extra cellular levels increased in tandem with biomass.

1.3.2 Amyloglucosidase: The Nature of Amyloglucosidase

Amyloglucosidase (E.C. 3.2.1.3, glucamylase, α-1.4 - D - glucan glucohydrolase, γ -amylase or AG) is an exoacting enzyme which cleaves β -D-glucose molecules from the

non-reducing end of amylose, amylopectin and glycogen, by hydrolysing α-1.4 links in a consecutive manner (Pazur and Ando, 1959). Other bonds are also broken, but at a much slower rate than α-1.4 links; α-1.6, 1% and α-1.3, 0.2% of the rate of hydrolysis of α-1.4 link (Pazur and Kleppe, 1962). Amyloglucosidases occur almost exclusively in fungi, and Aspergillus and Rhizopus spp. are used in commercial production (Godfrey and Reichelt, 1983) for conversion of malto-oligosaccharides into D-glucose. Rates of substrate hydrolysis are affected by molecular size and structure, and also by the next bond in sequence (Table 1.4) e.g. α-1.6 links close to α-1.4 links are attacked faster than the same linkages in isolation (Marshall, 1972).

Table 1.4 Relative rates of hydrolysis of α-D-glucosides by purified amyloglucosidase

	linkages		
	α-1.4	α-1.6	α-1.3
Di glucose	100	1.3	1.1
Tri glucose	360	8	3.7
Tetra glucose	770	15	2.6
Penta glucose	1000	23	-

Digestion of raw starch has been reported with a number of amyloglucosidases (Hayashida, 1975; Miah and Ueda, 1977 a, b).

The properties of a number of Aspergillus amyloglucosidases have been summarised in Table 1.5. The pH optima are generally in the range 4.5-5.0, while the temperature optimum is between 40° and 60°C. No amyloglucosidases have been found to be very active over 70°C.

Under conditions of submerged culture, amyloglucosidase is produced both in the medium and in the mycelium (Paszczynski et al, 1985). Maximum amyloglucosidase activity in the culture filtrate after 72h, and in the mycelium after only 48h growth.

All mould amyloglucosidase are glycoproteins containing between 5 and 20% carbohydrate and generally contain glucose,

TABLE 1.5 Properties of *Aspergillus* AMYLOGLUCOSIDASES

Organism		Optimum pH	Optimum temp.	Stability pH	Stability temp.	mw ($\times 10^{-3}$)	pI	References
A. awamori IFO 4033		4.5	60	5-9	50	83.7-88	3.7	Yamaski *et al* (1977)
A. awamori NRRL 3112	I	-	-	-	-	-	-	
	II	4.4	60	3-6	40-70	57.5	NR	Smiley *et al* (1971)
A. awamori	I	-	-	-	65	90c	3.55	Hayashida & Yoshino (1978)
var	I'	-	-	2-8	60	83	3.45	
kawachi	II	-	-	-	-	57	-	
A. niger C	I	-	-	-	-	263,290	-	Paszczynski *et al* (1982, 1985)
	II	-	-	-	-	209	-	
	III	-	-	-	-	136,134	-	
	IV	-	-	-	-	70	-	
A. niger	I	4.5-5.0	30-60	3-6	70	99	3.4	
	II	4.5-5.0	30-60	3-6	70	112	4.0	Lineback *et al* (1969)
A. niger	I				63.5			
	II				57			Lineback *et al* (1969)
A. niger NRRL 337		4.2-4.4	60	3-5	40-70	-	-	Moshin *et al* (1977)
A. oryzae Submerged	I	4.5	60	5-6	40	87	3.6	Saha *et al* (1979)
Solid	I	4.5	60	5-7	40	90	3.5	
	II	4.5	50	5-6	40	67	3.5	Mitsue *et al* (1979)
	III	4.5	50	5-6	40	54	3.4	
A. saitoi	MI	4.5	-	2.5-7.5	50	90	3.86	Takahashi *et al* (1981)
	MII	4.5	-	2.5-7.5	50	70	3.85	Inokuchi *et al* (1981)

glucosamine, mannose and galactose. In all Aspergillus amyloglucosidases, mannose is present in much larger quantities than galactose and glucose (Table 1.6). Hayashida and Yoshino (1978) showed that treatment of amyloglucosidase from A. awamori var. kawachi with α-mannosidase, decreased the enzyme's carbohydrate content by almost 50% and left it unstable to pH and temperature changes, although hydrolysis curves for soluble sunstrates and raw starches did not change.

Table 1.6 Carbohydrate content of amyloglucosidases (mole per mole of enzyme)

	A.candidus		A.foetidus		A.niger		A.niger		A.phoenicis	A.sartoi	
Author No.	(1)		(1)		(1)		(2)		(3)	(4)	
	I	II	I	II	I	II	I	II	II (or A)	M1	M2
Mannose	44.0	43.0	39.1	37.3	41.3	44.8	68.8	127.6	39.1	83.3	43.7
Glucose	-	-	6.9	4.8	8.0	4.2	16.0	19.6	6.9	4.4	4.2
Galact-ose	1.8	3.0	2.2	2.7	3.5	3.3	2.2	3.0	2.2	5.2	7.1
NAG*	7.1	6.9	4.0	3.7	2.9	3.4	-	-	4.0	4.1	2.3
Total	53.0	53.0	52.2	48.5	56.5	57.4	87.0	151.0	52.2	102.0	57.0

* NAG = N-acetyl glucosamine

Author Number
1. Manjunath and Raghavendra Rao (1979)
2. Pazur et al (1971)
3. Lineback and Baumann (1970)
4. Inokuchi et al (1981).

The amino acid composition of some amyloglucosidases from Aspergillus and Rhizopus spp. has been compared (Table 1.7). Shenoy et al (1985) reported that tryptophan groups were located at the active site of amyloglucosidase and that modification of these residues by photooxidation or by N-bromosuccinamide treatment resulted in loss of activity. Maltose, which is also a substrate, protects the enzyme against inactivation. Tryptophan residues were present in all amyloglucosidases examined except A. phoenicis (Lineback & Baumann, 1970).

Table 1.7 Amino acid composition of Amyloglucosidases from Aspergilli. (Summary of various authors)

Amino Acid	Average Range (number of residues per mole of enzyme)
Ala	38 - 82
Arg	11 - 23
Asp	49 - 85
Cys/2	5 - 11
Glu	27 - 56
Gly	30 - 63
His	3 - 6
Ile	9 - 27
Leu	16 - 56
Lys	8 - 16
Met	2 - 4
Phe	13 - 28
Pro	13 - 29
Ser	62 - 119
Thr	44 - 104
Trp	0 - 32
Tyr	14 - 30
Val	23 - 46
NH_3	21 - 63

Isolation and purification of fungal glucamylase is relatively easy because the enzymes are extracellular and quite stable. Early experimenters purified amyloglucosidase by ammonium sulphate precipitation followed by acid treatment to eliminate α-amylase. Recently, combinations of different procedures such as adsorption on raw starch, ammonium sulphate fractionation, gel filtration and ion exchange chromatography have been used (Svensson et al, 1982; Morita et al, 1966).

Bhella and Altosaar (1984 b) found that DEAE-cellulose alone showed very little separation of amyloglucosidase and α-amylase. Pazur and Ando (1960) first reported that the amyloglucosidase of A. niger could be resolved into two active fractions by DEAE-cellulose chromatography. A. foetidus (Lineback and Baumann, 1970), A. awamori and A. saitoi (Takahashi et al, 1981) have been reported to produce In some cases, more forms have been reported: three in A.

niger (Fleming and Stone, 1965, Miah and Ueda, 1977 a, b) and A. oryzae (Pazur and Ando, 1959) and four in A. niger (Paszczynski et al, 1985).

Hayashida (1975) reported that the existence of multiple forms of amyloglucosidase in a culture of A. awamori var. kawachi was due to the in vitro digestion of prototype (or native) amyloglucosidase. When grown in submerged culture, A. awamori produces three glucoamylases, GAI (native or prototype), GAI' and GAII having molecular weights of 90,000, 83,000 and 62,000 respectively. GAI was formed when the organism was grown on a medium containing no zinc. In the absence of zinc, protease production was depressed and GAI was formed in a culture protected from proteolysis. When zinc was present, such protelysis occurred to give GAI' and GAII. To support this suggestion, they prepared subtilisin modified (Hayashida et al, 1976) and acid-proteased modified (Hayashida and Yashino, 1978) GAI, and found these modified enzymes were similar in properties to GAI'.

Further, Yashino and Hayashida (1978) showed that GAI (mw 90,000) on treatment with protease, yielded GAI' (mw 83,000), and further treatment with glucosidases (α-mannosidase) gave rise to GAII (mw 57,000).

Very limited data is available concerning the secondary and tertiary structure of amyloglucosidase. The native enzyme contains 15-20% α-helix; in addition, it contained β-structure and disordered conformation. The carbohydrate content appears necessary to maintain the α-helix structure and with it pH and temperature stability (Manjunath et al, 1983).

Determination of amyloglucosidase activity is based on the measurement of reducing sugars liberated by the enzymatic hydrolysis of the substrate, usually starch (Hayashida, 1975). α-Amylase interferes directly in the determination of amyloglucosidases by the contributory production of reducing sugars, such as maltose and indirectly by providing more non-reducing ends of the substrate, which are then susceptible to attack by amyloglucosidase. The former effect can be readily eliminated by the use of a method for determining reducing sugars, specific for glucose, while the latter effect cannot be eliminated so readily.

Most amyloglucosidases totally convert starch into D-glucose but only when used in excess and over a long incubation period (Ueda et al, 1974). Although the initial rate of substrate hydrolysis is rapid, it subsequently decreases due to the lower affinity of the enzyme for α-1.6 bonds (Fogarty, 1983). If there is high concentration of substrate and prolonged incubation (as in industrial processes), there may be a reversion reaction, involving resynthesis of saccharides from D-glucose. The reversion reaction forms maltose, isomaltose and other oligosaccharides.

Glucoamylase preparations may also contain transglucosidase (E.C.2.4.1.24, transglucosylase, α-1.4-D-glucan -6-α-glycosyl-transferase, TG). This enzyme catalyses the synthesis of α-1.6-linked non-fermentable oligosaccharides (Pazur and Ando, 1961). Thus, significant amounts of panose and isomaltose may be synthesised, resulting in lower conversion of substrates into glucose. Many processes have been patented for the removal of transglucosidase from amyloglucosidase preparations but, in general, preparations from Aspergillus spp. show more transglucosidase activity than those of Rhizopus spp. (Benson et al, 1982).

1.3.3 Industrial Uses of α-Amylase and Amyloglucosidase

Conversion of starch into sugars, syrups and dextrins forms the major part of the starch processing industry (Marshall, 1974). These hydrolysates are used as carbon sources in fermentation, as sources of sweetness and also, because of their physical characteristics, in a range of food products and beverages. Hydrolysis of starch to produce these products, containing glucose, maltose or other oligosaccharides, is brought about by controlled degradation. The degree of hydrolysis may be controlled so that end products with the desired physical properties are obtained (Barfoed, 1976).

Preparation of starch hydrolysates may use either acid or enzymes as catalysts. The traditional acid-catalysis methods are now being replaced by enzymatic processes, which have several advantages over acid processes.

Enzymatic starch processing can be divided into various steps:

- gelatinisation - whereby insoluble starch is made into a slurry and then solubilised in water by heating the solution;

- liquefaction - the thinning of a starch colution, and

- saccharification - the breakdown of starch to the desired product.

Bacterial α-amylases are involved in liquefaction, while fungal α-amylases and amyloglucosidases are used in saccharification. Table 1.8 summarises the various end products of saccharification and enzymes involved in producing them.

The most important use of amyloglucosidase is the production of glucose syrups. Such syrups may be used directly, for example in fermentation, but transport and storage require heated conditions to prevent crystallisation and solidification. High glucose or dextrose syrups are usually used either for production of crystalline glucose or as a starting material for production of high fructose syrups (Zittan et al, 1975).

Amyloglucosidase is also used in the production of high conversion syrups (Harvey and Witt, 1986). Amyloglucosidase from A. awamori NRRL 3112 has successfully been used to produce high D.E. syrups by, among others, the Grain Processing Corporation, USA. These syrups are used in the brewing, baking, soft drinks, canning and confectionery industries. Their most important attribute is that they should have a high DE and yet be stable enough not to crystallise at temperatures down to 4°C at 80-82% dry substance. The maximum permissible glucose concentration in these syrups is 43%; above this level, glucose will crystallise out. Fungal amyloglucosidases, particularly from A. awamori have been used in distilleries to improve conversion of grain and starches in mashes in the production of potable alcohol (Aguino de Muro et al, 1984; Andreasen et al, 1976).

Table 1.8 Summary of Starch Processing

1. Gelatinisation — DE 5

2. Liquefaction/Thinning — DE 10 - 20
   A. acid
   B. enzyme - Bacterial α-Amylase

3. Saccharification
   Processing depends on the product required.

	Dextrose Syrups High glucose syrups	High Conversion Syrup High DE Syrup	High Maltose Syrup
Starch conc.	35 - 40%	30 - 45%	38%
pH	3.8 - 4.8	5.0 - 5.2	5.0 - 5.3
temp.	60 C	55 C	50 C
time	48 - 72 h	48 h	40 - 48 h
Enzyme(s) used	Amyloglucosidase + debrancher	Fungal α-Amylase + amyloglucosidase	Fungal β-Amylase
Produce DE	94-97	63 -67	30-50
Products	94-97%G_1	30-35%G_1 37-43%G_2 G_3	60%G_2
Further Processing			
	High Fructose Syrup pH 7.8 - 8.3 temp. 80°C Enzyme GI Substrate 45-55% G_1 Product 45-55% F		+ Pullulanase 80% G_2 + β-Amylase + Pullulanase 94% G_2

Notes: 1. debrancher = pullulanase or isoamylase
2. G_1 = glucose, G_2 = maltose, G_3 = Maltotriose, F = Fructose
3. GI = glucose isomerase
4. DE = dextrose equivalent.

There is an increasing demand in Japan for high maltose syrups (Maeda and Tsao, 1979), and it is expected that the world-wide demand will also grow. Fungal α-amylases are used in this process, but to increase maltose yields, pullulanase (a α-1.6 debranching enzyme), or pullulanase plus β-amylase can be added. The most important characteristics of these syrups are low viscosity in solution, low hygroscopicity, resistance to crystallisation, mildness (low sweetness), good heat stability and lack of colour formation. Such syrups are therefore very suitable in the confectionery and bakery industries, and because of the high concentration of fermentable sugars, are highly acceptable as brewing adjuncts in beer manufacture.

1.4 Production and Secretion of Extracellular Enzymes by Fungi

For commercial production of enzymes, it is particularly desirable that the enzyme be expressed extracellularly. Priest (1984) defined an extracellular enzyme as one which had passed through the cytoplasmic membrane of the organism.

Since the properties of an organism and its products are strain dependent, screening of organisms is very important. Increased yields of enzymes can be obtained by selecting for super secretors, organisms which do not produce feedback inhibitors or repressors, organisms which do not recognise inhibitors or repressors, organisms with increased gene dosage (both as plasmid copies and as integrated copies) and organisms whose enzymes have increased stability (Smith et al, 1985; Stanbury and Whitaker, 1984).

Levels of any extracellularly expressed biologically active protein depend on the efficiency of the following: transcription/translation, secretion and stability.

Initially, precursors of the protein to be transported usually contain an NH_4 terminal extension of some 15 to 30 amino acids called the signal sequence. As it emerges from the ribosome, the signal sequence directs the ribosome to the membrane, where a "pore" is formed and the protein is transported through the membrane. The signal sequence is removed from the newly-excreted protein by a signal peptidase. Most reports suggest that secretion occurs cotranslationally (e.g. α-amylase in B. subtilis, Davis and

Tai, 1980) although some suggest cases of post-translational secretion (e.g. ∝ -glucosidase in some Bacilli).

Aspergilli, due to their ability to secrete proteins, have been used as a model system for expression and secretion of mammalian gene products in filamentous fungi. Recently the secretory control regions of the A. niger amyloglucosidase gene were coupled to prochymosin cDNA, and the plasmid was inserted in A. nidulans. Synthesis of chymosin polypeptides, which were immunologically similar to bovine chymosin, was induced in a medium containing starch which also induced amyloglucosidase production (Cullen et al, 1987).

As stated previously (Section 1.3.2), multiple forms of amyloglucosidase have been found both within the mycelium and in the supernatent (Paszczynski et al, 1985). Svennson et al (1986) showed that the structure of the G2 form was generated by post-transcriptional modification (in this case, limited proteolysis) at the COOH-terminal region of G1, rather than being synthesised from another gene. Boel et al, (1984) found a single amyloamylase gene in the genome of A. niger and suggested that the G2 form of the enzyme was coded for by a unique G2 mRNA derived from G1 mRNA by differential splicing.

If the rate of enzyme synthesis is constant, irrespective of the presence of substrate in the environment, the enzyme is described as constitutive (Priest, 1984). Several extra cellular enzymes are constitutive, including the industrially important amylases of Bacillus amyloliquefaciens and B. licheniformis, but it is more common that enzyme synthesis is inducible.

Catabolic enzymes may be controlled by one or more of the following mechanisms:

- Induction: Enzyme is only produced in the presence of a compound termed the inducer, which is normally the substrate of the enzyme

- Feed back repression: Enzyme synthesis is repressed by products of its activity

- Catabolite repression: Enzyme synthesis is repressed when the organism grows rapidly on a readily utilised carbon source

- Feedback inhibition: Enzyme activity is inhibited by the end product of the pathway it catalyses.

1.4.1 Regulation of enzyme synthesis and activity in Aspergilli

Control systems in Aspergilli have been studied. In A. niger, synthesis of pectinases is induced by substances containing pectin (Perley and Page, 1971) and is subject to catabolite repression (Shinmyo et al, 1978; Aguilar and Huitron, 1986). ∝-Amylase biosynthesis is subject to induction by starch and maltodextrins, and catabolite repression by glucose and other monosaccharides (Yabuki et al, 1977; Fogarty and Kelly, 1979; Crueger and Crueger, 1982), although Reddy and Abouzeid (1986) showed that in one species of Aspergillus, amylase was not subject to catabolite repression but to feedback inhibition by glucose. Yurkevich (1985) also suggested that the ∝-amylase molecule itself totally suppresses its own synthesis when present in the medium above threshold levels. Tonomura et al (1961) showed that panose, koji-biose, isomaltose and maltose stimulated ∝-amylase formation.

Amyloglucosidase synthesis in A. niger is induced by maltose and isomaltose but not by polymeric glucans which are too large to enter the cell (Barton et al, 1972). The high level of amyloglucosidase may be due to the action of amyloglucosidase or ∝-amylase on starch (producing maltose and isomaltose) (Wang et al, 1979; Fujii and Kawamura, 1985).

Nunberg and co-workers (1983,1984) showed a several hundred fold increase in amyloglucosidase mRNA in cells of A. awamori grown on an inducing substrate compared with cells grown on a non-inducing substrate, xylose. They suggested that induction of amyloglucosidase synthesis was transcriptionally regulated. By comparing the total mRNA from both types of cells, they identified an inducible 2.3 kilobase mRNA sequence encoding amyloglucosidase (Boel et al, 1984).

1.4.2 Parameters affecting Enzyme Production in Aspergilli

Various carbon and nitrogen sources effect expression and secretion of amylolytic enzymes and also growth of Aspergilli. Maltose, starch and in some cases glucose, are the favoured carbon sources for amyloglucosidase production (El-Azhary et al, 1984; Attia and Ali, 1974; Barton et al, 1972), while maltose, isomaltose and panose induce α-amylase formation (Reese et al, 1969; Tonomura et al, 1961; Yabuki et al, 1977).

Nitrogen sources have been shown to influence fermentation patterns. In fungi, ammonium ions repress uptake of amino acids by general and specific amino acid permeases (Whitaker, 1976). In A. nidulans, ammonia also regulates production of alkaline proteases (Cohen, 1973). Corn steep powder, a by-product of corn processing, has been recommended for amylase production (Qadeer and Kausar, 1971; Moshin et al, 1977). Its high buffering capacity and the presence of amino acids, vitamins and minerals (Rhodes and Fletcher, 1966) make it an ideal source of nitrogen for fungi. Ramachandran et al, 1979) found ammonium ions in the form of ammonium phosphate or tartarate gave good yields of amyloglucosidase.

pH has a great effect on enzyme production. The optimum pH for growth of the organism may not correspond with that for enzyme activity, secretion, or expression (Kelly et al, 1983). In turn, optima for secretion/expression (i.e. "intracellular production") may differ from those for extracellular formation, since the internal pH of the organism may be independent of the medium pH. When production is considered, the pH stability of the enzyme in the fermentation broth must also be noted. For this reason, fungi generally produce enzymes with optimum pH stability in the range pH 2-6 (Godfrey and Reichelt, 1983). Both α-amylase and amyloglucosidase are stable in the acid range.

1.5 Cultivation of Filamentous Fungi

1.5.1 General Methodology for Cultivation of Filamentous Fungi.

Production of any fungal product involves a cultivation or fermentation stage, followed by a suitable product extraction stage (Aunstrup et al, 1979; Lambert, 1983). The fermentation may be conducted in either solid or submerged

culture. Solid culture is conducted using sterilised, moistened, solid substrates on trays, which are then inoculated and incubated at constant temperature and humidity. The organism grows aerially over the medium and generally sporulates readily. This method of growth is particularly favoured by filamentous fungi and allows a range of enzymes/acids to be formed. The technique is still used to produce Rhizopus amyloglucosidase, and Aspergillus acid protease and citric acid (Blain, 1975; Lockwood, 1979).

Submerged culture techniques were originally developed for acetone production by Clostridium acetobutyliticum and later for penicillin production by Penicillium (Hastings, 1978). The technique involves cultivation in stirred, liquid culture medium, which is well aerated (Gaden, 1981). Generally, only vegetative growth occurs under these conditions.

Submerged fermenter cultivation is the most widely used technique for aerobic fermentations (Stanbury and Whitaker, 1984). It allows greater control of temperature, pH, aeration and broth homogeneity than solid culture (Gaden, 1981). On a small scale (up to 2 l) shake flasks are used, but for larger volumes fermenters are used. The equipment usually consists of a baffled sterilisable tank, fitted with rotary impellers for agitation and piping for forced aeration (Solomons, 1969; Meyrath and Suchanek, 1972). Some fermenter designs have no impellers, and use aeration alone to provide mixing and oxygenation of the broth (Barker and Worgan, 1981; Greenshields and Smith, 1971).

Fungi present a number of problems when grown in submerged cultures in fermenters. These include ensuring adequate levels of aeration for the fungus; the non-Newtonian characteristics of the broth which may lead to inadequate mixing of the fermentation liquor; the disruption of the mycelia due to the high shear rates which may be required to give sufficient oxygen transfer; fouling of the baffles and tubing with growth; excessive sporulation in the fermenter head space and variations in fungal morphology (Wang et al, 1979, Stanbury and Whitaker, 1984). Morphology may be filamentous or pelleted (Metz and Kossen, 1977; Whitaker and Long, 1973), and is affected by strain, inoculum, medium and conditions of growth.

Growth morphology affects nutrient, oxygen and heat transfer within the fermentation broth (Wang and Fewkes, 1977; Atkinson and Daoud, 1976). Industrially, pelleted growth facilitates mixing, hence better mass transfer is obtained in the fermentation broth (Van Suijdam et al, 1980; Royer and Rouleau, 1985). The growth medium is also less viscous (Kim et al, 1983; Wittler et al, 1983). However, the main limitation comes from poor oxygenation within the pellet (Van Suijdam et al, 1982). This leads to growth and/or product formation occurring only in the outer zones of the pellet, and autolysis occurring in the centre.

In general, biomass levels formed by pelleted growth are substantially lower than those of the dispersed form of the same organism (Solomons, 1980).

Filamentous growth reduces mass transfer to the bulk fermentation fluid, and its increased viscosity requires a higher power imput to maintain adequate mixing. The sensitivity of filamentous fungi to shear forces limits the extent to which agitation may be increased to increase aeration rates (Hanisch et al, 1980).

Aspergilli readily form various morphological types depending on the strain, inoculum, conditions of storage, medium components pH, and culture conditions (Labanok et al, 1984; Trinci, 1983). Citric acid production by A. niger was favoured by heavy pelleted growth while dispersed/filamentous growth by A. flavus NRRL 3000 led to high levels of aflatoxin (Sodeck et al, 1981, Ciegler et al, 1966). Ramachandran and co-workers (1979) showed that carbon sources which induced filamentous growth in A. niger also gave high amylase yields.

1.5.2 Cultivation of Aspergillus spp.

A great number of medium ingredients have been shown to influence the growth of Aspergillus spp. in fermentations (Cocker and Greenshields, 1977). Starch, glucose, maltose, dextrins, maize and raw starch are among the recommended carbon sources for Aspergilli (Ustyuzhania et al, 1985; Ramachandra et al, 1979, Andrzejczuk-Hybel et al, 1985). Ammonium chloride, ammonium sulphate, sodium nitrate, ammonium nitrate, as well as complex nitrogen sources such as corn steep powder, yeast extract, urea and casein have proved

satisfactory nitrogen sources for microbial growth (Pestana and Castillo, 1985; Attia and Ali, 1973, 1974 a,b). Cocker and Greenshields (1977) have reviewed the effect of metals and other additives on growth of Aspergillus spp. and product formation.

Temperature has a marked effect on mycelial growth. Thus, the mycelial weight of A. oryzae increased with increasing temperature up to 35°C. No growth was detected at 40°C (Kundu et al, 1973). The specific growth rate of A. nidulans almost doubled with increase in temperature from 20° to 30° and from 30° to 37°C (Trinci, 1969).

Similarly, in regard to oxygen, adequate oxygen for fungal growth can be achieved by increasing air flow rate, agitation or fermenter back pressure (Unterkofler, 1969). Lasater and Smith (1978) suggested that Aspergilli required at least 3 ppm dissolved oxygen for adequate biomass formation.

pH has considerable effect on biomass formation. Kundu et al (1973) showed that optimal levels of A. oryzae were obtained at pH 7.5 which is slightly higher than values recorded elsewhere (pH 5-7, Berry, 1975).

1.5.3 Specific Growth Rate

The specific growth rate (μ) is used to refer to the growth rate per unit mass of organism. If x is the mass of the organism and t is the time, then

$$\mu = \frac{1}{x}\,\frac{dx}{dt}$$

The specific growth rate is also related to the doubling time (t_d) by the expression

$$\mu = \frac{\ln 2}{td}$$

Various authors have studied the influence of growth rate on metabolism and product formation in bacteria and filamentous fungi. Righelato et al (1968) showed that μ affected

penicillin production by Penicillium chrysogenum; the penicillin synthetic activity of the mould decreased to zero after growth was stopped, at a rate inversely related to the previous growth rate.

Temperature has an effect on specific growth rate and doubling time. Trinci (1969, 1970) showed that for A. nidulans doubled with the increase in temperature from 20° to 30° C and from 30° to 37° C. He also showed that A. nidulans had a shorter doubling time in submerged culture than P. chrysogenum and Mucor hiemalis.

Nutrition has an obvious effect on microbial growth. High levels of glucose can give higher yields of biomass, although the rate of biomass formation may not be greater. The specific growth rate of germ tubes of A. nidulans varied from 0.60 - 0.71 h^{-1}, as the glucose concentration increased from 0.04 - 41.0 g/l (Righelato et al, 1968).

pH variations may lead to qualitative differences in metabolism as well as variations in μ. Miles and Trinci (1983) suggested that variation of pH in the range of 6-8 did not have a significant effect on in P. chrysogenum, but did not record results at more acidic pHs.

The minimum oxygen requirement of P. chrysogenum was 0.74 mM O_2/g dry weight/h, irrespective of growth rate (Carter and Bull, 1971).

1.6 Scale-up of Fermentation

If the scale of operation of a fermentation is to be increased (so-called scale-up), it is important that conditions which lead to optimum product/biomass formation in a small scale process be maintained, as nearly as possible on the large scale. Criteria which have been considered useful for translation between two scales of operation (Stanbury and Whitaker, 1984, Wang et al, 1979) have been:-

1. Constant volumetric O_2 tranfer rate - K_La
2. Constant impeller tip speed
3. Constant volumetric power input
4. Equal mixing times
5. Similar Reynolds number (Nr_e) or momentum factors
6. Similar geometry/physical proportions.

Before 1948, most fermentations for the production of alcohol or organic acid, were scaled up maintaining geometric similarity and keeping the power/unit volume constant. Under these conditions, power imput per unit volume of a small and large fermenter can be related to impeller rotational speed (N) and impeller diameter (Di) of each as follows (Ruston et al, 1950):-

$$\frac{P}{V} \propto \left[N^3 Di^2\right]_S \simeq \left[N^3 Di^2\right]_L \qquad (1)$$

where P is power imput
V is fermentation broth volume
S is small fermenter
L is a large fermenter

Early penicillin fermentations were scaled up using this equation and geometric similarity (Gaden, 1961).

Fermentations in which there is large production of mycelial matter are affected by shear or the absolute value of tip speed. Constant tip speed can be used as a parameter for scale up from a small to a large fermenter (Hattori et al, 1972).

$$\text{Tip speed} = \left[\pi N Di\right]_S = \left[\pi N Di\right]_L \qquad (2)$$

Since shear is proportional to tip speed, then combined with similar volumetric power inputs (1), shear can be related to impeller diameter as follows:

$$\frac{S_S}{S_L} = \left[\frac{Di_S}{Di_L}\right]^{1/3} \qquad (3)$$

Thus the small impeller systems are higher shear input devices.

The most commonly used parameter for scale up is constant K_La or O_2 transfer rate. Bartholomew (1960) proposed that

due to the high O_2 demand of antibiotic fermentations, the volumetric O_2 transfer coefficient should be maintained constant on scale-up. In fact, as long as K_La is maintained above a certain value, reasonable results are obtained. (For derivation of formulae for K_La values, see Appendix 6).

Summary

Usually constant K_La is chosen as a basis for scale-up. However, in some fermentations, particularly the mycelial-type, pellet formation and hence shear, mixing and other factors are important. If the fermenter geometry is similar, one other scale up parameter can be used (Wang et al, 1979). It should be noted that some of these parameters for scale up are mutually incompatible e.g. constant shear (N Di) and Reynold's number (N Di^2). Oldshue (1983) suggested that similar geometry is not as important to scale up as maintaining constant K a and fluid shear.

It would appear that while use of the many formulae proposed for scale up are of considerable value, a large element of 'trial and error' may also be necessary (varying with the particular fermentation and the type of fermenter used) in order to arrive at optimum yield of the desired fermentation product.

Section 2 METHODS AND MATERIALS

2.1 Sources of Chemicals

Oxoid Ltd., London, U.K.: Nutrient Agar and Agar (technical) No. 3.
Falek Chemical Co., U.S.A.: Mazu DF Antifoam.
Roquette Freres, France: Maltodextrins (including MD05).
Sigma (London) Ltd.: Miscellaneous biochemicals
B.D.H. Ltd., England and Reidel-de-Haen A.G., Germany: All reagent grade and AnalaR grade laboratory chemicals.
Boehringer Mannheim GmbH, Germany: Glucose GOD-PAP kit.
Biocon Ltd., Co. Cork, Ireland: Yeast extract, malt extract, malt powder, maize, commercial grade starch, corn steep powder (CSP).

2.2 Fungal Culture

2.2.1 Source of Strains

The following strains of Aspergilli were used in this study:-

1.	A. niger	NRRL 3	(ATCC 9029)
2.	A. niger	NRRL 330	(ATCC 10864)
3.	A. foetidus	NRRL 337	(ATCC 10254)
4.	A. awamori	NRRL 3112	(ATCC 22342)
5.	A. niger mutant	NRRL 3122	(ATCC 22343)
6.	A. niger	CBS 10366	
7.	A. niger	CBS 26265	
8.	A. niger	CBS 26365	
9.	A. niger	ATCC 13496	
10.	A. niger	ATCC 13497	

Organisms 1 to 5 were kindly donated by the U.S. Department of Agriculture, Northern Regional Research Laboratory, 1815 North University Street, Peoria, Illinois 61604, United States. Organisms 6 to 8 were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, Netherlands. Organisms 9 and 10 were received from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, United States.

2.2.2 Culture Maintenance

Freeze dried cultures were opened aseptically and the pellet resuspended in 2 ml of Nutrient Broth (Oxoid). After several minutes, loopfuls of the rehydrated organism were streaked on yeast malt extract (YME) agar (4 gl^{-1} glucose, 4 gl^{-1} yeast extract, 10 gl^{-1} malt extract, 20 gl^{-1} agar, pH 7.2) or nutrient agar (Oxoid), and plates were incubated at 30°C for 7 days. Single colonies were subcultured on yeast malt extract agar for 7 days at 30°C prior to storage at 4° C. Subculturing was carried out on a monthly basis to maintain viability of cultures.

2.2.3 Detection of Contamination

Fungal/yeast contamination of stock cultures, spore suspensions or fermentor samples was detected by plating samples onto Nutrient (Oxoid) or Yeast Malt Extract Agar and incubating at 30°C for 3-4 days. Bacterial contamination was detected by plating samples on Nutrient Agar (Oxoid) containing 0.1 mg/l cyclohexamide, incubated overnight at 30° C.

Microscopic examination of spore suspension for bacterial contaminants was routinely carried out.

2.2.4 Inoculum Development / Standard inoculum procedure

Spores were harvested from 7 day old cultures, grown on yeast malt extract agar, using sterile diluent (Appendix 1) containing sterile 5mm diameter glass beads. Numbers were routinely determined by duplicate counting of samples in an improved Neubauer haemocytometer. Spore inocula, used to inoculate 100 ml of medium in a 250 ml conical flask, contained 2.0 - 2.2 x 10^7 spores.

For fermenter work, 2.2 x 10^7 spores were inoculated in 100 ml of inoculation medium (Medium A, section 2.5.1) in 250 ml conical flask and incubated on an orbital shaker (150 rpm, L.H. Engineering Ltd., Model MK II/III) at 30°C. After 24 hours, a suitable amount of this medium was aseptically transferred into the fermenter, to give a 1% inoculum, i.e. in the Laboferm 50 ml was used in 5 l, and in the Microgen 100 ml in 10 l.

2.3 Shake Flask Cultivation

Media was dispensed 90 or 100 ml, where stated, in 250 ml conical flasks, and sterilised at 15 psi (121° C) for 20 minutes. Incubation was conducted at 30° C, on L.H. Engineering orbital shaker (Model MK II/III) at 150 rpm.

