MICROBIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF LACTOSE FERMENTATION BY *Kluyveromyces fragilis*

by

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Abstract

Dimorphism in K. fragilis is a strain dependent phenomenon. Conditions have been established whereby Kluyveromyces fragilis NRRL y 2415 can be induced to grow either in a yeast-like (Y) or filamentous (F) mode of growth. Oxygen availability is a determining factor which induces the dimorphic change in batch culture conditions. Anaerobic culture conditions which favour fermentation also promote yeast-like morphology, whereas oxidative conditions promote filamentous development. Increased cellular cytochrome content and oxygen uptake rates accompany the Y-F transition which suggest that mitochondrial functions are needed for the morphological transformation. Additional evidence in support of mitochondrial control of morphogenesis in K. fragilis NRRL y 2415 came from studies using chloramphenicol. This inhibitor prevented filamentation and induced the formation of respiratory-deficient budding cells in semi-aerobic cultures.

When K. fragilis NRRL y 2415 was grown aerobically under lactose limited conditions, morphology was related to growth rate. A transition from round to cylindrical shaped cells occurred during a shift from low growth rates ($D = 0.1\ hr^{-1}$) to higher specific growth rates ($D = 0.4\ hr^{-1}$). It appeared that nutrient limitation had an influence on the morphogenetic expression of K. fragilis NRRL y 2415.

The filamentous form of K. fragilis NRRL y 2415 was characterised by a more rapid lactose utilisation and ethanol production rate than the yeast form although maximum ethanol concentrations were not significantly different. Yield coefficients for both ethanol and biomass production were higher for anaerobic (yeast phase) cultures than for aerobic (filamentous) cultures.

CONTENTS

1. GENERAL INTRODUCTION

1.1 ASPECTS OF WHEY FERMENTATION

1.1.1 Composition of whey

Whey is the solution remaining after the removal of butterfat and casein from milk. It is a by-product of both cheesemaking and the manufacture of casein and related products. Until very recent times, whey has been regarded as a process effluent. Figure 1.1 outlines the production of whey. The composition of this dairy waste varies according to its origin (ewe, goat, cow) and to the cheese making procedure employed. The coagulation with rennet or rennet preparations yields sweet whey (pH 4.5 - 6.7), with high lipid contents, whereas coagulation by lactic fermentation yields acid whey (pH 3.9 - 4.5), containing smaller quantities of lactose and proteins. The typical composition of an industrial whey permeate powder is given in Table 1.1. Cheese whey or whey ultrafiltration permeates are complete culture media containing all the minerals and trace elements required for the growth of micro-organisms (see Table 1.2). In addition, they contain water-soluble vitamins (see Table 1.3) and thus constitute an excellent culture medium for those micro-organisms capable of utilising lactose or its hydrolysis products (Moulin and Galzy, 1984).

1.1.2 Whey utilisation

Although considerable advances have been made recently in developing novel approaches for the utilisation of surplus

whey, production and utilisation trends indicate a steady increase in the future availability of whey (Friend and Shanani, 1979). The utilisation of whey and whey ultrafiltration permeates have been the subject of considerable study because of the high cost of disposal and the need to reduce environmental pollution of natural water resources (O'Leary et al., 1977a). Figure 1.2 shows the principal possibilities for the use of whey ultrafiltration permeate.

The enzymatic hydrolysis of lactose markedly changes the two properties of greatest commercial importance in this sugar, sweetness and solubility (Coton, 1980). It also makes milk available to lactose-intolerant individuals (Alum, 1982). Several microbial sources of lactase have been studied and the lactose fermenting yeasts are considered an excellent source of this enzyme (Gekas and López - Leiva, 1985).

The lactose fermenting yeast Kluyveromyces fragilis is considered suitable as a food yeast and has been grown in whey with reported satisfactory yields (Peppler, 1970). In a comprehensive review on the production of biomass from whey, Meyrath and Bayer (1979) remarked that K.fragilis was considered the organism of choice since it was the most efficient lactose utiliser.

Production of Vitamin B_{12} from whey has been suggested as another way of furthering the use of permeates (Marth, 1970). The production of vitamin B_{12} from whey by Propionibacterium shermanii is discussed by Bullerman and Berry (1966) and by Marwaha, Kennedy and Sethi (1983).

Table 1.1

Composition of Industrial Whey Permeate Powder (according to Vienne and Von Stockar, 1983).

Lactose	80.6%
Moisture	3.08%
Total Nitrogen	0.57%
Non protein Nitrogen	0.45%
Protein	0.72%
Ash	8.16%
Mg	0.12%
K	2.30%
P	0.51%

Fig.1.1 Production of Whey

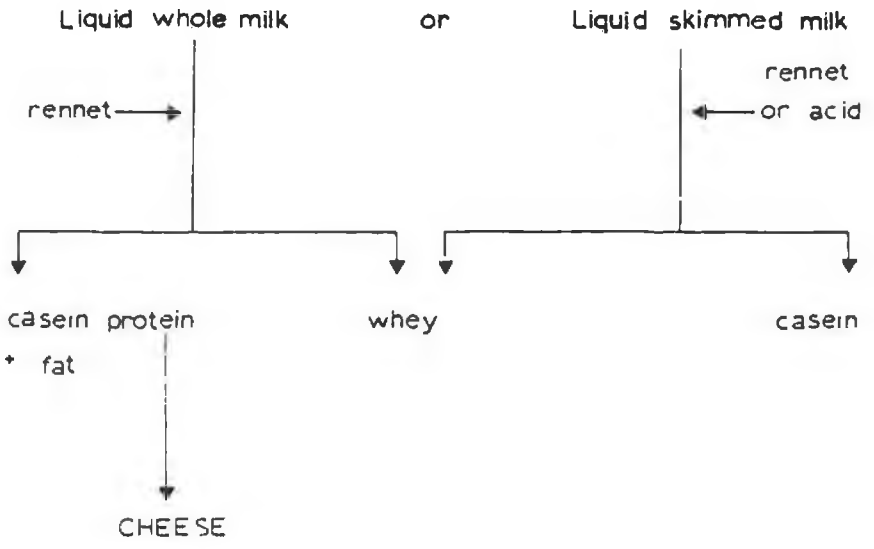

Table 1.2

Average content of the main trace elements in cheese whey and ultrafiltration permeate (mg per 100g dry matter) (according to Moulin and Galzy, 1984).

Element	Whey	Permeate
Iron	1 - 7	3 - 11
Copper	0.5 - 5	1 - 3
Zinc	5 - 9	30 - 33
Manganese	0.01 - 0.04	0.5 - 0.8

Table 1.3

Average vitamin composition of cheese whey and ultrafiltration permeate (mg/100g dry matter) (according to Moulin and Galzy, 1984).

Vitamin	Whey	Permeate
Vitamin A	100	80
Thiamin	4-6	5-6
Pyridoxin	6-10	5-10
Riboflavin	7-30	15-20
Ca Pantothenate	30-70	50-60
Biotin	0.2-0.3	0.1-0.3
Cobalamine	0.01-0.05	0.02-0.05
Vitamin C	30-50	20-40

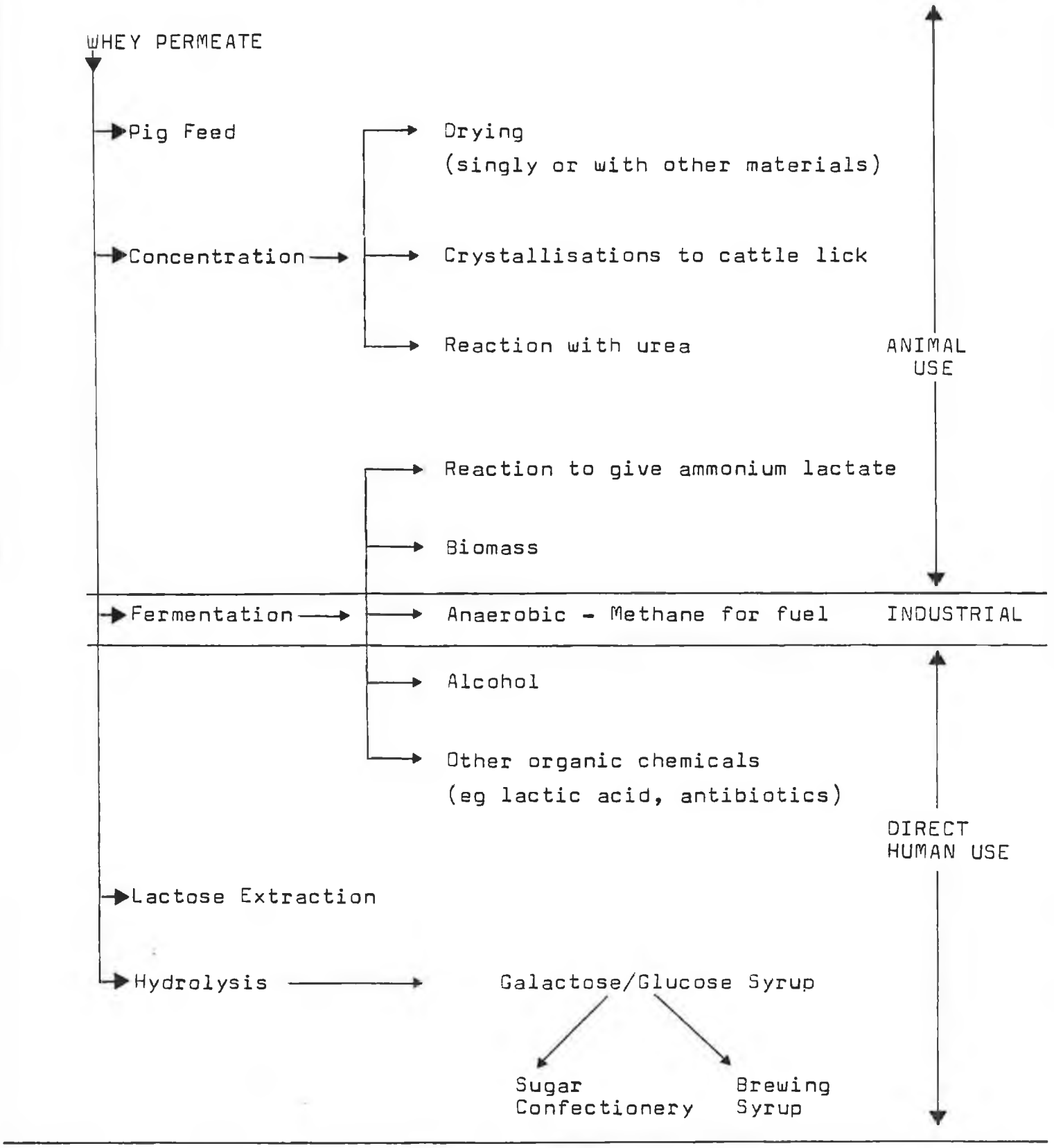

Fig.1.2 The principal possibilities for use of whey ultrafiltration permeate (from Coton, 1980).

1.1.3 Ethyl alcohol from whey

1.1.3.1 Optimisation studies

Strain Selection / Fermentation of concentrated whey:
It has been suggested by various investigators, for example Rogosa et al., (1947); Wilharm and Sack (1947) and Marth, (1970) that the fermentation of whey into alcohol or alcoholic beverages would lead to a greater utilisation of whey. Several selection studies of strains capable of fermenting lactose directly have been undertaken (Gawel and Kosikowski, 1978; Demott et al., 1981; Izaguirre and Castillo, 1982; Moulin, Guillaume and Galzy, 1980 and Vienne and Von Stockar, 1983). Not all authors are in agreement as to which organism is most satisfactory, which may be due to different growth conditions. A survey of 40 lactose-assimilating yeasts was made by Laham-Guillaume, Moulin and Galzy (1979) with respect to their ability for converting lactose to ethanol. Four strains having high ethanol yielding capacities of 110-120 gl^{-1} were selected - C. pseudotropicalis CBS 19384 and IP 513 in addition to Kluyveromyces fragilis CBS 397 and CBS 5795. Moulin and Galzy (1981) found that certain strains of the same species can ferment lactose up to a concentration of 200 gl^{-1} with over 90% of the theoretical yield. Other strains, on the contrary, only ferment lactose at very low concentrations. These observations showed a considerable variation in behaviour of strains of the same species.

The economic feasibility of distillation is directly dependent on an increase in ethanol concentration in the fermentation broth. This can be achieved by the fermentation of a

concentrated whey permeate (Demott et al., 1981; Vienne and Von Stockar, 1985b). Data from such studies is presented in Table 1.4. Yeast adaptation to concentrated whey permeate resulted in a high yield of approximately 80 gl^{-1} ethanol by K. fragilis NRRL y 2415 (Gawel and Kosikowski, 1978). Burgess and Kelly (1979) investigated the alcoholic fermentation of 3 lactose fermenting yeasts on whey permeate solutions containing up to 150 gl^{-1} lactose. In their study they showed that C. pseudotropicalis NCYC 744 and K. fragilis CBS 5795 were able to completely ferment the lactose in a 150 gl^{-1} solution within 36 hours. The inability of K. fragilis NRRL y 1109 to completely ferment a 150 gl^{-1} lactose solution was consistent with the low ethanol tolerance of this yeast. This corresponds with reports from Vienne and Von Stockar (1983) and O'Leary et al. (1977b), and with the findings of Wendorff et al. (1970) who showed that 40 gl^{-1} alcohol reduced the activity of the enzyme lactase in K. fragilis NRRL y 1109. Under optimised growth conditions the specific growth rate, lactose consumption rate and the ethanol yielding capacity of K. fragilis NRRL y 665 were all markedly reduced when the lactose concentration was increased from 50 to 150 gl^{-1} (Vienne and Von Stockar, 1985b). Whey-lactose fermentation studies undertaken by Gawel and Kosikowski (1978) indicated that fermentation rate and ethanol yield are limited largely by the physiological abilities of lactose fermenting yeasts.

Media optimisation:

Data from recent studies on the optimisation of physiological conditions for whey fermentations is presented in Table 1.5.

Table 1.4

Effect of lactose concentration on the fermentative abilities of different yeast types

Yeast Strain	Lactose gl^{-1}	Maximum Ethanol Conc $.gl^{-1}$	Reference
K. fragilis CBS 397	50	23.8	Moulin *et al.*(1980)
	100	47.7	
	150	69.2	"
	200	90.6	"
	250	90.6	"
	300	87.0	"
K. fragilis CBS 397	50	25.4	Janssens *et al.*, (1983)
	100	47.7	
	150	71.5	"
	200	84.3	"
K. fragilis CBS 397	200	95.4	Moulin and Galzy,(1981)
K. fragilis CBS 5795	200	93.0	
C. pseudotropicalis IP 513	200	94.6	"
C. pseudotropicalis CBS 19384	200	92.2	"
K. fragilis NRRL y 2415	240	95.4	Kosikowski and Wzoreck,(1977).
K. fragilis NRRL y 2415	150	53.0	Linko *et al.*, (1984).
K. fragilis NRRL y 2415	240	80.5	Gawel and Kosikowski, (1978)
K. fragilis ATCC 8635	240	58.1	
K. fragilis NRRL y 1193	240	41.3	"
K. marxianus NCYC 179	98	42.6	Marwaha and Kennedy,(1984a)

Table 1.5. Medium Optimisation Studies: Whey Alcohol production

Yeast Strain	Initial Lactose Conc (gl^{-1})	Maximum Ethanol Conc.(gl^{-1})	Initial pH	Temp. ^{o}C	Media Supplementations	References
Bath Culture						
K. fragilis						
(strain not specified)	100	36.5		32	$7gl^{-1}$ yeast extract $10mgl^{-1}$ ergosterol	Chen and Zall (1982)
K. fragilis CBS 5795	150	74.4	4.6	35	$1gl^{-1}$ yeast extract $0.5gl^{-1}$ Urea	Burgess and Kelly (1979)
C.pseudotropicalis NCYC 744	150	69.9	4.6	35		"
K.fragilis NRRL y 1109	50	22.78	4.6	35		"
C.pseudotropicalis ATCC 8619	201	98.6	4.5	30	$0.1gl^{-1}$ yeast extract $0.1gl^{-1}$ corn steep liquor	Izaguirre and Castillo (1982)
K.fragilis CBS 397	200	86.6		30	lipid and sterol stock solution: 0.6g ergosterol 1ml linoleic acid 100 ml Tween 80; used at a rate of 5 ml l^{-1}	Janssens et al.,(1983)
Continuous Culture						
K. fragilis NRRL y 665 (Dilution rate 0.1 hr^{-1})	46	22.9	4.0	38	$3.75gl^{-1}$ yeast extract	Vienne and von Stocker. (1983)

Note: The ethanol concentration was calculated on the basis of the theoretical conversion of 100% lactose to ethanol according to the Gay-Lussac equation; (1 gram of lactose theoretically yields 0.54g ethanol).

Castillo et. al (1982) reported that complete fermentation of the available lactose in whey took place without supplementary nutrients; additions of nitrogen, phosphorus salts, yeast extract or corn steep liquor resulted in increased biomass yet lower alcohol yields. These results suggest that deproteinised whey contains all the required nutrients for total lactose fermentation which is in agreement with the studies of Maddox (1980), Moulin, Guillaume and Galzy (1980), and Moulin and Galzy (1981). Apparently, the presence of high ash inhibits the production of biomass and alcohol (Mahamoud and Kosikowski, 1982). This effect had previously been observed in studies by Gawel and Kosikowski (1978).

It is always difficult to compare data in the literature concerning the optimisation of whey alcohol fermentations because of the lack of information regarding the composition of the whey permeate used in the studies. For this reason, Vienne and Von Stockar (1983) investigated the composition of the industrial whey permeate used in their studies. They showed that the whey permeate alone sustained the growth of yeasts yet the medium had two growth limitations. The first was a stoichiometric one due to a shortage of nitrogen in the medium. This was demonstrated by the addition of 1.7 gl^{-1} $(NH_4)_2SO_4$ which increased the biomass yield. The second was a kinetic limitation which was shown by the increase in specific growth rate upon the addition of 1 gl^{-1} yeast extract to the permeate. Both types of limitation could be overcome by the addition 3.75 gl^{-1} yeast extract. Consequently, the maximum specific growth rate (μ max) in batch cultures

increased from 0.164 hr^{-1} to 0.310 hr^{-1} and the alcohol productivity of continuous culture could be improved from 2.0 to 5.1 gl h^{-1} (Vienne and Von Stockar, 1983).

The effect of lipid supplementation on the fermentation kinetics of K. fragilis CBS 397 has been studied by Janssens et al. (1983). The rate of ethanol production and the maximum ethanol concentration obtained was significantly enhanced by the addition of a mixture of oleic and linoleic acids and ergosterol to the fermentation broth. Plasma-membrane lipid composition has been shown to be an important aspect of end-product tolerance by yeast (Hayashida and Ohta, 1980; Thomas et al., 1978). The incorporation of unsaturated fatty acids, sterols or both into the cellular membrane leads to an increased membrane fluidity (Ingram, 1982), which theoretically overcomes the decrease in membrane fluidity attributed to ethyl alcohol. An investigation of ethanol inhibition on the growth of K. fragilis NRRL y 665 in a concentrated whey permeate was undertaken recently by Vienne and Von Stockar (1985 a). They suggested that the adverse effects of ethanol on the growth rate and ethanol yielding capacity of the yeast was dependent on the time of exposure to ethanol which may be amplified by the high osmotic pressure of whey. Panchal and Stewart (1980) reported that when cells of a lager brewing yeast S. uvarum were grown in a medium of high osmotic pressure, increased levels of intracellular ethanol were obtained. The high levels of intracellular ethanol resulted in a reduction in ethanol yield and in cell viability.

Temperature optimisation:

An optimum temperature range of 32-37°C has been reported for cell growth and ethanol production by K. fragilis by Chen and Zall (1982). This result is consistent with the results of Rogosa et al. (1947) and Burgess and Kelly (1979). Burgess and Kelly (1979) found a high rate of fermentation for both K. fragilis CBS 5795 and for C. pseudotropicalis NCYC 744 at 35°C. Castillo et al. (1982), determined an optimum temperature of 30°C for C. pseudotropicalis ATCC 8619 which is similar to that reported for the fermentation of concentrated whey by K. fragilis (Kosikowski and Wzoreck, 1977). Vienne and Von Stockar (1983) determined an optimum temperature of 38°C for whey-alcohol production by K. fragilis NRRL y 665. Although this value is higher than values reported earlier it is the same as the value reported by Moulin, Malige and Galzy (1981) for a K. fragilis strain cultivated for single cell protein. This high temperature favours the production of higher alcohols (fusel oils) which is disadvantageous in the beverage industry but advantageous for industrial ethanol fermentation processes (Vienne and Von Stockar, 1983). Temperature optimisation for ethanol production in whey permeate was performed with both immobilised and free cells of K. marxianus NCYC 179 by Marwaha and Kennedy (1984 b). They found that the temperature tested had no significant influence on the fermentation ability of immobilised cells but an increase in yield and the rate of alcohol production was observed in free cells when the temperature was raised from 25°C to 40°C. These results

are in agreement with the results of other workers (Brown and Oliver, 1982; Lee et al., 1980 and Lee et al., 1981).

pH optimisation (batch culture):

Optimal conditions for ethanol production in 70 gl^{-1} whey solutions for C. pseudotropicalis ATCC 8619 included an initial pH of 4.57 (Castillo et. al., 1982). Initial pH values in the range 4.6 to 5.6 had no significant effect on the rate of lactose fermentation by either C. pseudotropicalis NCYC 744 or for K. fragilis CBS 5795 (Burgess and Kelly,1979). A standard pH of 4.6 was chosen by Burgess and Kelly (1979) for the fermentation since a pH value of less than 5 inhibits lactic acid bacteria (Cassida, 1968). Studies on the effect of pH on immobilised K. marxianus NCYC 179 indicated a pH optimum of 5.5 , (Marwaha and Kennedy,1984b). Chen and Zall (1982) studied the effect of initial pH in batch culture of Saccharomyces (Kluyveromyces) fragilis. They found that no pH control appeared necessary in whey fermentation studies as the pH dropped only slightly from the initial pH of 5.0 to a value between 4.5 and 4.6. The fall in pH value was considered to have no effect on the fermentation rate of the yeast as lactose fermenting yeasts were reported to have an optimum pH range of 4.7 to 5.0 (Rogosa et al., 1947).

pH optimisation (continuous culture):

Experiments carried out by Zertuche and Zall (1985) to observe the effect of pH on the continuous ethanol production and fermentation rates showed that ethanol increased from

16 to 26 gl^{-1} when the pH was decreased from 7 to 4 (50 gl^{-1} initial lactose). Vienne and Von Stockar (1985a) found that both substrate and ethanol exhibited a maximum between pH 4 - 5 during continuous whey fermentations.

1.1.3.2 Lactose hydrolysis

The choice of organisms for utilisation of whey is limited since comparatively few organisms are able to ferment lactose (Moulin and Galzy, 1984). The pre-hydrolysis of whey lactose has improved alcohol yields obtained from whey fermentations by using traditional strains of Saccharomyces cerevisiae (O'Leary et al., 1977b). However, the galactose which comprised half of the available carbohydrate in lactase hydrolysed whey, was not fermented. The diauxic fermentation pattern resulting from lactase-hydrolysed whey has led to lengthened fermentation times and consequently has stimulated the selection of catabolite resistant mutants which are not subject to the diauxic effect (Terrel et al., 1984; Bailey, Benitez and Woodward, 1982). There are a number of yeasts, however, which have the efficiency to ferment and assimilate lactose. They transport the disaccharide lactose across the yeast cell membrane by means of a lactose permease. The lactose is then hydrolysed by β-Galactosidase (lactase) into it's constitutive monosaccharides, glucose and galactose, which then enter the common glycolytic pathway (Stewart and Russel, 1983). Galactose enters the glycolytic pathway following a series of reactions as illustrated in Figure 1.3.

The structural and regulatory genes for lactose utilisation from Kluyveromyces lactis have recently been cloned and expressed in an industrial polyploid strain of S. cerevisiae by Hanley and Yocum (1986). The resulting strain of S. cerevisiae had ethanol tolerance and osmo-tolerance superior to that of Kluyveromyces strains.

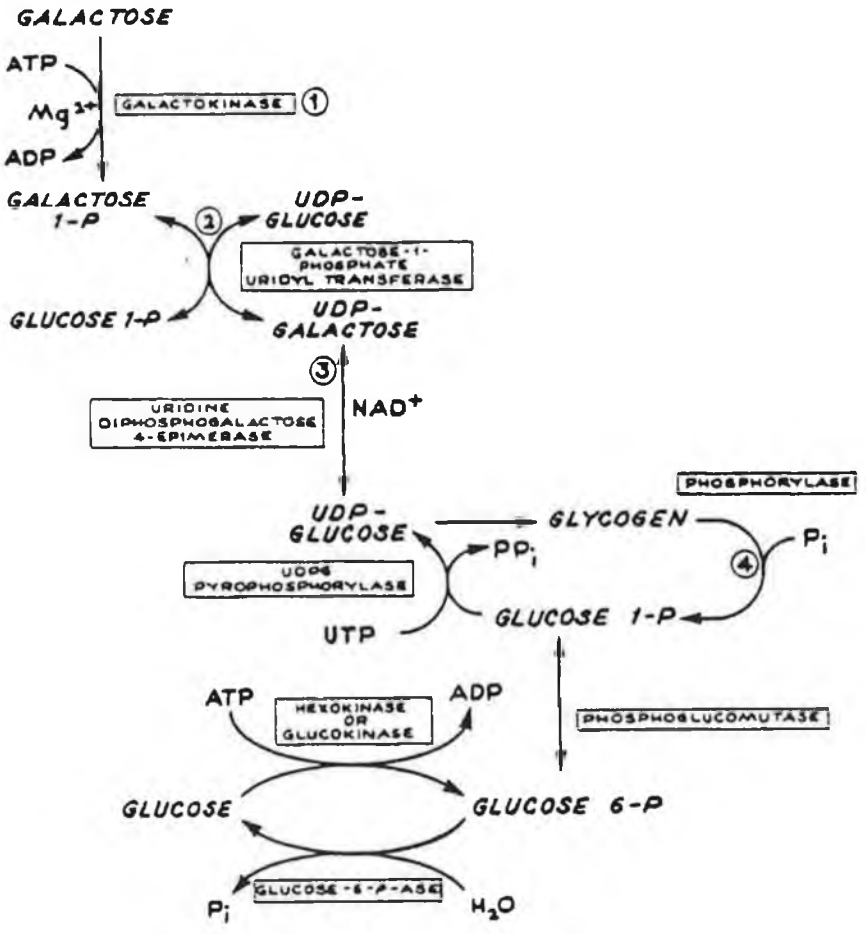

Fig. 1.3 The pathway for the conversion of galactose to glucose (from Mayes, 1981)

1.1.3.3 Continuous culture: (whey fermentation)

Many papers have been published recently reporting sophisticated developments of cheese-whey alcohol fermentations (Chen and Zall, 1982; Linko and Linko, 1981 ; Cheryan and Mehaia, 1983; Marwaha and Kennedy, 1984b; and King and Zall, 1983). Kinetic and stoichiometric parameters for conventional optimised batch and continuous fermentation of non-concentrated whey permeate have been studied by Vienne and Von Stockar (1983). Their study defined optimum growth conditions which overcame the kinetic and stoichiometric limitations of whey permeate so that a complete and efficient fermentation of the permeate could be achieved. Furthermore, attempts to increase the ethanol yields in the fermentation of concentrated whey permeate in continuous culture resulted in decreased lactose consumption rates and low alcohol yields. Vienne and Von Stockar (1983) suggest that the decrease in specific productivity be counterbalanced by the use of high cell density cultures obtained by cell recycling. Kinetic data from recent continuous whey fermentation studies is presented in Table 1.6.

One of the most recent technologies used to improve the economics of ethanol fermentation is the use of immobilised biocatalysts and bioreactors (Marwaha and Kennedy, 1984 a,b; Linko *et al*.1981 a,b ; Chen and Zall, 1982; and Kierstan and Bucke, 1977). King and Zall (1983) successfully entrapped *K. fragilis* in calcium alginate beads and showed that the continuous immobilised cell system had an increased ethanol productivity over a continuous culture with free

growing cells.