2.4 Fermenter Cultivation

2.4.1 Microgen Fermenters

This fermenter, (Microgen Model SF116, New Brunswick Scientific) had a gross capacity of 16 l. Working volume was 10 l. Inoculation and sampling of the fermenter were carried out under sterile conditions. The medium was sterilised *in situ* for 20 minutes at 15 p.s.i., using direct steam injection. The Microgen fermenter was run at 5 p.s.i. back-pressure to control foaming and to reduce the risk of contamination. Level of aeration was 0.5 l of air/l of medium/minute and agitation, which was varied, was reported in individual experiments. Temperature was controlled by circulating hot or cold water through a hollow baffle heat exchanger immersed in the culture fluid. Fermentations were conducted at 30° C, unless otherwise stated. pH was automatically controlled (where necessary) by addition of sterile 1 M NaOH, by means of a pump which was activated when the fermentation pH fell below predetermined levels.

2.4.2 Laboferm Fermenters

The Laboferm fermenters (New Brunswick Scientific, UK) were glass walled vessels, with gross capacity of 7.5 l and a working volume up to 5 l. Agitation and aeration could be varied in individual fermenters. Temperature was maintained at 30° C (unless otherwise stated) by standing the fermenter vessels in a heated circulating water bath. Sterilisation was achieved by removing the glass vessels and autoclaving them in a large autoclave, at 15 p.s.i. (121° C) for 20 minutes; after cooling, the vessels were connected to agitation and aeration. Air was sterilised by passing it through Gelman filters (ACRO 50, hydrophobic 0.45 μm PTFE). pH was not controlled in these fermentations. The dimensions of both fermenters are given in Table 2.1, and differences between the two fermenters are shown in figure 2.1.

A

B

Figure 2.1 Laboratory Fermenters used in these experiments.
(a) Microgen fermenters
(b) Laboferm fermenters.

Table 2.1 Dimensions of the Fermenters Used
(Volumes measured in l, length in cm)

	Microgens	Laboferms
Total volume	16	7.5
Medium volume	10	5
Vessel diameter	22	14
Impeller diameter	7.2	5
Impeller number	3	3
Inter-impeller distance	10	8
Number of baffles	4	4
Baffle width	-	1.5
Depth of sampling point	33	-

2.4.3 Scale up/down

Changing the scale of a fermentation from one fermenter to another is not a random process. It involves varying all the fermentation parameters so that the same conditions, and therefore similar fermentation products, will occur at the larger or smaller scale.

During this research project, two types of fermenters were used. In an attempt to reduce the variation between results with each type, the fermentation was scaled down, using the following criteria:

a) Similar geometry

Although some of the geometric parameters of the fermenters were fixed, some changes could be made e.g. liquid height, impeller diameter, inter-impeller distance. The following ratios were compared:-

	Microgens	Laboferms
$\frac{\text{Vessel diameter}}{\text{Impeller diameter}}$	$\frac{22}{7.2} = 3.1$	$\frac{14}{5} = 2.8$
$\frac{\text{Liquid height}}{\text{Impeller diameter}}$	$\frac{26}{7.2} = 3.6$	$\frac{32.5}{5} = 6.5$

Although the liquid height in the Laboferms could have been reduced to bring the ratio of liquid height to impeller diameter closer to that in the Microgens, the volume, and therefore the number of samples which would be taken, would have been greatly reduced.

b) Constant tip speed

Hattori and co-workers (1972) suggested that constant tip speed was a useful parameter for scale up, particularly in fungal fermentations where shear had a large effect. Tip speed = agitation (in rpm) x impeller diameter (in cm). To maintain tip speed, the agitation

rates of 400 rpm and 250 rpm in the Microgens corresponded to 580 and 360 rpm in the Laboferms.

c) Constant oxygen transfer

Constant oxygen transfer rate (O.T.R.) or K_La values have often been used as a dimension in scale up/down. The derivation of the formulae is given in Appendix 6. Simply K_La values can be given by the following formula (Fukuda et al, 1968):-

$$K_La \propto (2 + 2.8\,Ni)\left(\frac{Ni}{V}\right)^{0.56}\left(\frac{1}{D}\right)^{1.4}(N)^{2.464}(Di)^{3.276}(Q)^{0.56}$$

where Ni = number of impellers
V = liquid volume
D = vessel diameter
Di = impeller diameter
N = agitation
Q = volumetric flow rate of air.

From this formula, and the previous constraints (i.e. similar geometry and constant tip speed), an aeration rate for the Laboferms can be chosen to give them a similar K_La value to the Microgens. Table 2.2 shows the relative K_La values which occur in the fermentations described later in this report.

Table 2.2 Relative K_La values for fermentations in laboratory fermenters

Microgens		Laboferms			
400 rpm	5l/min 100%	580 rpm	1.4 l/min 100%	360 rpm	1.4l/min 31%
250 rpm	5l/min 30%	580 rpm	2.5 l/min 139%	360 rpm	2.5l/min 43%
		580 rpm	5l/min 204%		

2.5 Fermentation Media

2.5.1 Media for shake flask trials.

The following media were used in this study, under the conditions previously described in section 2.3. All were sterilised at 15 p.s.i. (121°C) for 20 minutes.

Medium A - Inoculation medium (after Cadmus et al, 1966)
15 g/l ground maize
10 g/l corn steep powder
NaOH to pH 6.0
post-sterilisation pH ca 5.7

Medium B - Initial production medium
100 g/l ground maize
20 g/l corn steep powder
NaOH to pH 6.0
Made up to 90 ml with distilled water.

All the following media C - Q were nutritionally equivalent to medium B. Carbon, nitrogen, sulphur, phosphate, calcium, magnesium, and potassium were all balanced in the complex, semicomplex and defined media described in Table 2.3. All had an initial pH of 6.0, prior to sterilisation. (Miller and Churchill, 1986).

Table 2.3 Composition of Media C - Q.

Medium	Carbon Source (g/l)	Nitrogen source(s) (g/l)	Buffer	Minerals
C	Maize (100)	NH Cl (3.08)	-	-
D	Starch (50)	CSP(20) + NH_4Cl (1.53)	-	-
E	Starch (50)	CSP(20) + NH_4Cl (1.53)	Na Citrate (3)	-
F	Starch (50)	NH_4Cl (4.61)	-	+
G	Starch (50)	NH_4Cl (4.61)	Na Citrate (5)	+
H	Glucose (55.5)	CSP(20) + NH_4Cl (1.53)	-	-
J	Glucose (55.5)	CSP(20) + NH_4Cl (1.53)	Na Citrate (3)	-
K	Glucose (55.5)	NH_4Cl (4.61)	-	+
L	Glucose (55.5)	NH_4Cl (4.61)	Na Citrate (5)	+
M	Maltose (53)	CSP(20) + NH_4Cl (1.53)	-	-
N	Maltose (53)	CSP(20) + NH_4Cl (1.53)	Na Citrate (3)	-
P	Maltose (53)	NH_4Cl (4.61)	-	+
Q	Maltose (53)	NH_4Cl (4.61)	Na Citrate (5)	+

CSP = Corn steep powder

The concentrated mineral solution used in some of the media had the following composition:-

$ZnSO_4.7H_2O$	-	0.003 M	(0.863 g/l)
$MnSO_4.4H_2O$	-	0.02 M	(4.46 g/l)
$CuSO_4.5H_2O$	-	0.003 M	(0.75 g/l)
$FeSO_4.7H_2O$	-	0.009 M	(2.50 g/l)

One ml of the mineral concentrate was added to 100 ml of medium.

To study the effect of carbon : nitrogen ratio, the following variations of medium H were developed (Table 2.4). Each had an initial pH of 6.0 before autoclaving.

Table 2.4 Composition of Media H1 - H5.

Medium	Carbon source (g/l)	Nitrogen source (g/l)	C/N ratio
H1	Glucose (55.4)	CSP (5)	111
H2	Glucose (55.4)	CSP (10)	55
H	Glucose (55.4)	CSP (20)	28
H3	Glucose (55.4)	CSP (30)	19
H4	Glucose (55.4)	CSP (40)	14
H5	Glucose (55.4)	CSP (50)	11

CSP = corn steep powder

Variations on medium H, with different levels of tri-sodium citrate were developed (Table 2.5) to study the effect of pH and citrate on enzyme production and microbial growth. Again, each medium had pH of 6.0, before autoclaving.

Table 2.5 Composition of Media H6 - H9.

Medium					
H 6	=	Medium H	+	citrate	(3 g/l)
H 7	=	Medium H	+	citrate	(5 g/l)
H 8	=	Medium H	+	citrate	(7.5 g/l)
H 9	=	Medium H	+	citrate	(10 g/l)

To test the effect of initial pH on growth and biomass, the pH of medium H was adjusted to the appropriate pH, by addition of 1M NaOH. The pH, before and after sterilization, is recorded in Table 2.6.

Table 2.6 Effect of Sterilisation on Medium H

Flask	pH Values	
	Before Autoclaving	After Autoclaving
pHi 6.0	6.00	5.77
pHi 5.5	5.51	5.47
pHi 5.0	5.00	4.84
pHi 4.5	4.50	4.51
pHi 4.0	4.03	4.06

Medium R - non inducing or peptone-glycerol medium (Yabuki et al, 1977).

20 g/l Mycological peptone (Oxoid)
30 g/l Glycerol
5 g/l KH_2PO_4
2.5 g/l $MgSO_4 . 7H_2O$
post sterilisation pH 5.18

Medium S - peptone-starch medium (Yabuki et al, 1977)
- as medium R except glycerol is replaced by 30 g/l soluble starch. Post sterilisation pH is 5.20.

2.5.2 Media for fermenter trials

The following media were used in the laboratory fermenter trials (sections 3.4 and 3.5).

Basic medium for laboratory fermenters.

50 g/l carbon source
20 g/l Corn steep powder
1 ml/l MAZU antifoam (See appendix 5).
Add NaOH to bring to the appropriate pH (in most cases, 6.0)
Bring to volume with distilled water.

The following variations of this basic medium were used:-

Medium T - use 50 g/l Maltodextrin (MD05) as the sole carbon source (Appendix 4)
Medium U - use 50 g/l soluble starch (BDH) as the carbon source
Medium T2 - use 25 g/l MD05 as the sole carbon source
Medium T3- This is a modified form of Medium T used to determine biomass. The concentrations of all nutrients were similar to medium T, except that the nitrogen source was non-particulate. To make the non-particulate corn steep powder, the usual quantities of corn steep powder (i.e. 20 g/l) were dissolved in half the volume of distilled water. The solution was then autoclaved at 15 p.s.i. (121°C) for 20 minutes. After cooling, it was filtered through Whatmann No. 1 filter paper. (To speed up this process, the solution was centrifuged in a Damon/IEC PR 6000 centrifuge at 2000 rpm for 15 minutes), before the supernatent was filtered. The soluble corn steep powder was then used in the normal way.

These media were each sterilised at 15 p.s.i. (121°C) for 20 minutes. Those in the Microgen fermenters were sterilised by direct steam injection, while the Laboferm vessels were sterilised in a large autoclave. The advantage of direct steam injection sterilisation is that the media continue to be agitated during the sterilisation cycle.

2.6 Analytical Procedures

2.6.1 Estimation of Reducing Sugars

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

DNS Reagent:

3,5-dinitrosalicylic acid (10 g/l), potassium sodium tartarate (300 g/l), and sodium hydroxide (16 g/l) were dissolved in 600 ml distilled water by heating, without boiling. The solution was cooled and diluted to 1 litre, and stored in a sealed dark glass container at room temperature.

Procedure: A standard curve was prepared using a range of glucose solutions from 0.1 to 1.5 g/l. Unknown glucose solutions were determined within this range.

Method:

1. Tubes were prepared as follows:-

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

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

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

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

5. The absorption was plotted against glucose concentration (g/l).

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

2.6.2 Estimation of α-Amylase activity.

α-amylase activity was estimated using an iodine staining method (Biocon Ltd., Ireland, Product Information sheet, AM-011-80).

Procedure:-

The following stock solutions were made:

1. Acetate Buffer, pH 5.0
   1 M Acetic Acid - (57.2 ml/l) 30 parts
   1 M Sodium Acetate - (135 g/l) 70 parts.

2. Enzyme diluting solution
   0.585g of NaCl and 2.2g $CaCl_2.2H_2O$, were added to 20 ml of 1 M acetate buffer, pH 5.0, and diluted to 1 l.

3. Starch solution:
   1.74g of Lintner's Starch was made up in a slurry with 10 mls of distilled water. This was added to 50 mls of boiling water, and boiled for a further 3 minutes. After cooling, 26.25 ml of 1 M Acetate buffer, pH 5.0 was added and the solution was diluted to 250 ml.

4. Stock Iodine Solution (Horwath, 1984).
   5.5g of crystalline iodine and 11 g of KI were dissolved and diluted to 250 ml. The solution was stored in a sealed dark bottle, and remained stable for up to 1 month.

5. Dilute Iodine Solution (Horwath, 1984).
   1 ml of the stock iodine solution was mixed with 10 g of KI and diluted to 250 ml. This solution should be made daily.

6. Standard Colour Solution:
   25g cobaltous chloride hexahydrate and 3.84g of potassium dichromate were dissolved in 100 ml of 0.01 M HCl. The solution is stable indefinitely, if stored in a dark stoppered container.

Method:

Boiling tubes containing 10 ml of starch substrate and test tubes containing at least 5 ml of enzyme sample diluted with enzyme diluting solutions were pre-incubated for 10 minutes at 40°C. To start the reaction, 5 ml of sample was added to 10 ml of substrate. After 10 minutes, 1 ml samples were removed, added to 5 ml of dilute iodine solution and thoroughly mixed. Samples were removed at various time intervals up to 20 minutes after the start of the enzyme reaction. The absorbance at 620 nm of each iodine sample compared to that of distilled water was measured. The end point of the reaction was determined to be 0.470, using the standard colour solution. This corresponds to a light brown colour, and samples should be taken frequently (no more than 30 seconds apart) around this end point.

Calculations:

One fungal α-amylase unit is the amount of enzyme which breaks down 5830 mg of Linters starch per hour at 40°C and pH 5.0. (This assumes that the average Lintners Starch molecule contains 36 glucose units).

$$\text{Activity} = \frac{1}{T} \times \frac{S}{E} \times \frac{A}{B} \times D$$

where T = time taken in hours for the unknown enzyme to reach the critical absorbance.
= t (in minutes) X 60 (minutes/hour)
S = ml of substrate i.e. 10 ml
E = ml of enzyme i.e. 5 ml
A = initial amount of starch i.e. 6.95 mg/ml
B = amount of starch which must be broken down from the initial 6.95 mg/ml of starch, to give the critical absorbance i.e. 5.832 mg.
D = dilution of the unknown enzyme solution.

The equations can be simplified to:

$$\text{Activity} = 143 \times \frac{D}{t}$$

2.6.3 Estimation of Amyloglucosidase activity

Amyloglucosidase (AG) breaks down polymers such as starch by releasing glucose units. Activity of the enzyme can be determined by quantifying the glucose released. Pazur and Ando (1959) measured the glucose indirectly by quantifying the reducing sugars, using the D.N.S. method. Pazur and co-workers (1971) later suggested using a method which only measured glucose - the glucose oxidase-peroxidase-o-dianisidine (GOPOD) method. In this study, a modification of the latter method was used.

Procedure:

The following solutions were made:-

1. Starch solution.
2g of soluble starch, made into a slurry, were added

to 50 ml of boiling distilled water. After boiling for a further 5 minutes, the solution was diluted to 100 ml.

2. Acetate Buffer
   25 ml of 0.2 M Acetic acid (11.44 ml/l) was mixed with 20 ml of 0.2 M sodium acetate (27.16 g/l). The pH was checked and adjusted to pH 4.5 with either solution if necessary.

3. Enzyme substrate
   The starch solution and the acetate buffer were mixed in equal amounts to give a 1% starch solution in 0.1M acetate buffer pH 4.5.

Enzyme Assay:

1. 1 ml of substrate was dispensed into test tubes.

2. Substrate and enzyme solutions were allowed to equilibrate at 40°C for 10 minutes.

3. The tubes for the assay were prepared as follows:-

	Enzyme Analytical	Enzyme Control
Substrate	1 ml	1 ml
Enzyme solution	1 ml	-
Boiled Enzyme solution	-	1 ml

4. After 10 minutes, the reaction was stopped by putting tubes into a boiling water bath, and boiling for 5 minutes.

5. These samples can be kept for several days if they are sealed and stored at 4°C.

GOD-PAP Assay

Glucose produced by the enzyme was measured using an enzymatic colorimetric method based on Trinder (1969). The test is based on the following reactions:-

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

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{4-(p-benzoquinone-mono-imine)} + 4H_2O$$

Note: GOD = Glucose oxidase
POD = Peroxidase

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

Reagent 1: phosphate buffer, 100 mmol/l, pH 7.0; GOD ≥ 18 u/ml; POD ≥ 1.1 u/ml and 4-aminophenazone, 0.77 mmol/l.
Reagent 2: phenol 11 mmol/l.

The contents of one bottle containing reagent 1 were dissolved in 200 ml distilled water and the contents of one bottle of reagent 2 were added. The reagent mixture was then filtered through a Gelman 0.45 µm filter, and the mixture was stored in a dark bottle at 4°C for up to 1 month.

Method:

1. The tubes were prepared as follows:

	Analytical	Standard	Blank
Distilled water	-	0.1 ml	0.2 ml
Glucose standard (0.5mg/ml)	-	0.1 ml	-
Sample	0.2 ml	-	-
Reagent mixture	2.0 ml	2.0 ml	2.0 ml

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

3. The absorbance at 510 nm was determined, using the reagent blank to zero the spectrophotometer.

4. The glucose concentration of the unknown solutions was determined from the glucose standard and the standard curve.

5. One unit of amyloglucosidase is the amount of enzyme which releases 1 mg of glucose per minute, at 40°C and pH 4.5.

6. To calculate the activity of an unknown enzyme:

$$\text{Activity} = \frac{(A - B)}{2x} \times \frac{D}{t}$$

where A = Average absorbance of enzyme analytical
B = Absorbance of enzyme control
x = Absorbance of glucose standard (i.e. 0.5 mg/ml)
D = Dilution of enzyme
t = Length of the reaction i.e. 10 minutes.

2.7 Biomass Assessment

Biomass determinations were carried out routinely in most experiments. The procedure was as follows (Calam, 1969): filter papers (Whatman No. 1, 9 cm diameter) were dried at 105° C to constant weight (ca 17 hours). They were then transferred to a dessicator and cooled under vacuum. The papers were then weighed on a balance to 4 decimal places. Biomass was harvested from cultures using the filter papers on a Buchner funnel. Growth was washed with three culture volumes of distilled water. The filters were then dried, cooled, and weighed as before. Dry weight was determined by the difference in the two readings. In all cases, units of biomass/growth were given in g dry weight/l of medium or mg/ml.

In shake flask work, 100 ml of culture medium (i.e. all the flask contents) was filtered, while in the fermenter trials, 20 ml samples were used. All determinations were carried out in duplicate.

Growth type was assessed visually throughout the work. In most media, filamentous, dispersed growth occurred, except media R and S, which gave pelleted growth.

2.8 Preparation of Intracellular Samples

Preparation of intracellular samples of enzymes was conducted as follows:- Mycelium was grown on appropriate medium for a suitable length of time. The contents of a 250 ml volumetric flask were harvested by centrifugation (Hereus Christ bench centrifuge) at 4000 rpm for 10 minutes. The cell free supernatant was decanted, and the extracellular level of enzyme was assayed.

The biomass was washed with distilled water and recentrifuged twice, and the excess water was removed. The mycelium was divided into samples of known wet weight. Some of these were dried (as in Section 2.7) to determine the dry weight. To break open the fungus, the sample (ca 1g wet weight) was put into a Bead Beater (Biospec Products Ltd., P.O. Box 722, Bartlesville, Oklahoma 74005, U.S.A.) containing 10 g of 0.5 mm glass beads, and 0.01 m potassium phosphate-sodium phosphate buffer (pH 5.6). The sample was homogenised for 15 seconds, cooled, and then homogenised for another 15 seconds. The supernatant was decanted, and the beads were washed twice with phosphate buffer. The washings were added to the supernatant, which was then centrifuged at 4000 rpm for 5 minutes (Hereus Christ bench centrifuge) to remove cell debris. The clear liquid was decanted and made up to a known volume with buffer, before being assayed.

2.9 Routine Measurements and Instrumentation

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

Balances routinely used included a Sartorius 121 9MP electronic balance (600g $\pm$ 0.01g) and Precisa 80A electronic balance (30g $\pm$ 0.0001g).

Centrifugation was carried out using a bench-top Heraeus Christ model 600, and a floor centrifuge DuPont Instruments Sorvall RC-5B Refrigerated Superspeed Centrifuge, or Damon/IEC PR 6000 centrifuge.

2.10 Definitions of Terms Used

Production of enzyme: Amount of enzyme which can be assayed. Unless otherwise stated, enzyme production refers to extracellular enzyme production, which is the sum of the enzyme excreted into the medium less the amount of enzyme which is inactive. (Expressed in units/ml).

Yield of enzyme: Total amount of enzyme which can be assayed in the supernatent, at a particular time. (Expressed in units/ml).

Enzyme production per mg biomass or dry weight: This term relates the amount of enzyme assayed in a known volume of medium, to the amount of growth (expressed as dry weight) which occurs in that volume of medium. (Expressed in units/mg or units/g).

Enzyme activity: The amount of enzyme which is capable of acting on its appropriate substrate under given conditions.

Specific growth rate and doubling time (see Section 1.).

Section 3.1 Screening of Aspergilli for Production of Amylolytic Enzymes.

3.1.1 Screening of *Aspergillus* spp. for production of α-amylase and amyloglucosidase.

To obtain an organism which produces superior levels of extracellular α-amylase and/or amyloglucosidase, various strains of *Aspergillus* spp. were inoculated into the inoculation medium (medium A, section 2.5.1) using the standard inoculation technique (section 2.2.4). All organisms used had been cited in the literature as "amylase producers" and some had been patented for industrial amyloglucosidase production. After an incubation of 24 hours, under the usual conditions (30° C, 150 rpm), these flasks were used to inoculate flasks of medium B, the production medium (section 2.5.1). This medium, with a presterilization pH of 6.0, solidified after sterilization, so that the post-sterilization pH could not be determined. The inoculum pH varied from 4.1 to 4.7, and as in all shake flask experiments, the inoculum used was 10% of the final fermentation broth volume. All flasks were harvested after 96 h and results are tabulated in Table 3.1.

Table 3.1 Production of α-amylase and amyloglucosidase by various *Aspergillus* spp. on medium B, after 96 h.

Organisms*		pH	Reducing sugars (g glucose/l)	α-amylase (units/ml)	amylo-glucosidase (units/ml)	Ratio αA/AG**
A. niger	NRRL 3	4.19	20.5	97	2.8	34
A. niger	NRRL 330	4.54	6.1	880	4.6	190
A. foetidus	NRRL 337	4.53	12.6	456	4.4	104
A. awamori	NRRL 3112	4.18	26.7	1004	21.8	46
A. niger	NRRL 3122	3.90	32.1	231	13.5	17
A. niger	CBS 10366	4.49	33.2	2569	20.9	123
A. niger	CBS 26265	4.50	24.6	2457	12.9	190
A. niger	CBS 26365	4.51	1.8	2383	16.0	149
A. niger	ATCC 13496	4.29	1.3	1066	5.3	203
A. niger	ATCC 13497	4.26	1.0	1119	6.8	165

* full details of organisms are given in section 2.2.1

** α-amylase/amyloglucosidase

pH values in all flasks fell from 6.0 to between 3.9 and 4.6, lower pH values being recorded in known acid producers such as A. niger NRRL 3 (gluconic acid producer). The 96 h fermentation produced biomass and enzyme, accompanied by a liquefaction of the starch in medium B. The control flask (medium B + no organism) remained solidified for the full incubation period.

Highest levels of amyloglucosidase were obtained from A. awamori NRRL 3112, A. niger CBS 10366, and A. niger CBS 26365 while highest α-amylase levels were produced by A. niger CBS 10366, A. niger CBS 26265 and A. niger CBS 26365. Due to the poor sporulation of A. niger CBS 26363 and A. niger CBS 10366 in comparison to all the other organisms tested, A. awamori NRRL 3112 and A. niger CBS 26265 were chosen for further study (Figure 3.1).

Biomass levels could only be determined visually due to the particulate nature of the maize. In all flasks, growth was heavy and dispersed.

The ratio of α-amylase:amyloglucosidase in these organisms varied between 17 and 203, with the majority in the range 150-200. The lowest ratio recorded, 17, by A. niger NRRL 3122, also had the lowest pH. This would suggest that pH has a large effect on the ratio of each enzyme produced, and that acidic conditions affected α-amylase production particularly. Although the pH value of 3.9 might be expected to cause enzyme instability, subsequent experiments showed that at this pH, the enzyme exhibited greater than 80% of its maximum activity. Therefore, it would appear that this organism (i) has not got the genetic capability to produce higher levels of α-amylase, (ii) the pH adversely affects transcription/translation, or (iii) the pH adversely affects secretion of α-amylase.

Section 3.2 Preliminary Characteristics of α-Amylase and Amyloglucosidase of Aspergillus awamori NRRL 3112.

3.2.1 pH and temperature optimum of α-amylase.

The optimum pH for α-amylase activity was determined using universal buffer (Appendix 2) at various pH values in place of sodium acetate-acetic acid buffer, pH 5.0, (Section

Figure 3.1 Dispersed growth of *A. awamori* NRRL 3112

2.6.2) in the enzyme diluting solution, and the substrate. The results are tabulated in figure 3.2. The temperature optimum was determined by incubating the enzyme-substrate mixture at various temperatures. Results are presented in Figure 3.3.

Optimum activity for α-amylase occurred at pH 4.0 - 5.0 with 60% activity at pH 6.0 and 78% at pH 3.0. For this reason, all amylase assays were conducted with the substrate at pH 5.0. Temperature optimum occurred at 60°C, with greater than 50% of this activity between 40° and 70° C. Above 70°C no α-amylase action was detected. This was to be expected since fungal amylases are, in general, more thermolabile than those produced by bacteria (Fogarty, 1983), and this is reflected in their use in low temperature saccharification.

3.2.2 pH and temperature optimum of amyloglucosidase.

The optimum pH for amyloglucosidase activity was determined by substituting acetate buffer in the substrate (pH 4.5, 0.1M) with universal buffer (Appendix 2) at various pH values. The relationship between pH and amyloglucosidase activity is illustrated in Figure 3.4. Optimum activity was observed at pH 3.5, but values greater than 75% of this maximum were obtained in the range pH 2.5 - 5.5. Only 11% of activity remained at pH 7.5.

The temperature optimum was determined by incubating the enzyme with the substrate at various temperatures for ten minutes. Results are given in Figure 3.5. Maximum activity was obtained at 50°C, with 48% of this activity at 40° C. Activity declined sharply above 70° C, with only trace activity above 80°C.

For convenience, both amylase and amyloglucosidase were assayed at 40°C, although this was not their optimum for activity.

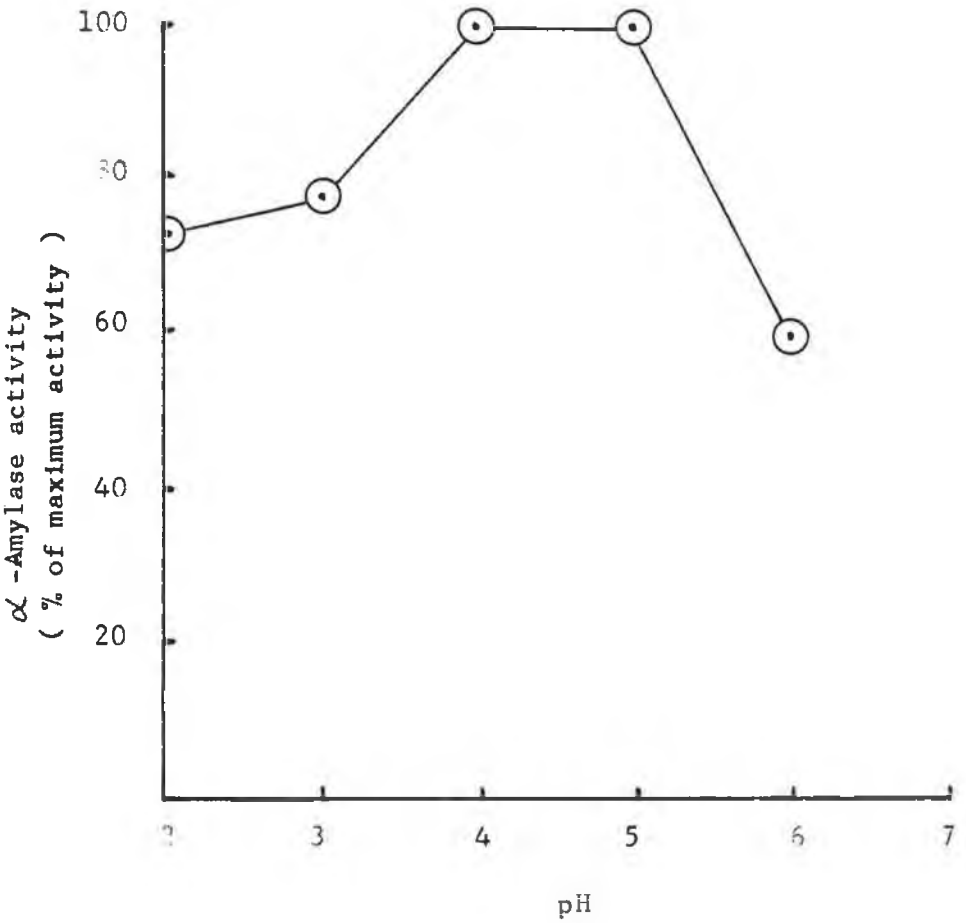


Figure 3.2 Effect of pH on α-amylase activity of A. awamori NRRL 3112

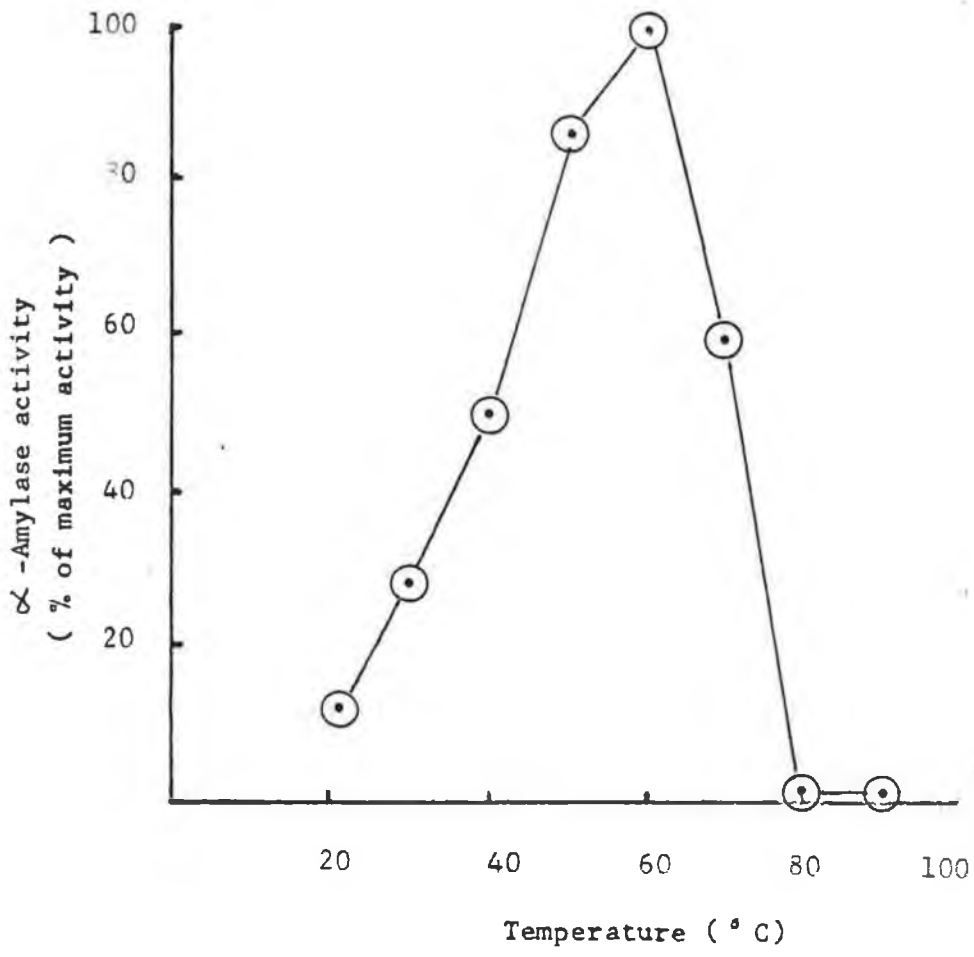


Figure 3.3 Effect of temperature on α-amylase activity of A. awamori NRRL 3112

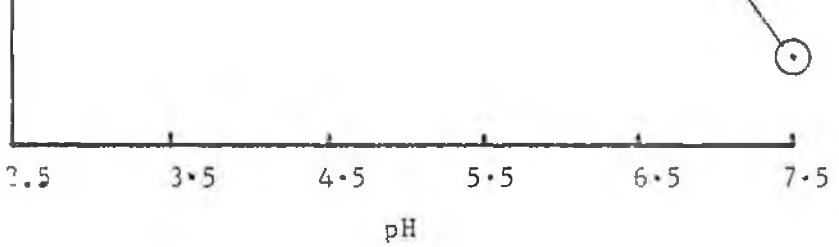
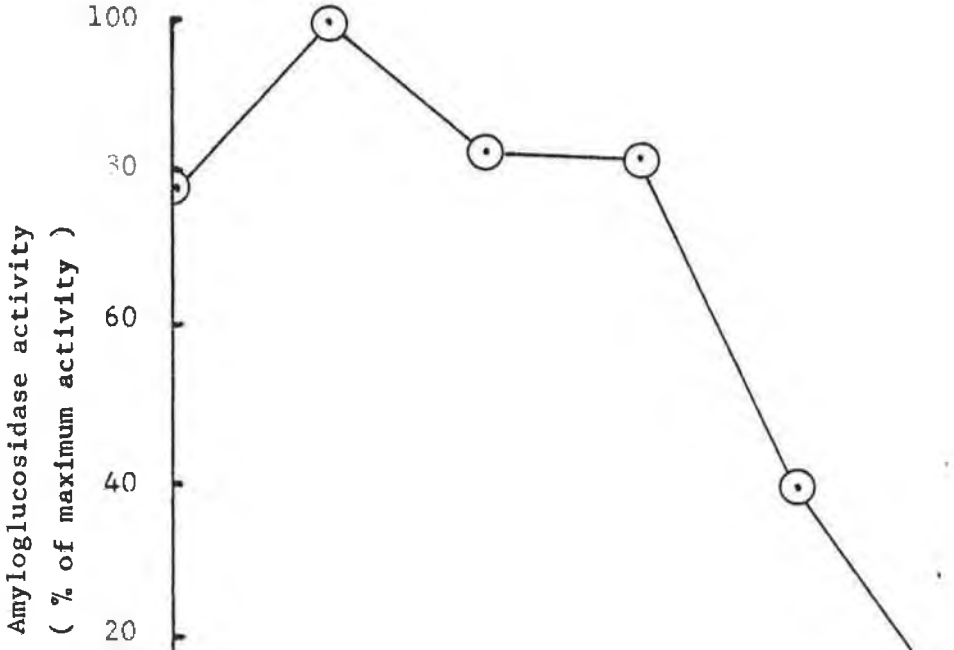


Figure 3.4 Effect of pH on amyloglucosidase activity of A. awamori NRRL 3112


Amyloglucosidase activity
(% of maximum activity)
100
80
60
40
20

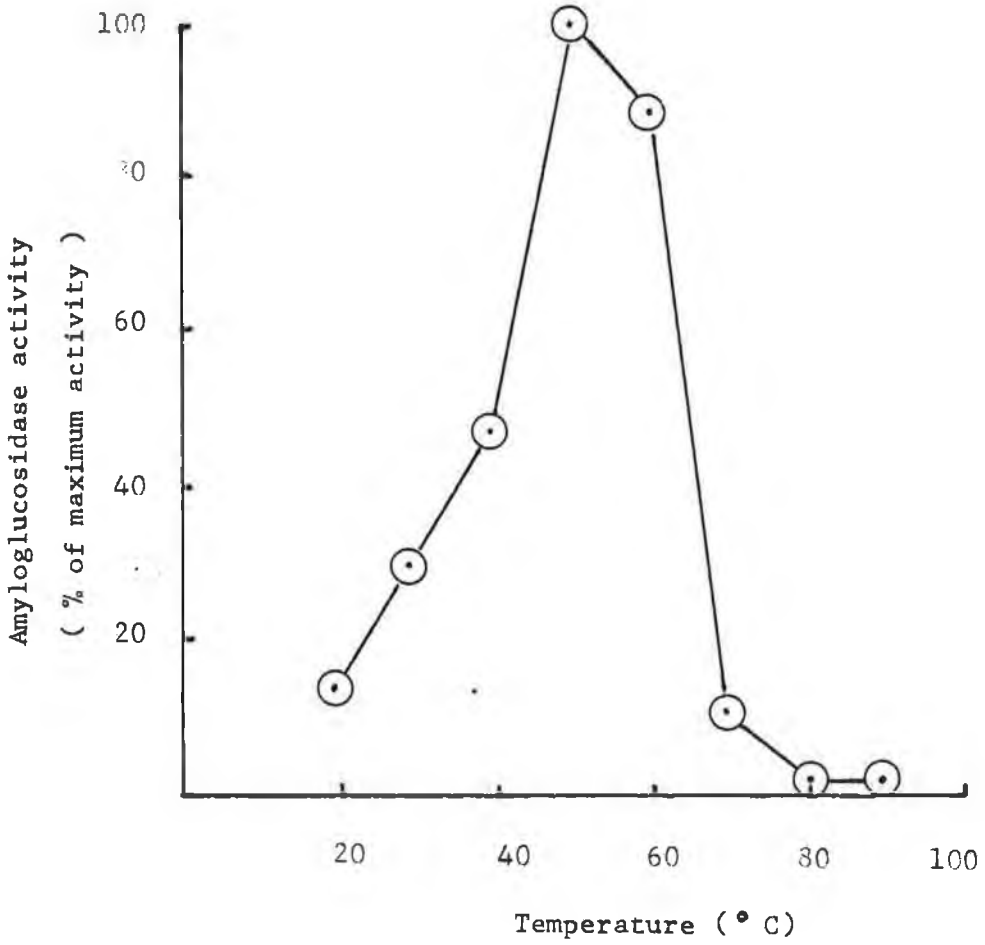


Figure 3.5 Effect of temperature on amyloglucosidase activity of A. awamori NRRL 3112

Section 3.3 Microbial Growth and Production of α-Amylase and Amyloglucosidase by A. niger CBS 26265 and A. awamori NRRL 3112 in Shake Flask Culture.