Table 1.6 Whey Alcohol Production: Continuous Culture Studies

Yeast Strain	S_o Initial Lactose	D Dilution Rate	r_p Productivity	P Max Etoh Conc.	Conversion Efficiency	Reference
	gl^{-1}	h^{-1}	$gl.h^{-1}$	gl^{-1}	%	
Continuous Culture						
K.fragilis NRRL y 665		0.27	5.1		90	Vienne and Von Stockar, (1983)
K.fragilis NRRL y 665		0.18		22.9		
K.fragilis NRRL y 665	70.03	0.201	5.26	25.26		Vienne and Von Stockar, (1985 b)
K.fragilis CBS 397	100	0.15	7.1	47.3	88	Jannsens, Bernard and Bailey,(1983)
K.fragilis CBS 397	120	0.15	7.1	47.3	81	
S.cerevisiae SR.Mutant	150	0.2	13.6	70	92	Terrel et al., (1984)
S.cerevisiae SR.Mutant	150	0.3	16.8	56	84	
Continuous Culture with cell immobilisation						
K.fragilis NRRL y2415	150	6	240		60	Cheryan and Mehaia,(1983)
S.fragilis (strain not specified)	10	1.1	6.9			Chen and Zall, (1982).
K.fragilis NRRL y2415	225			7.1		Linko et al., (1984)
K.fragilis NRRL y2415	45	0.09	1.1	13	57	Hahn-Hagerdal, (1985)
S.cerevisiae YI + β-galactosidase)[a]	100	0.09	2.5	52		"
S.cerevisiae YI(β-Gal) + Galactose adapted S.cerevisiae)[b]	150	0.09	2.5	71		"

Note: 'a' S.cerevisiae co-immobilised with β-Galactosidase

'b' A galactose adapted immobilised S.cerevisiae column in series with a co-immobilised (S.cerevisiae + β-Galactosidase) column.

1.2. ASPECTS OF YEAST DIMORPHISM

1.2.1 Introduction to dimorphism

Under certain conditions some yeasts will grow as elongated cells in a filamentous fashion. Conversely, some mycelial fungi can grow as budding yeast-like cells. "The yeast-mycelium (Y ⇆ M) dimorphism may be described as the process by which the mycelial habit of growth (with cells in hyphal or in a filamentous form) is transformed by some change in cultural or environmental conditions so that a yeast-like or unicellular morphology is adopted at the cellular level" (Stewart and Rogers, 1978). The yeast-mycelium dimorphism exhibited by a number of pathogenic and non-pathogenic fungi from a wide range of taxonomic groups has been investigated. For general reviews on different aspects of dimorphism, see San-Blas and San-Blas (1984), Scherr and Weaver (1953) and Romano (1966). Amongst the literature the terms mycelial, hyphal and filamentous are used equivalently. Criteria for the description of yeast species have been described by Kreger-van Rij (1984), and by Odds (1979).

The following is a brief description of some morphological terms used in the characterisation of yeasts.

blastospore: a vegetative spore formed as a bud on true- or pseudomycelium.

germ tube: The germination of a blastospore gives rise to extension of new cellular material known as a germ tube, (Fig. 1.4).

arthrospore: an asexual spore resulting from the division of a hypha or a single cell by fission.

true-mycelium: true-mycelia arise as separate long thread like cells that eventually branch or as septate branched filaments in which the single limbs are separated by cross walls in the filament, (Fig. 1.5).

Fig. 1.4 Germ tube

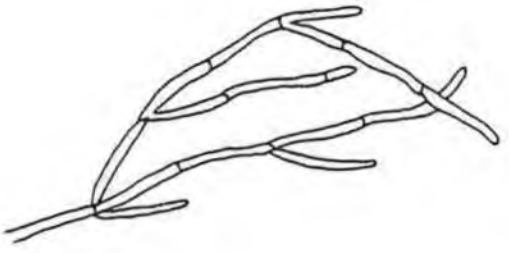

Fig. 1.5 True-mycelium

pseudomycelia: pseudomycelia or pseudohyphae refer to the formation or elongation of cells which arise by budding. Pseudomycelia may be rudimentary in which case they consist of elongated cells of more or less equal dimensions or they may be differentiated into elongated cells each of which may produce blastospores in a regular or characteristic arrangement. Langeron and Talice (1932) classified the pseudomycelium forming yeasts into several genera, the names of which are useful in the standard description of the various types of pseudomycelial formation. The definitions are according to Kreger-van Rij (1984) and are as follows:

(a) 'Mycotorula-type': The blastospores are arranged in compact spherical structures around the junction of

adjacent elongated pseudomycelial cells.

(b) 'Mycotoruloides-type': The blastospores are arranged in loose branched verticils around the pseudomycelium.

(c) 'Candida-type': Pseudomycelium with chains of blastospores arising at the junctions of adjacent pseudomycelial cells.

(d) 'Mycocandida-type': Strongly branched pseudomycelia with the blastospores often symmetrically arranged as pairs or as small whorls or verticils at the junction of adjacent pseudomycelial cells.

(e) 'Blastodendrion-type': Stalagmoid blastospores are arranged in a penicillium- like structure.

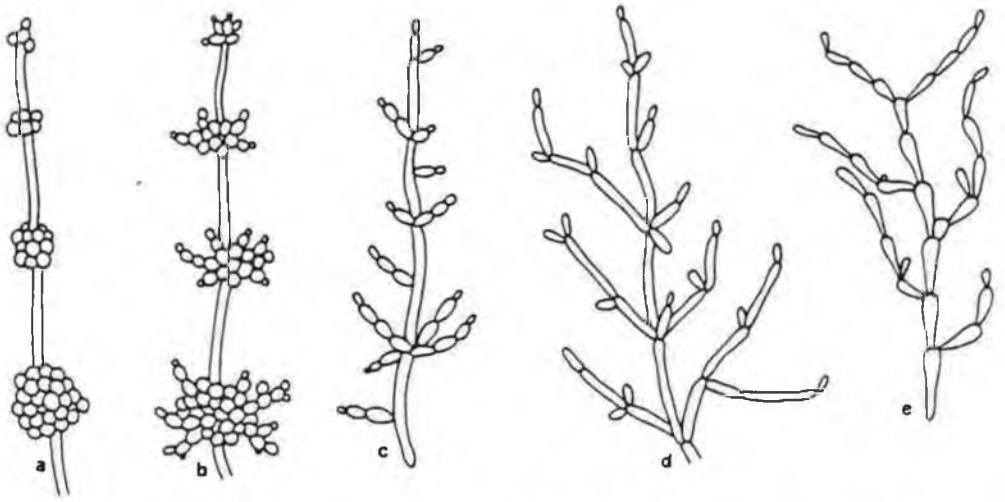

Fig. 1.6 Various types of pseudohyphae: a, Mycotorula; b, Mycotoruloides; c, Candida; d, Mycocandida; e, Blastodendrion. (Redrawn from Diddens & Lodder, 1942)

The terms used to describe the dimorphic state, yeast and mycelium, tend to indicate that two exclusive states exist. This is not the case as it is often difficult to decide when elongation of the cells major axis ceases to be regarded as mere elongation and constitutes the formation of a pseudomycelium. Filamentous cells of dimorphic fungi may convert to the yeast phase by one of three basic

mechanisms: (i) by lateral budding, (ii) by terminal budding or (iii) by arthrospore formation resulting from fragmentation. Yeast phase cells convert to filamentous forms by germ tube formation and elongation (Stewart and Rogers, 1978).

1.2.2 Environmental factors affecting yeast-mycelia conversion. Candida albicans:

This is a well studied example of a dimorphic yeast and has been used as a model for many morphogenetic investigations (Odds, 1979). A variety of environmental and cultural conditions have been implicated in the control of dimorphism in C. albicans, many of which are listed in table 1.7.

Table 1.7 Some environmental factors that have been considered to affect C. albicans morphogenesis (from Odds, 1979 - numbers quoted refer to references therein).

1 Factors that favour filamentation or suppress blastospore formation	temperature ≥35°C [109, 325, 328, 400, 541, 1153, 1279, 1454, 1876, 2014]
	temperature <35°C [1308]
	pH ≥ 7.0 [415, 540, 541, 932, 1279, 1883, 2014, 2015]
	pH < 7.0 [716, 1308, 1876]
	low oxygen tension [932, 1784, 1883]
	controlled ratio of CO_2, O_2 [1277]
	inoculum ≤10^6 yeasts/ml [152, 402, 540, 541, 945, 949, 1229, 1279]
	liquid growth media [69, 1153, 1729, 1784, 1883]
	'impoverished' growth media [historically – see 2192]
	nonfermentable carbon source [1308]
	polysaccharide carbon source [168, 413, 415, 1445, 1784]
	low sulphydryl content [1434, 1440, 1445, 1446]
	'suboptimal concentration of yeast extract' [2224]
	nitrate as nitrogen source[1] [415]
	N-acetyl glucosamine [1876]
	albumin [109, 152, 328]
	amino acids [328, 402, 415, 1122, 1123, 1153, 1277, 1454]
	L-α-amino-*N*-butyric acid [1279]
	biotin (concentration <1 μg/l) [2225, 2226]
	cobalt salts [1434, 1446]
	β-indole acetic acid [1386]
	iron (Fe^{II}) salts [1127]
	maltose [1308, 1857]
	phosphate [1308, 2165]
	zinc salts (concentration 3–5 μM) [2227]
2. Factors that favour blastospore formation or suppress filamentation:	temperature <35°C [109, 328, 541, 1153]
	pH < 7.0 [415, 540, 541, 1279, 2014, 2015]
	inoculum >10^6 yeasts/ml [402, 541, 1229]
	ammonium salts [1122, 1123]
	biotin (concentration >1 μg/l) [1122, 1123, 2226]
	cysteine or other thiol-containing compound[2] [1434, 1440, 1445, 1446, 1876]
	lactate [1857]
	2-phenyl ethanol [780, 1193]
	phosphate [1122, 1123]
	polyene antifungals [209, 1873]

1 In fact, *C. albicans* does not assimilate nitrate as a source of nitrogen (see appendix)

2 some authors have found no effect of cysteine on morphogenesis [1454, 1857, 2122]

Attempts to identify environmental factors which induce or stimulate the conversion from the yeast to the mycelial phase have yielded a plethora of often contradictory or unrelated results. Consequently, little insight has been gained into the mechanisms underlying the transformation from yeast-like to mycelial forms of this yeast.

Mucor rouxii :

This and various other species of Mucor have the capacity to develop vegetatively as either a typical mycelium with branched hyphae or as round yeast-like cells. Vegetative morphogenesis of Mucor rouxii is dependent on a variety of environmental factors such as oxygen, carbon dioxide, hexoses, heavy metals, dicarboxylic acids and uncharacterised factors present in complex media (Bartnicki-Garcia and Nickerson, 1962 a,b,c; Bartnicki-Garcia, 1963, 1968; Haidle and Storck, 1966; Elmer and Nickerson, 1970). The morphogenetic effect of each factor is influenced by the concentration of the others. For example, in anaerobic cultures of M. rouxii, morphogenesis is strongly dependent on hexose concentration as well as pCO_2. High hexose and pCO_2 levels favour yeast-like development; however, if the hexose concentration is below 0.1% the mycelial form predominates (Bartnicki-Garcia, 1968).

True-yeasts:

A number of factors appear to influence the elongation of yeast cells, notably temperature, nutrition, age and pH value (Scherr and Weaver, 1953). Brown and Hough (1965) studied the elongation of yeast cells in continuous culture under conditions where ammonium sulphate, methionine or asparagine were growth limiting. They found that on the addition of sodium thioglycolate or sodium selenate, the nitrogen

limited elongated cells reverted to oval shaped cells. In analogy with earlier studies of Nickerson and Mankowski (1953) who prevented filamentation in C. albicans by the addition of sulphydryl compounds, Brown and Hough (1965) concluded that the morphological change they observed in S. cerevisiae was also due to an alteration in the cellular balance of sulphydryl-disulphide (see Fig. 1.8). There has been much interest in the relationship between oxygen supply and cell shape. Curtis and Clarke (1960) noted that yeast propagators which were aerated produced elongated cells with some strains of yeast. Similar changes in cell shape resulted from an alteration in the ratio of assimilable carbon to assimilable nitrogen (Brown and Hough, 1965). Since the extent of oxygenation was found to alter the ability of yeasts to utilise nitrogenous compounds (Jones and Pierce, 1964) it appears that the effects of oxygen on yeast cell shape are probably indirect.

Kluyveromyces fragilis:

Kluyveromyces fragilis NCYC 100 has been reported by Kreger-van Rij (1984) and by Nisbet (1979) to be a dimorphic yeast. Nisbet found that pseudomycelial production in this yeast was related to poor carbon sources and to the availability of oxygen. Ergosterol was reported to enhance pseudomycelial production under semi-anaerobic conditions. Nisbet (1979) concluded that pseudomycelial development was a response to the environment and not an intergral part of the life cycle of the yeast. Halter (1973) monitored the change in morphology of S. fragilis during a whey fermentation. It was reported that the cells reached their maximum size (4.0 x 3.7 μ) after

a fermentation time of 7 hours. The above mentioned publications appear to be the only reports relating to the morphology of K. fragilis in liquid culture.

1.2.3 Biochemical differentiation of yeast and mycelial phases.

1.2.3.1 Cell wall composition

The morphological alterations demonstrated during yeast-mycelial conversion are associated with marked changes in cell wall composition. Detailed information on the biochemical differences between yeast and mycelial cell walls is found in the reviews by Nickerson (1963) and Bartnicki-Garcia and McMurrough (1971).

Polysaccharides: In a study of cell wall composition of the mycelial and blastospore forms of C. albicans, Chattaway and co-workers (1973) found quantitative differences in the amounts of cell wall polysaccharides. They reported that the alkali-insoluble fraction from hyphae had three times more chitin than the yeast form. Furthermore, the cell walls of the yeast phase cells contained only marginally more polymerised mannose than mycelial cells. The cell walls of Mucor rouxii yeast forms were found to be considerably richer in mannose containing polymers than mycelial walls (Bartnicki-Garcia and Nickerson, 1962 b). Changes in morphology may not always be correlated with gross changes in cell wall composition. For instance, chemical analysis of the cell walls of elongated and ellipsoidal forms of S. cerevisiae revealed the absence of significant qualitative differences in glucan and mannan content (McMurrough and Rose, 1967).

Protein: There are several lines of evidence supporting the idea that sulphydryl groups play a role in dimorphism (Nickerson and Falcone, 1956, 1959; Cortat et al., 1972). The possible roles of disulphide linkages within the cell will be discussed in relation to cellular morphogenesis in Section 1.2.3.3. Chattaway et al., (1968) noted qualitative differences in amino-acid composition in cell wall proteins derived from blastospore and filamentous forms of C.albicans.