3.3.1 Production of α-amylase and amyloglucosidase in complex media.

Having obtained suitable α-amylase and amyloglucosidase producers (Section 3.1), the effect of various complex media components was examined, with an aim of optimising extracellular enzyme production. All the following media (Medium C-G) are theoretically equivalent nutritionally to medium B. Carbon, nitrogen, potassium, phosphorous, calcium, sulphur, magnesium, zinc, copper, iron and manganese were balanced using theoretical calculations for the average composition of the complex and semi-complex media components (Section 2.5.1). The buffer used in some of the media (E and G), citrate, had previously been shown not to be utilised by either A. awamori NRRL 3112 or A. niger CBS 26265, as their sole carbon source. Results for A. niger CBS 26265 and A. awamori NRRL 3112 are given in Tables 3.2 and 3.3 which follow.

Maize combined with corn steep powder (medium B) provided a well buffered medium. Using it, maximum production of α-amylase with A. niger CBS 26265 and amyloglucosidase with A. awamori NRRL 3112 were obtained. The second most successful medium for amyloglucosidase production was the buffered medium E; with A. awamori NRRL 3112, it yielded 84% the amount of amyloglucosidase produced on medium B, while with A. niger CBS 26265 it produced only 20%.

In most other cases, media containing NH_4Cl were poorly buffered, even if citrate buffer was added, suggesting that stronger buffers would be required to maintain pH. The low pH values obtained led to low enzyme levels. (Cell-free supernatents with pH values below 2.1 were not assayed due to the poor stability of both enzymes, under highly acidic conditions).

Due to the particulate nature of liquefied maize, biomass could not be determined on media B and C. Maximum biomass levels with both organisms were obtained on corn steep powder /starch media (media D and E). This was probably due to the presence in this complex nitrogen source of small

Table 3.2: Growth and production of ∝-amylase and amyloglucosidase on complex media by A. niger CBS 26265 after 96 h.

	Medium (g/l)			pH	pH	Dry Weight	Reducing Sugar	∝-amylase	amylo-glucosidase
	Carbon	Nitrogen*	Buffer	Initial	Final	g/l	g/l	units/ml	units/ml
B	Maize (100)	CSP (20)	No	Solid	4.51	-	33.3	1936.7	9.8
C	Maize (100)	NH_4Cl (3.1)	No	Solid	2.05	-	ND	ND	ND
D	Starch (50)	CSP (20)	No	4.04	2.82	1.70	16.9	Trace	2.0
E	Starch (50)	CSP (20)	Yes	4.49	3.61	2.45	11.2	71.5	3.0
F	Starch (50)	NH_4Cl (4.6)	No	4.94	1.54	1.26	10.2	ND	ND
G	Starch (50)	NH_4Cl (4.6)	Yes	5.70	1.96	1.08	14.0	ND	ND

N.D. = Not Determined. Trace = less than 70 units ∝-amylase/ml.

* indicates major nitrogen source. CSP (corn steep powder) or ammonium chloride.

Table 3.3: Growth and production of ∝-amylase and amyloglucosidase on complex media after 96h by A. awamori NRRL 3112

	Medium (g/l)			pH	pH	Dry Weight	Reducing Sugar	∝-amylase	amylo-glucosidase
	Carbon	Nitrogen	Buffer	Initial	Final	g/l	g/l	units/ml	units/ml
B	Maize (100)	CSP (20)	No	Solid	4.18	-	39.5	780.0	14.4
C	Maize (100)	NH_4Cl (3.1)	No	Solid	2.07	-	24.0	Trace	0.9
D	Starch (50)	CSP (20)	No	4.04	2.63	2.35	10.2	Trace	7.4
E	Starch (50)	CSP (20)	Yes	4.49	3.40	1.57	4.9	Trace	12.1
F	Starch (50)	NH_4Cl (4.6)	No	4.94	1.50	1.41	8.2	Trace	0.1
G	Starch (50)	NH_4Cl (4.6)	Yes	5.70	1.95	1.60	13.8	ND	ND

N.D. = Not Determined. Trace = less than 70 units ∝-amylase/ml.

quantities of chemicals which stimulate growth (e.g. amino acids, trace elements). Non-particulate simple and semi-complex media were used for further experiments in order that biomass yields could be related to other culture parameters.

3.3.2 Production of ∝-amylase and amyloglucosidase in simple media, by A. niger CBS 26265 and A. awamori NRRL 3112.

The effects on biomass and enzyme production of glucose and maltose used in combination with corn steep powder or NH_4Cl were examined. As in the previous experiment, these media (H - Q) were nutritionally equivalent, in terms of carbon, nitrogen, sulphur, potassium, phosphorus, calcium, magnesium, zinc, copper and iron, to medium B and similarly equivalent to media C - G. Again, sodium citrate was used as a buffering agent. Results are shown in Tables 3.4 and 3.5.

Large reductions in pH were observed in culture media containing ammonium chloride, both with and without buffer. The pH after four days was 1.53 - 1.93. Neither enzyme was detected in these fermentation broths. Corn steep powder was a good nitrogen source for biomass production in A. awamori NRRL 3112. Addition of buffering agent to media with corn steep powder enhanced ∝-amylase and, in most cases amyloglucosidase levels after four days, in both organisms, due to the increased stability of the enzymes (particularly ∝-amylase) at higher pHs.

A. awamori NRRL 3112 produced higher levels of amyloglucosidase than A. niger CBS 26265. Medium N gave highest biomass levels (2.16 g/l), greatest amyloglucosidase production (13.3 units/ml) and highest pH value (3.57) recorded with A. awamori NRRL 3112.

Maltose proved to be a good carbon source, giving in general more biomass than glucose. However, when media J and N (both buffered with corn steep powder) were compared in A. niger CBS 26265, glucose (medium J) gave substantially higher levels of ∝-amylase than medium N, while amyloglucosidase levels were equal. In A. awamori NRRL 3112, maltose yielded increased levels of both enzymes.

Table 3.4: Growth and production of α-amylase and amyloglucosidase on simple media by A. niger CBS 26265 after 96h.

	Medium (g/l)			pH	pH	Biomass	Reducing Sugar	α-amylase	amylo-glucosidase
	Carbon	Nitrogen*	Buffer	Initial	Final	g/l	g/l	units/ml	units/ml
H	Glucose (555)	CSP (20)	No	4.03	2.85	1.77	16.9	72.2	3.2
J	Glucose (555)	CSP (20)	Yes	4.48	3.73	1.77	15.1	131.2	4.1
K	GLucose (555)	NH_4Cl (4.6)	No	4.94	1.52	0.87	25.6	ND	ND
L	Glucose (555)	NH_4Cl (46)	Yes	5.62	1.93	0.88	16.6	ND	ND
M	Maltose (53)	CSP (20)	No	4.02	2.90	1.95	21.1	Trace	2.0
N	Maltose (53)	CSP (20)	Yes	4.44	3.58	1.83	13.8	96.9	4.2
P	Maltose (53)	NH_4Cl (4.6)	No	4.94	1.55	0.90	23.3	ND	ND
Q	Maltose (53)	NH_4Cl (4.6)	Yes	5.64	1.92	0.97	17.1	ND	ND

* major nitrogen source only. CSP = corn steep powder

Table 3.5: Growth and production of α-amylase and amyloglucosidase on simple media by A. awamori NRRL 3112 after 96h.

	Medium (g/l)			pH	pH	Biomass	Reducing Sugar	α-amylase	amylo-glucosidase
	Carbon	Nitrogen*	Buffer	Initial	Final	g/l	g/l	units/ml	units/ml
H	Glucose (555)	CSP (20)	No	4.03	2.60	1.60	15.1	Trace	8.5
J	Glucose (55.5)	CSP (20)	Yes	4.48	3.52	1.52	7.5	Trace	7.8
K	Glucose (55.5)	NH_4Cl (4.6)	No	4.94	1.55	1.27	13.3	ND	ND
L	Glucose (555)	NH_4Cl (4.6)	Yes	5.62	1.80	1.44	15.1	ND	ND
M	Maltose (53)	CSP (20)	No	4.02	2.56	1.60	18.2	Trace	9.6
N	Maltose (53)	CSP (20)	Yes	4.44	3.57	2.16	13.4	80.8	13.3
P	Maltose (53)	NH_4Cl (4.6)	No	4.94	1.57	1.10	13.3	ND	ND
Q	Maltose (53)	NH_4Cl (4.6)	Yes	5.64	1.80	1.47	16.0	ND	ND

* major nitrogen source only. CSP = corn steep powder.

3.3.3 Effect of carbon: nitrogen ratio on α-amylase and amyloglucosidase production.

The effect of carbon: nitrogen ratio on enzyme production was monitored. Medium H was chosen for this experiment; the glucose concentration was maintained, while the amount of corn steep powder was varied (Section 2.5.1). Results after 4 days are given in Tables 3.6 and 3.7.

Table 3.6: Effect of carbon:nitrogen ratio on growth and enzyme production in A. niger CBS 26265 after 96 h.

	C/N ratio	pH Final	Dry Weight g/l	Reducing sugar g/l	α-Amylase units/ml	Amylo-glucosidase units/ml
H1	111	2.31	1.05	6.4	Trace	1.18
H2	55	3.21	2.22	10.3	157.3	5.09
H	28	4.32	2.28	11.0	841.9	8.63
H3	19	4.60	2.47	6.5	1848.0	10.14
H4	14	4.77	2.44	11.1	2540.0	9.32
H5	11	4.82	2.54	7.2	2359.0	9.91

Trace = less than 70 units/ml α-amylase.

Table 3.7: Effect of carbon:nitrogen ratio on growth and enzyme production on A. awamori NRRL 3112 after 96 h.

	C/N ratio	pH Final	Dry Weight g/l	Reducing sugar g/l	α-Amylase units/ml	Amylo-glucosidase units/ml
H1	111	2.65	1.84	16.6	Trace	1.33
H2	55	3.03	2.07	7.2	96.4	7.59
H	28	4.12	2.25	5.6	209.9	15.76
H3	19	4.33	2.38	4.8	589.7	16.28
H4	14	4.50	2.43	4.4	1118.1	14.77
H5	11	4.65	2.66	9.3	1544.5	15.70

Trace = less than 70 units/ml α-amylase.

The most notable feature was the increase in final pH, with increasing corn steep powder concentration. After autoclaving, all flasks had pH values of 5.7; after 4 days' growth, pH values varied from 2.31 to 4.82 with A. niger CBS 26265 and 2.65 to 4.65 with A. awamori NRRL 3112. There was an increase in biomass with increased corn steep powder concentration, and this was paralleled with greater α-amylase level per ml of supernatent. When α-amylase levels per mg biomass are compared, there was an increase from 70.9 to 1041.4 units of α-amylase per g dry weight as the corn steep powder concentration increased from 10 to 40 g/l in A. awamori NRRL 3112. With A. niger CBS 26265, the increase was less dramatic, i.e. 46.6 to 460.1 units/g dry weight.

There was an increase in amyloglucosidase levels in both organisms, up to 30 g/l corn steep powder (i.e. C:N of 19:1). The maximum amyloglucosidase level per mg biomass recorded with A. awamori NRRL 3112 was 7.00 units/g dry weight on medium H. This medium also gave the best productivity for input of corn steep powder, i.e. 788 units per g corn steep powder.

In this experiment, it was difficult to attribute any change in the fermentation to changes in the medium, since corn steep powder was responsible for (1) changes in nitrogen concentration, (2) changes in the medium's buffering capacity and pH and (3) changes in nutrient concentrations.

3.3.4 Effect of citrate on α-amylase and amyloglucosidase production.

Since many of the effects recorded in the previous experiments depended on pH of the medium, particularly the media involving corn steep powder and its concentration (i.e. Section 3.3.3), this experiment tried to separate the pH buffering effects of corn steep powder from its nutritional effects. Medium H, with various amounts of tri sodium citrate dihydrate (Section 2.5.1), were inoculated with A. awamori NRRL 3112 and A. niger CBS 26265. The medium was adjusted to pH 6.0, prior to addition of sodium citrate. Post-sterilisation pH values of the media H 6-9 are presented, with other results in Tables 3.8 and 3.9.

Table 3.8: Effect of citrate on α-amylase and amyloglucosidase production in A. niger CBS 26265 after 96 h.

	Sodium Citrate g/l	pH Initial	pH Final	Dry Weight g/l	Reducing Sugar g/l	α-amylase units/ml	Amylo-glucosidase units/ml
H	0	5.74	4.32	2.28	11.0	814.9	8.63
H6	3	5.89	4.65	2.25	9.9	1978.8	10.87
H7	5	5.67	4.61	2.33	6.8	ND	10.46
H8	7.5	5.99	4.98	2.41	8.0	2393.4	9.70
H9	10	6.05	5.12	2.67	8.5	2388.4	9.90

ND = not determined

Table 3.9: Effect of citrate on α-amylase and amyloglucosidase production in A. awamori NRRL 3112, after 96 h.

	Sodium Citrate g/l	pH Initial	pH Final	Dry Weight g/l	Reducing Sugar g/l	α-amylase units/ml	Amylo-glucosidase units/ml
H	0	5.74	4.12	2.25	5.6	209.9	15.76
H6	3	5.89	4.32	2.13	8.2	381.3	14.13
H7	5	5.67	4.37	ND	2.1	468.9	14.95
H8	7.5	5.99	4.52	2.04	1.7	704.1	11.98
H9	10	6.05	4.68	2.21	1.5	903.2	12.26

Addition of citrate to medium H gave increasing initial pH values and also increased final pHs. With both organisms, more α-amylase was detected in less acidic fermentation broths. Again, this effect is more than can be accounted for solely by an increase in enzyme stability. As before, A. niger CBS 26265 produced considerably more α-amylase than A. awamori NRRL 3112. Citrate itself (or the resultant buffering effect) stimulated biomass formation in A. niger

CBS 26265, but had no appreciable effect on growth in A. awamori NRRL 3112.

Amyloglucosidase production in A. awamori NRRL 3112 was at a maximum when no citrate had been added (medium H; 15.8 units/ml) while in A. niger CBS 26265 it occurred at 3g/l citrate, but with 80% or more of this amount produced at all other citrate levels tested, showing that A. niger CBS 26265 was insensitive to citrate concentration.

Due to the high levels of amyloglucosidase produced by A. awamori NRRL 3112 on medium H, and the acceptable levels of both enzymes demonstrated on this medium by A. niger CBS 26265, it was decided to use it for further studies.

3.3.5 Time curve for the growth and production of α-amylase and amyloglucosidase on medium H.

From the previous experiments (Section 3.3.3 and 3.3.4) medium H appeared to be a good medium for growth and production of enzymes by both organisms, especially amyloglucosidase from A. awamori NRRL 3112. Samples were taken from the flasks of medium H every 24 hours and assayed for reducing sugars, as well as α-amylase and amyloglucosidase activity, to determine the optimal time for enzyme production. Results are presented in figures 3.6 and 3.7.

During the 7 day fermentation, the pH fell from 5.7 to 4.1-4.2 with both organisms and then rose again slowly, indicating the start of cell lysis. Reducing sugar curves were similar in both fermentations, with most of the available sugars being used up by 96 h.

Amyloglucosidase production was again greater in A. awamori NRRL 3112 than A. niger CBS 26265. In the latter, this enzyme was produced at an almost constant rate over the 7 day period, with 9.7 units/ml recorded on the final day. The rate of production by A. awamori NRRL 3112 was almost 150% that of A. niger CBS 26265. Maximum enzyme activity occurred on day 6 (13.1 units/ml), in A. awamori NRRL 3112.

A. niger CBS 26265 showed a maximum rate of α-amylase production at 24 h (32.6 unit/ml of medium / h) with a second production phase between 144 and 168 h (22.9

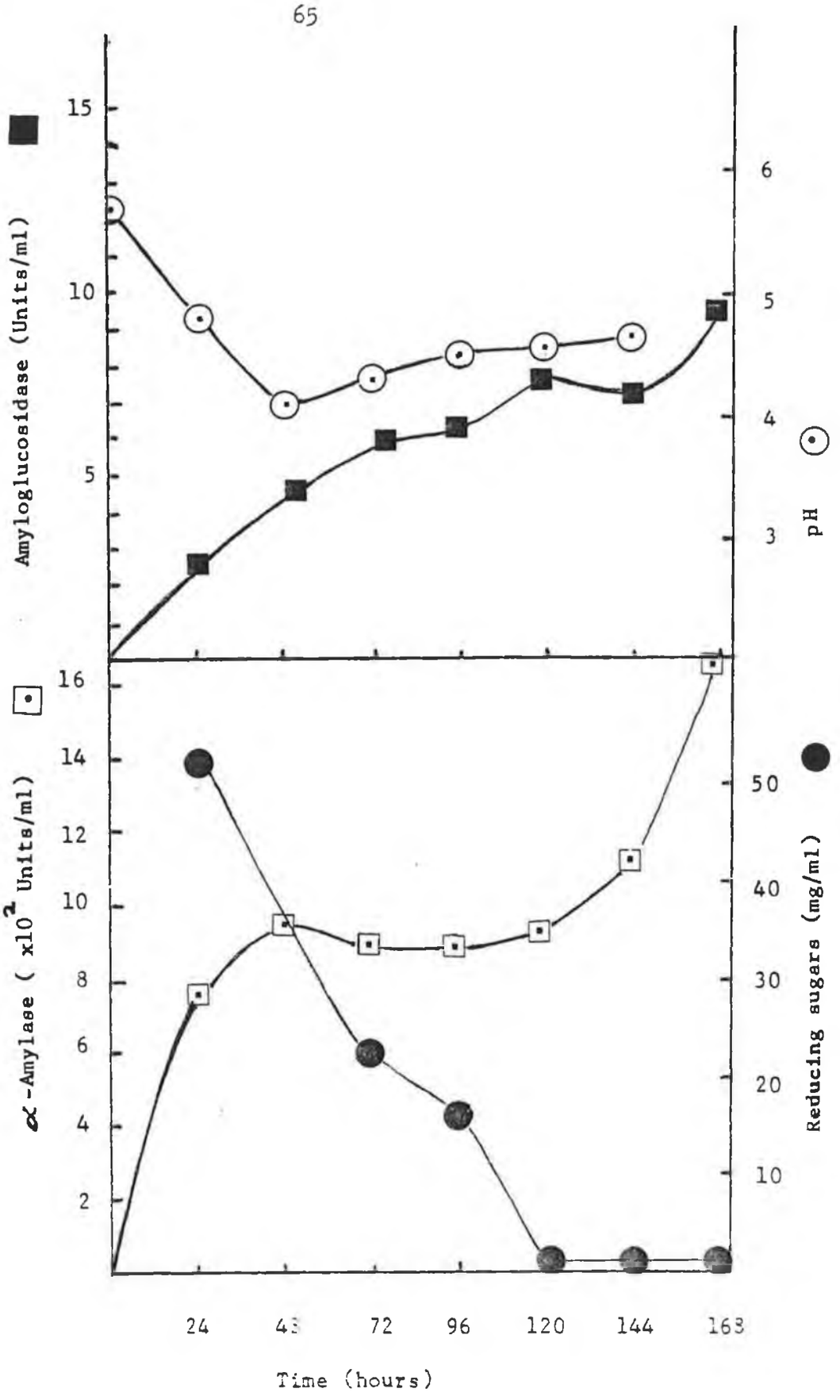


Figure 3.6 Time curve for α-amylase and amyloglucosidase production by A. niger CBS 26265 in medium H

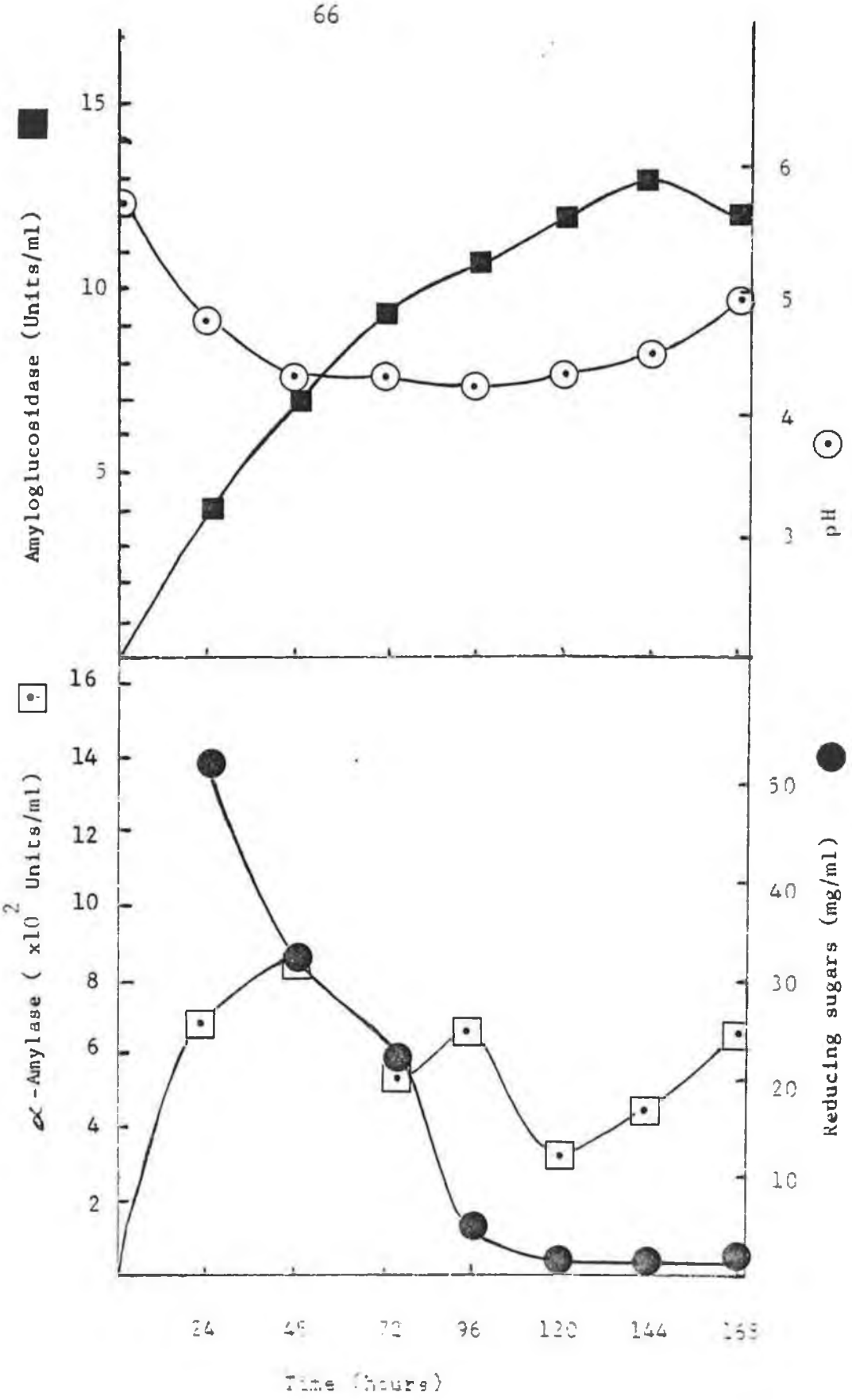


Figure 3.7 Time curve for α-amylase and amyloglucosidase production by A. awamori NRRL 3112 in medium H

units/ml/h). In comparison, α-amylase production in A. awamori NRRL 3112 was erratic, with a maximum of 885 units/ml on the second day and decreasing after that.

Biomass produced by A. awamori NRRL 3112 was in the range 2.1 - 2.5 g dry weight/l.

3.3.6 Time curves for growth and α-amylase and amyloglucosidase production on medium H5.

From section 3.3.3, medium H5 showed optimal α-amylase production for A. awamori NRRL 3112, and very high levels for A. niger CBS 26265 (2359.0 units/ml at 96 h). It was decided to compare the two organisms grown on medium H5 (the 'α-amylase medium') and also to compare these results with those obtained on medium H (the 'amyloglucosidase medium') in the previous section (Section 3.3.5). Results are presented in Figures 3.8 and 3.9.

With both organisms, pH of the fermentation broths fell from 5.7 to 4.8 (48 h) in A. niger CBS 25265 and 4.6 (72 h) in A. awamori NRRL 3112, and later rose again to 5.5 in both organisms at 196 h. These values were higher than in the previous experiment (3.3.5) but predictable due to the higher concentration of corn steep powder (Section 3.3.3).

The reducing sugar curves were similar in both organisms and in good agreement with the previous experiment.

A. awamori NRRL 3112 produced biomass in the range 3.2 - 3.6 g dry weight/l. This was greater than that recorded with this organism on medium H (2.1 - 2.5 g/l), and can probably be attributed to the extra nutrition available for growth in medium H5 (i.e. 30g/l extra corn steep powder).

A. awamori NRRL 3112 showed diphasic production of α-amylase, with the initial rate of production (24 h) 26.8 units/ml of medium/h, and the second peak (120-144h) 22.7 units/ml/h. This was similar to the results recorded with this organism on medium H (Section 3.3.5). In contrast, A. niger CBS 26265 showed an almost constant rate of production with a maximum of 3011.8 units per ml, produced at 144 h. This was almost double the amount produced by A. awamori NRRL 3112, on medium H5, and 2.6 times the α-amylase level produced by A. niger CBS 26265 on medium H.

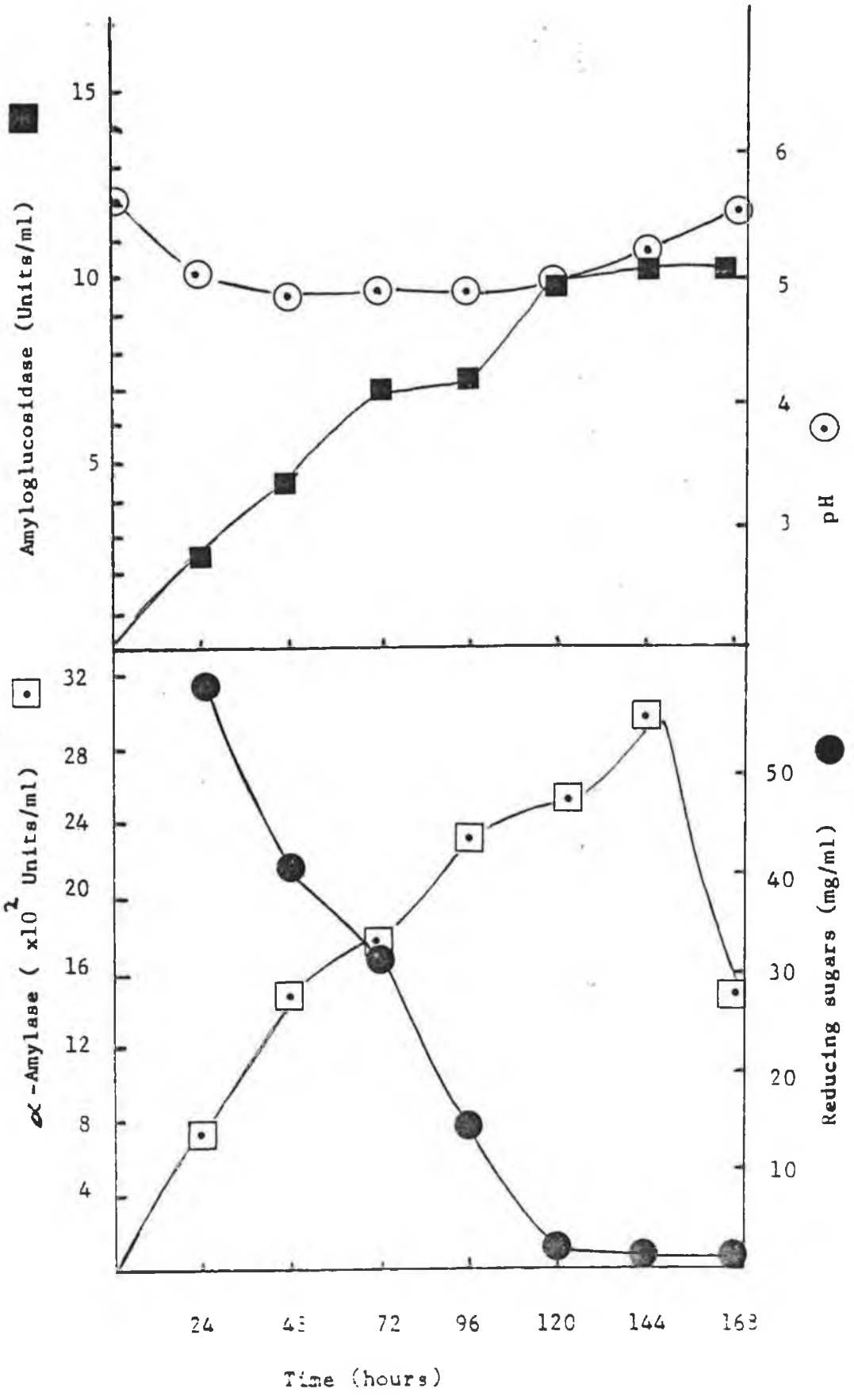


Figure 3.8 Time curve for α-amylase and amyloglucosidase production by A. niger CBS 26265 in medium H5

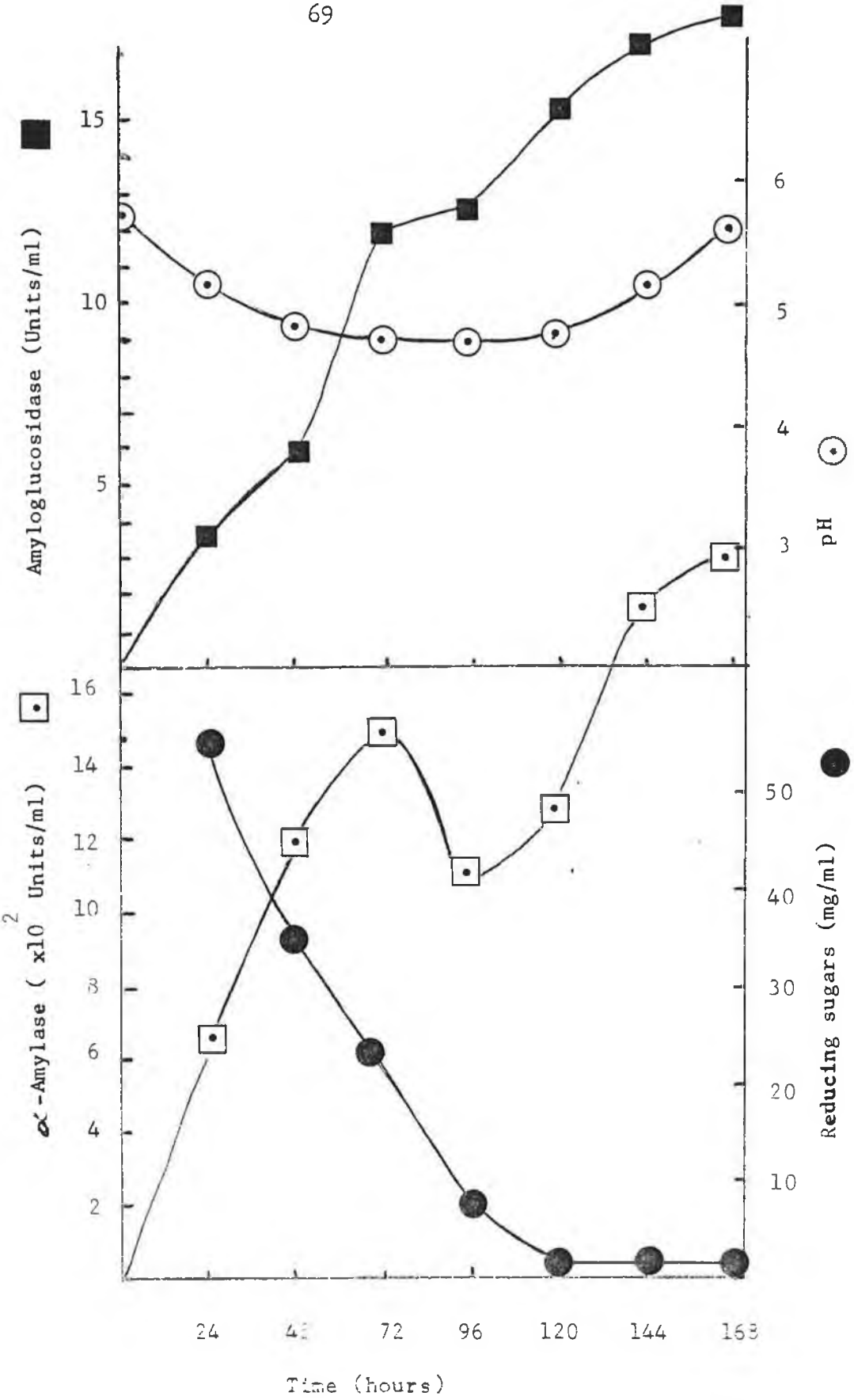


Figure 3.9 Time curve for α-amylase and amyloglucosidase production by A. awamori NRRL 3112 in medium H5

Amyloglucosidase as usual was produced in greater quantities by A. awamori NRRL 3112 than A. niger CBS 25265; at 168 h, the former had produced 17.8 units/ml, while the latter had given only 57% of this amount. In both fungi, the rate of production was almost linear. As with α-amylase, the more nutritious medium (H5) gives higher amyloglucosidase levels than medium H (Section 3.3.5).

3.3.7 Effect of initial pH on growth and enzyme production in A. awamori NRRL 3112.

In fungal fermentations, pH is a constantly changing variable. Previous experiments monitored the effect of pH on growth and enzyme production. This experiment aimed to show the effect of initial pH on these variables. Medium H was adjusted to the required initial pH, i.e. pHi 6.0, 5.5, 5.0, 4.5 and 4.0, with NaOH, prior to sterilization. Samples were taken every 24 hours over a 7 day period.

A. awamori NRRL 3112 was chosen for this study due to its ability to produce high levels of amyloglucosidase and adequate levels of α-amylase on medium H. Results are shown in Figures 3.10, 3.11 and 3.12.

With A. awamori NRRL 3112, medium pH values dropped 1.5-2.0 pH units by 120h, and then rose sharply, probably due to autolysis. In each case, sugars were completely assimilated by this time.

Production of α-amylase was greatest at 48h; at pHi 6.0 352 units/ml, while the lowest pHi had 30% of this value. A similar trend occurred in units per g biomass. At 48h, 282, 235, 159, 118 and 56 units of amylase/g dry weight were recorded in flasks with pHis of 6.0, 5.5, 5.0, 4.5 and 4.0. Although some of this variation can be related to enzyme stability (see section 3.2.1 and 3.2.2), it would also appear that pH affects the yields of enzyme through pH control of transcription/translation and secretion.

The production of α-amylase/g dry weight varies with the age of the fermentation (Figure 3.10), reaching a maximum at 24-48h. This would suggest that expression and/or secretion of the enzyme are time dependent, or that inducers are only formed at that time in the growth/production cycle.

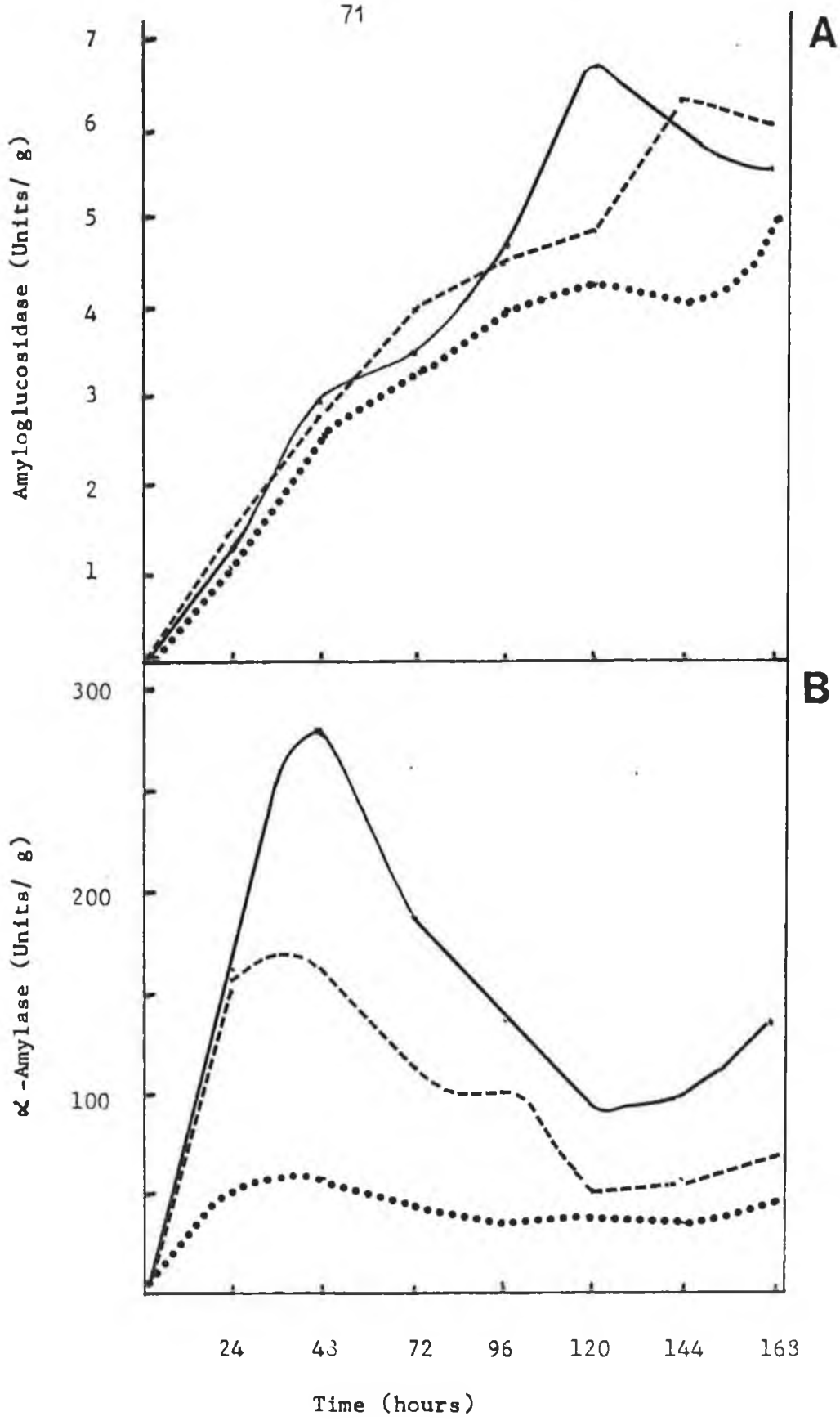


Figure 3.10 The effect of initial pH on enzyme production per g dry weight by A. awamori NRRL 3112, in medium H.
A. Amyloglucosidase production. B. ∝-Amylase production.
pHi 6.0 ——— pHi 5.0 - - - - - - - pHi 4.0 •••••••

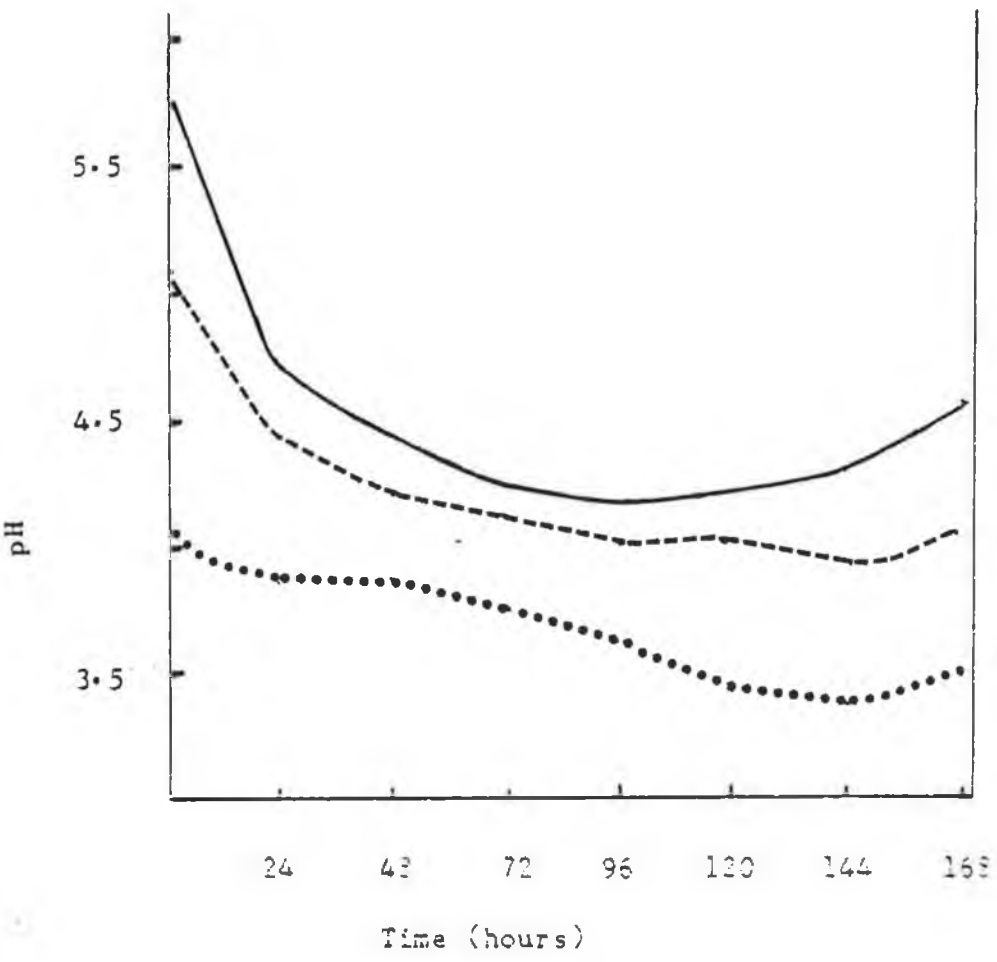


Figure 3.11 The effect of initial pH on pH curve of A. Awamori NRRL 3112 in medium H

pHi 6.0 ——— pHi 5.0 ------- pHi 4.0 ••••••••

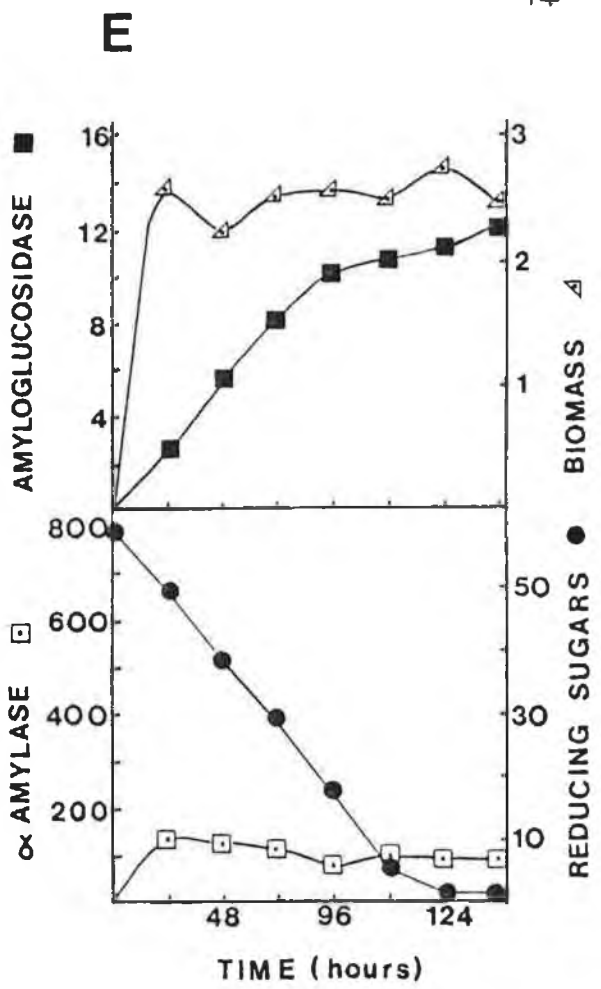


Figure 3.12 Growth and enzyme production by A. awamori NRRL 3112 in medium H, at various initial pH values.
A = Initial pH 6.0;
B = Initial pH 5.5;
C = Initial pH 5.0;
D = Initial pH 4.5;
E = Initial pH 4.0.

Rates of production of α-amylase by A. awamori NRRL 3112 over the initial 48h are given in Table 3.10, while rates of production of amyloglucosidase (up to 144h) are given in Table 3.11.

Table 3.10. Rate of production of α-amylase by A. awamori NRRL 3112 on medium H, at various initial pH values, over the initial 48h period.

Initial pH	α-Amylase units/ml/h		
	0-24h	24-48h	0-48h
			Average
6.0	22.1	15.9	19.0
5.5	23.1	13.8	18.4
5.0	17.8	9.0	13.4
4.5	15.9	- 2.2	6.9
4.0	5.5	- 3.3	1.1

Table 3.11. Rate of production of amyloglucosidase by A. awamori NRRL 3112 on medium H, with various initial pH values.

Initial pH	amyloglucosidase units/ml/h						
	24h	48h	72h	96h	120h	144h	Average
6.0	0.18	0.17	0.13	0.12	0.13	0.11	0.14
5.5	0.19	0.17	0.14	0.12	0.13	(0.03)	0.15
5.0	0.17	0.16	0.14	0.12	0.11	0.11	0.135
4.5	0.17	0.14	0.12	0.11	0.11	0.10	0.125
4.0	0.12	0.12	0.12	0.11	0.09	0.08	0.107

Amyloglucosidase production was less affected by initial pH than α-amylase. Maximum yields were obtained late in the fermentation, 120-144h. Production at pH 4.0 was noticeably lower than in the other flasks. This can be accounted for by (i) poor biomass yields, and (ii) low pH values which adversely affect enzyme stability and/or expression secretion.

The first 24h shows the greatest increase in biomass. This appears to correspond with the period of exponential growth.

3.3.8 Intra- and extracellular production of ∝-amylase and amyloglucosidase by A. awamori NRRL 3112, on medium R and S.

Since some of the phenomenon observed in the previous experiments could be explained if it was known whether the enzyme was produced in the cell (if at all) or whether it was being transported out of the cell, it was decided to measure both the levels of intracellular and extracellular enzyme in A. awamori NRRL 3112.

Yabuki and co-workers (1977) in an experiment to elucidate the control mechanism involved in formation of ∝-amylase by A. oryzae, designed two media for growth and amylase production. Medium R, called peptone-glycerol or non-inducing medium, contained glycerol as sole carbon source, while medium S contained starch.

In the following experiments, both intra and extracellular levels were recorded daily (Section 2.8). Results are recorded in Figures 3.13 and 3.14.

On medium R, ∝-amylase production (both intra and extracellular) peaked at 48 h, while on medium S, both peaks occurred at 24h. Production of extracellular ∝-amylase on starch medium was twenty seven times greater than on glycerol. Both of these phenomena can be attributed to the inductive powers of starch. Extracellular amyloglucosidase levels were six times greater on medium S, than medium R, again probably due to the presence of inducers.

Both media yielded similar biomass levels, ca 11 g/l, although medium S appears to have reached exponential growth earlier (24-48h) than medium R (48-96h). Unlike previous experiments where all growth was mycelial and dispersed, A. awamori produced a variety of morphological types on medium R and S, (Table 3.12 and Figure 3.15).

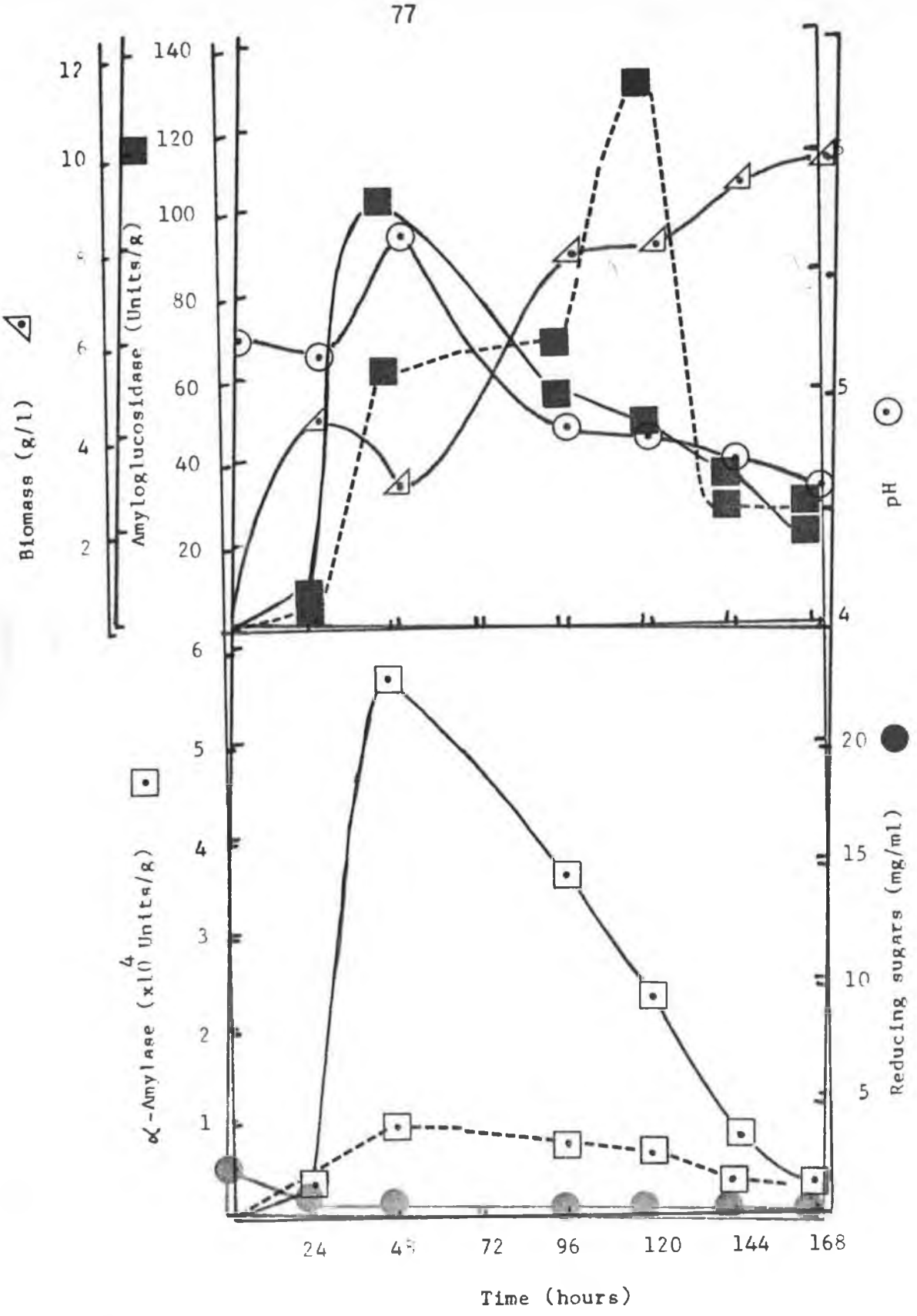


Figure 3.13 Intra and extracellular production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 on medium R

——— extracellular

--------- intracellular

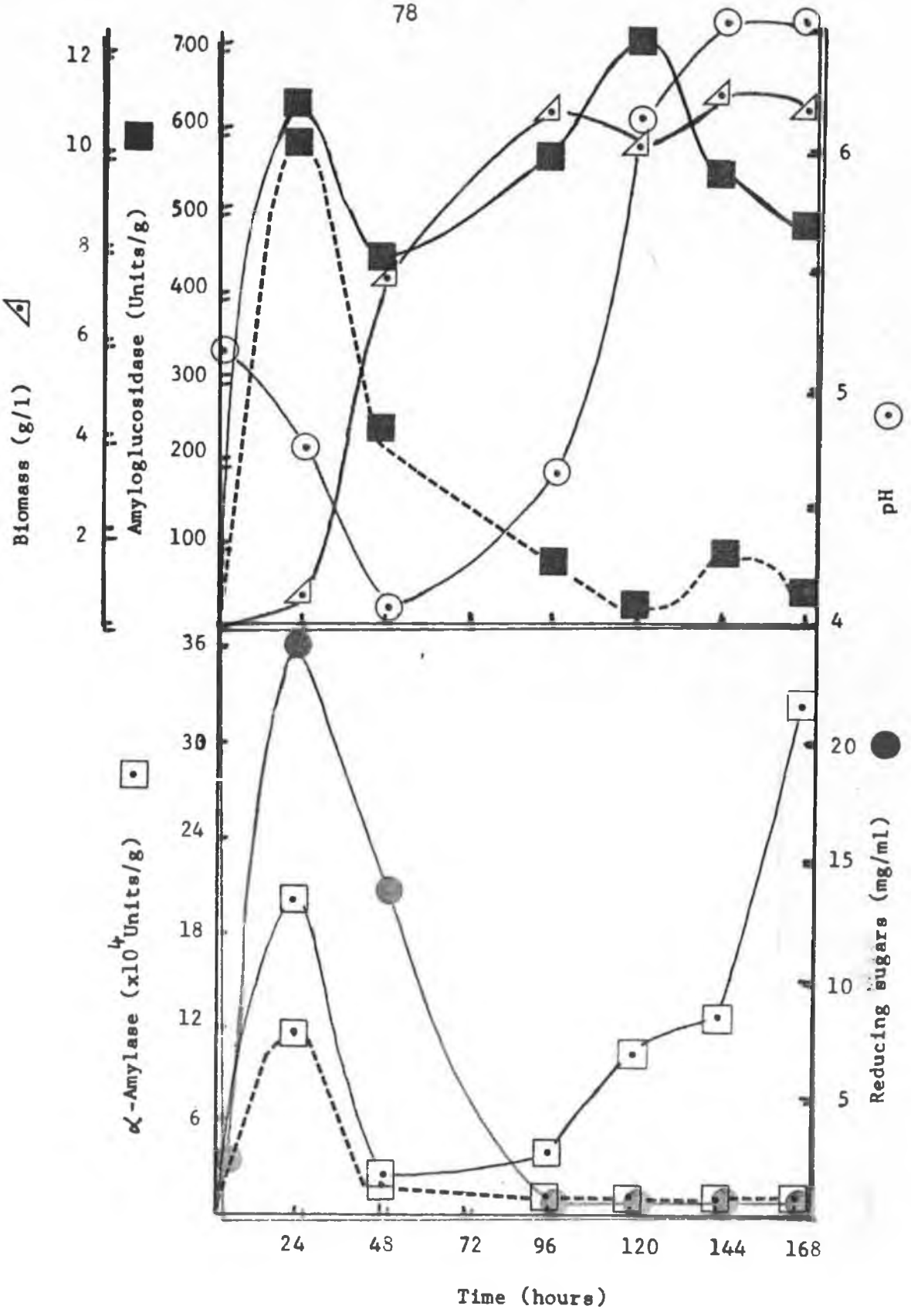
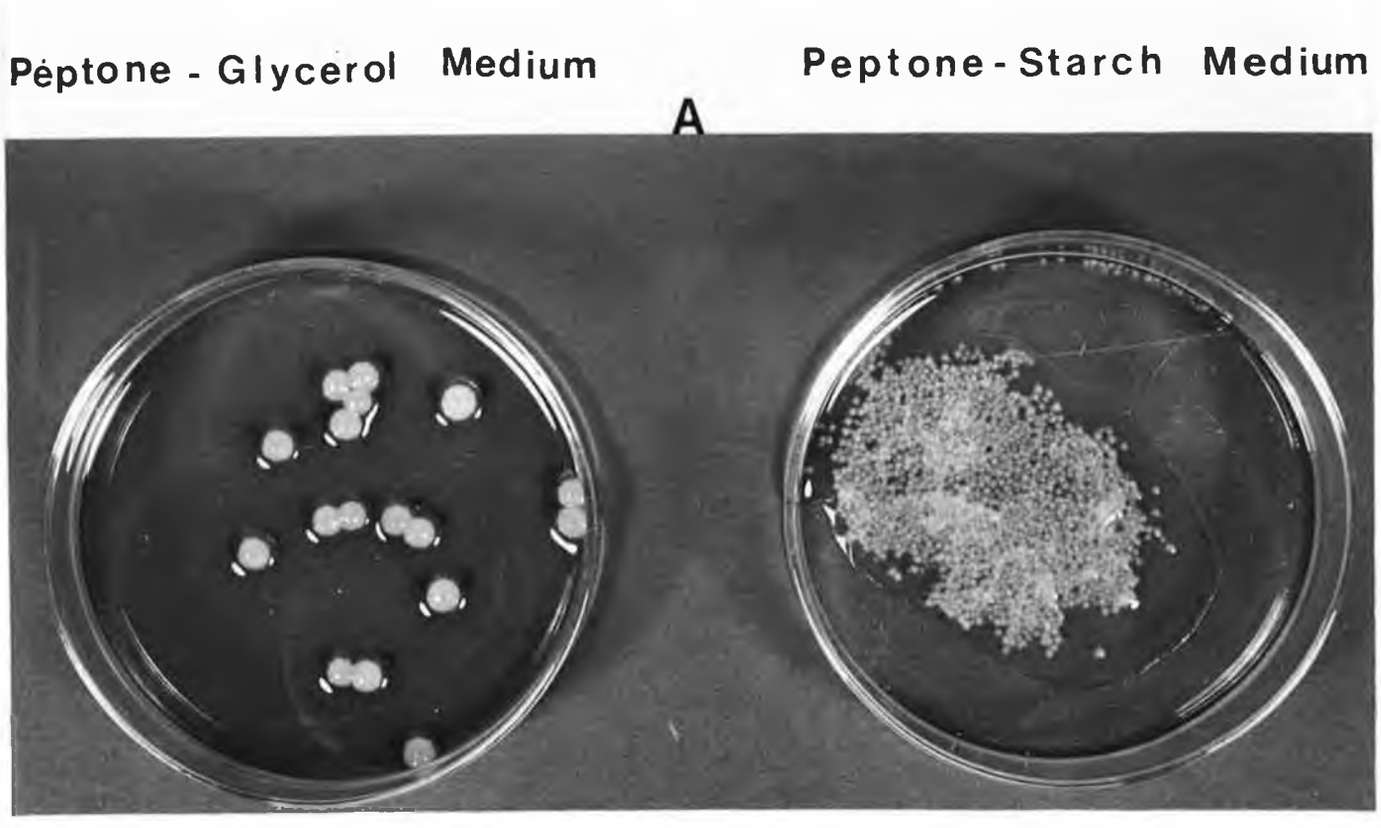


Figure 3.14 Intra and extracellular production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 in medium S

——— extracellular

-------- intracellular



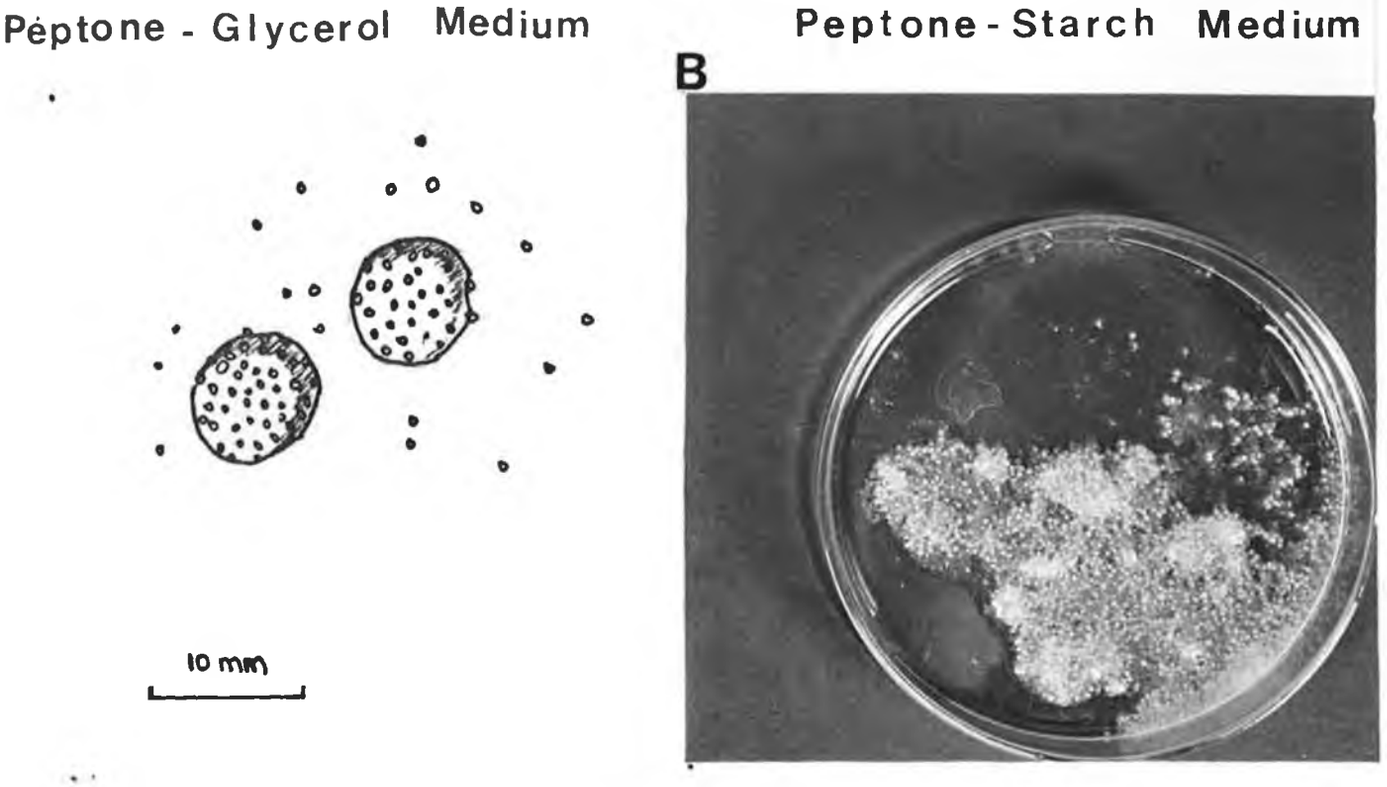


Figure 3.15 Morphological variations occurring in A. awamori NRRL 3112 grown on medium R and S.

A. 24 h B. 72 h C. 96 h.

Peptone - Glycerol Medium

Peptone - Starch Medium

C

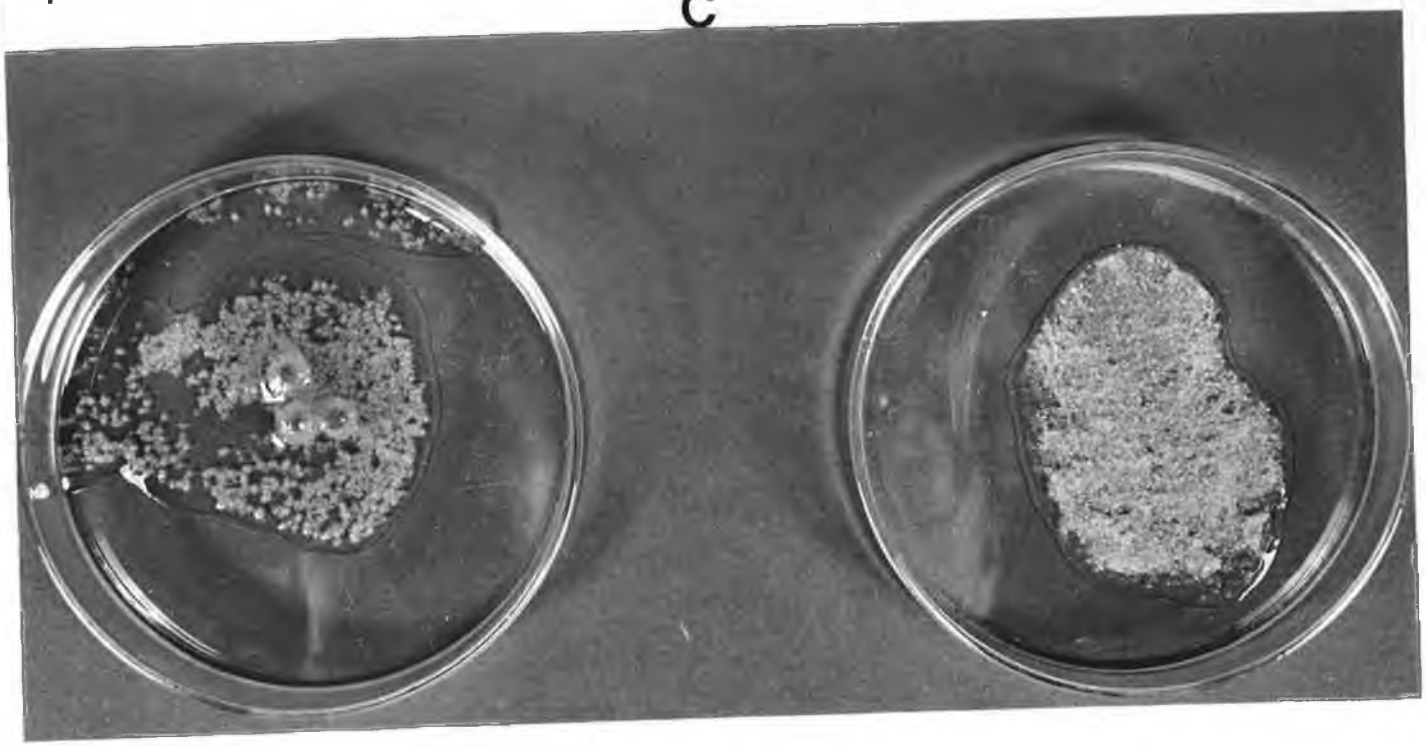


Table 3.12. Morphological variation occurring in A. awamori NRRL 3112 grown on Medium R and S.