Lipids: Changes in total lipid content and the proportion of different lipid fractions have been noticed in comparative studies of the wall composition of different cellular forms of C. albicans (Bianchi, 1968) and S. cerevisiae (McMurrough and Rose, 1967).

1.2.3.2 Relationship of metabolic status of the cell to dimorphic transformation.

Changes in carbohydrate metabolism and an interruption of electron transfer within the cell have been closely associated with yeast morphology (Nickerson, 1954, 1963; Chattaway et al., 1973). Nickerson (1954) postulated that a continuous flow of electrons from flavoprotein is necessary for yeast morphology and that a build up of reducing potential occurred during filamentation. Furthermore, he suggested that carbohydrate metabolism (particularly available glucose), by providing an adequate supply of sulphydryl groups within the cell maintained the yeast-like form of C. albicans. Land et al., (1975 b) examined glucose metabolism and respiration in C. albicans during filamentation. Filamentous cultures produced more ethanol, evolved less CO_2 and consumed less oxygen than yeast

cultures. Changes from aerobic to fermentative metabolism resulted in a yeast to mycelial transformation. This finding supported the groups original hypothesis that a Crabtree-like repression of mitochondrial activity (by high glucose concentrations) may be correlated with the onset of filamentation in Candida albicans (Land et al., 1975a). There now appears to be strong evidence in relating mitochondrial function to morphogenesis in Mycotypha. For instance, the influence of certain inhibitors of the respiratory chain or mitochondrial protein synthesis inhibitors on the phenotypic expression of dimorphism in Mycotypha can be interpreted on the basis of a coupling between fermentation and yeast growth and respiration and mycelial growth (Schulz et al., 1974). The involvement of cAMP in the dimorphic process has been documented by Stewart and Rogers (1978) and by San-Blas and San-Blas (1984). Possible effects of cAMP on dimorphism are listed in figure 1.7.

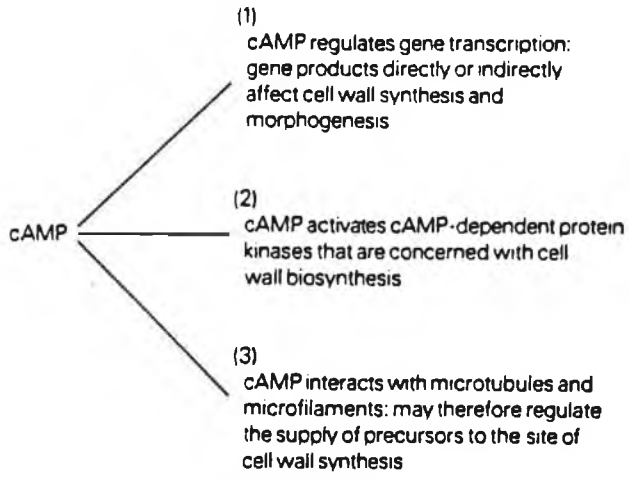

Fig. 1.7 Possible roles for cAMP in the regulation of morphogenesis in dimorphic fungi (From Stewart and Rogers, (1978).

1.2.3.3 Mechanisms of dimorphism

Many studies have sought to elucidate the underlying biochemical mechanisms by which yeast-mycelial conversions occur and several hypotheses have been proposed. The development of filamentous growth in C. albicans was described by Nickerson and Mankowski (1953) as the result of an interruption in the budding process without a concomitant interruption of growth. Since hyphal formation in C. albicans can be arrested and reversed to yeast development by the addition of cysteine, a reducing agent, to the medium, the sulphydryl groups in cell wall complexes were envisaged to play a role in yeast morphogenesis (Nickerson, 1963). A mycelial mutant of C. albicans which is unable to form yeast-like cells under standard growth conditions has been investigated by Nickerson and Edwards (1949) and by Nickerson (1954). This mutant had an impaired reductase system but could produce yeast-like cells when cysteine was incorporated into the medium (Winstein and Murray, 1956). Nickerson and Falcone (1956) suggested that protein disulphide reductase is responsible for cleaving disulphide covalent bonds in the cell wall. The biochemical process is illustrated in Figure 1.8.

The presence or absence of apical growth has been proposed as the key event in determining whether Mucor develops into a mycelial or a yeast-like form (Bartnicki-Garcia, 1963). The repression of hyphal morphogenesis may be mediated by a yeast promoting agent, such as hexose or CO_2, or both or by the formation of an inhibitor of apical growth (see Fig. 1.9).

Bartnicki-Garcia and Lippman (1972) later suggested that cellular form in Mucor rouxii is dependent on the balance between cell wall synthesis and wall lysis. Accordingly, hyphal tip growth involved an active chemical process of apical wall weakening which may be due to the activation of cell wall lytic enzymes in the apical tip.

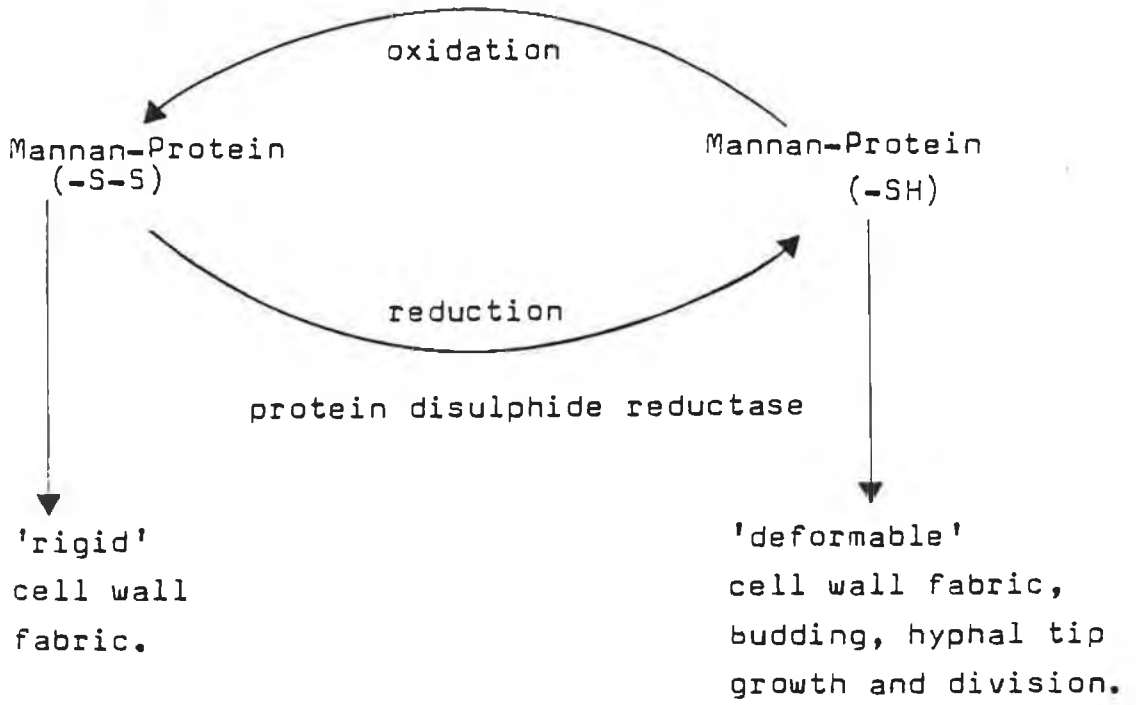

Fig.1.8 Outline of the role of protein disulphide reductase in the chain of events resulting in cellular division of yeasts (modified from Nickerson and Falcone,1959).

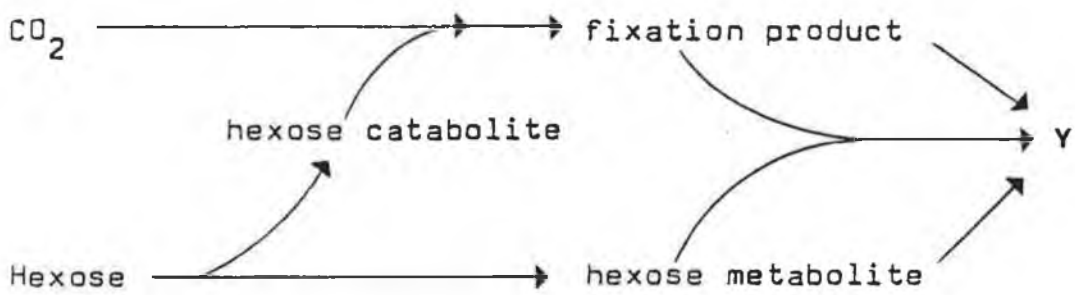

Fig.1.9 Hypothetical schemes for the participation of of hexoses and CO_2 in the formation of 'Y' morphogen, the presumed internal effector of yeast development (Bartnicki-Garcia,1968).

1.3. *Kluyveromyces fragilis* - DISCUSSION OF THE SPECIES

The lactose fermenting species, *Kluyveromyces fragilis* was originally isolated by Jorgensen (1909) from a fermented milk product known as kéfir. *Kluyveromyces fragilis* (Jorgensen) van der Walt is characterised by ascospores which are reniform in shape and are easily liberated from the ascus. Usually diploid vegetative cells are directly transformed into asci, but the conjugation of independent cells may precede ascus formation; one to four ascospores are formed. Cells are spheroidal to cylindrical and reproduce by budding. Pseudomycelia are usually formed on corn meal agar (van der Waalt, J.P., 1970).

The exact classification of this yeast has proved to be rather difficult. It was originally classified as *Saccharomyces marxianus* and later reclassified as *S.kéfir*. Synonyms since employed in the naming of this yeast include *Saccharomyces fragilis*, *Guilliermondella fragilis*, *Dekkeromyces fragilis* and *Kluyveromyces marxianus* (Kreger-van Rij, 1984). *K. fragilis* was believed to be a natural hybrid between *Kluyveromyces lactis* and *Kluyveromyces marxianus*,(Wickerham and Burton, 1956). *K. fragilis* and *K. marxianus* are closely related and a sharp division between the species is not always possible. Typical, however, of *K. fragilis* is its rapid fermentation of lactose; *K. marxianus* weakly ferments this sugar. *K. fragilis* appears to be stabilised in the diplophase whereas in *K. marxianus* the diplophase is less prominent. *K. fragilis* is considered to be the perfect form of *Candida pseudotropicalis* (Lodder and Kreger-van Rij, 1952).

K. fragilis is a versatile species and is of great commercial and industrial interest. It is the source of the enzyme lactase (Wendorff *et al.*,1970; Mahoney *et al.*, 1974) and it can excrete a constitutive polygalacturonase (Phaff, 1966). *K. fragilis* has inulinase activity (Bourgi *et al.*, 1986) which enables the yeast to hydrolyse and ferment the polyfructoside inulin present in the roots and tubers of the Jerusalem artichoke (Bajpai and Margaritis, 1985, 1986).

1.4 AIMS OF THIS RESEARCH

Objectives:

(a) To establish conditions for the production of yeast and filamentous forms of *K. fragilis*.

(b) To extend the initial studies to determine the nature of the underlying biochemical events occuring during morphological transformation of *K. fragilis*.

(c) To develop the morphological studies and purposely manipulate the cellular form of *K. fragilis* during lactose fermentations to evaluate the fermentative capabilities of both morphological types.

2. MATERIALS AND METHODS

2.1 SOURCES OF CHEMICALS

Aldrich Chemical Company, England: Ergosterol, Hydrogen peroxide

Oxoid Ltd.,: Mycological peptone, Malt extract broth, Agar Technical No. 3.

Reidel-de-Haen, Germany: Calcium Chloride, Ammonium sulphate, Sodium azide, Potassium sodium tartrate (Analytical reagent grade)

Falek Chemical Co., U.S.A.: Mazu DF Antifoam.

Sigma (London) Ltd.,: Miscellaneous biochemicals.

B.D.H., England: Reagent grade and AnalaR grade laboratory chemicals.

2.2 ORGANISMS

Source of strains used in this study are outlined in Table 2.1.

Table 2.1 Source of strains used in this study

Strain	Code	Source
Kluyveromyces fragilis	NRRL y 1109	USDA [a]
Kluyveromyces fragilis	NRRL y 2415	USDA
Kluyveromyces fragilis	NRRL y 665	USDA
Kluyveromyces fragilis	NCYC 100	NCYC [b]
Candida pseudotropicalis	NCYC 744	Carbery [c]
Kluyveromyces fragilis	CBC 5795	Carbery

Adresses

(a) U.S. Department of Agriculture, Northern Regional Research Centre, 1815 North University Street, Peoira, Illinois 61604, United States.

(b) National Collection of Yeast Cultures, Norwich, England.

(c) Carbery Milk Products Ltd., Balineen, Co.Cork.

2.3 MEDIA

Except where indicated, medium was sterilised at 15 psi and at 121^{o}C for 15 mins.

2.3.1 Cheese whey permeate medium

Spray-dried whey permeate was obtained as a gift from Carbery Milk Products Ltd., Cork. The whey permeate was reconstituted in distilled water to give a lactose concentration of approx 50 gl^{-1} then boiled for 1 min. to precipitate residual protein. The medium was clarified by vacuum filtration through Whatman No.1 filters followed by filtration through 0.45 µm pore size Millipore filters. Unless otherwise stated the permeate was adjusted to pH 4.5 . After the medium was autoclaved, the pH was checked and re-adjusted to give the desired pH using sterile HCL or NaOH.

2.3.2 Defined lactose medium

The defined medium used was a modification of the chemically defined medium described by Wickerham and Burton (1948) and Wickerham (1951). The constituents of the medium are shown in Table 2.2 .

Table 2.2 - LACTOSE BASED DEFINED MEDIUM

COMPOUND TYPE	NAME	QUANTITY PER LITRE
Carbon Source	Lactose	50g (unless otherwise stated)
Nitrogen Source	$(NH_4)_2SO_4$	5g
Growth factors	p-Aminobenzoic acid	200µg
	Biotin	20µg
	Folic acid	2µg
	myo-inositol	10mg
	Nicotinic acid	400µg
	Pantothenate (Ca)	2mg
	Pyridoxine HCL	400µg
	Riboflavin	200µg
	Thiamine HCL	400µg
Trace element sources.	H_3BO_3	500µg
	$CuSO_4.5H_2O$	40µg
	KI	100µg
	$FeCl_3.6H_2O$	200µg
	$MnSO_4.4H_2O$	400µg
	$Na_2MoO_4.2H_2O$	200µg
	$ZnSO_4.7H_2O$	400µg
Salts	KH_2PO_4	850mg
	K_2HPO_4	150mg
	$MgSO_4.7H_2O$	500mg
	NaCl	100mg
	$CaCl_2.2H_2O$	100mg

Note: a mixture of amino acids was also used in some experiments; L-Histidine 10 mgl^{-1}, DL-Methione 20 mgl^{-1} DL - Tryptophan 20 mgl^{-1}.