Day	Medium R	Medium S
1	3-4 mm 1 pellets	2-3 mm fluffy pellets
2	7-8 mm 1 pellets	2-3 mm very fluffy pellets/ almost dispersed
4	**	No visible pellets, totally dispersed
5	1 mm 2 pellets	Dispersed
6	1 mm 2 pellets	Dispersed
7	1 mm 2 pellets	Dispersed

** Between days 2 and 4, the primary pellets become more fluffy and start to form 1 mm pellets on their surface. Some of these secondary pellets fall from the primary pellets. By day 5, the primary pellets have shattered to give heavy growth of the secondary pellets.

Patterns of enzyme location are recorded in Table 3.13. Secretion patterns were very similar for both α-amylase and amyloglucosidase on starch medium. After recording intracellular levels of between 37 and 49% for both enzymes, for 48h, intracellular levels fell to less than 14% of the total produced. This suggests that (a) secretion is time dependent (which is not borne out by results on medium R); (b) secretion is dependent on the morphological form of the fungus since the shift from predominantly intracellular to extracellular coincides with the shift in morphology from pelleted to dispersed in medium S. Results on glycerol medium would agree since growth remains pelleted for the entire fermentation and intracellular levels remain high (up to 73% of total enzymes).

Table 3.13. Localisation of α-amylase and amyloglucosidase of A. awamori NRRL 3112, in media R and S, during a 168h fermentation.

% of total enzyme which is located intracellularly

Time (hours)	α-Amylase		Amyloglucosidase	
	Medium R	Medium S	Medium R	Medium S
24	-	38.5	39.0	49.3
48	21.7	37.3	38.3	35.6
96	16.4	5.7	48.1	9.3
120	23.0	10.6	72.7	3.6
144	33.9	8.8	45.7	13.6
168	53.9	3.8	55.9	8.6

3.3.9 Appraisal of results

Although media B to Q were all nutritionally equivalent, large differences in enzyme production were recorded. When sections 3.3.1 and 3.3.2 were compared, the following trends emerged:

- Buffered media gave higher levels of α-amylase and amyloglucosidase than unbuffered. Some of this increase in enzyme production can be attributed to increased enzyme stability at higher pH values.

- Media containing corn steep powder, due to its high buffering capacity, gave only a slight decrease in pH, when compared with ammonium chloride. This in turn led to increased enzyme levels.

- A. niger CBS 26265 produced more α-amylase than A. awamori NRRL 3112, while A. awamori NRRL 3112 was the superior amyloglucosidase producer.

- In both organisms, the complex carbon source maize was the preferred substrate for enzyme production, due to the probable presence of minute quantities of inducing substances.

- Of the soluble media, the easily utilised carbon source glucose gave best fungal growth.

The effect of increased corn steep powder and carbon:nitrogen ratio was studied in Section 3.3.3. Increased nitrogen led to improved buffering of the medium, increased biomass (due to increased nutrition) and higher levels of α-amylase (partly due to increased enzyme stability).

To distinguish the effects of corn steep powder as a nutrient and as a buffer, citrate was added to medium H (Section 3.3.4). α-Amylase production increased with increasing pH, up to pH 5.0. This was to be expected, as it coincides with increased enzyme activity/stability. Citrate had an adverse effect on biomass production.

Time curves for the production of α-amylase and amyloglucosidase by both A. niger CBS 26265 and A. awamori NRRL 3112 on medium H and H5 showed some interesting variations.

- Medium H favoured amyloglucosidase production while medium H5 produced α-amylase.

- As expected, A. niger CBS 26265 gave higher levels of α-amylase, while A. awamori NRRL 3112 gave better yields of amyloglucosidase.

- Amyloglucosidase production reached its maximum late in the fermentation (ca. day 7) while α-amylase showed diphasic production; the first peak around 48h, and the second at the end of the fermentation.

- Amyloglucosidase production was greatest in the medium with the smallest fall in pH.

Conditions for production of ∝-amylase and amyloglucosidase are different, with amylase being more sensitive to changes in pH than amyloglucosidase (Section 3.3.7).

Section 3.3.8 studied location of ∝-amylase and amyloglucosidase in A. awamori NRRL 3112. Medium constituents had a large effect; starch or its breakdown products stimulated amylase and amyloglucosidase expression as well as secretion, while glycerol was not as effective an inducer of expression or secretion.

In conclusion, it would appear that A. awamori NRRL 3112 is the superior amyloglucosidase producer.

Section 3.4 Factors affecting production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 in laboratory scale fermenters with and without pH control.

Section 3.3 described how medium components and fermentation conditions were varied in an attempt to optimise amylolytic enzyme production, by A. awamori NRRL 3112 and A. niger CBS 26265, in shake flask culture. A. awamori NRRL 3112 was chosen for further study, because of its superior amyloglucosidase producing capacity, and its ability to grow as 'dispersed mycelium', which should aid growth in a fermenter (Figure 3.1). Media containing corn steep powder as nitrogen source had proved satisfactory for this purpose, therefore, all media prepared contained 20 g/l corn steep powder, and 0.5 ml of Mazu antifoam. Investigations were carried out on the production of enzymes in Microgen fermenters (section 2.4.1). The advantages of using these fermenters were that pH could be controlled, agitation could be varied, and aeration could be adjusted. Initially aeration was set at 0.5 v/v/m, and agitation at 250 rpm.

3.4.1 Comparison of the effect of starch and dextrin on production of α-amylase and amyloglucosidase by A. awamori NRRL 3112.

In this experiment, two carbon sources were compared for their effect on production of biomass, α-amylase and amyloglucosidase. Medium T contained maltodextrin, MD05, and medium U contained soluble starch, as sole carbon source (section 2.5.2). The maltodextrin MD05 is a hydrolysate of starch, which is widely recommended as a substitute for starch in fermentations (Appendix 4).

In both media, the pH was not controlled, and it fell from the initial 4.5 in both, to 4.0 in medium T (Figure 3.16) and 4.1 in medium U (Figure 3.17) after 96h. In the starch medium the small fall in pH was accompanied by a slight decrease in reducing sugars, while in the medium containing MD05, the decrease in reducing sugars was more noticeable, particularly between 72 and 96h.

On both media, α-amylase levels peaked after two days at 258 and 174 units/ml on medium T and U respectively. The initial rate of α-amylase production on MD05 was 24% greater than that on starch, due to the presence of glucose and maltose in the

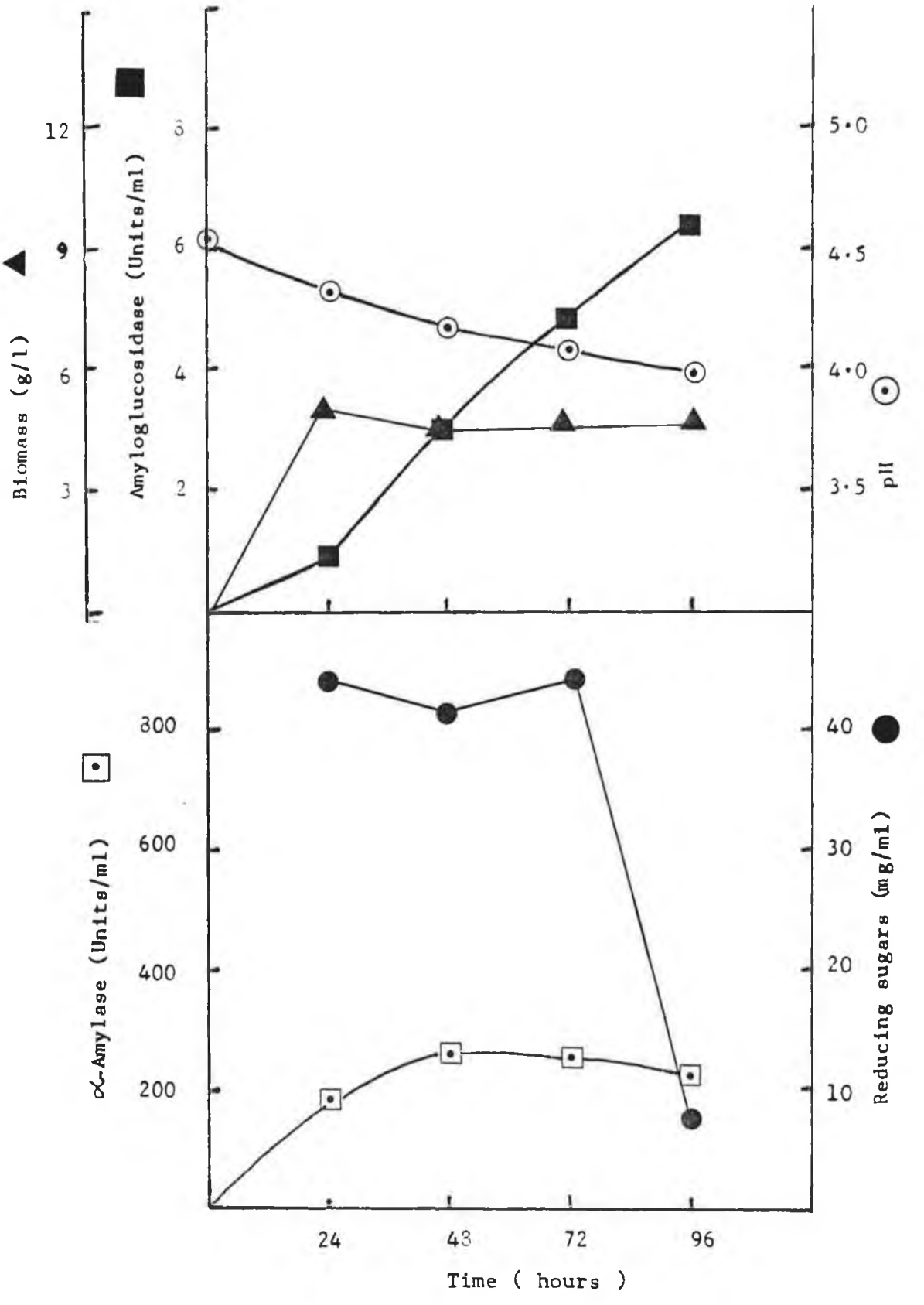


Figure 3.16 Production of α-amylase and amyloglucosidase by A. awamori NRRL 3112, in medium T, initial pH 4.5, 250 rpm.

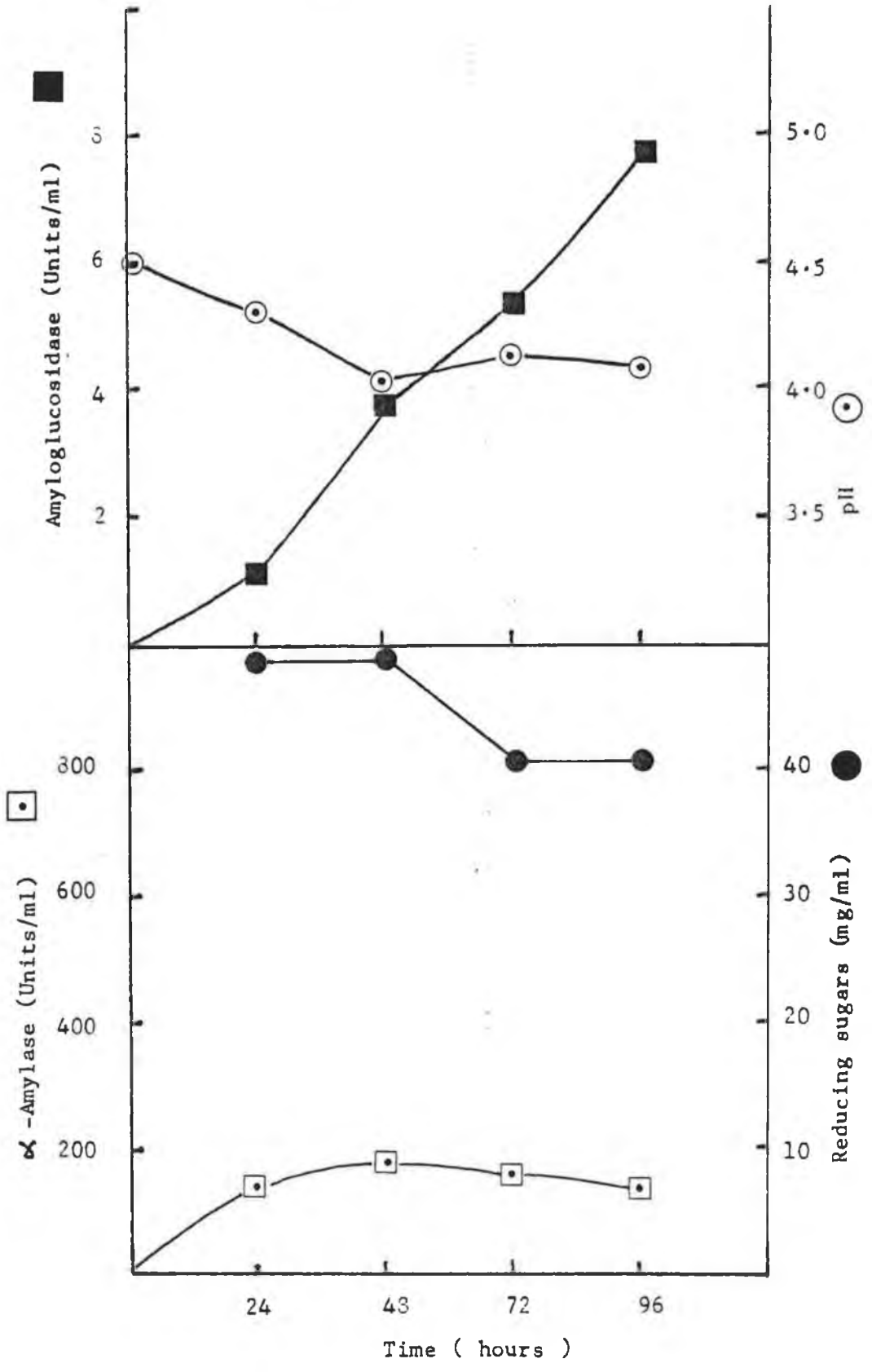


Figure 3.17 Production of α-amylase and amyloglucosidase by A. awamori NRRL 3112, in medium U, initial pH 4.5, 250 rpm.

maltodextrin, which has been noted as an inducer of α-amylase production (Ustyuzhamina et al, 1985). The production of amyloglucosidase showed a very different pattern, with ever increasing levels shown over the four day period. At 96h, medium U produced only 17% more than medium T, and in both cases the rate of production steadied to an almost constant rate of production of 60 - 70 units/l/h.

Growth on both media was heavy and gave "dispersed growth". MD05 gave ca 4.5 - 5.0 g dry weight/l.

Due to the high levels of α-amylase produced on medium T, and the slight difference in amyloglucosidase levels between medium T and U, it was decided to use MD05 as the sole carbon source for further experiments.

3.4.2 Studies on the effect of agitation and pH on enzyme production by A. awamori NRRL 3112 on medium T, in laboratory fermenters.

The medium used in the following experiments contained 50 g/l dextrin (MD05) as carbon source and 20 g/l corn steep powder. Parameters which were varied were initial pH and agitation rate. In all cases aeration was 0.5 volumes of air per volume of medium per minute (v/v/m). Experiments were carried out both under condition of pH control and no control. The actual experiments carried out are outlined in the following table (Table 3.14).

Table 3.14. Parameters varied in Section 3.4.2

Figure	Initial pH	pH control	Agitation (rpm)
3.16	4.5	No	250
3.18	4.5	No	400
-	4.5	No	500
3.19	4.5	Yes	250
3.20	4.5	Yes	400
3.21	5.5	Yes	400
3.22	5.5	No	400

The effect of agitation at 250, 400 and 500 rpm was investigated. However, an agitation rate of 500 was only tried once since rates higher than 400 rpm lead to disintegration of the mycelium; this was shown by the

inability of the inoculum to "take off", and poor final biomass levels.

Comparing figures 3.16 and 3.18, the most striking difference in these pH uncontrolled fermentations, was biomass production. At 400 rpm, the amount produced after 96 hours was almost 3 times that produced at 250 rpm (i.e. 13.2 and 4.8 g dry weight/l respectively), suggesting that oxygen for growth is limited at 250 rpm.

Both fermentations had an initial pH of 4.5, but in the case of 400 rpm, the pH fell more sharply to 3.7 at 96h. The lower final pH of 400 rpm was probably due to the greater amount of biomass produced, and with it, increased sugar utilisation and organic acid formation.

The amylase production patterns varied under different conditions of agitation. 400 rpm produced higher levels of α-amylase, on its second and greatest day of production, than did 250 rpm, i.e. 504.7 and 258.3 units/ml respectively. However, when units per g dry weight were considered, both 250 and 400 rpm gave similar amounts (57.2 and 53.3 units/mg dry weight), therefore the increased levels of amylase in the supernatant are solely due to increased biomass.

There was little difference between the amyloglucosidase levels in the supernatant, up to 72h, at agitation rates of 250 and 400 rpm. However, at 96h, 250 rpm showed a 53% increase in enzyme production over 400 rpm. This late decrease in the rate of amyloglucosidase production at 400 rpm coincides with low levels of reducing sugars, which may indicate that inducers (such as starch) may not be present, resulting in the slight decrease in amyloglucosidase levels.

Reducing sugars at 400 rpm showed an almost linear decrease with time between 24 and 96h, while at 250 rpm, they remained constant, followed by rapid utilisation after 72h. The difference in reducing sugar patterns could be related to the growth of the organism. The high growth rate at 400 rpm was only sustained by rapid utilisation of available sugars.

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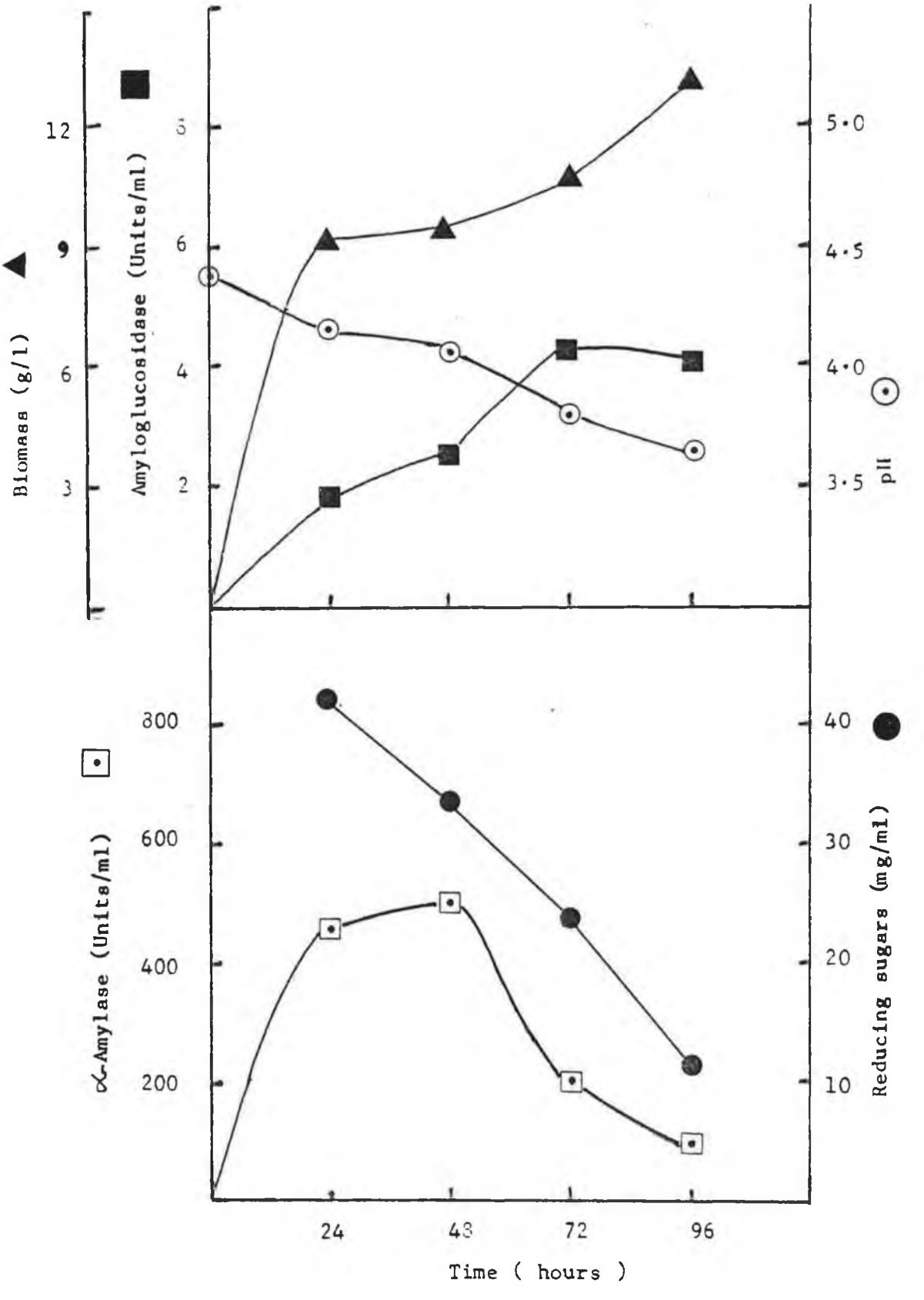

Table 3.18 Growth and production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 in medium T
Initial pH 4.5, 400 rpm.

When Figures 3.19 and 3.20 are compared, the effect of agitation on pH-controlled fermentations can be seen. PH control eliminates the effect of variations in pH, which were observed in Figures 3.16 and 3.18. As in the case of the pH-uncontrolled fermentations, biomass levels at 400 rpm were greater than at 250 rpm, due to increased available oxygen (cf Table 2.2). The initial rate of production at 400 rpm was 1.7 times that at 250 rpm (i.e. 0.31 and 0.18 dry weight/l/h respectively).

Amyloglucosidase production at both agitation rates gave the same yield at 96h, 3.8 units/l ml. When the enzyme activity per g dry weight of these two fermentations was compared, 400 rpm produced 0.34 units of amyloglucosidase/g dry weight, while 250 rpm gave 0.81 units/g. This would suggest that amyloglucosidase above a certain level in the medium causes inhibition of further enzyme production, thus the variations in amyloglucosidase/g dry weight.

α-Amylase production patterns showed major differences in time scale of production. At 400 rpm, maximum production occurred at 48h (644.9 units/ml), and fell after that. At 250 rpm, it took 96h to reach this level, the average by a rate of production being 6.8 units/ml/h.

* * * * * * * *

Figures 3.16 and 3.19 show that pH control, or lack of it, did not have much effect on biomass levels at 250 rpm. In both cases, a plateau of ca 4 g/l was reached by 24h and was maintained over the fermentation period. The greatest increase in growth occurred in the first 24h period, 0.21 g/h (Figure 3.16, 250 rpm) and 0.18 g/h (Figure 3.19, 400 rpm), which coincided with the phase of exponential growth.

Amyloglucosidase levels were significantly higher in the pH uncontrolled fermentation. As the pH fell below 4.3, the rate of production increased to 0.08 units/ml of supernatant/hr, while the rate in the controlled fermentation remained at 0.03-0.04 units/ml/hr. This would suggest that low pH values (i.e. less than 4.3) increased amyloglucosidase activity up to a maximum at pH 3.5 (see Figure 3.4). But increased enzyme stability alone could not account for increased production, so pH also appears to stimulate enzyme production (i.e. increased

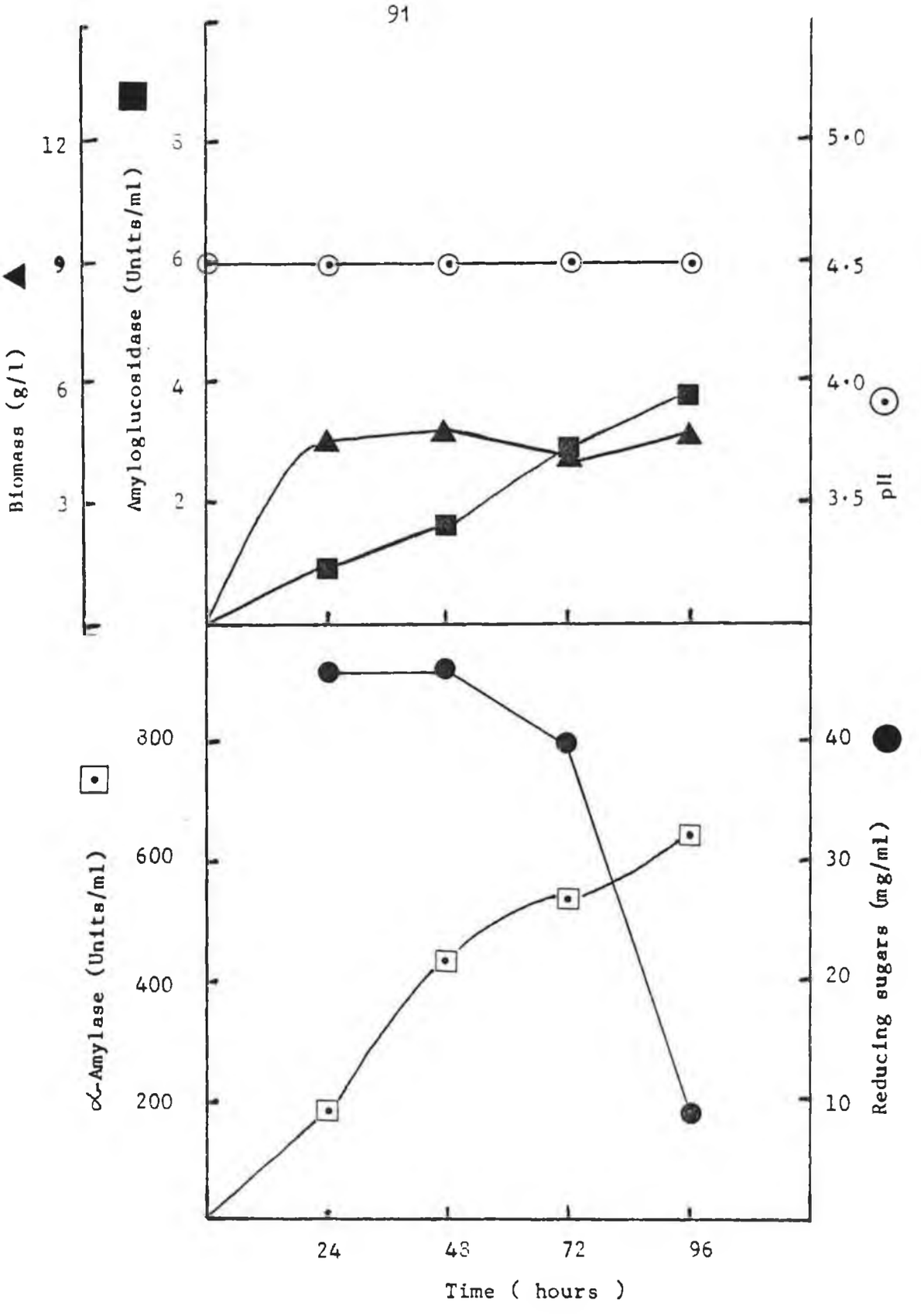

Figure 3.19 Growth and produciton of∝-amylase and amyloglucosidase by A. awamori NRRL 3112 in medium T pH control 4.5, 250 rpm.

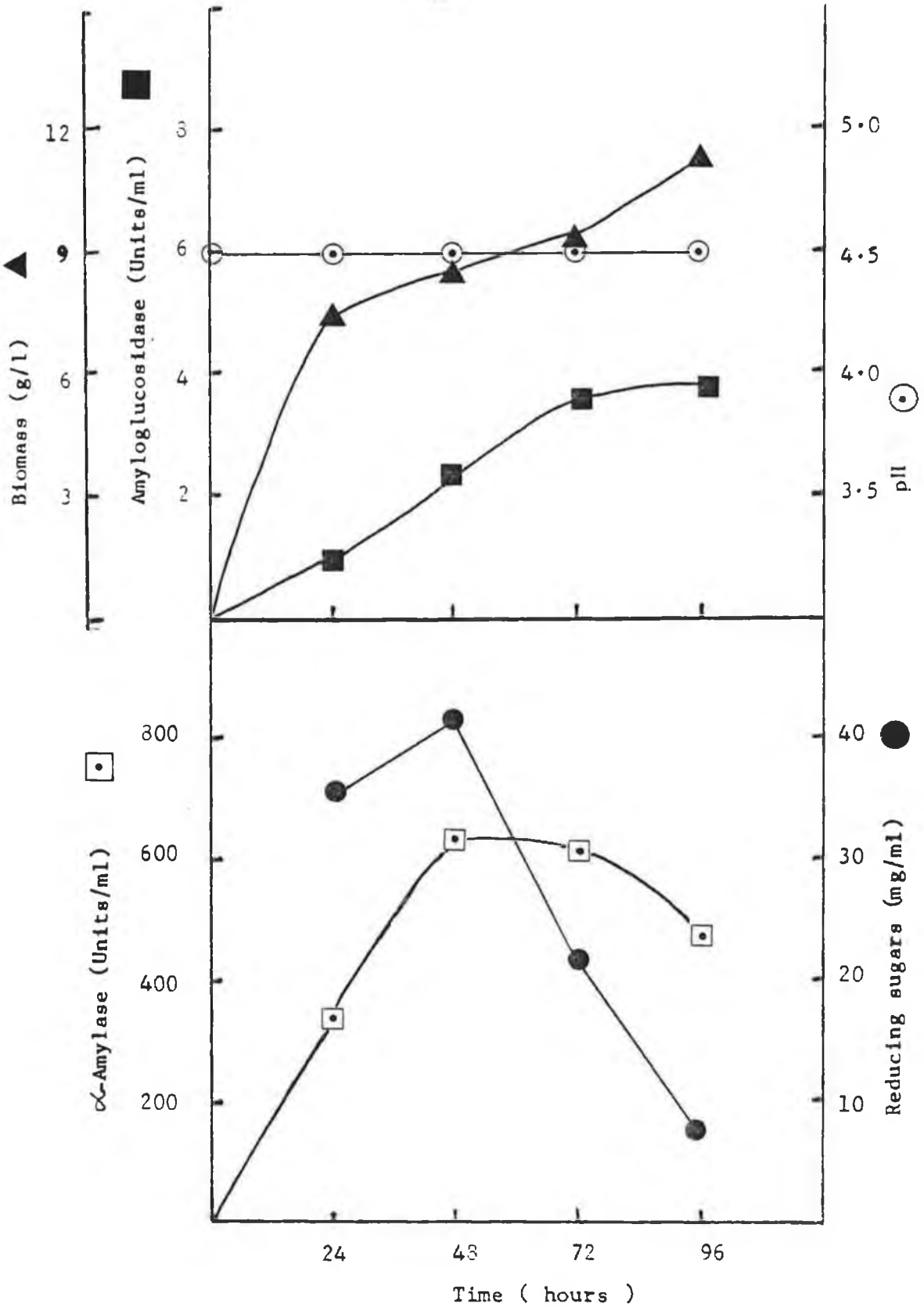

Figure 3.20 Growth and production of ∝amylase and amyloglucosidase by A. awamori NRRL 3112 in medium T pH control 4.5, 400 rpm.

transcription/translation or increased secretion). The average rate of production over the 96h fermentation in the controlled fermentation was 60% of that in the uncontrolled fermentation.

Control of pH at 4.5 enhanced the α-amylase levels obtained in these fermentations. After 96h, the pH controlled fermentation showed 650 units of amylase/ml while the uncontrolled one gave 143 units/ml. This cannot be accounted for by increased enzyme stability, since the pH values of both fermentations remain within the limits of maximum enzyme activity (Figure 3.2). Therefore, some other factor other than pH must be affecting α-amylase production; or the pH range for α-amylase transcription/translation and/or secretion must be narrower than that for maximum activity.

Production of amylase/mg biomass with and without pH control was therefore studied, the results being shown in Table 3.15.

Table 3.15 Comparison of α amylase production per mg dry weight of cells in fermentations with and without pH control (initial pH 4.5).

Time (hours)	Activity (units α amylase/mg dry wt)	
	Control	No Control
24	40.7	35.1
48	92.7	57.2
72	127.9	53.4
96	139.8	48.3

It was noted that the production of α-amylase per mg dry weight remained almost constant in the uncontrolled fermentation, while it increased with time in the controlled fermentation. This is not expected since either (a) both would be expected to show time dependence of production per mg biomass or (b) that pH control would lead to a (relatively) constant environment and therefore constant stimulation/inhibition, in this fermentation.

* * * * * * * *

When Figures 3.18 and 3.20 were compared, the effect of pH control at 4.5 and 400 rpm on biomass and enzyme production could be seen. Biomass profiles were similar; the maximum rate of production occurred in the first 24h, with a gradual increase to 13.2 g/l in the uncontrolled fermentation, and 11.3 g/l in the fermentation maintained at pH 4.5, at 96h.

Peak α-amylase production occurred earlier in uncontrolled fermentations (24-48h) than in controlled ones (48-72h). The later enzyme peak coincides with a late increase in reducing sugars and perhaps inducers. The final yield in the uncontrolled was less than the controlled (i.e. 505 and 645 units/ml respectively), due to differences in enzyme stability, according to the final pH of the fermentation broth.

Amyloglucosidase production increased in parallel over the 4 day fermentation, with the uncontrolled experiment being about 10% greater than the controlled (pH 4.5) experiment, again accounted for by increased activity.

* * * * * *

The effect of pH control at 5.5 on biomass and enzyme production was examined (Figures 3.21 and 3.22). Both fermentations were agitated at 400 rpm. Biomass levels showed very little difference between the two fermenters, and formed a plateau from 24-72h of 7-8g dry weight/l. The uncontrolled fermentation gave a second growth phase, at 96h, (11.3g/l).

Equal amounts of amyloglucosidase were produced at 48h by both fermentations, despite differences in pH values of 1.2. After this, however, the levels of enzyme in the uncontrolled fermentation fell as the medium became more acidic, while this decrease was less noticeable when the pH was maintained at 5.5.

Reducing sugars fell more quickly in the controlled fermentation, reaching low levels, (less than 10g glucose equivalents/l) by 72h.

Maintenance of pH at 5.5 had an effect on α-amylase production. Under both conditions of control and no control, maximum yields were obtained at 48h, but the controlled fermentation produced 160% more enzyme than the uncontrolled.

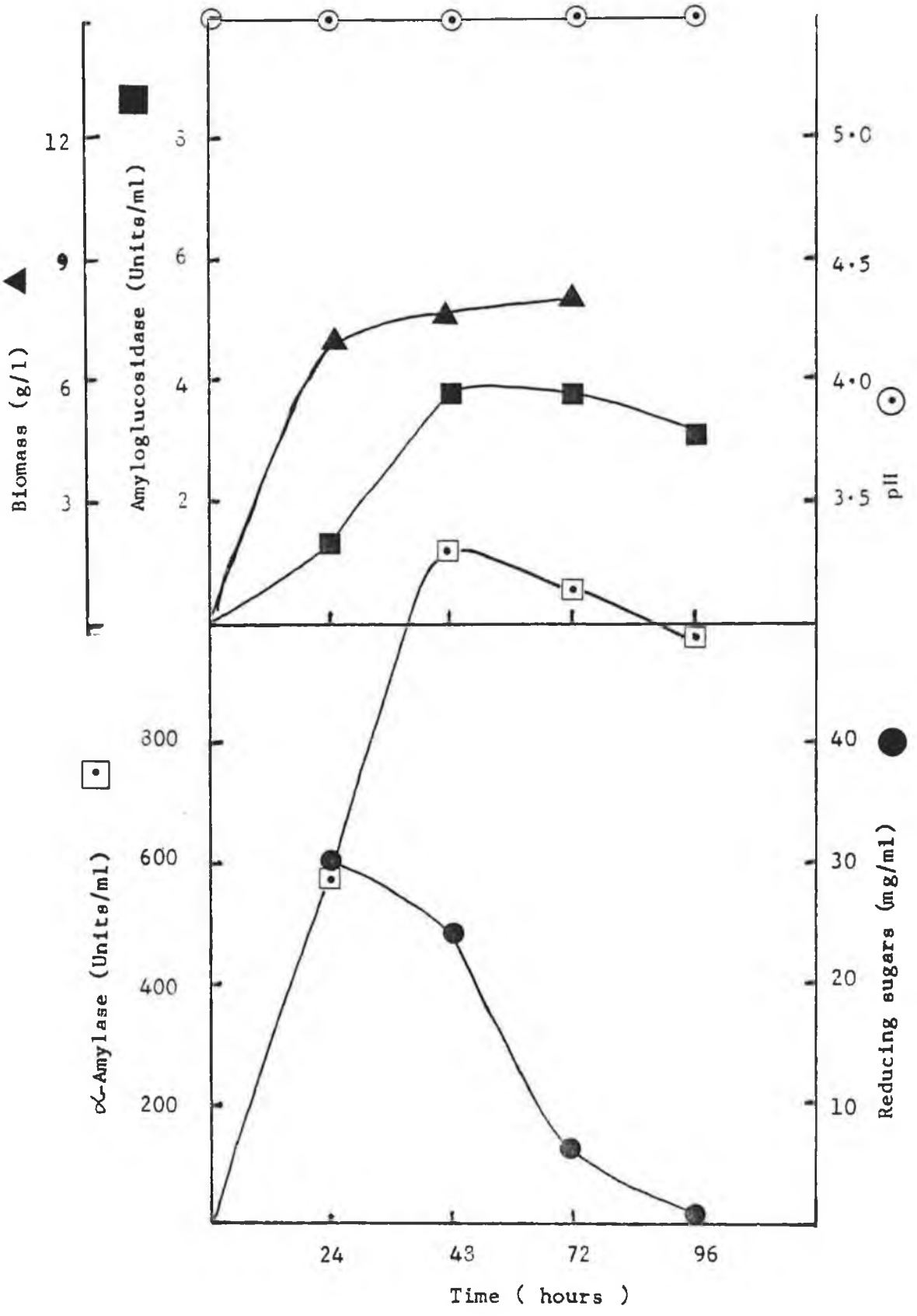

Figure 3.21 Growth and production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 in medium T pH control 5.5, 400 rpm.

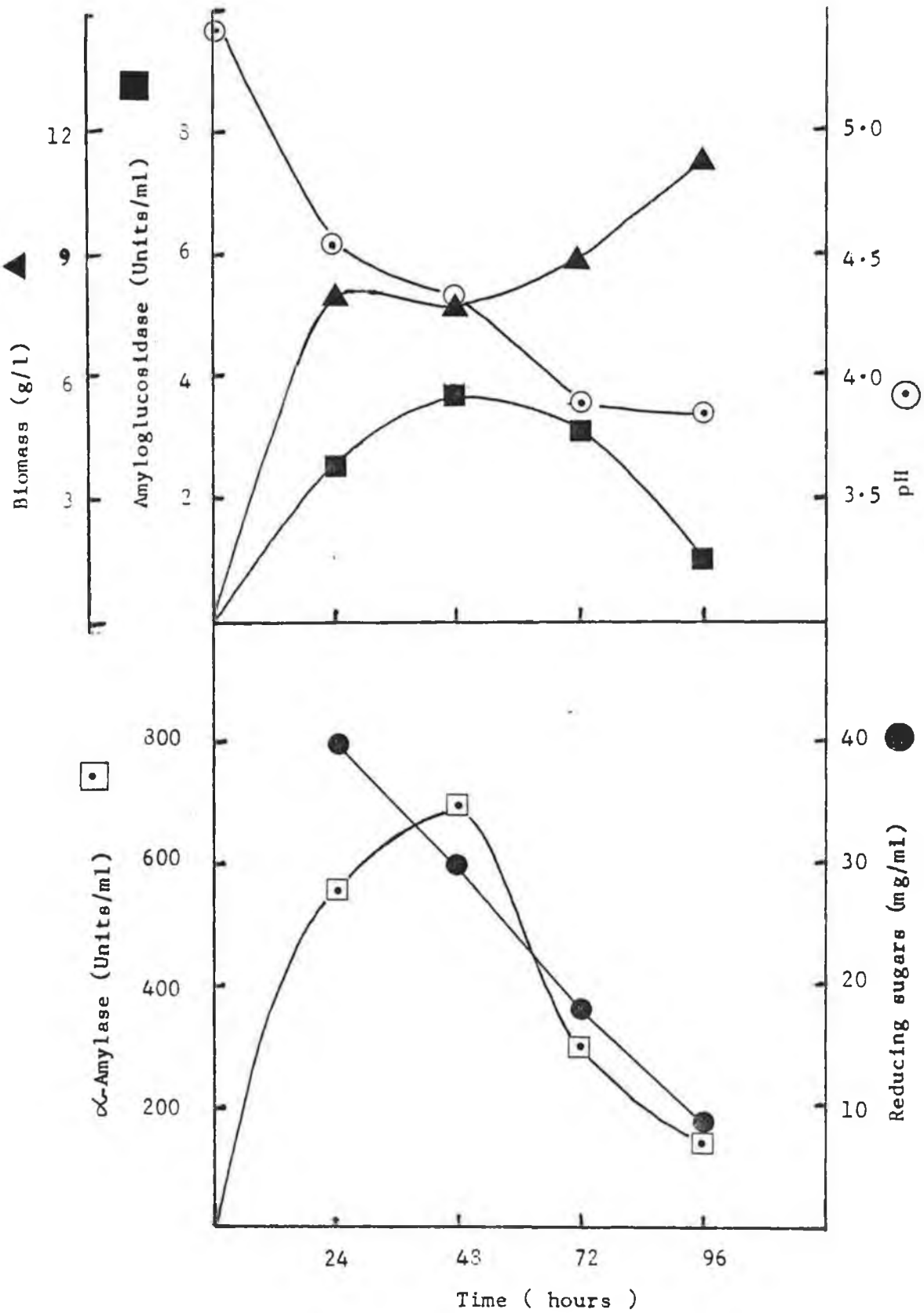

Figure 3.22 Growth and production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 in medium T
Initial pH 5.5, 400 rpm.

This cannot be related solely to stability, but suggests that high pH values (ca 5.5) increase expression/secretion of amylase. The highest yield of α-amylase in these fermenter trials was obtained at 400 rpm and a constant pH of 5.5, i.e. 1120 units/ml, 1.46 x 10^5 units/g dry weight. The initial rate of α-amylase production under both conditions was 23 units/ml/h. However, without pH control, the rate fell to 6 units/m/h over the second day, suggesting again that expression/secretion rates were dependent on pH, and that a pH fall of even 1 unit (to 4.5) had detrimental effects on expression/secretion, but not on activity.

* * * * * * *

When Figures 3.18 (initial pH 4.5, no control) and 3.22 (initial pH 5.5, no control) were compared, some notable differences were observed. An initial pH (pHi) of 4.5 gave higher biomass levels than pHi of 5.5. The 96 h yield of the latter was only 86% that of the former, with similar variation in initial rates of growth (0.39 and 0.34 g/l/h respectively).

α-Amylase levels peaked at 48h with 504.7 units/ml for pHi 4.5 and 703.2 units/ml for pHi 5.5, i.e. 40% more α-amylase was produced at the higher pH value. Since both fermentation broths were in the maximum α-amylase activity range, the increase must be due to increased expression/secretion at higher pH values. In both cases, the enzyme levels decrease after 48h.

Unlike α-amylase, amyloglucosidase production was favoured by the lower pH values, i.e. the fall in pH from 4.4 to 3.7, corresponding solely to increased enzyme stability. Peak production of 4.37 units/ml occurred at 72h in the fermentation at pHi 4.5.

* * * * * * *

The effect of pH control at various pH values can be studied by comparing figures 3.20 (pH control at 4.5) and 3.21 (pH control at 5.5). Biomass at pH 4.5 was about 8-12% more than that at pH 5.5. Utilisation of reducing sugars by the fermentation at 5.5 was substantially quicker than at 4.5.

No difference was recorded at 72h in the levels of

amyloglucosidase; both were ca 4 units/ml. Amyloglucosidase yields reached a maximum at 48h. The higher pH yielded 1122 units/ml while the lower value gave 645 units/ml.

3.4.3 Comparison of growth and production of α-amylase and amyloglucosidase by A. awamori NRRL 3112 at 23° and 30° C.

The effect of temperature on enzyme production and other fermentation parameters was examined. Two fermenters containing medium T, with an initial pH of 4.5, no pH control and agitated at 250 rpm were used, run at 30°C (figure 3.16) and at 23°C (figure 3.23).

pH profiles for both fermentations were similar: both fell from 4.5 to 4.0 over the 96h fermentation.

The 30°C fermenter showed the "normal" reducing sugar curve, with a fall from 40 - 45 g glucose equivalent/l to less than 10 g/l at 96h. At 23°C this pattern was not repeated. Reducing sugars had only fallen to 41 g/l by the final day, suggesting that production and utilisation of sugars was diminished at lower temperatures.

αAmylase levels peaked at 48h at the higher temperature, with 258.4 units/ml at 30°C, while at the lower temperature levels rose to 430.0 units/ml by 96 h. The rate of production over the first 48h was 28% greater at 23°than at 30°C.

In both fermentations, amyloglucosidase levels increased over the four day period. The production at 23°C was 26% greater than that at 30°C, i.e. 8.2 units/ml/96h compared with 6.5 units/ml/96h. The increased rates of production of both α amylase and amyloglucosidase at 23°C were unexpected, since most authors show increasing production with increasing temperature (up to 37-40°C).

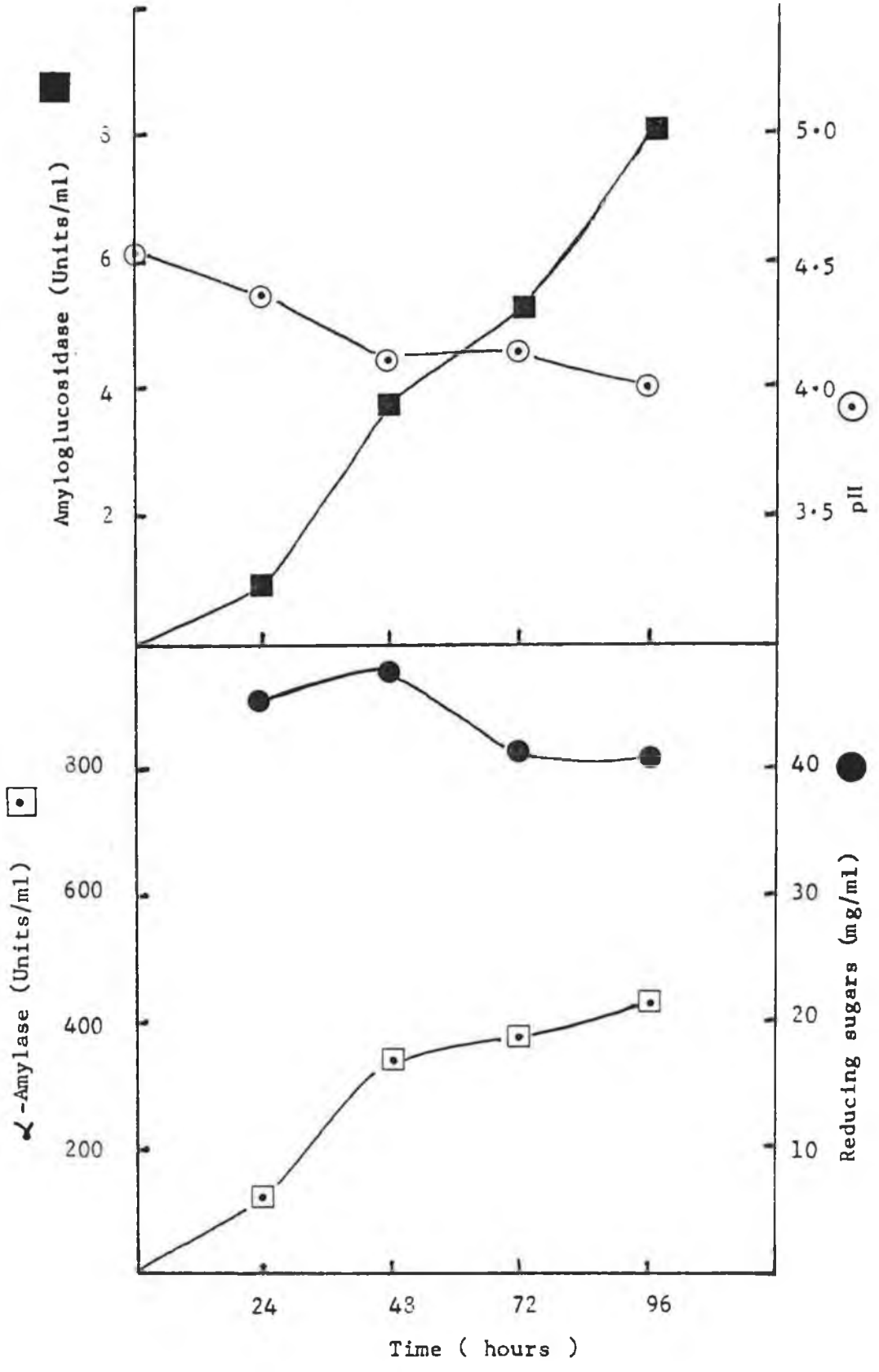

Figure 3.23 Effect of reduced temperature on enzyme production by A. awamori NRRL 3112, initial pH 4.5, 250 rpm.

3.4.4 Effect of reduced maltodextrin on growth and enzyme production by A. awamori NRRL 3112, initial pH 5.5, 400 rpm.

From previous experiments, 400 rpm was shown to be beneficial for both growth and enzyme production. Using these conditions and an initial pH of 5.5, with no pH control, the level of carbon source, MD05, was reduced to see if similar yields could be obtained with lower energy imput. The media used for this series of experiments were the standard medium T, with 50 g/l MD05 and medium T2, with half this amount of dextrin, but the usual amount of corn steep powder and antifoam.

Biomass levels were unaffected by the level of MD05 (Figures 3.22 and 3.24). At 72h, both media produced 8.7 - 9.1 g dry weight/l, while at 96h the standard medium gave 13% more biomass than medium T2. This suggested that only part of the excess maltodextrin in medium T was converted into biomass (i.e. 13% of 25 g/l) but the richer medium could continue to support a higher rate of growth than the poor medium.

Reducing sugar levels on T2 medium were much lower than those on medium T. At 96h, only 0.74g glucose equivalent per 1 remained available in the poorer medium; another suggestion that this medium was unlikely to sustain further new growth. At this stage, medium T had ten times this amount of available sugar. Both α-amylase and amyloglucosidase demonstrated diphasic enzyme production on the poor medium T2, and monophasic production on medium T. At 72h, the poor medium produced twice the amyloglucosidase levels seen on medium T, suggesting that inhibitors are absent, or inducers are present in medium T2 at this time. (Due to the complex nature of MD05 and the possible breakdown products of MD05 following action of amylase and/or amyloglucosidase, it is not possible to be certain of the nature of the inducer/repressor).

By comparison, the standard medium gave better α-amylase yields than the poorer medium, although there was little difference in the initial rates of production, 23 and 21 units/ml of supernatent/hr respectively. When amylase levels per mg dry weight are examined (Table 3.16), the levels remained almost constant at 55-59 units/mg dry weight in medium T2, while in the standard medium, they varied with time.

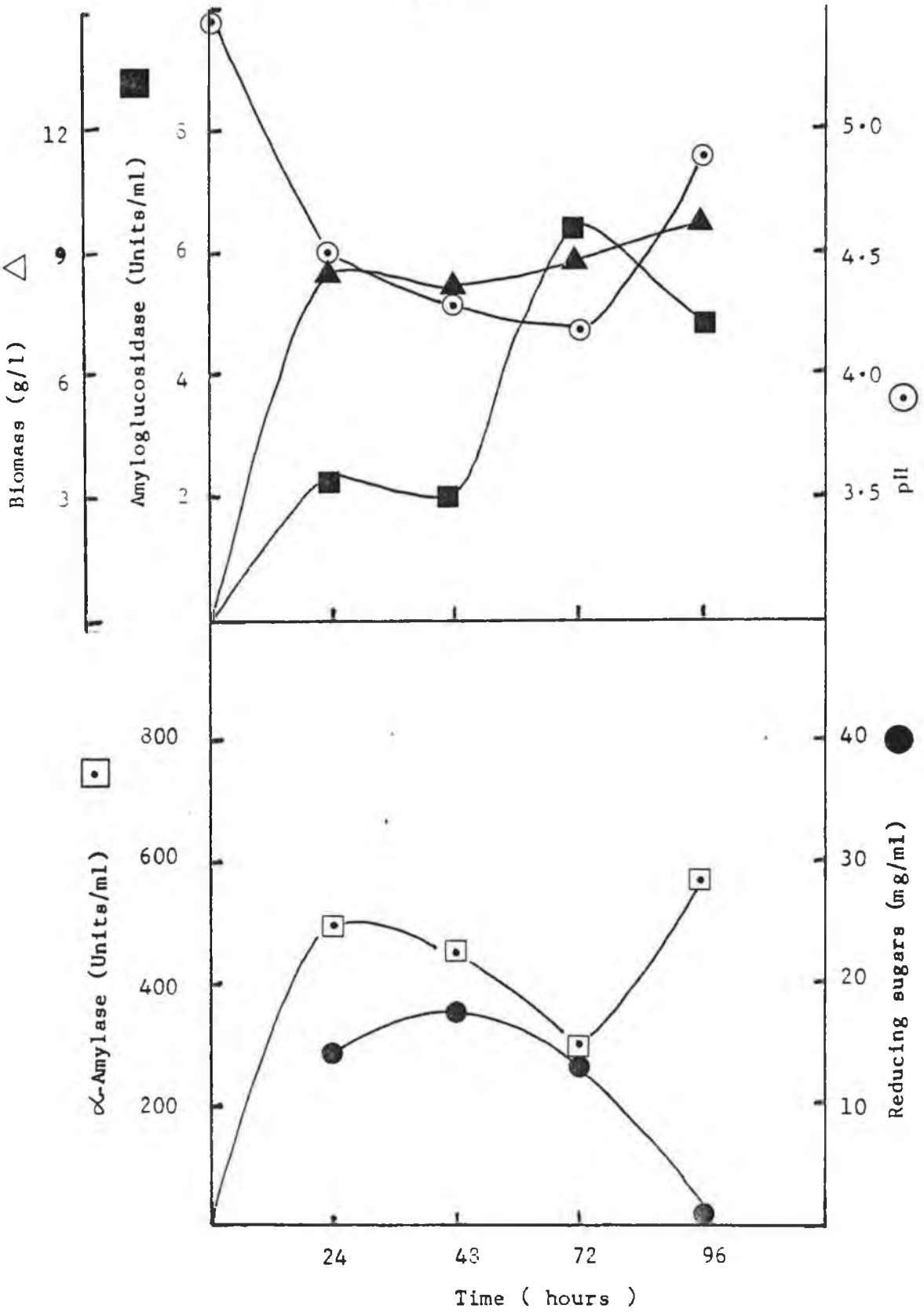

Figure 3.24 Effect of reduced maltodextrin on growth and enzyme production by A. awamori NRRL 3112, initial pH 4.5, 400 rpm.

Table 3.16 Effect of levels of maltodextrin (MD05) on ∝-amylase production per mg biomass produced by A. awamori NRRL 3112, initial pH 5.5, 400 rpm.

Time (hours)	∝-Amylase levels (units/mg dry wt) Medium T	Medium T2
24	68	59
48	92	55
72	35	32
96	12	58

The pH profiles of both media were similar; both fell from 5.5 to 3.9-4.2 by 72h. After that, the standard medium continued its fall, while the poorer medium rose sharply to 4.9, which may indicate the start of autolysis.

3.4.5 Appraisal of results

Results of this section can be summarised as follows:

1. Medium T, containing dextrin, produced 48% more α-amylase than medium U (soluble starch) at 48h.

2. Rates of production of amyloglucosidase were almost equal on media containing dextrin and soluble starch.

3. Agitation rates greater than or equal to 500 rpm caused disintegration of the mycelium.

4. 400 rpm produced 2-3 times more biomass than 250 rpm (irrespective of pH control or not) due to increased availability of oxygen.

5. pH control led to little or no increase in biomass.

6. More biomass was produced in fermenters with initial pH values of 4.5 than 5.5, both with and without pH control.

7. The maximum growth rate of the fungus occurred in the first 24 h, corresponding with the phase of exponential growth.

8. 400 rpm yielded higher levels of α-amylase per ml than 250 rpm although enzyme/mg dry weight levels were similar.

9. pH control (both at 4.5 and 5.5) increased α-amylase production.

10. In general, slightly higher levels of α-amylase were obtained at 5.5. than at 4.5. This cannot be accounted for solely by increased enzyme activity/stability, so it must be assumed that increased pH (ca 5.5) increases α-amylase expression/secretion.

11. Similar levels of amyloglucosidase were obtained at 250 and 400 rpm, therefore oxygen levels do not adversely affect enzyme stability or production.

12. Yields in pH controlled amyloglucosidase fermentations were only 10% or less, greater than those in uncontrolled

fermentations.

13. Both in pH controlled and uncontrolled experiments, yields of amyloglucosidase were greater at 4.5 and 5.5, reflecting the enzyme's known activity/stability pattern.

14. In general, reducing sugar profiles fell linearly from around 40 g residual glucose equivalents at 24h, to less than 10 g/l at 96 h.

15. Yields of both ∝-amylase and amyloglucosidase were 26-28% greater at 23° C than at 30° C.

16. At lower temperatures (23° C) reducing sugars were consumed more slowly than at 30° C.

17. Medium containing half the normal amount of dextrin (i.e. medium T2 - 25 g/l MD05) gave double the levels of amyloglucosidase at 72h, while ∝-amylase production was favoured by medium T (50 g/l MD05). This and other phenomena can be explained by the presence/absence of inducers/repressers, following the utilisation of the substrate MD05 by the organism, and the activity of ∝-amylase and/or amyloglucosidase on the substrate.

In conclusion, different conditions are required for production of ∝-amylase, amyloglucosidase and biomass in laboratory fermenters. Optimal biomass levels were obtained with high available sugars, and good oxygen transfer through adequate agitation. Maintenance of the fermentation broth at high pH values (5.5), with good oxygen transfer and high initial sugar levels, favoured ∝-amylase production. In contrast, amyloglucosidase production was stimulated by low pH values and low agitation levels. Ideally, pH was initially 4.5 and fell slowly to 4.0-4.1 over 96h, while the available reducing sugars remained at around 40 g glucose equivalents/l, over the initial 72h period.

Section 3.5. Studies on the Relationship between Biomass Formation and Enzyme Production by A. awamori NRRL 3112 in Laboratory Fermenters.

In previous fermenter trials, biomass rose to ca 7 g/l in the first 24h, after which the growth rate declined. The relationship between pH, reducing sugars, enzyme levels and biomass was investigated during this initial growth period. Laboferm fermenters were used, due to their smaller volume and availability of multiple units. Therefore, the fermentation was scaled down from 10 l to 5 l using similar geometry, constant tip speed and constant K_La to maintain optimal conditions observed in previous experiments (see Section 3.5.1 for scale down procedures).

3.5.1 Effect of initial pH on specific growth rate of A. awamori NRRL 3112

Effect of initial pH values of 5.9, 5.2, 4.7, 4.1 and 3.2 of the medium on specific growth rate, as well as reducing sugars, biomass and enzyme production was investigated in this experiment. To obtain more exact biomass readings, medium T was modified to remove all particles (see Section 2.4.2, medium T3). Biomass and pH were determined at 4-hourly intervals and enzyme activity and reducing sugars at 8-hourly intervals. Results are shown in Figures 3.25 - 3.29.

In all five fermentations, the pH profiles were similar: a steady decrease from 0-12 h, a period of rapid decline from 12-16 h, and a period of little or no increase in pH subsequently. The greatest decrease in pH was recorded in the fermenter with the highest initial pH.

Production of amylase were greatest in the fermenter with highest initial pH (pHi 5.9) (393 units/ml, 24 h) and decreased with lowering initial pH, to pHi 3.2 (trace levels). This trend was also shown with amylase production per mg biomass (Table 3.17). When the results are adjusted to take into account enzyme activity/stability, this increased activity/stability alone cannot account for the variation observed. As previously suggested, increase in pH must also affect expression/secretion of αamylase, to account for increased levels observed.

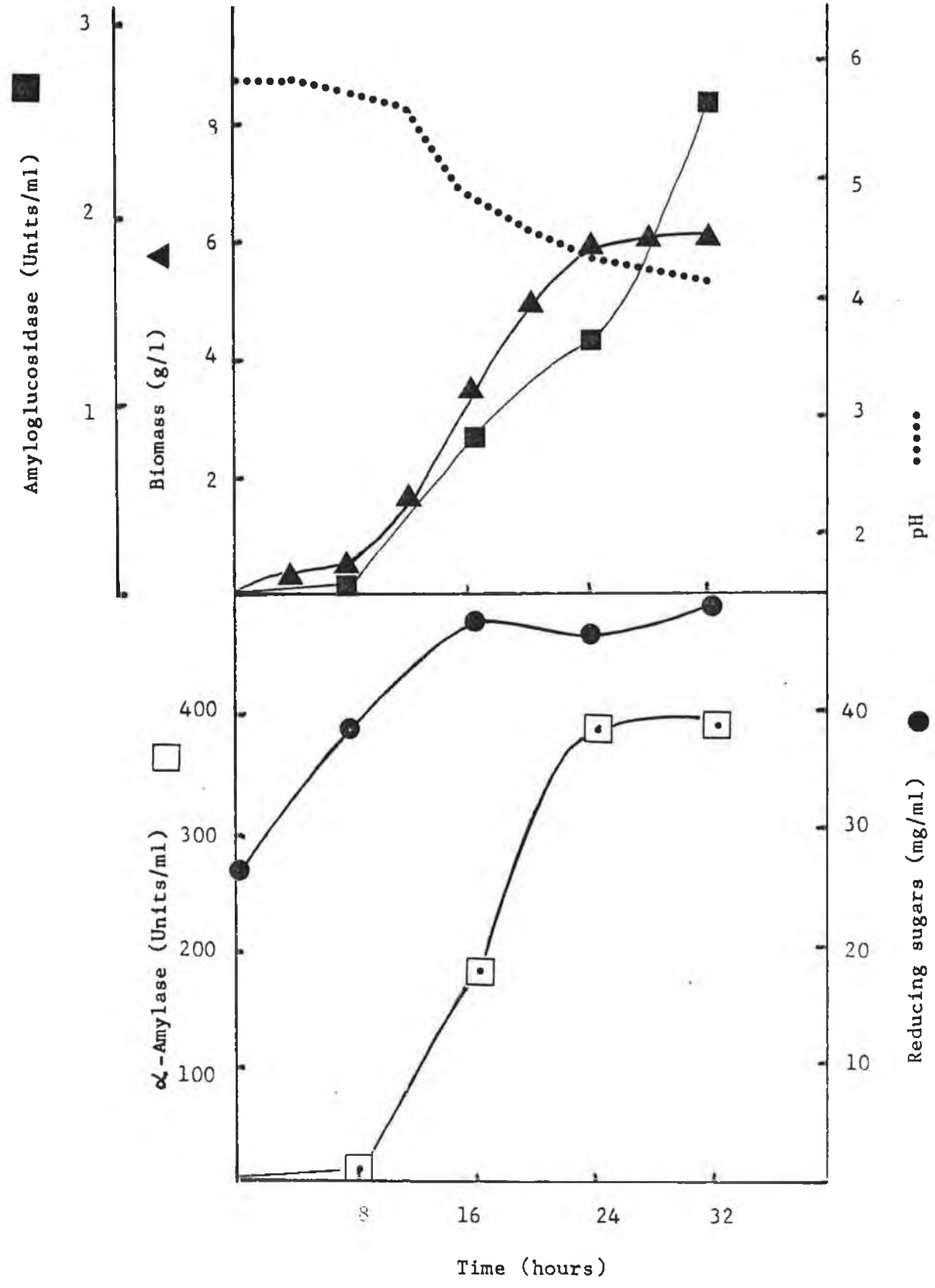

Figure 3.25 Effect of initial pH on growth and enzyme production by A. awamori NRRL 3112, on medium T3, pHi 5.9

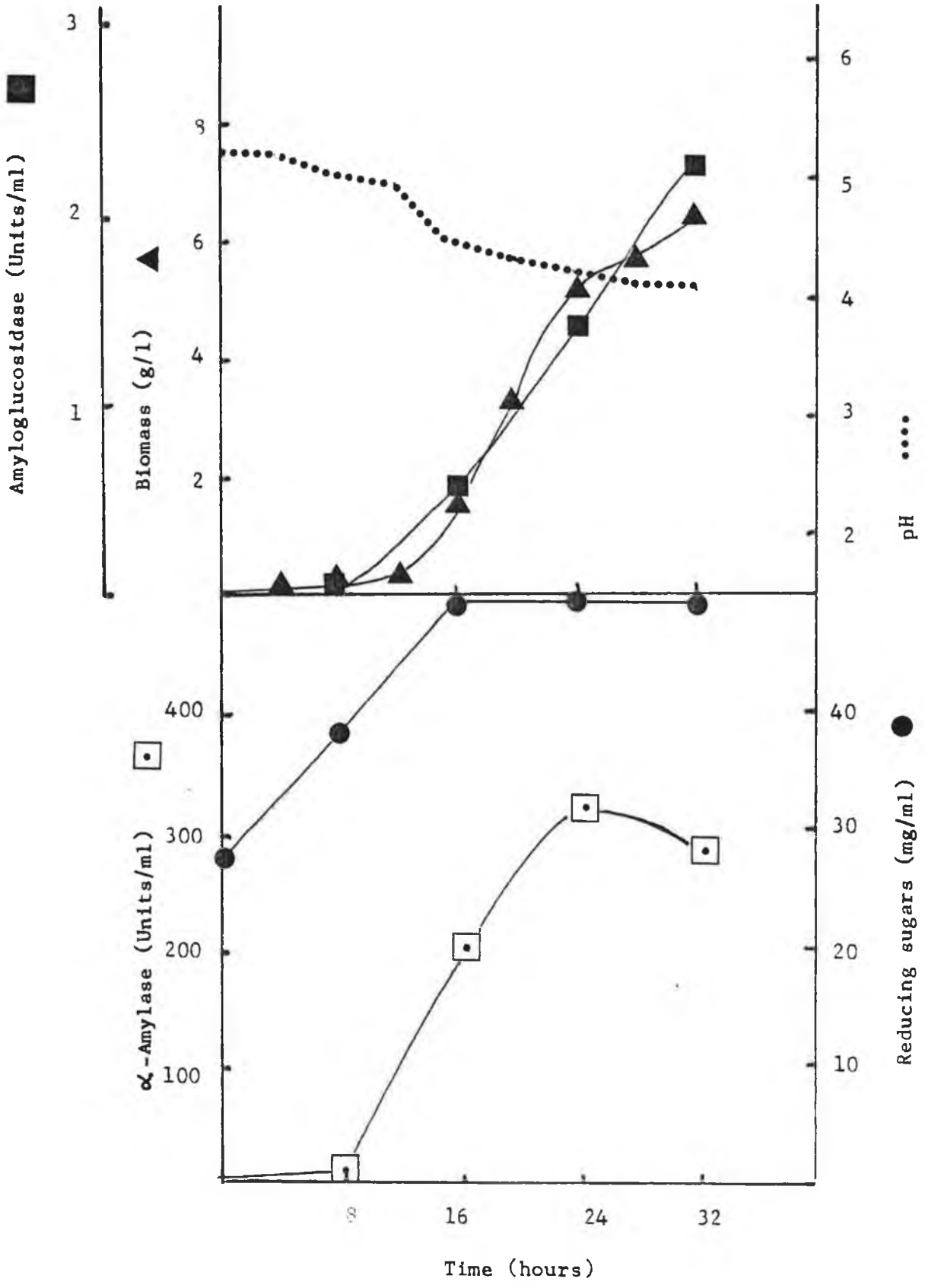

Figure 3.26 Effect of initial pH on growth and enzyme production by A. awamori NRRL 3112, on medium T3, pHi 5.2