The lactose and phosphate sources were sterilised seperately for 15 min at 121°C, and 15 psi and then added aseptically to the remainder of the sterilised medium.

2.4 CULTIVATION CONDITIONS

2.4.1 Culture maintenance

Freeze dried preparations of yeast were opened aseptically, and the pellet resuspended in 2 ml of Y-M broth ($3gl^{-1}$ yeast extract, $3gl^{-1}$ malt extract, $5gl^{-1}$ peptone, $10gl^{-1}$ glucose). Loopfuls of the sterile suspension were streaked on Y-M agar (Y-M broth plus $20gl^{-1}$ technical agar) and the plates incubated at 30°C for 3 days. Single colonies were subcultured on malt extract agar slopes for 3 days at 30°C, prior to storage at 4°C. Subculturing was carried out on a monthly basis to maintain viability of cultures.

2.4.2 Inoculum preparation

Pre-cultures for experiments were prepared by inoculating a loopful of cells from a malt extract agar slope into a cotton plugged 250 ml Erlenmyer flask containing approximately 100 ml of sterile medium. Semi - aerobic propagation of a starter culture was carried out overnight in a reciprocating water-bath operating at approximately 160 rev. min^{-1}. The pre-culture was then used to inoculate the experimental medium. Unless indicated elsewhere, inoculum sizes were of the order of 1×10^6 cells ml^{-1}.

2.4.3 Fermenter cultivation

Fermenters used on a laboratory scale were of 7.5 litre (Labroferm, New Brunswick Scientific) and of 2 litre (Biolaffite) gross capacities. Operating volumes and dimensions of the various fermenters are listed in Table 2.3 Labroferm fermenters were steam sterilised for 25 min at 121^{o}C and 15 psi. The Biolaffite fermenters were sterilised for 15 min at 121^{o}C and 15 psi. Foaming was controlled by the addition of 0.5 ml l^{-1} of 'Mazu' (an organic based antifoam), prior to inoculation. Levels of aeration and agitation for aerobic fermentations were as reported in the results of individual experiments.

2.4.4 Continuous cultivation technique

Basic Concepts

The differential equation

$$\frac{dX}{dt} = \mu X \qquad (1)$$

describes the exponential growth of a population of microorganisms; the rate of increase of biomass is constant with respect to time, where:

X = cell concentration in gl^{-1}

t = time (hr)

μ = specific growth rate in hr^{-1} (mass)

In chemostat the specific growth rate is a function of some single growth limiting nutrient ie. $\mu = \mu(S)$.

Table 2.3 DIMENSIONS OF FERMENTERS USED

(volumes measured in litres, length in cm.)

PARAMETER	FERMENTERS A	B	C
Total Volume	2.0	7.5	1.2
Medium Volume	1.0	5.0	0.44
Vessel diameter	13.0	14.0	10.0
Impeller Number	2	3	2
Impeller diameter (Di)	4.5	5.0	4.5
Impeller spacing	5.0	9.5	1.0
Impeller speed r.p.m. (N)	400.0	400.0	550.0
Impeller tip speed $(\pi . \frac{N}{60} . Di)$ (a) cms^{-1}	94.2	104.7	129.6
Impeller shear $(\frac{N}{60})^2 . Di^2$ (b) $\times 10^3$ $cm^2 s^2$	0.90	1.11	1.70
Number of baffles	2	4	2
Baffle width	1.5	2.0	1.5
Distance baffle-wall	2.0	1.5	1.0
Distance impeller tip-wall	4.25	4.0	3.25

Note: Fermenter A: Biolaffite, England ; Fermenter jar supplied by Corning, England.

B: Labroferm fermenter, New Brunswick, U.S.A.

C: Chemostat; Biolaffite fermenter, A Corning 1 litre fermenter vessel was modified to include a side arm as an overflow device.

References:

(a) Wang et al., 1979

(b) Wang and Fewkes, 1977

Monod (1942) described this relationship by the following equation

$$\mu = \mu_{max} \quad \frac{S}{K_s + S} \qquad (2)$$

where S = concentration of the growth limiting substrate

μ_{max} = maximum specific growth rate

K_s = saturation constant which equals the substrate concentration when the growth rate (μ) is at 0.5μ max

During chemostat operation medium containing the substrate (S_o) is fed at a constant flow rate (F) to a homogeneous stirred vessel containing a constant volume (V) of culture. The specific growth rate is determined by the flow rate of medium divided by the culture volume. This ratio is defined as the dilution rate (D) or

$$D = \frac{F}{V} \qquad (3)$$

Theoretically, when the rate of production of cells (μX) through growth equals the rate of loss of cells through the overflow (DX), the steady state condition is reached and may be expressed by the following:

change in biomass = growth - output concentration

$$\frac{dX}{dt} = \mu X - DX \qquad (4)$$

At steady state when dX/dt = 0, the specific growth rate (μ) becomes equal to the dilution rate (D) of the system. Consequently, the growth rate of an organism is controlled by adjusting the rate of limiting nutrient feed to the culture vessel and allowing the system to come to steady state. For further reading on the theory of continuous

cultivation see Tempest (1970 a) and Pirt (1975).

Components of Continuous Culture Fermenter

Figure 2.1 describes the overall assembly of the chemostat apparatus which consists of the following components:

Growth vessel:

Laboratory scale continuous cultivation was carried out in a 1 litre fermenter (Biolaffite, England). The fermenter jar (Corning, England) was modified so as to incorporate a side arm as a 'weir type' overflow device. Dimensions of the fermenter are listed in Table 2.3 , column C. The fermenter was equipped with a number of ports to accommodate the following:

(a) inflowing medium from the reservoir

(b) return of circulating culture from the flow-through cuvette used for the measurement of culture absorbance (λ = 550nm)

The fermenter had a working volume of 0.442 litre.

Nutrient supply:

A 10 litre pyrex flask was used as a medium reservoir which was vented to the atmosphere so as to allow medium to flow from the vessel without setting up a partial vacuum. A regulated flow of medium into the chemostat was maintained with a peristaltic pump (model 501U, Watson - Marlow, England).

Mixing and aeration:

The vessel was aerated at a flow rate of 200 cc min^{-1} by means of an air pump (ASEA, Electrolux Electromotor,

Denmark). Baffles within the fermenter aided the aeration and mixing properties. Agitation was maintained at 550 rpm using an overhead 'Biolaffite' motor drive unit.

Measuring culture absorbance:

The culture was continuously circulated from the growth vessel through an optical flow-through cell using a peristaltic pump (model 301, Watson Marlow, England) at a flow rate of approximately 4 $l.hr^{-1}$. After measurement, the culture was returned to the vessel through a port at the top of the fermenter. The culture absorbance was monitored at λ = 550nm using a PYE - UNICAM SP6-550 spectrophotometer which was connected to a pen recorder (model 2001, Vitatron, England) so as to give a visual representation of the state of culture growth.

Collecting reservoir:

A cotton plugged 25 litre vessel was used to collect spent medium and cells at room temperature.

Atemperation:

An operating temperature of $30^{o}C$ was maintained by coupling a flow through coil within the fermenter to a water circulator (model C-400, Techne, Cambridge, England).

pH Control:

No pH control was used in the continuous culture studies.

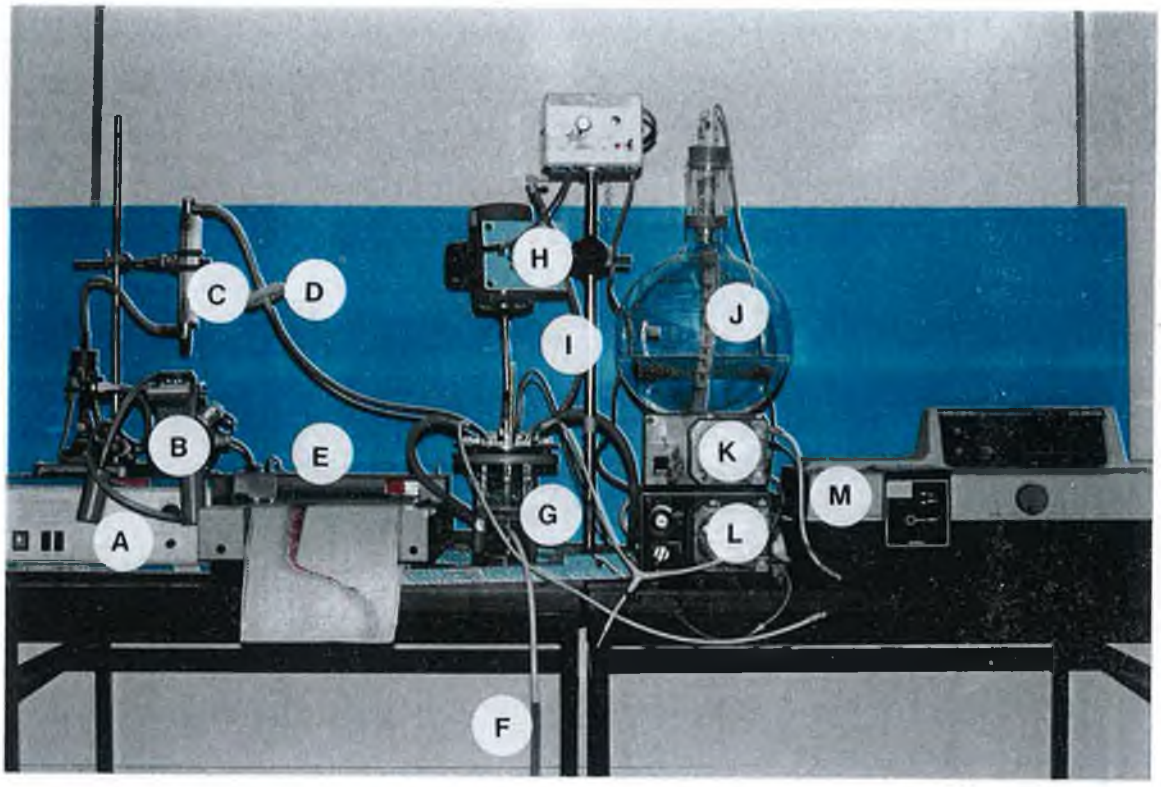

Fig. 2.1 Chemostat: Continuous Cultivation Apparatus

LEGEND:

A - Circulator

B - Air pump

C - Air flow meter

D - Air filter

E - Pen recorder

F - Overflow tube

G - Fermentation vessel

H - Motor drive unit

I - Air vent from vessel

J - Medium reservoir

K - Peristaltic pump (Culture absorbance measurement)

L - Peristaltic pump (Medium supply)

M - Spectrophotometer

Operation

Calibration of 'nutrient supply' pump.

Procedure:

1. The pump setting was adjusted to give different flow rates and a calibration curve was constructed plotting pump setting against flow rate (F) $l.hr^{-1}$.
2. The working volume (V) of the chemostat was measured. It included the volume of media in the tubing to and from the spectrophotometer.
3. In order to determine the exact flow rate (F) of the medium into the growth vessel for a particular dilution rate (D), the following formula was used:

$$D = \frac{F}{V}$$

where D = Dilution rate (hr^{-1})

F = Flow rate ($l.hr^{-1}$)

V = Vessel working volume (l)

example

Vessel volume (V) = 0.442 l.

Desired Dilution rate (D) = 0.1 hr^{-1}

$$0.1\ hr^{-1} = \frac{F}{0.442\ l.}$$

$F = 0.0442\ l\ hr^{-1}$

= exact flow rate for a dilution rate of 0.1 hr^{-1}

The corresponding pump setting for the calculated flow rate of 0.0442 l hr^{-1} was read off the previously constructed calibration curve.

Medium:

The lactose based defined medium as described in Table 2.2 was employed with lactose as the growth limiting nutrient. The initial pH of the medium used was 5.5. The criteria used to define lactose limitation were as follows:

1. An increase in the concentration of lactose in the medium reservoir should result in a proportional increase in cell concentration.
2. At low growth rates, the limiting nutrient should not be detectable in the culture vessel, indicating 'complete' exhaustion of that nutrient.

The growth limiting nutrient was established experimentally by showing that at a fixed dilution rate, the biomass was directly proportional to the growth limiting substrate concentration in the medium reservoir. (see Section 3.15)

Sterilisation:

The reservoir medium was prepared in 10 litre pyrex flask and autoclaved for 25 min at 121^{o}C and at 15 psi. The chemostat vessel was filled with 400 ml of medium and sterilised (together with attachments) for 15 min at 121^{o}C and at 15 psi. The appropriate amounts of sterilised phosphate and lactose were later added to the sterile media. The air supplied to the fermenter was sterilised on line by passage through a sterile ACRO 50, 0.45 μm pore size filter, (Gelman, U.S.A.).

Inoculation:

An overnight culture was used to inoculate the medium in

the chemostat vessel at a concentration of 1 x 10^6 cells ml^{-1}. The batch culture in the chemostat vessel was allowed to reach stationary phase after which the overflow device on the vessel was opened and the medium pumped into the culture vessel at a dilution rate of 0.1 hr^{-1}. Once the cells reached a steady state condition further increases in dilution rate were made.

Sampling:

A steady state was established before samples were removed. The steady state condition was generally achieved after about five replacements of the culture volume. Large samples for cytochrome analysis (500 ml- 1L) were withdrawn from the culture effluent stream into an ice-cooled receptacle. Smaller samples (1-10ml) were directly taken from the culture effluent for oxygen uptake measurements, lactose and ethanol analysis.

The use of continuous culture for studying microbial physiology has many distinct advantages (Tempest 1970b; Tempest and Neijssel,1976; Light and Garland, 1971) but one possible disadvantage arises from the effect of collecting substantial quantities of biomass over several hours. This is particularly the case when chemostats of moderate size (0.5 litre working volume) are used at low dilution rates (D = 0.1 hr^{-1}). Thus, "one of the objects of using continuous culture, namely that ofobtaining a highly homogeneous population whose physiological properties depend solely on the environmental conditions imposed in the chemostat, could be negated by changes during storage" (Aiking et al., 1977).

2.4.5 Batch cultivation techniques

Unless otherwise stated, cultures contained 100 ml of medium in a cotton plugged 250 ml Erlenmeyer flask.

Semi-aerobic:

Semi-aerobic conditions were obtained using shake-flask cultures incubated at 30°C in a reciprocating water-bath at 160 rev. min^{-1}. Occasionally, baffled flasks were employed (4 indentations on the side of an Erlenmeyer flask) to achieve increased turbulence and O_2 transfer in the culture.

Strictly aerobic:

Strictly aerobic conditions were achieved by sparging the medium with air. Flow rates were as described in the results of individual experiments.

Semi-anaerobic:

Semi-anaerobic conditions were achieved by using still cultures equipped with fermentation locks.

Strictly anaerobic:

Anaerobic cultivation of yeast was performed by purging fermentation broths with oxygen-free nitrogen; flow rates were as described in the results of individual experiments. An initial purging of the medium with N_2 (500 cc/l/min) for 5 min was performed to establish anaerobic conditions. Lipid supplementation was necessary for the anaerobic cultivation of the yeast in the defined lactose medium (Tyagi, 1984).

The stock solution of ergosterol was prepared by adding 0.6g of ergosterol to 10 ml of ethanol followed by 100 ml of Tween 80. The stock solution was then sonicated at 14 microns for 25 min, using a Soniprep 150 (M.S.E., England). The lipid solution was sterilised for 15 min at 121°C, and at 15 psi and added to the fermentation medium prior to inoculation at a rate of 5 ml per litre.
An anaerobic jar (Oxoid) was used for the cultivation of yeast in some experiments.

2.4.6 Micro-aerophilic environment

A micro-aerophilic environment was generated in solid media by an agar overlay technique. An agar plate containing 10 ml of molten agar was seeded at 40°C with 1×10^4 yeast cells ml^{-1}. The solidified malt extract agar was overlaid with a further 5 mls of technical agar. The plates were then incubated at 30°C for approximately 1 week. Sampling for morphological examinations was carried out by removing a section of agar with a sterile cork-borer and solubilising the agar in distilled water with gentle heating.

2.5 MORPHOLOGICAL DETERMINATION

The following qualitative criteria were used in descriptions of yeast cell morphology:

Morphology	Description
Elongated cells	Unicellular, elongation of the cells major axis.
Pseudomycelia	Elongated cells where each generation of buds remains attached to its parent.
Yeast-like	Oval to ellipsoidal shaped cells, ie. normal budding cells.
Filamentous	Elongated cells/Pseudomycelia.

Different morphological forms of *K. fragilis* were photographed using a Nikon Optiphot phase-contrast microscope. Unless otherwise stated, the magnification used was x 544 (phase 3) Photographic records were taken using an Olympus OM - 10 quartz camera which was set at 160 A.S.A and at automatic. The film used was Kodak Ektachrome 160 A.S.A Tungsten.

2.6 CELL NUMBER DETERMINATION

Cell numbers were routinely determined by duplicate counting of cultures in an improved Neubauer haemocytometer. For a single count a total of 160-200 yeast cells (filamentous or yeast-like) were tallied according to the following criteria:

(1) A single yeast or a yeast with a bud less than 2/3 the size of the parent mother cell was counted as one.

(2) Two large yeasts together were counted as two separate units.

(3) Pseudomycelia both in a branched or unbranched form were regarded as filaments.

(4) As far as possible, each cell within a septated filament was counted as a single cell.

The specific growth rate, (μ) was determined by the formula

$$\mu = \frac{\ln Z - \ln Z_o}{t - t_1}$$

where μ = specific growth rate and the values Z and Z_o correspond to yeast cell number determinations in the culture at time t and t_o respectively.

The mean doubling time (t_d) was computed using the formula

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

where μ = specific growth rate constant.

2.7 CELL DRY WEIGHT DETERMINATION

Clean glass centrifuge tubes were placed in an oven at 105^{o}C for 21 hours after which they were removed and placed in a desiccator to cool. The tubes were then weighed to four decimal places and stored until required. A 20 ml sample of

culture was centrifuged in a pre-weighed centrifuge tube at 4,500 rpm (4250 g) for 5 minutes at room temperature using a Haereus Christ centrifuge. The supernatant was aspirated off and the pellet was resuspended in distilled water. After re-centrifuging the supernatant was aspirated off and the tubes were placed in an oven at 105°C for 21 hrs. The difference in weights between the sample tube and the empty tube were noted and the result expressed as mg dry wt per ml.

2.8 ANALYSIS OF LACTOSE

Three methods of analysis were employed (a) Phenol in the presence of sulphuric-acid (Dubois et al.,1956) for quantitative colorimetric lactose determinations; (b) Total reducing sugars were estimated using the D.N.S. method of Miller, (1959); (c) An enzymic method of lactose analysis (Boehringer Lactose Assay Kit) where the amount of NADH formed from a coupled reaction is stoichiometric with the amount of lactose present. The increase in NADH was measured by means of its absorbance at 340nm.

Comparison of Assays:

The concentration of lactose was determined using the above assays in whey permeate, reconstituted and prepared in four different ways A,B,C,D.

Sample A: Reconstituted whey permeate (see Section 2.3.1)

Sample B: Reconstituted whey permeate boiled for 1 minute and centrifuged at 5,000 rpm (4250g) for 10 minutes.

Sample C: Reconstituted whey permeate autoclaved for 10 minutes at 10 psi and centrifuged at 5,000 rpm (4250g) for 10 minutes.

Sample D: Reconstituted whey permeate autoclaved for 15 minutes at 15 psi and centrifuged at 5,000 rpm (4250g) for 10 minutes.

The whey permeate solutions were prepared by dissolving 6.1g of whey powder in 100 mls. of distilled water.

Results are presented in Table 2.3

Table 2.3

Lactose Assay System	Grams per litre (Lactose)			
	Sample A	Sample B	Sample C	Sample D
(a) Phenol-Sulphuric Acid Assay	52	53	59.5	49.5
(b) Dinitrosalicylic Acid Assay	54	54	48	48
(c) Enzyme Assay	56	55	47.5	42.5

All methods of analysis showed that steam sterilisation of whey permeate for 10 psi/10 min (Sample C) or 15 psi/15 min (Sample D) reduced the availability of lactose or reducing sugars in the medium. This may be due to lactose degradation or caramelisation during sterilisation.

The phenol-sulphuric acid assay was rejected on the basis that it was the least sensitive of the assays present. The dinitrosalicylic acid assay compared very well with the

enzymic method of analysis in terms of accuracy and reproducibility and was the method of choice for routine analysis of lactose (reducing sugar equivalents) both in whey and in lactose defined medium.

2.9 ANALYSIS OF ALCOHOL

Instrumentation:

Ethanol was determined by Gas-Liquid Chromatography (G.L.C) using a flame ionisation detector (f.i.d.). The instruments used were a Carlo Erba HRGC 5300 Mega Series chromatograph coupled to a Mega Series Integrator (Carlo Erba Strumentazione, Italy). A 2 metre glass column (outside diameter 6mm) was packed with 5% Carbowax 20M on Chromosorb WAW 80/100 Mesh. The packing was supplied by Phase - Separations Ltd., Queensferry, Clwyd CH5 2 LR, England and the glass column was supplied by A.G.B. Scientific, Dublin.

Preparation of samples:

Samples for ethanol analysis were periodically taken throughout the course of fermentation. The samples were immediately centrifuged at 5,000 rpm (4,250g) for 5 minutes. The supernatant was then decanted off the pellet and stored in an eppendorf tube at - 4^{o}C for future analysis. Ethanol standards (5 to 30 gl^{-1}) were prepared in distilled water using AnalaR grade absolute ethanol.

Operational parameters:

Operational parameters for the gas chromatograph were as follows:

Injector temperature	130^{o}C
Detector temperature	180^{o}C
Oven temperature	130^{o}C

Air pressure at	: cylinder head	3 bar
	: gas chromatograph (G.C) needle valve	1.0 Kg/cm^3
H_2 pressure at	: cylinder head	2 bar
	: G.C. needle valve	0.5 Kg/cm^3
N_2 pressure at	: cylinder head	3 bar
Carrier Gas (N_2) flow rate measured at the detector		40 ml min^{-1}
Injection volume		1 µl

2.10 ANALYSIS OF OXYGEN UPTAKE

Oxygen uptake measurements of whole yeast cell suspensions were made using a Rank electrode (Rank Brothers, Cambridge, England).

Preparation of the electrode:

1. The base of incubation vessel was detached and enough 1M KCL was added to wet the silver and platinum electrodes.
2. A 1cm square of Whatman lens tissue with a 1mm hole in the centre was centred over the platinum electrode.
3. A 1cm square piece of teflon was placed over the lens tissue and secured in place by screwing down the perspex locking nut taking care that no air bubbles were trapped beneath the teflon membrane.

4. The temperature of the incubation vessel was maintained at an operating temperature of 30°C, using a water circulation pump.

Recording:

The oxygen electrode was connected to a Linseis potentiometric pen recorder (model L 6512, Linseis, Germany) at a sensitivity 1 volt and using full scale deflection.

Calibration of the electrode:

1. The electrode was filled with 3 ml of air-saturated distilled water and a potential of - 0.6 v was applied to the platinum (Pt) electrode relative to the Ag - AgCl electrode.
2. The perspex plug was placed in position after checking that there were no air bubbles in the sample chamber. The magnetic stirrer was then turned on and the recorder activated.
3. When a steady state response for 100% oxygen saturation was achieved the sensitivity control was adjusted to give a deflection of 95 units on the pen recorder.
4. Zero per cent oxygen activity was found by adding a few grains of sodium dithionite to the incubation chamber. The pen recorder 'zero' was adjusted to give a deflection of 5 units when a steady state response for 0% oxygen content was achieved. The

chamber and stopper were thoroughly rinsed after contact with the sodium dithionite.

The electrode was calibrated so that full scale deflection on the recorder corresponded to an oxygen content of 237n moles per ml H_2O (Cooper, 1977).

Routine use:

A 3 ml volume of air-saturated culture medium was added to the magnetically stirred incubation vessel. The perspex stopper was put in place ensuring that the solution reached up to the constricted entrance inside the stopper and that no air bubbles were left remaining in the chamber. The pen-recorder was activated, the desired chart speed selected and the pen deflection was measured.

Calculation of the rate of oxygen consumption:

The rate of oxygen consumption by the cells was calculated by the following equation:

$$\frac{X \times C \times V}{S} \text{ ng atoms } O_2 / \text{ reaction volume (V) /min} \qquad (1)$$

where X = Pen deflection (chart divisions per min)

C = Oxygen content in ng atoms O_2 per ml

V = Reaction volume (ml)

S = Span of recorder calibrated for an air-saturated solution.

Worked example:

The oxygen content of air saturated water at $30^{\circ}C$

= 237 nmoles O_2 per ml (Cooper, 1977)

= 474 ng atoms O_2 per ml

= (C)

Reaction volume (V) = 3.0 ml

Dry weight of cell suspension = 0.5mg/ml

100% air-saturation water = 95 recorder divisions

0% oxygen = 5 recorder divisions

Span of recorder (S) = 90 recorder divisions

Therefore each recorder division = $\frac{474 \times 3}{90}$ ng atoms O per 3 ml

Pen deflection chart divisions per min (X) = 40

substituting into Equation (1)

Rate of oxygen uptake = $\frac{40 \times 474 \times 3}{90}$ ng atoms O_2/3ml/min

= 632 ng atoms O_2 min^{-1} per 3 ml suspension

= 632 ng atoms O_2 min^{-1} per 3 x 0.5 mg dry weight

= 632 ng atoms O_2 min^{-1} per 1.5 mg dry wt

= 421.3 ng atoms O_2 min^{-1} per mg dry wt

The oxygen electrode was calibrated using air-saturated distilled water. Exposure of electrodes to solutions equilibrated with known oxygen tensions cannot give accurate calibrations unless the solubility of oxygen is known for the particular medium (Beechey and Ribbons, 1972). Hence, accurate electrode measurements were not achieved since the fermentation broth was used as the incubation medium in all measurements of cellular oxygen uptake rates. Ideally, cells should have been resuspended in a medium of known oxygen activity.

2.11 ANALYSIS OF CYTOCHROMES

Cytochrome spectra were recorded at room temperature using a Pye-Unicam SP 1800 double beam spectrophotometer fitted with a turbid sample holding facility. Cells grown in continuous culture were harvested when steady state conditions were reached in the culture vessel. Biomass was accumulated by collecting the effluent culture into an ice cooled receiver. The culture was then centrifuged at 5,000 rpm (4250g) at 4°C for 5 min using a Sorvall RC 5B centrifuge. The cells were washed in distilled water, recentrifuged and the pellet resuspended in 0.1 mol l^{-1} phosphate (KH_2PO_4 - NaOH) buffer (ph 7.5) containing 0.65 mol l^{-1} sorbitol. Cells grown in batch culture were harvested and prepared for cytochrome analysis as above. The turbid cell suspensions for cytochrome analysis were approximately 35-50 dry wt/ml.

The parameters used for the recording of spectra were as follows:

Wavelength speed	1 nm sec^{-1}
Chart speed	10 sec cm^{-1}
Bandwidth	2 - 5 nm
Absorbance range	0.2 Absorbance units
Scanning range	650-500 nm

To each of two identical cuvettes (1 cm light path), 3.0 ml

of whole cell suspension was added and the baseline recorded in triplicate. The sample cell was reduced with a few grains of sodium dithionite and the reference cell oxidised with 5 µl of 30% H_2O_2. After 5 min another 5 µl of H_2O_2 was added to the reference cell. The sample and reference cuvette were carefully mixed and the spectrum recorded. The cytochrome contents were calculated from the wavelength pairs and the extinction co-efficients as quoted in Table 2.5.

Table 2.5 Millimolar extinction co-efficients at wavelength pairs for yeast cytochromes.

Cytochrome	Wavelength pair (nm)	mM extinction coefficient	Reference
cytochrome aa_3	605-630	24	Wilson and Epel, 1968
cytochrome b	560-540	22	Wilson and Epel, 1968
cytochrome c	550-540	19	van Gelder, 1966

Absorption spectra were recorded at least in triplicate. The difference in absorbtion at particular wavelength pairs was calculated for each particular cytochrome, after correction for the baseline. A typical reduced minus oxidised absorption spectrum is illustrated in Fig 2.2.