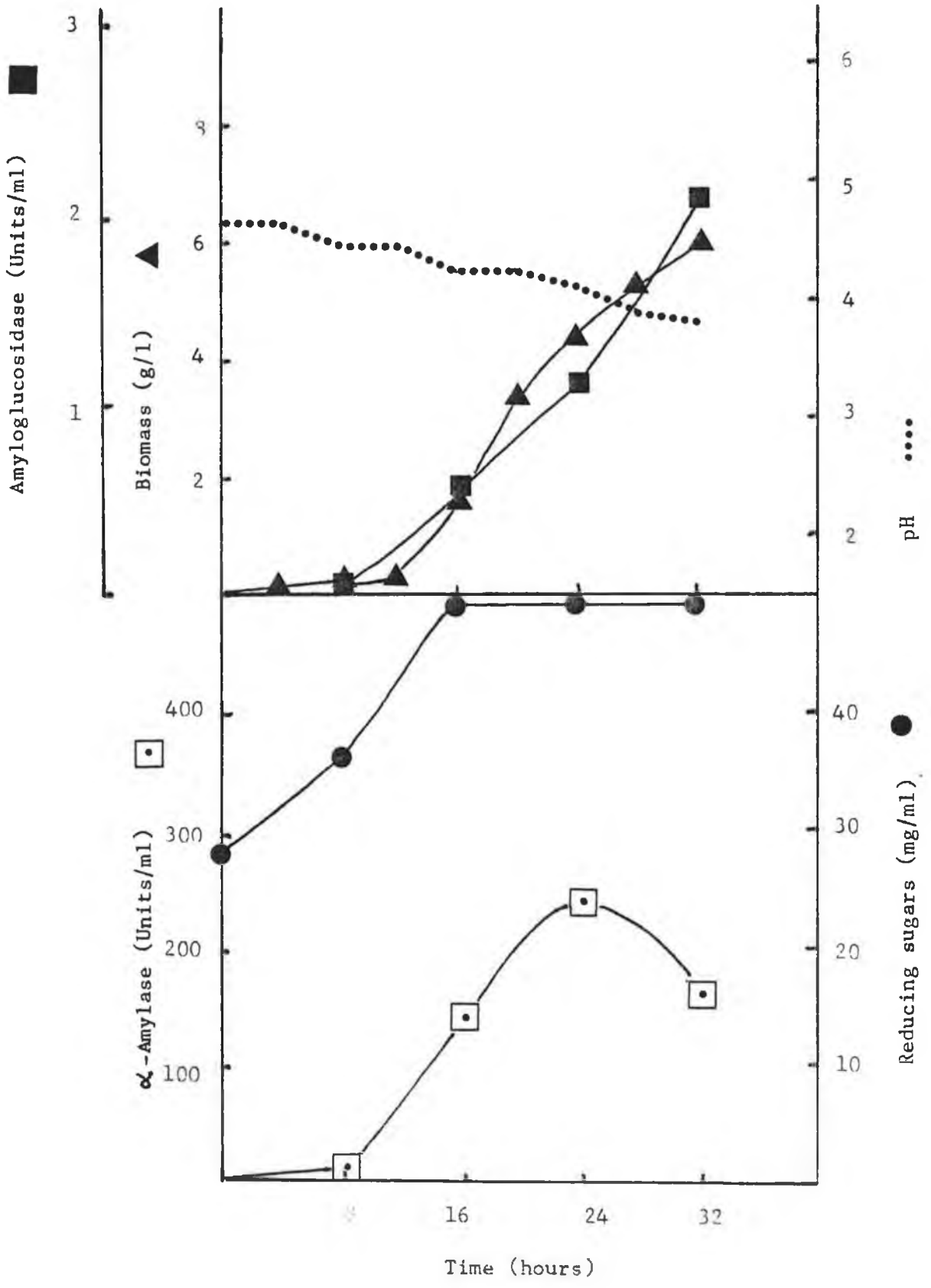

Figure 3.27 Effect of initial pH on growth and enzyme production by A. awamori NRRL 3112, on medium T3, pHi 4.7

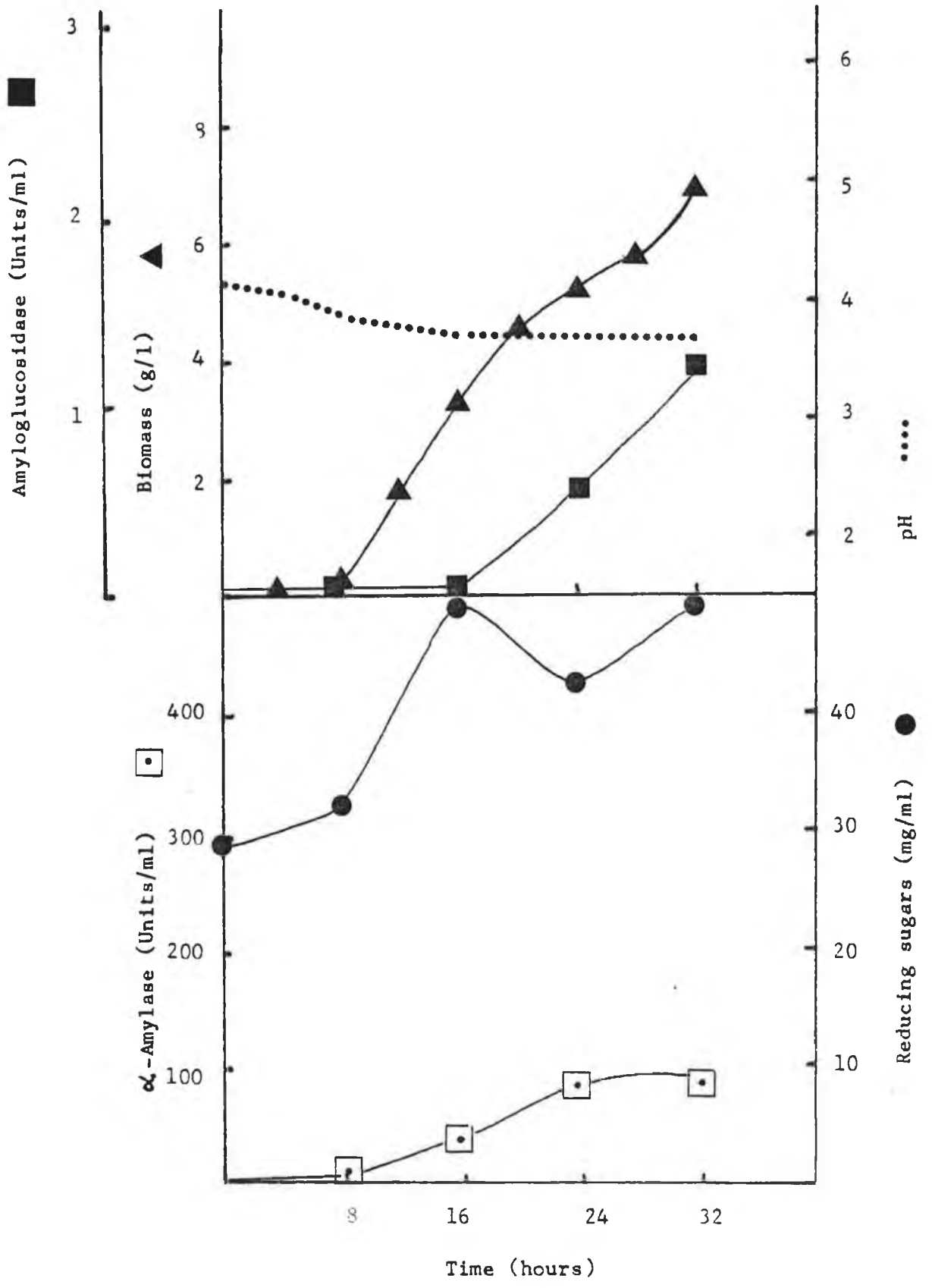

Figure 3.28 Effect of initial pH on growth and enzyme production by A. awamori NRRL 3112 on medium T3, pHi 4.1

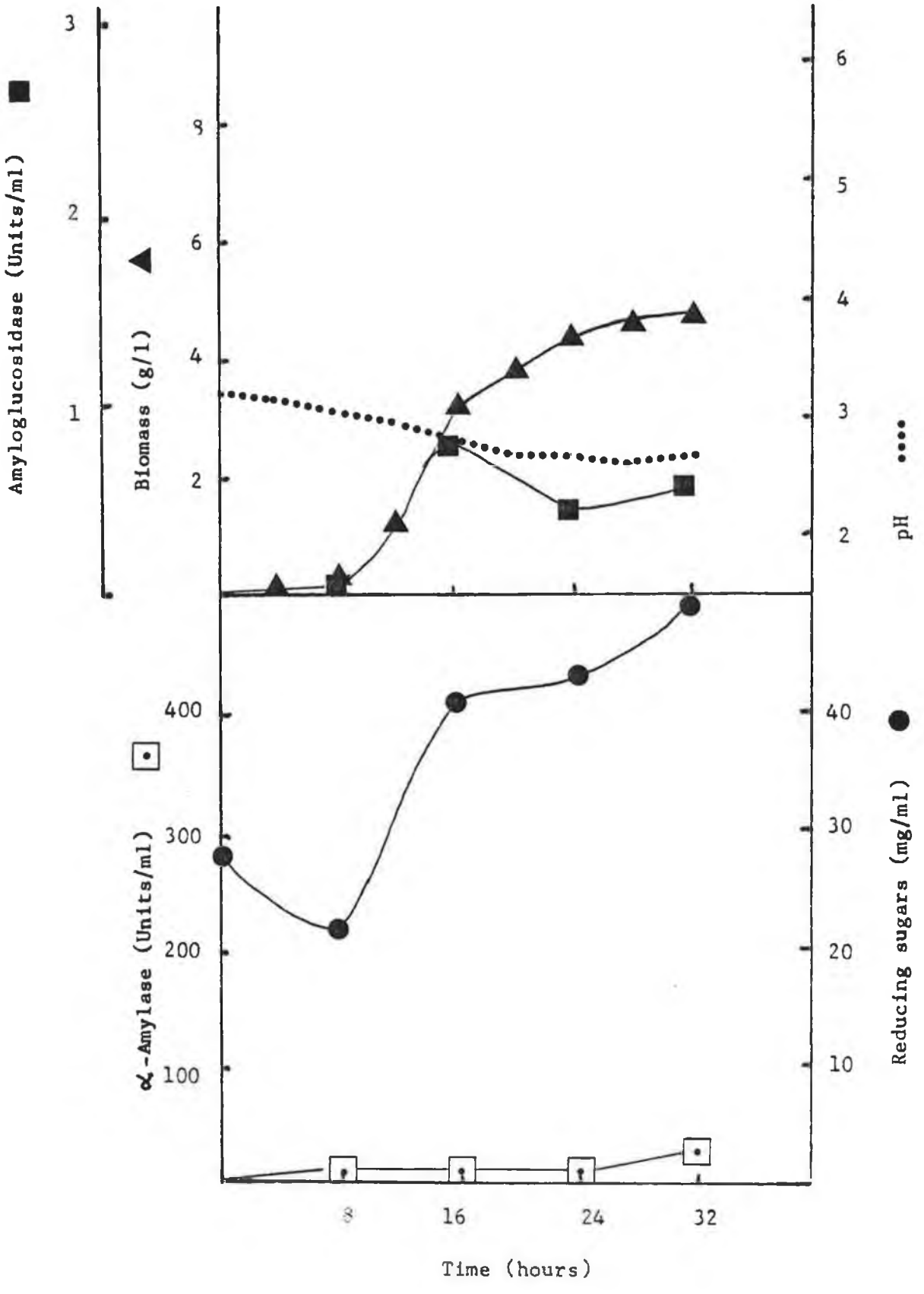

Figure 3.29 Effect of initial pH on growth and enzyme production by A. awamori NRRL 3112, on medium T3, pHi 3.2

In all cases, except pHi 3.2, the maximum production/mg dry weight was recorded at 24 h.

Table 3.17. Effect of initial pH on levels of ∝-amylase/mg biomass produced by A. awamori NRRL 3112 on medium T3 ; 580 rpm.

Time (hours)	∝-Amylase (units/mg dry weight)				
	pHi 5.9	pH 5.2	pHi 4.7	pHi 4.1	pHi 3.2
8	Trace	Trace	Trace	Trace	Trace
16	52.3	60.1	40.5	10.6	Trace
24	65.7	62.0	46.9	15.5	Trace
32	61.1	43.4	27.1	11.8	2.1
Average	59.7	55.2	38.2	12.6	-

Biomass levels of A. awamori grown on medium T3, were in the range 4.3-6.0 g/l, after 24 h. The fermenter with the lowest initial pH, pHi 3.2, gave the poorest rate of growth, due to the acidic conditions. The specific growth rate μ, of each of the fermentations reached a maximum over the period 12-16 h (Table 3.18). This corresponds with the period of greatest change in pH in the fermentation. As predicted from shake flask trials, the doubling time at pHi 4.1 was less than at pHi 5.9 and 5.2 (Table 3.19).

Table 3.18. Effect of initial pH on the specific growth rate of A. awamori NRRL 3112 on medium T3, 580 rpm.

Time (hours)	Specific growth rate ($X 10^{-3}$ g/l/h)				
	pHi 5.9	pHi 5.2	pHi 4.7	pHi 4.1	pHi 3.2
4 - 8	6	5	5	5	5
8 - 12	27	29	32	34	24
12 - 16	46	45	45	40	50
16 - 20	38	28	25	30	12
20 - 24	25	20	20	17	16
24 - 28	6	13	12	14	5
28 - 32	5	19	7	34	5
Average	21.9	22.7	20.9	24.9	16.7

Table 3.19. Effect of initial pH on average doubling times of A. awamori NRRL 3112, on medium T3, 580 rpm.

Fermenter pHi	Average doubling time (hours)
5.9	3.2
5.2	3.1
4.7	3.3
4.1	2.8
3.2	4.2

As with the overall biomass yield, initial pH was not a critical factor in growth, except when it was at pH 3.2 or less.

Amyloglucosidase levels increased with time up to 32 h, and with increasing pHi up to 5.9. The maximum production (both unit/ml and units/g) level recorded in these fermentations occurred at 32 h (Table 3.20). When the enzyme stability/activity is considered, it would be predicted that at 24 h, the fermentation with pHi 4.1, would show maximum amyloglucosidase production. This was not the case, again

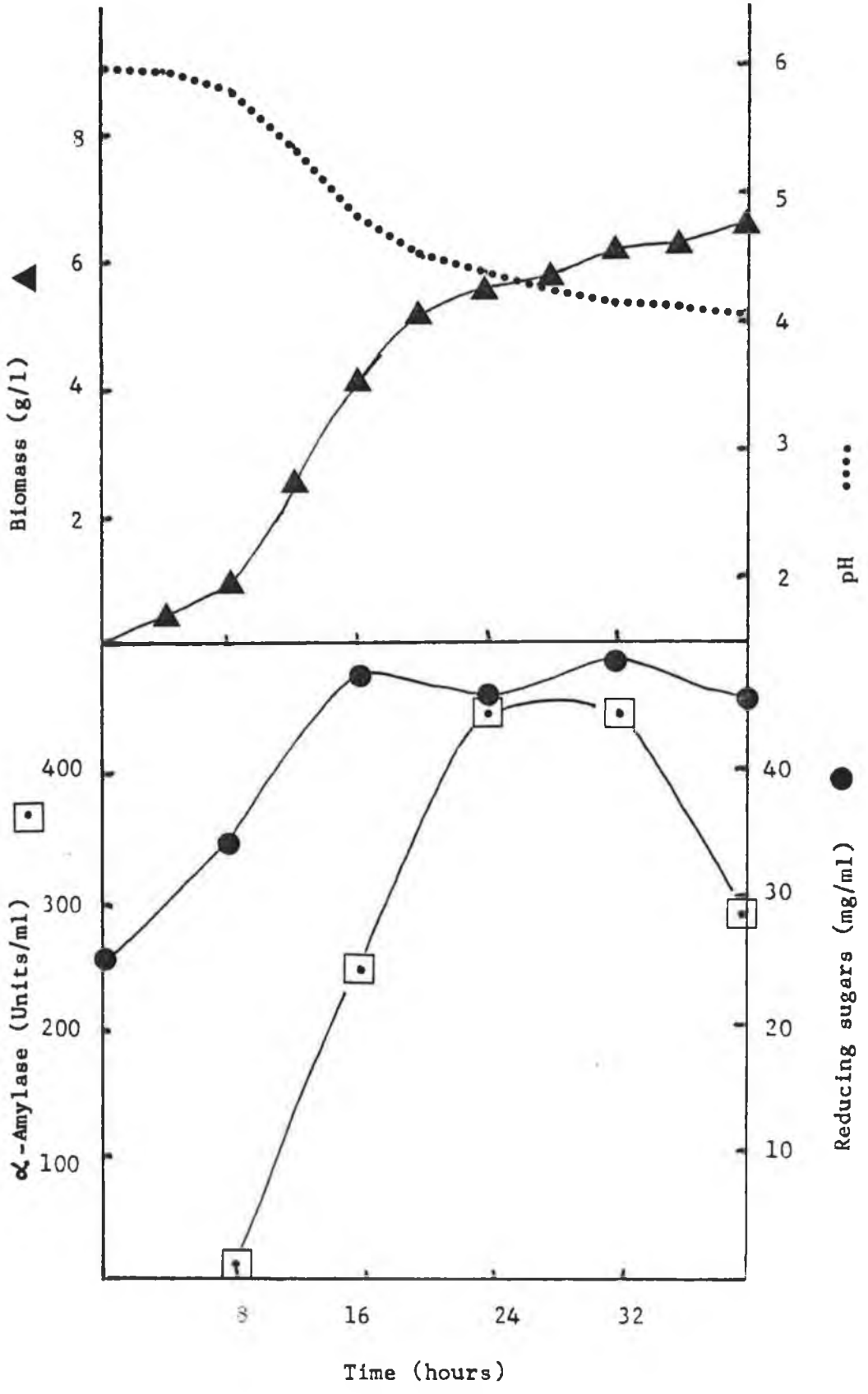

Figure 3.30 Effect of aeration and agitation on growth and enzyme production, by A. awamori NRRL 3112 on medium T3, at 0.28 v/v/m, 580 rpm.

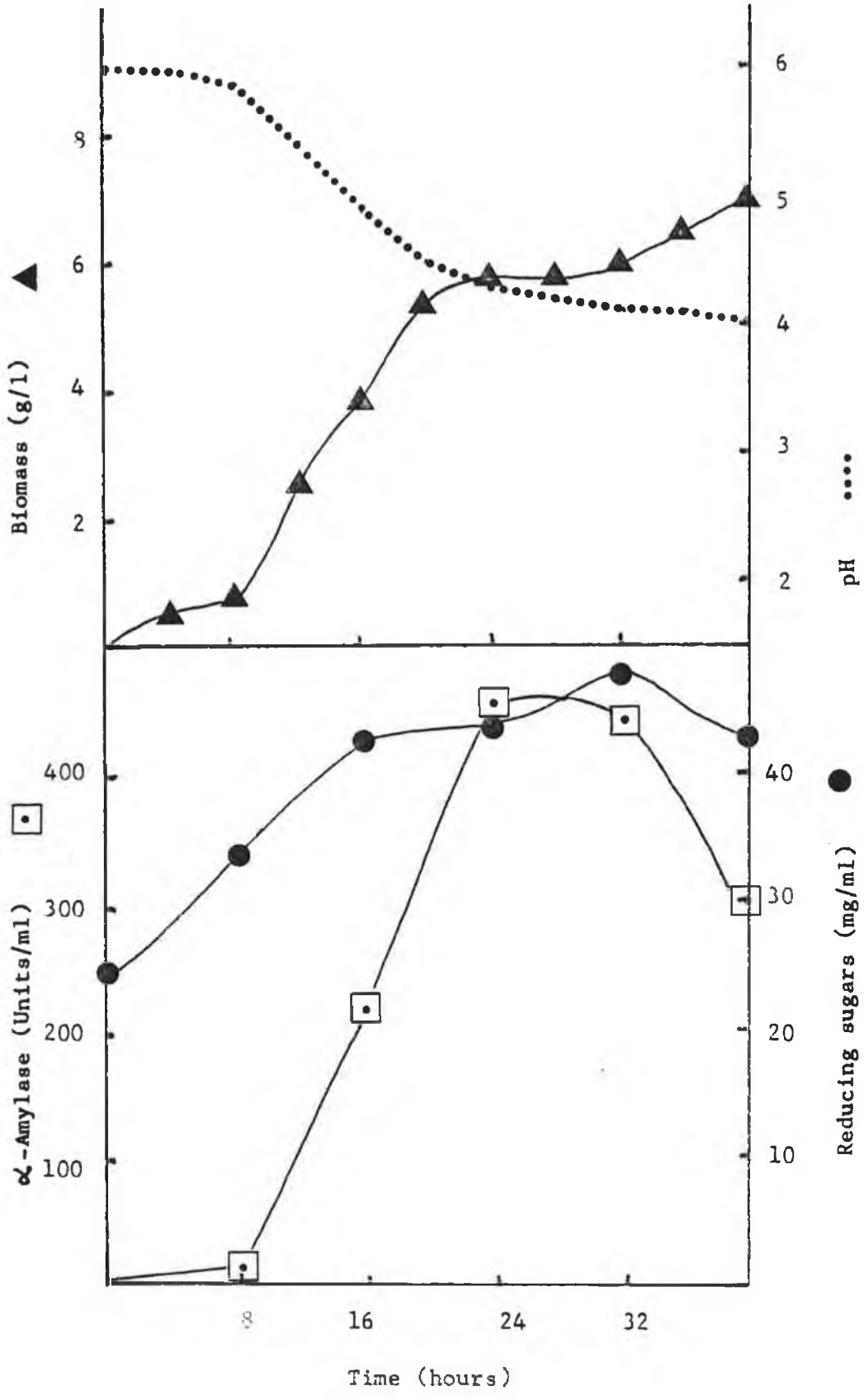

Figure 3.31 Effect of aeration and agitation on growth and enzyme production by A. awamori NRRL 3112 on medium T3, at 0.5 v/v/m, 580 rpm.

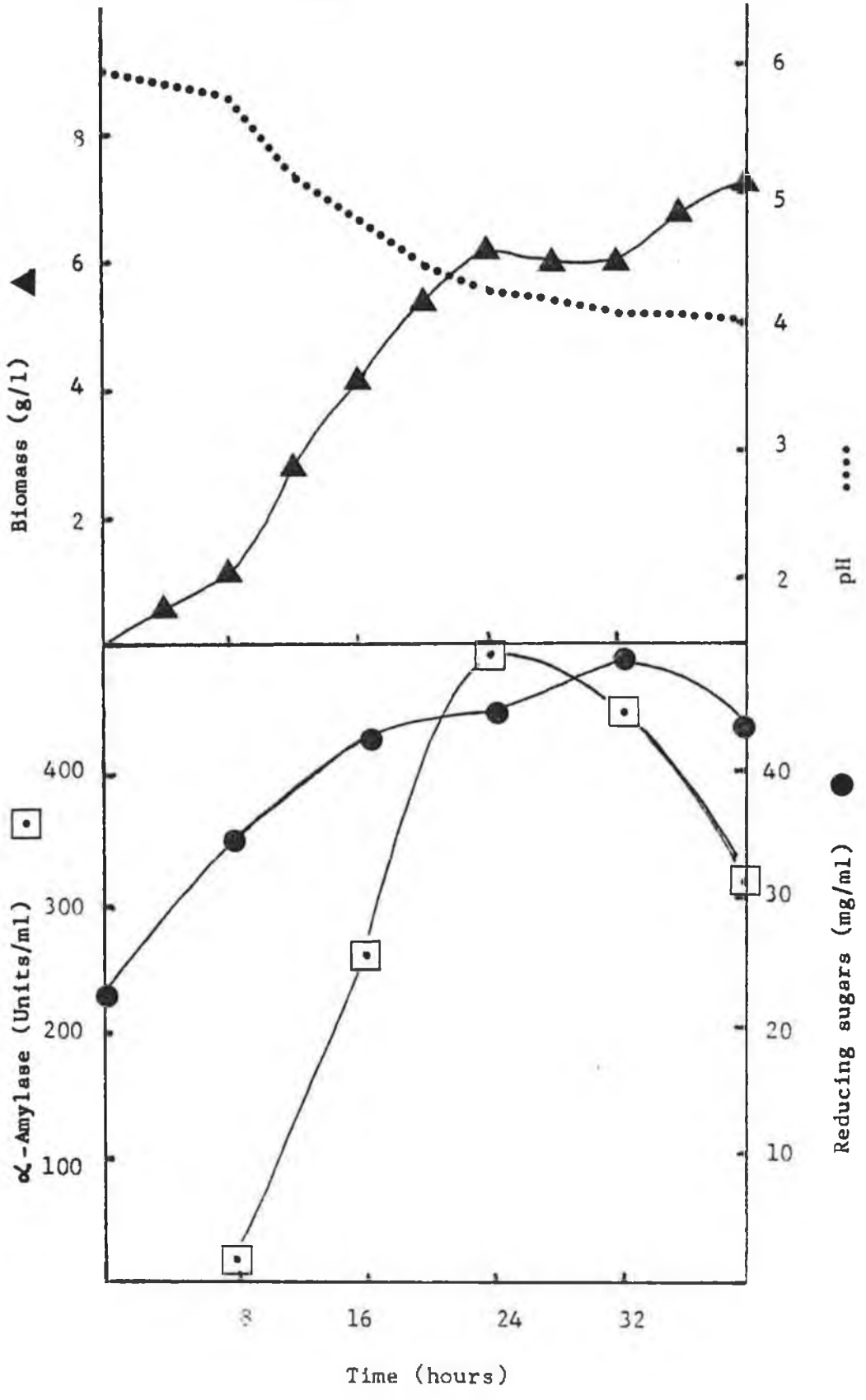

Figure 3.32 Effect of aeration and agitation on growth and enzyme production, by A. awamori NRRL 3112 on medium T3, at 1.0 v/v/m, 580 rpm.

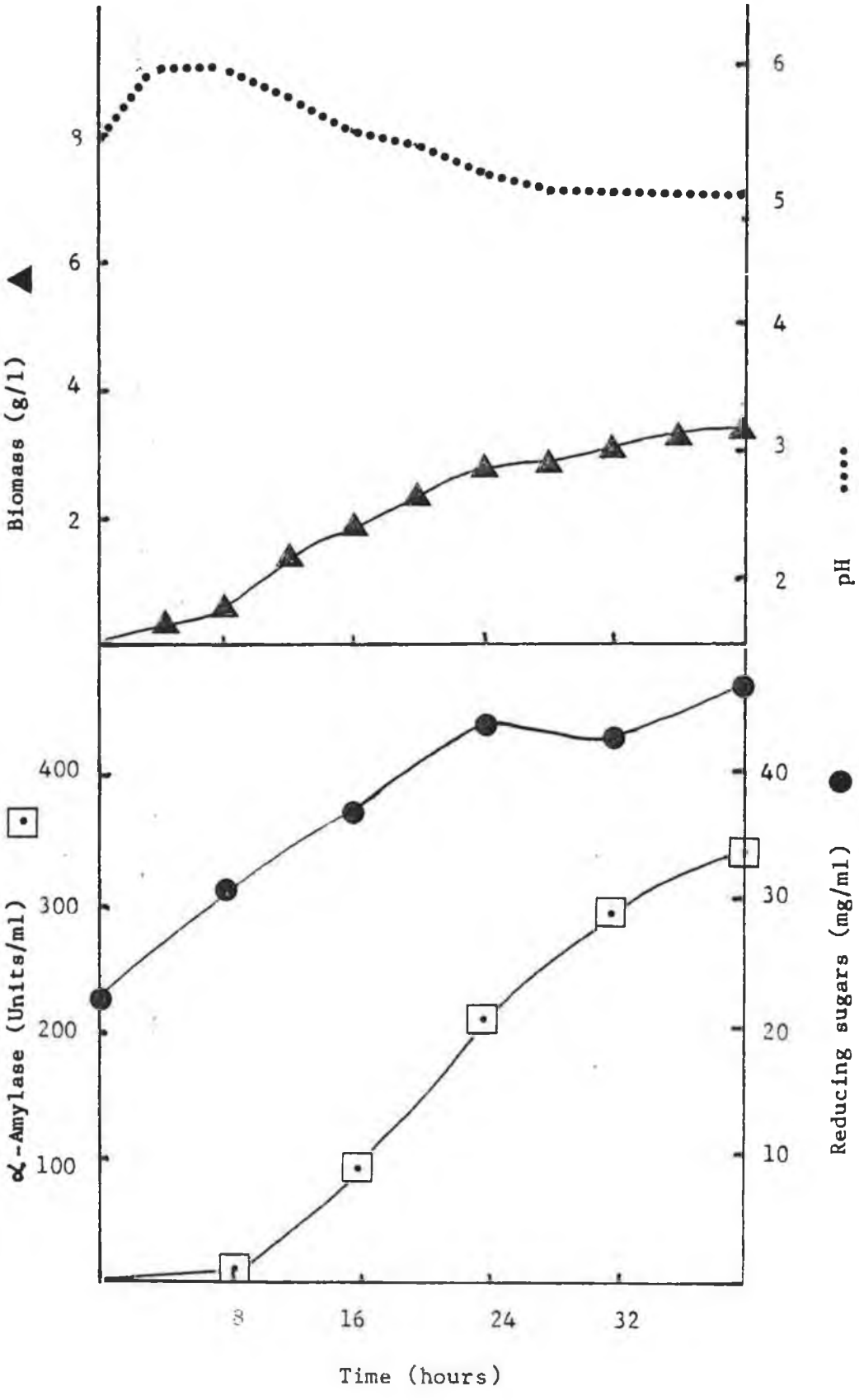

Figure 3.33 Effect of aeration and agitation on growth and enzyme production, by A. awamori NRRL 3112 on medium T3, at 0.28 v/v/m, 360 rpm.

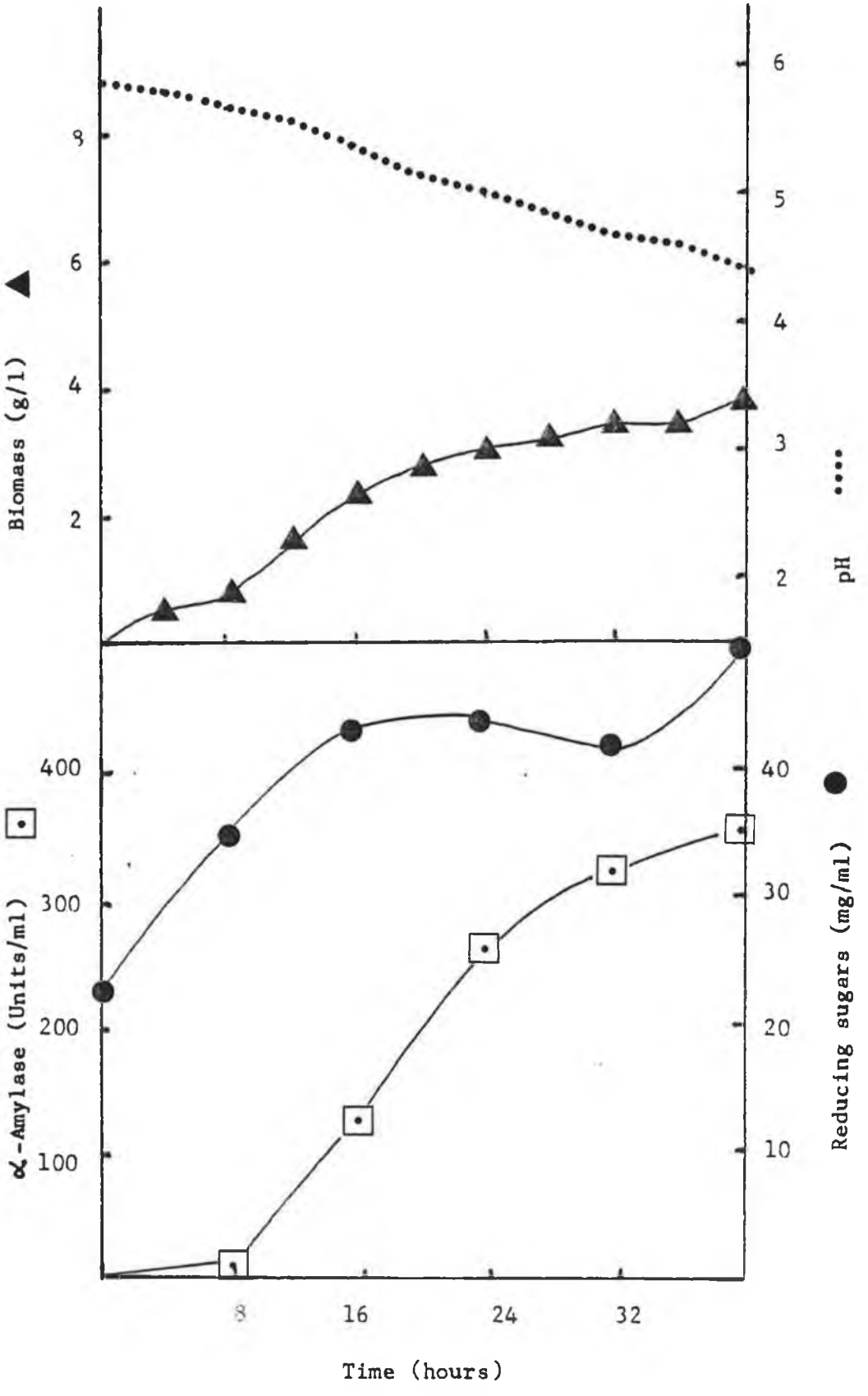

Figure 3.34 Effect of aeration and agitation on growth and enzyme production, by A. awamori NRRL 3112 on medium T3, at 0.5 v/v/m, 360 rpm.

suggesting that pH optima for expression/secretion are higher than those for activity.