Sample calculation of cytochrome aa_3

$$A = e_{mM}\ c\ l \qquad \text{Beer's Law} \quad (1)$$

where A = Absorbance (nm)

e_{mM} = millimolar extinction coefficient (mM)

c = concentration (mM)

l = light path (cm)

rearranging equation (1) $c = \dfrac{A}{e_{mM} \times l}$ (2)

cytochrome aa_3:

wavelength pair: 605 - 630nm

millimolar extinction co-efficient: $e_{mM} = 24$

605 - 630 nm = 0.004 Absorbance Units
(from baseline)

605 - 630 nm = 0.036 Absorbance Units
(from reduced minus oxidised difference spectrum)

corrected absorbance (605-630nm) = 0.032

light path length (l) = 1 cm

substituting into equation (2)

cytochrome aa_3 $= \dfrac{0.032}{24 \times 1}$ mM

= 0.000133 mM

= 0.133 μM

= 0.133 nmol/ml

Dry weight of whole cell suspension used for analysis

= 48.43 mg dry weight/ml

cytochrome $aa_3 = \dfrac{0.133}{48.43}$ nmol/mg dry weight

cytochrome aa_3 = 0.027 nmol/mg dry weight

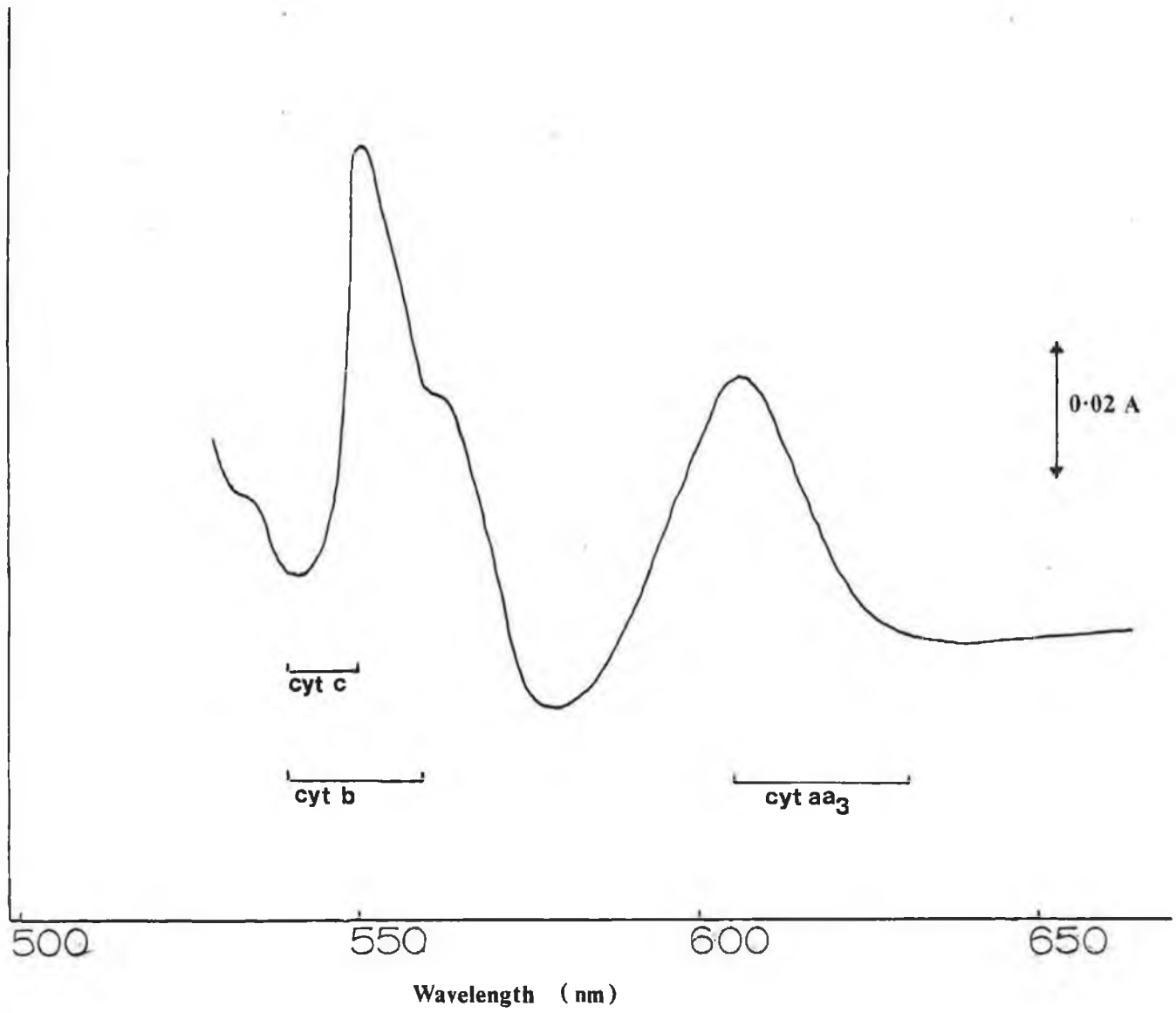

Fig. 2.2 Cytochrome reduced-minus-oxidised difference spectrum of K. fragilis NRRL y 2415 cells grown aerobically. Cells were harvested under steady state conditions at a dilution rate of 0.3 hr^{-1} in defined lactose medium (1.2 gl^{-1}). Spectra were recorded as outlined in Section 2.11 .

3. RESULTS AND DISCUSSION

3.1 CHAPTER 1 : MORPHOLOGICAL STUDIES

3.1.1 Media effects

3.1.1.1 Effect of different media on the morphology of *K. fragilis* and *C. pseudotropicalis*

Microscopic examination of *K. fragilis* strains on Y-M agar plates (see Section 2.4.1) indicated a variation in the degree of pseudomycelium production. In an attempt to examine the influence of various growth media, inocola were prepared in Y-M broth (24 hr) and the following media were inoculated:

(a) Y-M broth (YM)

(b) Cheese whey (CW)

(c) Defined lactose medium (DLM)

The use of different media was employed to maximise pseudomycelium production and to investigate if the strain dependent nature of dimorphism was related to the growth medium. The morphology of five *K. fragilis* strains was evaluated subjectively after growth in liquid shake-flask cultures after 24 hr and the results are presented in Table 3.1 . *K. fragilis* NRRL y 2415 was characterised by a strong predominance of the F-form in all media tested. However, the defined lactose media did not favour filamentation to the same extent as that observed in the complex media such as Y-M broth and cheese whey. A small percentage

of cells from K. fragilis NRRL y 1109 and NCYC 100 developed elongated cell types in both cheese whey and Y-M broth but not in defined media. The remainder of the strains tested grew entirely as oval to round shaped normal yeast-phase cells. Results indicated that media had an influence on the morphology of K. fragilis (rich media favouring filamentation) and that dimorphism is a strain dependent phenomenon in this yeast.

Table 3.1 Effect of different media on the morphology of K. fragilis and C. pseudotropicalis

Yeast Strain	MEDIA		
	Y-M	CW	DLM
	% of Total Growth in Filamentous Form		
K. fragilis NCYC 100	5	4	0[a]
K. fragilis NRRL y 2415	85	80	65
K. fragilis NRRL y 1109	1	2	0
K. fragilis NRRL y 665	0	0	0
K. fragilis CBS 5795	0	0	0
C. pseudotropicalis NCYC 744	0	0	0

(a) 0% Filamentation indicates 100% budding yeast forms.

3.1.1.2 Effect of carbon source on the morphology of *K. fragilis*

It has been shown that the degree of pseudomycelial production in *K. fragilis* is very variable and depends on the growth conditions (eg. fermentable sugar) (Scherr and Weaver, 1953; Nisbet, 1979). The effect of glucose, galactose, lactose and glycerol ($50gl^{-1}$ in defined medium) on the production of elongated cells and pseudomycelia by *K. fragilis* NRRL y 2415 was therefore investigated. Inocula were prepared under standard conditions in each of the experimental media and morphology was evaluated after 5 and 10 hr incubation. From the data in Table 2, it appears that the morphology of *K. fragilis* is significantly influenced by the nature of the carbon source. Cells grew well on all carbon sources, except glycerol, which is generally regarded in *Saccharomyces* spp. as being a non-fermentable substrate. The proportion of filamentous forms to normal yeast-phase forms was also considerably lower for the glycerol-based defined medium compared with the other sugars tested. For example, after 10 hr incubation the ratio of filamentous forms to oval or round yeast forms was 80:20 for the lactose defined medium as opposed to 25:75 for the glycerol-based medium. Table 3.2 also shows that filamentation of *K. fragilis* increases with duration of incubation.

Table 3.2 Effect of carbon sources on the morphology of *K. fragilis* NRRL y 2415

Carbon Source	5 Hr	10 Hr
	% of Total Growth in Filamentous Form	
Glucose	45	85
Galactose	30	70
Lactose	35	80
Glycerol	15	25

3.1.1.3 Effect of amino-acid supplementation

The induction of morphological transition in fungi by amino acids has been reported. Proline (Land *et al.*, 1975a; Dabrowa *et al.*, 1976), leucine (Gupta *et al.*1971), cysteine (Wain *et al.*, 1975) and methionine (Mardon *et al.*, 1969) have been the most frequently cited amino acids influencing dimorphism in *C. albicans*, *H. capsulatum* and *B. dermatitidis*. Filamentation of *K. fragilis* NRRL y 2415 appeared to be suppressed in defined lactose medium as compared to cheese whey (see Table 3.1). The possibility that this was in part due to a deficiency of amino acids was studied by supplementing the defined lactose medium with a mixture of amino acids; histidine, methionine and tryptophan. Table 2.2 shows that no significant changes in morphological form in shake-flask cultures were observed with amino acid supplementation. This leaves open to question the possibility of some alternative nutrient- limited suppression of filamentation in defined lactose medium.

3.1.1.4 Effect of varying initial medium pH

Schulz et al.(1974) studied the yeast-mould dimorphism (Y-M) in Mycotypha and found the effect of pH on morphology was pronounced with maximal Y-growth stimulation, reaching or exceeding 90% Y-form within the pH range 5.8 to 6.5. Hence, the influence of initial pH of the culture medium on the morphology of K. fragilis NRRL y 2415 was investigated. Initial pH in the range 2.5 - 7.5 was found to have no significant morphological effect after 6, 12 and 24 hr incubation (data not shown). Large deviations from pH occurred during growth in all cultures, but the general trend was one of medium acidification with progression of fermentation. Ideally, a buffer system should have been used to maintain constant pH values over the entire experiment, for example: HCl : K-phthalate buffer over the range 5.4 to 7.5. Therefore, in the absence of more definitive pH conditions, no firm conclusions can be drawn concerning the influence of pH on K. fragilis morphogenesis.

3.1.2 Oxygen effects

3.1.2.1 Morphological changes of lactose-fermenting yeasts under micro-aerophilic conditions.

Protoplasts of K. fragilis when allowed to regenerate form pseudomycelia when embedded in agar, but not on the surface of the agar (Dempsey, R. 1985). The possibility of oxygen-limiting conditions influencing the dimorphism of K. fragilis

was therefore investigated with a view to maximising pseudomycelium production. A micro-aerophilic environment was created using an agar overlay technique as described previously (see Section 2.4.6). Microscopic examination of colonies was carried out after 24 and 48 hr incubation. Results were compared to a 'control' yeast culture grown on the surface of the agar plate and are shown in Table 3.3.

Table 3.3 Effect of micro-aerophilic conditions on the morphology of *K. fragilis* and *C. pseudotropicalis* grown on agar.

Yeast Strain	Colony Location	24 Hr	48 Hr
		% of Total Growth in Filamentous Form	
K. fragilis NCYC 100	Embedded	20	15
	Surface	0	30
K. fragilis NRRL y 1109	Embedded	0	0
	Surface	0	0
K. fragilis NRRL y 665	Embedded	5	1
	Surface	0	0
K. fragilis NRRL y 2415	Embedded	85	55
	Surface	65	80
K. fragilis CBS 5795	Embedded	0	0
	Surface	0	0
C. pseudotropicalis NCYC 744	Embedded	0	0
	Surface	0	0

The effect of micro-aerophilic conditions on the morphology of K. fragilis highlights the strain dependent nature of dimorphism in this species (see Table 3.3). For example, K. fragilis NRRL y 2415 and NCYC 100 were truly dimorphic in nature (particularly under micro-aerophilic environments), whereas the other strains tested grew predominantly as oval to round budding yeasts, irrespective of colony location. All aerobic 'control cultures' grew predominantly as oval shaped cells with the exception of K. fragilis NRRL y 2415 which was characterised by a high level of cells in the filamentous form. K. fragilis NRRL y 665 had a tendency to produce a small percentage of elongated thread-like cells under micro-aerophilic conditons. Candida pseudotropicalis never deviated from growth as a true budding yeast.

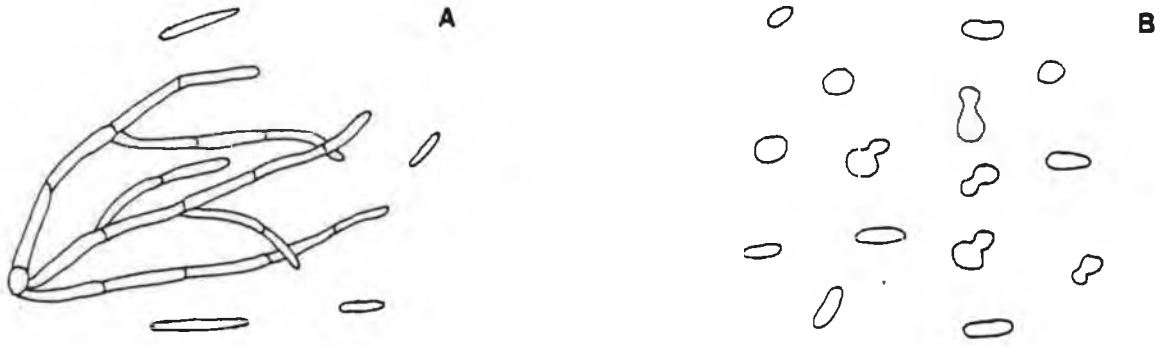

Fig. 3.1 Diagrammatic representation of the dimorphic nature of K. fragilis NCYC 100

A. Micro-aerophilic environment- pseudomycelium and elongated cells embedded in malt extract agar.

B. Aerobic environment - oval/round/ellipsoid cells from a colony grown on the surface of malt-extract agar.

3.1.2.2 Influence of oxygen limiting conditions on the morphology of *K. fragilis* NCYC 100

Microscopic examination of *K. fragilis* NCYC 100 cells embedded in malt extract agar after 24 hr revealed a mixed morphology: 10 per cent of cells were pseudomycelia of the 'Mycocandida type'; and a further 10 per cent were elongated thread-like cells (see Fig 3.1). Using a solubilised section of agar (as described in Section 2.4.6), propagation of the filamentous form was attempted in malt extract broth under the following conditions:

(a) Still culture overlaid with 10 ml liquid paraffin.