Table 3.20.Effect of initial pH on levels of amyloglucosidase per mg of biomass produced by A. awamori NRRL 3112 on medium T3, 580 rpm.

Time (Hours)	Amyloglucosidase (units/mg dry weight)				
	pHi 5.9	pHi 5.2	pHi 4.7	pHi 4.1	pHi 3.2
8	Trace	Trace	Trace	Trace	Trace
10	0.24	0.17	0.17	-	0.25
24	0.22	0.28	0.21	0.10	0.10
32	0.41	0.36	0.36	0.17	0.11
Average	0.29	0.27	0.25	-	0.15

3.5.2 Effect of aeration on the specific growth rate of A. awamori NRRL 3112.

The effect of aeration and agitation on specific growth rate and other parameters was examined to optimise the oxygenation of the fermentation broth. Table 3.21 gives details of individual experiments.

Table 3.21 Parameters varied in fermentations in section 3.5

Figure	Agitation (rpm)	Aeration (v/v/m)	Aeration (l/min)
3.30	580	0.28	1.4
3.31	580	0.5	2.5
3.32	580	1.0	5.0
3.33	360	0.28	1.4
3.34	360	0.5	2.5

Considering the three fermentations at 580 rpm, reducing sugar, pH and biomass plots were similar at 0.28, 0.5 and 1.0 v/v/m. In each case, the mean doubling time was 3.8-4.2h,

and the average specific growth rates over 40 h were 164.3, 176.1 and 183.1 mg/l/h, at 0.28, 0.5 and 1.0 v/v/m respectively (Table 3.22).

Table 3.22 Effect of aeration and agitation on specific growth rate of A. awamori NRRL 3112, on medium T3.

Time (hours)	Specific growth rate (mg/l/h)				
	580 rpm			360 rpm	
	0.28 vvm	0.5 vvm	1.0 vvm	0.28 vvm	0.5 vvm
4-8	78	44	116	59	61
8-12	405	438	419	161	194
12-16	425	338	357	117	224
16-20	232	377	279	163	85
20-24	118	84	62*	90	64
24-28	16	29	62*	19	28
28-32	134	34	62*	55	36
32-36	1	127	184	51	0
36-40	17	114	109	25	115
Average	164.3	176.1	183.1	82.2	89.7

* Average over the period 20-32h.

α-Amylase production (both units/ml and units/g dry weight) were similar at 460-510 units/ml and 80-83 units/g at 24 h. (Table 3.23).

Table 3.23 Effect of aeration and agitation on levels of α-amylase per mg biomass, produced by A. awamori NRRL 3112, on medium T3.

Time (hours)	α-Amylase (units/mg dry wt.)				
	580 rpm			360 rpm	
	0.28 vvm	0.5 vvm	1.0 vvm	0.28 vvm	0.5 vvm
8	ND	19.1	18.9	ND	0
16	57.8	57.2	61.5	52.3	52.0
24	80.6	79.8	83.2	73.9	83.7
32	71.7	73.4	74.7	95.3	95.3
40	43.6	43.4	43.9	99.9	91.9

ND = Not Determined

At 360 rpm, slight differences occurred between 0.28 and 0.5 vvm. At 0.25 1/min., the pH fell more sharply than 0.14 1/min. (i.e. to 4.3 and 4.6 respectively at 24h). This corresponded with the slightly higher biomass yields and also the higher (ca 7%) specific growth rate at the higher aeration rate. Levels of α-amylase (units/ml) were up to 37% greater at the higher aeration rate, but the effect diminished with time until, at 40 h, the difference was only 2.4%.

In summary, at 580 rpm, increasing aeration had no effect on biomass, pH fall, reducing sugars or enzyme production. At 360 rpm, the higher aeration rate gave slightly higher (ca 10%) biomass and specific growth rate while α-amylase production was not affected. These results would suggest that at 580 rpm, oxygen availability was sufficient for growth, while at 360 rpm, increasing the air flow rate increased the biomass, indicating that 360 rpm and 1.4 1/min. led to sub optimal aeration conditions.

3.5.3 Effect of agitation on specific growth rate of A. awamori NRRL 3112.

In this section, the effect of various agitation rates on specific growth rate, biomass, pH and enzyme formation were considered; the following comparisons being made:-

0.28 vvm	360 rpm	Figure 3.33
	580 rpm	" 3.30
0.5 vvm	360 rpm	" 3.34
	580 rpm	" 3.31

At 1.4 1/min, biomass yields were almost double at the higher agitation rate (6.6 and 3.4 g dry weight/l). This corresponded to a greater drop in pH at 580 rpm than at 360 rpm. In both cases the period of greatest decrease in pH was at 12 - 16 h.

α-Amylase production reached a maximum at 24 h at 580 rpm (456.6 units/ml) and at 40 h at 360 rpm (338.8 units/ml). When α-amylase production per mg dry weight was considered, the lower agitation rate was more productive at 80.2 units/mg compared with 63.4 units/mg at 580 rpm.

At 2.5 1/min., the trends seen above were repeated; higher

agitation rates led to higher biomass yields, shorter doubling times, greater specific growth rates, larger fall in pH, greater α-amylase production per ml, and earlier α-amylase production peak, although greater productivity of α-amylase per g dry weight was recorded at 360 rpm.

Many of these observations can be linked to the availability of oxygen in the fermentation. At both air flow rates, the K_La value at 580 rpm is 3.2 times that at 360 rpm. This would suggest that oxygen for growth was limited at lower agitation rate.

3.5.4 Appraisal of results

The specific growth rate, μ, varied with time during the fermentation, reaching a maximum at 12-16h. The maximum average specific growth (μmax) was recorded in medium with an initial pH of 4.1, and it decreased with increasing and decreasing pHi values. The average doubling time at pHi 4.1 was less than at pHi 5.9 and 5.2; this was predicted in shake flask work.

Both α-amylase and amyloglucosidase production increased with increasing initial pH. This suggests that expression/secretion mechanisms are enhanced by increasing pH, and that the optimum for enzyme stability/activity does not correspond to the optimum for expression/secretion.

The effect of aeration and agitation on various fermentation parameters at 40 h is summarised in Table 3.24.

Biomass levels were substantially higher at higher agitation levels, but increasing air flow rate from 1.4 to 5 l/min. did not have the same effect. When relative K_La values were considered, it would suggest that agitation is required for some other purpose, as well as increasing oxygen availability, probably adequate mixing of nutrients, or removal of toxic products/inhibitors. For productive biomass formation, it would appear that 580 rpm and 0.28 vvm are adequate.

Table 3.24 The effect of aeration and agitation on various fermentation parameters at 40 h.

Agitation (rpm)	Fermentation parameters (in appropriate units)		Aeration (l/min.) 1.4	2.5	5.0
580	relative K_La	(%)	100	139	204
	biomass	(g/l)	6.6	7.0	7.2
	Average μ	(g/l/h)	0.164	0.160	0.183
	α-Amylase	(units/ml)	286	302	316
	α-Amylase	(units/g)	43.6	43.4	43.9
	pH (final)		4.0	4.0	4.0
360	Relative K_La	(%)	31	43	
	Biomass	(g/l)	3.4	3.8	
	Average μ	(g/l/h)	0.082	0.090	
	α-Amylase	(units/ml)	339	347	
	α-Amylase	(units/g)	99.9	91.9	
	pH (final)		4.6	4.4	

α-Amylase levels per mg biomass are substantially higher at low agitation rates, and decrease with increasing K_La. This suggests that oxygen has a harmful effect on enzyme stability, or that at high K_La values (which led to higher specific growth rates) the organism concentrates more of its resources on growth than on enzyme production.

Section 4 DISCUSSION

4.1 Screening

To obtain superior producers of α-amylase and amyloglucosidase, various Aspergilli, all of which had been shown to produce these enzymes, were compared to each other.

The relative amounts of amyloglucosidase produced by various Aspergilli are given in Table 4.1. Five of the six studies which screened A. awamori NRRL 3112 found it the largest producer of amyloglucosidase. Bhumibhamon (1983) suggested that A. niger ATCC 13497 produced four times more enzyme than A. awamori NRRL 3112, while this study found the former produced only one quarter the levels of amyloglucosidase of the latter (Table 3.1). This variation could be due to the individual isolates.

In this study, maximum yields of α-amylase were obtained from A. niger CBS 10366, CBS 26265 and CBS 26365.

4.2 Properties of Amylase and Amyloglucosidase

The optimum temperature and pH for α-amylase activity found in this work were similar to those of other fungal α-amylases (Bhella and Altosaar, 1984; Bhumibhamon, 1983) at pH 4-5 and 60°C.

The optimum temperature for amyloglucosidase activity of 50° C was in good agreement with the work of Pestana and Castillo (1985), but other studies showed a slightly higher optimum, 60-65°C (Voronova et al, 1976; Saha et al, 1979). In general, the pH optima for Aspergillus amyloglucosidases was in the range 4.4-5.8, (Ramachandran et al, 1978), while this study showed that the enzyme of A. awamori NRRL 3112 was more active at lower pH values, (pH 3.5). This confirmed the finding of Ziobra-Rykala and Kaczkowski (1978), which suggested the A. awamori NRRL 3122 and A. niger NRRL 3122 had optima in the range 2.8-3.7

Although the optimum for enzyme activity is important, it may not coincide with the optimum for stability or for production. Kelly et al (1983) found an α-glucosidase in Bacillus sp ATCC 21591, which was produced at pH 9.7 but had

Table 4.1 Relative rates of Amyloglucosidase Production by Various Aspergilli

Organism	% of Maximum Amyloglucosidase Yield								
	Unterkofler (1969)	Cadmus (1966)	Smiley et al (1964)	Pestana & Castillo (1985)	Ziobra-Rykala & Kaczkowski (1978)	Bhumibhamon (1983)	Armbruster (1961)	Anstrup (1967)	This STUDY (Table 3.
A. niger NRRL 330	47	64-95	-	-	-	-	40	-	21
A. niger NRRL 337	37	25	30	-	-	-	47	-	20
A. awamori NRRL 3112	100	100	100	100	100	24	-	-	100
A. niger NRRL 3122	-	-	-	44	53-60	-	-	-	62
A. niger CBS 10366	-	-	-	-	-	-	-	100	96
A. niger CBS 26265	-	-	-	-	-	-	-	48	59
A. niger CBS 26365	-	-	-	-	-	-	-	48	73
A. niger ATCC 13496	-	-	-	-	-	100	39	-	24
A. niger ATCC 13497	-	-	-	-	-	-	100	-	31

an optimum for activity at 7.0. Some results with A. awamori NRRL 3112 in this study suggest that the pH range for ∝-amylase activity does not coincide with the range for expression/secretion, (Section 3.3-3.5) and that the temperature for optimum activity of ∝-amylase (60 ° C) and amyloglucosidase (50°C) is not that for production (30°or 23°C).

4.2 Shake Flask Trials

Data obtained in the shake flask trials using A. awamori NRRL 3112 and A. niger CBS 26265 (Section 3.3) was compared with previously recorded results under the following headings:

- nutrition
- pH
- time
- intracellular and extracellular production.

It must be noted that it is difficult to separate each of these variables, since in batch culture they are all constantly varying, and effecting each other.

Nutrition

The effects of various carbon and nitrogen sources, and other additives on enzyme and biomass production were examined, so that ∝-amylase and amyloglucosidase production could be better understood and, therefore, production increased.

There is disagreement in the literature about which simple carbon source was favoured by Aspergilli for amyloglucosidase production. Qadeer and Kauser (1971) favoured glucose, Sreekantiah et al (1973) starch, while Attia and Ali (1974) favoured maltose. Of the two organisms tested in this research (section 3.3.1 and 3.3.2), A. awamori NRRL 3112 showed highest production on maltose but the highest production per mg of biomass was obtained on starch with only 78% of this amount on maltose. A. niger CBS 26265 showed almost equal production (in units/mg and units/ml) on glucose and maltose with 50% of this level produced on starch. Ustyuzhanina and co-workers (1985) found that active synthesis of ∝-amylase (as well as other

enzymes such as protease) was associated with good growth of the producer; with A. niger CBS 26265, this study showed that good growth was required but that maximum biomass did not lead to maximum α-amylase production. In other words, production of α-amylase per g dry weight was not constant, but appeared to depend on the carbon source and other factors.

In these experiments, glucose was the favoured carbon source for α-amylase production followed by maltose and starch (Sections 3.3.1 and 3.3.2). This was unexpected, and contrasts with previously recorded data suggesting that starch, then maltose and glucose, stimulated α-amylase production (Ustyuzhanina et al, 1985; Ramachandran et al, 1979; Attia and Ali, 1974).

The effect of two nitrogen sources, one complex and one simple - ammonium chloride and corn steep powder was examined (Sections 3.3.1 and 3.3.2). Various authors favoured corn steep powder for biomass production (Ramachandran et al, 1979), α-amylase formation and amyloglucosidase production (Attia and Ali, 1974; Pestana and Castillo, 1985). After seven days, Qadeer and Kausar (1971) showed that A. awamori NRRL 3112 produced 200-224% more amyloglucosidase, on medium containing corn steep powder, than that with ammonium chloride, irrespective of the carbon source. Barton et al (1969) showed a similar but smaller trend in A. niger NRRL 330. The effect was attributed to the pH buffering capacity of the complex nitrogen source and the presence of unidentified chemicals which may increase enzyme production. In Section 3.3.1, A. awamori NRRL 3112 confirmed these findings with 74 times amyloglucosidase levels in medium with corn steep powder, compared with ammonium chloride.

Andrzejczuk-Hybel et al (1985) found that use of ammonium chloride in combination with starch showed a large production of acidity, which stopped mycelial growth, and showed no amyloglucosidase activity. Similar results were obtained by A. awamori NRRL 3112 and A. niger CBS 26265 (Tables 3.2-3.5), where pH values fell below 2.0, and biomass levels were lower than expected.

In general, fungi produced greater quantities and more types of enzymes on organic media than on synthetic media

(Sreekantiah et al, 1973), probably due to the presence in the former of minute amounts of beneficial substances (e.g. amino acids, trace elements). The very high levels of α-amylase and amyloglucosidase produced on medium B (maize and corn steep powder) confirm these findings.

Barton and co-workers (1969) claimed that higher levels of amyloglucosidase were produced when the carbohydrate:nitrogen levels were reduced. Andrzejczuk-Hybel et al (1985), showed that increasing the corn steep powder concentration maintained the pH at a higher level; a result which was confirmed by this study using A. niger CBS 26265 and A. awamori NRRL 3112 (Section 3.3.3). With both of these organisms, the maximum amyloglucosidase level was obtained at 3% corn steep powder; this corresponded to a C/N ratio of 19, which was not the lowest ratio tested. Pestana and Castillo (1985) using A. awamori NRRL 3112 grown on rice flour/sodium nitrate medium found the optimal ratio for amyloglucosidase production to be 35, with 94% of this level of enzyme obtained at a ratio of 17. Again, increasing nitrogen levels increased the final pH values.

Ramachandran et al (1978) showed that citrate inhibited amyloglucosidase activity. Results in section 3.3.4 showed that addition of sodium citrate to the medium did not increase amyloglucosidase production, although pH was more favourable for production. This suggests that the increased pH may have increased amyloglucosidase production, but the high levels of citrate lead to its inactivity. Increasing α-amylase levels were recorded as the citrate concentration increased, probably due to increased pH.

Initial pH

pH plays an important part in any fermentation. It affects the enzymes' stability, activity, expression and secretion. Therefore, various authors have investigated the effect of pH on amylolytic fermentations. Pestana and Castillo (1985) showed that initial pH of the medium had a marked effect on amyloglucosidase production in A. awamori NRRL 3112, with a narrow optimum range of 6-7, while Kvesitadze et al (1981) favoured the range pH 5-6. Unterkofler (1969) on the other hand showed that after 24 hours growth, A. foetidus produced almost identical amounts of amyloglucosidase in shake flasks

with initial pH values of 6.0, 5.0 and 4.0. Results on the effect of initial pH on A. awamori (Section 3.3.7) showed that amyloglucosidase was less affected by initial pH values than α-amylase, with almost equal amounts produced in the flasks at initial values of pH 6.0, 5.5, 5.0 and 4.5. These results confirm the observations of Barton et al (1969) and Lineback et al (1966) which suggested that a specific pH value was not necessary for amyloglucosidase production, since enzyme production occurred over a wide range of pH values.

Grigorov et al (1976) showed that amylase accumulation in the medium of A. awamori at pH 4 or 3 was 50 and 25% respectively, of that at pH 6. These results were in very close agreement with those for production of α-amylase by A. awamori NRRL 3112 on medium H (Section 3.3.7). Some of the pH variation can be explained by the increase in enzyme stability at values around their maximum (e.g. α-amylase pH 4-5), but as stated in Section 3.3.7, this does not account for the phenomenon entirely. Therefore, it would appear that decreasing pH stimulates α-amylase transcription/translation and/or secretion, although it must be recalled that extracellular pH may not be similar to the intracellular value, and that changes in extracellular pH have an (as yet) undetermined effect on intracellular pH values.

Kundu and co-workers (1973) showed a significant increase in biomass with increasing initial pH with A. oryzae. After 96 h, it produced 8.3 g/l at pHi 6.0, 6.8 g/l at pHi 5.0 and 5.5 g/l at pHi 4.0. In contrast, A. awamori NRRL 3112 showed very little variation with between 2.54-2.60 g/l at pHi 6.0, 5.0 and 4.0 after 96h (Section 3.3.7).

Time

To optimise biomass and enzyme production with respect to time, the time course of A. awamori NRRL 3112 and A. niger CBS 26265 was followed. Rate of production of amyloglucosidase by A. awamori NRRL 3112 and A. niger CBS 26265, was almost constant over a 7-day fermentation (Section 3.3.5 and 3.3.6). This was similar to the pattern of production of the amylolytic systems of A. oryzae described by Kujawski (1966) and A. niger 337 described by Cadmus et al (1966) which showed amyloglucosidase activity

steadily increasing until the seventh day. However, it contrasted with the results of Barton and co-workers (1969) which showed a diphasic amyloglucosidase production pattern in A. niger NRRL 330 grown on 1% glucose: the first maximum was at 72 hours and the second at 168 hours. The latter gave 40% greater levels of amyloglucosidase than the former. Barton et al (1969) and Ebertova (1966) attributed the diphasic growth to the organic nitrogen source.

In his study of the growth and production of amylolytic enzymes by A. oryzae, Kujawski (1966) noted that the initial 36 hours showed intense development of mycelium, and considerable utilisation of carbohydrate, which occurred simultaneously with rapid synthesis of all amylolytic enzymes. Reducing sugars rose from their initial value, to a maximum after 24 hours, falling to low levels (less than 10% reducing sugar in the medium) by 72 hours. Similar reducing sugar patterns, with very little residual sugar detected after 96 h were found with both A. awamori NRRL 3112 and A. niger CBS 26265 in shake flask cultures (Section 3.3.).

α-Amylase production by both A. niger CBS 26265 and A. awamori NRRL 3112 was erratic over the seven day fermentation. In most cases, a peak was reached at 48 hours; after this the α-amylase levels fell. Reddy and Abouzeid (1986) suggested that this might be due to feedback inhibition by glucose released from hydrolysis of starch or, in this case, an over supply of available glucose in the medium. They showed that α-amylase levels in the medium rose only when reducing sugars fell below the inhibitory level (ca. 10mg/ml). This theory fits the α-amylase production pattern shown in figures 3.6 - 3.9.

Section 3.3.5 showed that the maximum rate of amylase production with A. terreus occurred in the first 48 hours; similar results were obtained by Sharma & Joseph (1984).

Intracellular and extracellular production

From findings in these experiments, and by other authors, it was thought useful to examine the intracellular as well as the extracellular levels of enzymes, to get a better understanding of expression and secretion of the enzyme. Parameters so far examined led to increased extracellular

production but did not take into account changes in enzyme transcription/translation or secretion.

Medium containing glycerol as sole carbon source produced low levels of α-amylase and amyloglucosidase, and the percentage of both enzymes which was expressed intracellularly remained high over the four-day fermentation (in these trials up to 72% of the total enzyme yield). Starch medium produced greater levels of extracellular enzyme (89-96% extracellular α-amylase and 86-96% extracellular amyloglucosidase after 48 h). This is in good agreement with the results of Yabuki et al (1977) who suggested that starch or its breakdown products stimulated secretion of α-amylase and amyloglucosidase and also induced enzyme formation.

4.4 Fermenter Trials

Data obtained in the series of experiments using A. awamori NRRL 3112 in laboratory scale fermenters (Sections 3.4 and 3.5) was compared with previously published data under the following headings:

- medium
- temperature
- pH
- aeration and agitation
- time
- growth.

Again, it must be noted that all these factors (and others), act together, meaning that a fermentation is a dynamic, complex biological system.

Medium

In current experiments, dextrin (DE 5) was found to produce higher levels of α-amylase than starch, while amyloglucosidase levels were almost equal in both of these media (Section 3.4.1). In shake flask trials, using A. oryzae, Kundu et al (1973) showed little difference between α-amylase levels on both carbon sources, over a 96 h fermentation, although dextrin gave a 10% increase in biomass yields. Andrzejczuk-Hybel et al (1985) found that many types of starch (rice, potato and corn) gave 1.5-2.5 times the yield obtained on corn modified to DE value of 5%.

Amyloglucosidase is an inductive enzyme synthesised in the presence of appropriate inducers, mainly the products of starch degradation (Andrzejczuk-Hybel et al, 1978), although inclusion of high concentrations of monosaccharides was found to reduce amyloglucosidase levels in most cases (Barton et al, 1969). Various carbohydrates at 1 and 2% level, yielded higher levels of amyloglucosidase than at 5% levels (Barton et al, 1969). This result was borne out in the present study where 2.5% dextrin was shown to give higher yields than 5% dextrin (Section 3.4.4). Also α-amylase production was stimulated by increased levels of maltodextrin and thus increased levels of residual sugars (Section 3.4.4). Similar results which would appear to confirm these findings were observed by Ziobra-Rykala and Kaczkowski (1978) using A. niger NRRL 3122 and Vallier et al (1977) with A. oryzae.

Temperature

In these experiments (Section 3.4.3) the fermenter was maintained at 23°C rather than the usual 30°C. The economic saving from the use of non-heated water to control temperature would be significant, particularly in a large scale fermentation. The decrease in temperature led to slight increases in α-amylase and amyloglucosidase production, with reducing sugars being consumed at a slow rate (again, a saving). This confirms the recent studies by Michelena and Castillo (1984), with A. foetidus ATCC 10254, which showed that amylase production was 1.5 times greater at 24° than 30°C, but contrasts with the work of Sreekantiah et al, (1973) where amyloglucosidase production by various Aspergilli increased with increasing temperature, and Kundu et al (1973) where α-amylase and biomass increased almost threefold when temperature was increased from 20°C to 30°C.

Many authors conducted amylolytic fermentations with Aspergilli at 35° (Moshsin et al, 1979; Cadmus et al, 1966; Smiley et al, 1964; Kundu et al, 1973; Smiley, 1967) but such high temperatures were not used in the present study because Andrzejczuk-Hybel and co-workers (1978) suggested that although high yields of amyloglucosidase were obtained, higher temperatures intensified synthesis of transglucosidase, an undesirable side product of this Aspergillus fermentation.

pH

Amyloglucosidase production in A. awamori NRRL 3112 was favoured at pH 4.5 over pH 5.5 (Section 3.4.2) while α-amylase levels were greater at higher pH values (Sections 3.4.2 and 3.5.1).

This is in agreement with the work of Van Lanen and Smith (1968), who showed, using A. awamori NRRL 3112, that fermenters with lower initial pH values (4.7), yielded 39% more amyloglucosidase than those at pH 5.6, although the final pH of both was 2.8-3.0 after 5 days.

Dworschack and Nelson (1972) recommended that pH should be controlled to increase amyloglucosidase production. The findings of this study agree with these results (Figures 3.19, 3.20 and 3.21).

Lasater and Smith (1978) obtained high levels of both α-amylase and amyloglucosidase using A. awamori NRRL 3112 and A. niger NRRL 3122, with the pH of the fermentation between 4.5 and 5.5; they suggested maintaining the pH between 4.7 and 5.2 by addition of gaseous ammonia.

As occurred in these experiments (Figure 3.22 and section 3.5.1) Vallier et al (1977) showed that α-amylase production was stimulated in fermentations maintained at high pH values.

Aeration and agitation

The function of aeration is to provide adequate oxygen to the microorganism for growth and/or product formation, while agitation provides adequate mixing and a homogeneous environment within the fermenter. Agitation also affects oxygen transfer in that increased agitation leads to smaller air bubbles in the medium and therefore a greater surface for oxygen transfer. Formulae derived in Section 1 and Appendix 6 show that oxygen transfer rate (OTR), or K_La can be increased by increasing agitation or air flow. Stanbury and Whitaker (1984) stated that aeration conditions necessary for optimum product formation may be different from those favouring biomass production, and indeed

production of specific microbial products may be directed by oxygen conditions (Hirose and Shibai, 1980).

Cadmus et al (1966), Zieba-Kostka (1983) and Unterkofler (1969) showed that increased agitation gave better amyloglucosidase production, and also a more rapid pH drop. Results using A. awamori NRRL 3112 indicate that increasing agitation rate had more effect on pH, biomass and α-amylase, than on amyloglucosidase production (Sections 3.4.2 and 3.5.3). This agreed with the findings of Shreekantiah et al (1973).

Aeration levels of 0.5 v/v/m were recommended by Dworschack and Nelson (1972) (and were used in experiments in Section 3.4) but in many cases 1.0-1.5 v/v/m were preferred (Smiley 1967, Cadmus et al, 1966).

Increased aeration, through greater air flow or higher agitation rates, increased biomass, and caused a greater fall in pH levels (Sections 3.4.2 and 3.5.2). Andrzejczuk-Hybel et al (1985) showed that higher aeration not only intensified amyloglucosidase synthesis, but also resulted in considerably increased enzyme yield. From Section 3.5.4, it would appear that although biomass production requires oxygen saturation (which may have been achieved in the Laboferm fermenters at 580 rpm and 1.4 l/min), optimal α-amylase production occurred at K_La values which were 15-21% of this value. Optimal K_La values vary with the organism and the enzyme produced (Sharma and Joseph, 1984).

In general, it was difficult to compare aeration and agitation rates in various fermenters, since details such as impeller type, tank diameter and impeller diameter were not available, hence shear rates, oxygen transfer rate etc. could not be calculated.

Time

Previously reported fermentations of A. awamori NRRL 3112, in laboratory fermenters, showed a general pattern: amyloglucosidase levels increased almost linearly over 120 h; maximum α-amylase activity was noted at 48h, and followed by a rapid decrease; and pH dropped over the fermentation period until lysis occurred (Andrzejczuk-Hybel

et al, 1978; Ziobro-Rykala and Kaczkowski, 1978; Cadmus et al, 1966). Kujawski (1966) noted a period of intensive mycelial development associated with rapid utilisation of carbohydrates, which was particularly notable in the first 36 h. After 48 h, 70% of the utilisable carbohydrate had been assimilated and the nutrient pH decreased most rapidly over this period also.

In general, results in section 3.4 and 3.5 agree with these findings, although they disagree with the suggestions of Vallier et al (1977) that production of α-amylase is dependent on cell lysis. The initial increase in reducing sugars, over 0-24h, recorded in Section 3.5, was also noted by Kujawski (1966) who suggested that reducing sugar level depended on rate of hydrolysis of polymers in the medium and the rate of assimilation of the carbohydrates formed by the microorganism, which in turn depended on the intensity of growth of the organism and the ratios of α-amylase to amyloglucosidase.

Growth

Specific growth rates and mean doubling time have been successfully used as 'markers' of fungal growth (Trinci, 1970). Maximum specific growth rates of Aspergilli occurred within the first 36 h (Kujawski, 1966), and in the case of A. awamori NRRL 3112 from 12-16 h (Table 3.22). Miles and Trinci (1983) found that specific growth for Penicillium chrysogenum was $0.29h^{-1}$ at an initial pH of 6.0; and did not increase significantly with increased pH. Results with A. awamori NRRL 3112 showed an average of $0.22h^{-1}$ at initial pH 6.0, with no significant changes in the initial pH range 5.9 - 4.1.

In conclusion, I feel that these trials have demonstrated the suitability of Aspergilli, especially A. awamori NRRL 3112, for production of amylolytic enzymes in submerged culture. They also have shown the ease with which the process could be scaled up to commercial levels.

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Appendix 1: Spore Diluent

The following stock solutions were made up:

1. 10% Triton-X-100 solution
   10 ml of Triton X-100 was dissolved in 50 ml of warm distilled water, and diluted to 100 ml.

2. Stock buffer
   0.34g of KH_2PO_4 was dissolved in 50 ml of distilled water. The solution was brought to pH 7.2 with NaOH and diluted to 100 ml.

The diluent consisted of 1ml of 10% Triton-X-100 solution and 1.25 ml of stock buffer, diluted to 1000 ml.

Appendix 2: Universal Buffer

To make 0.2 M universal buffer, the following chemicals were mixed:

11.44 g Acetic acid
12.36 g Boric acid
31.21 g Sodium dihydrogen orthophosphate (NaH_2PO_4)

The solution was made up to 700 ml with distilled water. It was adjusted to the required pH with NaOH, and then made up to 1l, with distilled water.

This buffer (0.2M) is a mixture of 0.2M acetate, 0.2M borate and 0.2M phosphate. It has a range of pH values from 2.5 to 12.

To make 1 M universal buffer, the above mentioned quantities of the chemicals were mixed, the pH determined, and the volume brought to 200 ml.

Appendix 3: Corn Steep Powder - Manufacturers' Specifications

(Biocon Ltd., Co. Cork.)

Water (2 h, at 105°C)	5% maximum
pH	3.8 - 4.2
Reducing sugars	2% maximum
Amino nitrogen	1.6 - 2.3%
Total nitrogen	7.2 - 8.2%
Protein	45 - 51%
Minerals	13 - 16%
Total phosphorus	2.4 - 3.2%

Appendix 4: Maltodextrin MD05 - Manufacturers' Specifications

Dextrose equivalent (DE)	20 - 23
Carbohydrate composition	
- glucose	2 - 4%
- maltose	6 - 8%
- oligo and other polysaccharides	92 - 88%
pH (in solution)	4.8 - 5.2%
Protein (maximum)	0.15%
Ash (maximum)	0.3%

Manufactured by: Roquette Freres
4, rue Patou
R 59022
Lille Cedex
France

Appendix 5: MAZU Antifoam - Manufacturers' Specifications

Mazu DF 8005 is a non-ionic low foam wetting agent, which is also a very effective foam control agent in fermentations.

Typical Properties

Composition	Polyoxyalkylene glycol
Active content	100%
Appearance at 20°C	Clear water white liquid
Specific gravity at 20°C	1.02
Viscosity at 20°C	700 cps

Mazu DF 800 is chemically stable to alkalis and to acids, except in the presence of strong oxidising agents.

Manufactured by: Mazer Chemicals Ltd.,
Martens Road,
Northbank Industrial Estate,
Manchester M30 5BL,
United Kingdom.

Appendix 6: Calculation of the Oxygen Transfer Rate and K a Values

The rate of oxygen transfer from air bubble to the liquid phase follows the equation:

$$\frac{dC}{dt} = K_L A (C^* - C)$$

where C is the concentration of dissolved oxygen in the fermentation broth,

t is time in hours,

$\frac{dC}{dt}$ is the change in oxygen concentration over time

K_L is the mass transfer coefficient

a is the gas/liquid interface area per liquid volume

C^* is the saturated dissolved oxygen concentration.

K_L may be considered as the "driving force" across the liquid interface. It is extremely difficult to measure both K_L and a in a fermentation, and therefore both terms are generally combined in the term K_La, the volumetric transfer coefficient, the units of which are normally h^{-1}. K_La is a measure of the aeration capacity of a fermenter.

Factors affecting K_La include airflow rate, degree of agitation, rheological properties of the culture broth and presence of antifoaming agents. The air flow rate has a relatively small effect on K_La values in conventional agitated systems.

Many attempts have been made to correlate power consumption (and hence, agitation) with K_La values, so that mathematical descriptions may be used for predicting power requirements in design and scale-up exercises. However, a major difficulty occurs in correlating Newtonian fluid models and the more complex non-Newtonian fluids, which include mycelial fermentation broths.

Richards (1961) stressed the probability that any relationship between K_La and power consumption per unit volume is affected by many variables including impeller speed and size, rheology, and superficial air velocity. He derived the following relationship which includes some of the extra variables:

$$K_La \propto \left[\frac{Pg}{V}\right]^{0.4} Vs^{0.5} N^{0.5}$$

where Pg is power absorbed in an aerated vessel
V is liquid volume in the vessel
Vs is superficial air velocity
N is impeller rotational speed (in rpm).

Fukuda et al (1968) also incorporated the effect of the number of impellers (Ni) on oxygen transfer in non-Newtonian mycelial fermentations.

$$K_La \propto (2 + 2.8\ Ni) \left[\frac{Pg}{V}\right]^{0.57} Vs^{0.7} N^{0.7}$$

To calculate K_La values from the above formula, Pg must be known. Michel and Miller (1962) demonstrated the relationship between gassed and ungassed power consumption (Pg and P respectively) in the turbulent flow region as:

$$Pg = kNi \left[\frac{P^2 N Di^3}{Q^{0.56}}\right]^{0.45}$$

where Q is the volumetric air flow rate
k is a constant relating to fermenter geometry
Di is impeller diameter
Ni is the number of impellers.

In this formula, there is still one unknown, P.

Ruston et al (1950) investigated the relationship between power consumption and operating variables in baffled, agitated, non-aerated Newtonian fluids. They concluded that the ungassed power consumption, P, varied as follows:

for viscous flow $P = c \mu N^2 Di^3$

for turbulent flow $P = c \rho N^3 Di^5$

where c is a constant depending on vessel geometry but independent of vessel size

μ is liquid viscosity

ρ is liquid density.

(Winkler, 1983).

So, by combining the previous formulae, P and thus Pg, and finally K a can be calculated for a baffled, agitated, aerated non-Newtonian fermentation broth as:

$$K_L a \simeq (2 + 2.8\ Ni) \left(\frac{Ni}{V}\right)^{0.56} \left(\frac{1}{D}\right)^{1.4} N^{2.46}\ Di^{3.28}\ Q^{0.56}$$

where D is vessel diameter.