(b) Still culture in an Oxoid anaerobic jar.

(c) Still culture equipped with a fermentation lock.

A shake-flask culture was used as a control. Morphological examination after 24 and 48 hr incubation under semi-aerobic shake-flask conditions showed 100% oval cell formation. The experimental still cultures produced less than 0.5% pseudomycelia and after 48 hrs, the cells were predominantly oval in shape. It appears, therefore, that oxygen availability does not play a major role in influencing morphological change in *K. fragilis* NCYC 100. *K. fragilis* NRRL y 2415 on the other hand, appeared from preliminary studies to have a greater potential than *K. fragilis* NCYC 100 for filamentation under shake-flask conditions and so was selected for further morphological investigation.

3.1.2.3 The effects of oxygen availability on the morphology of K. fragilis NRRL y 2415.

Vegetative development of Mucor rouxii may follow one or two patterns of morphogenesis depending on the growth environment. Under anaerobiosis spores germinate producing yeast-like cells (Y cells) which reproduce by budding, whereas aerobic development leads to the formation of a typical mycelium (M) (Bartnicki-Garcia and Nickerson.,1962a). The yeast to mycelium transformation is accompanied by the synthesis of cytochrome oxidase, indicating that respiratory adaptation accompanies the transformation (Haidle and Storck, 1966). These observations prompted an investigation into the effects of oxygen availability on the morphology of K. fragilis NRRL y 2415 cultured in cheese whey over a 24 hr incubation period. Stationary phase cultures were established in vessels equipped with fermentation locks to maintain oxygen limiting conditions whereas a 'baffled' shake-flask cultures were used to increase oxygen availability. An overnight shake-flask culture was used as the source of inoculum. Fig. 3.2 shows that limited oxygen availability (as generated by stationary phase culture conditions) had a pronounced effect on the morphology of K. fragilis NRRL y 2415 over the 3 to 24 hr incubation period. For example, the degree of filamentation (elongated and pseudomycelia cells) decreased with a corresponding increase in the level of yeast forms (ovoid and ellipsoid cells). After 24 hr incubation in stationary culture, 90% of total growth existed as ellipsoid or ovoid yeast forms. On the contrary, growth in the semi-aerobic culture showed a different morphological development pattern. After 3 hrs the percentage of

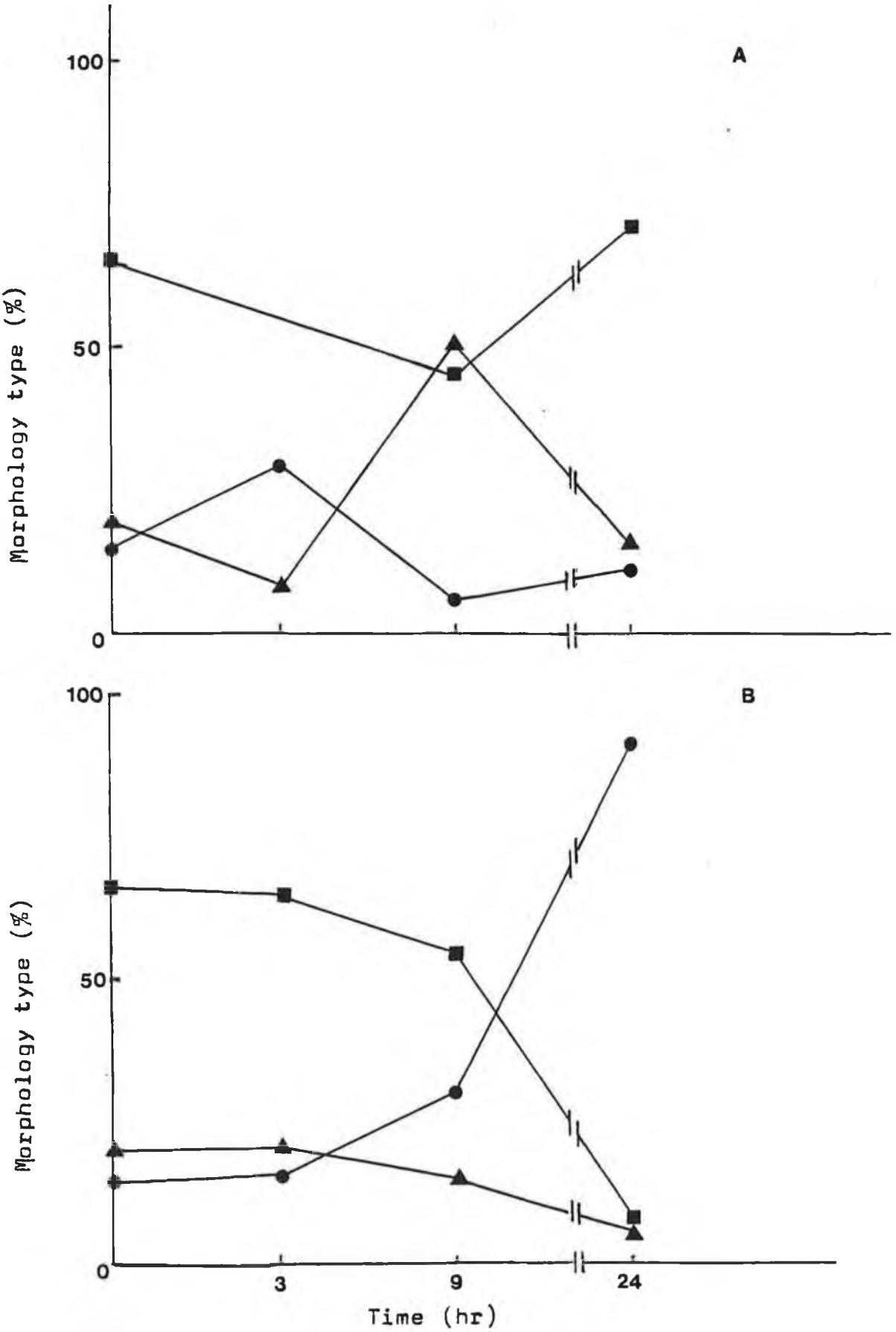

Fig. 3.2 Effect of oxygen availability on the morphology of K. fragilis NNRL y 2415 cultured in cheese whey. (A) Semi-aerobic shake-flask culture (B) Semi-anaerobic, stationary culture. Filamentous forms; (■) elongated cells (▲) pseudomycelia. Yeast-like forms; (●) oval to ellipsoid cells.

filamentous forms decreased while the percentage of budding yeast forms increased from 15 to 30% of total growth. It appeared that some reversion of elongated cells to oval ellipsoidal cells occurred during the initial 3 hr of incubation. Thereafter, these cells grew and developed into pseudomycelia and elongated cells as shown in the peak in filamentation at 9 hr incubation.

Pseudomycelial production in batch culture appeared to be a transient phenomenon with oxygen availability playing an important role in the morphological transformations of K. fragilis NRRL y 2415. Microscopic examination of pseudomycelial development in batch shake-flask culture conditions revealed that the yeast had a morphological 'life cycle' (see Fig. 3.3) in which budding yeast phase cells, elongated into pseudomycelia and reverted back to oval or ellipsoidal shaped cells. Elongated cells and pseudomycelia were characteristic of mid-log to early stationary phase conditions. This concept of 'morphological reversibility' is discussed further in Section 3.1.4.

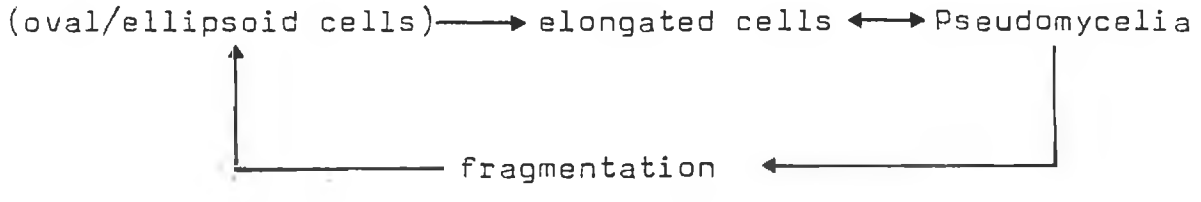

Fig. 3.3 A model depicting morphological 'life cycle' transitions during aerobic-batch cultivation of K. fragilis NRRL y 2415.

3.1.2.4 Effect of strictly aerobic and strictly anaerobic conditions on morphology of *K. fragilis* NRRL y 2415

A comparative study on growth and on morphological expression of *K. fragilis* NRRL y 2415 in strictly aerobic and anaerobic conditions was undertaken. Cheese whey fermentations were carried out using 2 litre Biolafitte fermenters from which samples were withdrawn periodically for morphological examination. An aeration level of 1/v/v/m was used for maintenance of strictly aerobic conditions while strictly anaerobic conditions were maintained by purging the fermentation broth with nitrogen at a rate of 0.5 v/v/m. Agitation was set at 400 rpm for both fermentations.

Results are presented in Fig. 3.4 and Table 3.4

Table 3.4 Kinetic data from *K. fragilis* NRRL y 2415 aerobic and anaerobic fermentation cheese whey fermentation.

	Fermentation	
	Aerobic	Anaerobic
Initial cell No. (cells ml^{-1})	6.1×10^6	6.1×10^6
Final cell No. (cells ml^{-1})	2.3×10^8	2.0×10^7
Specific growth rate (μ), (hr^{-1})	0.50	0.26
Mean doubling time (td), (hr)	1.39	2.66

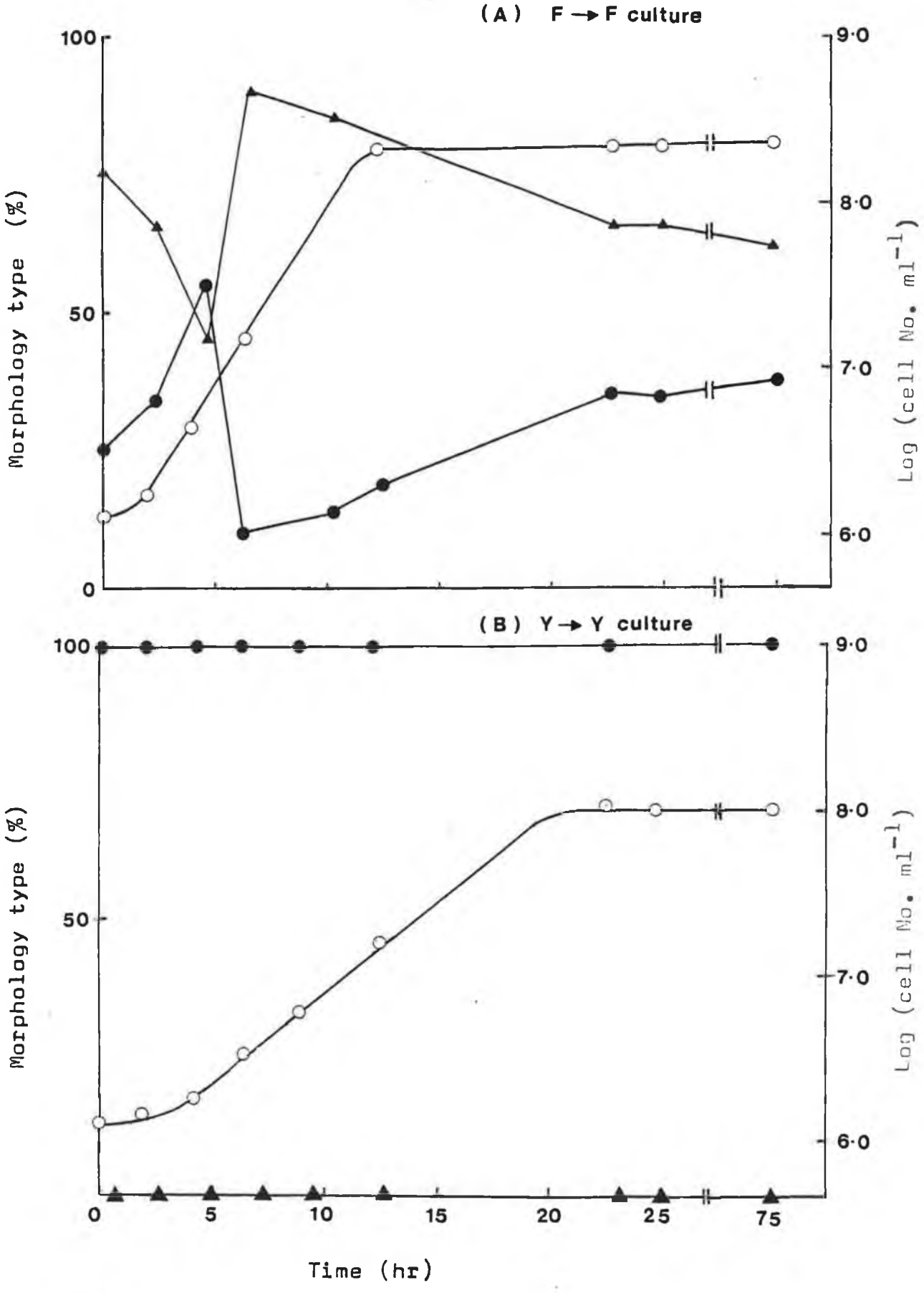

Fig. 3.4 Effect of (A) aerobic and (B) anaerobic environments on growth and morphology of K. fragilis NRRL y 2415 during cheese whey fermentation. Symbols : (○) log (cell No. ml^{-1}), (●) yeast phase, (▲) filamentous phase

Cells from the stationary pre-culture inoculum were entirely oval to round in shape and this morphology was maintained throughout the anaerobic fermentation. During the exponential phase of growth, cells became distinctly rounded in shape and many characterised by a bipolar mode of budding. Fig. 3.4 shows that filamentation in the aerobic fermentation peaked at approximately 7 hr with 90% of total growth in an elongated or pseudomycelial form. After this time, the degree of branching in the pseudomycelia decreased and at 24 hr, the aerobic culture was composed mainly of elongated cells with few rudimentary pseudomycelia. Budding yeast cells were the predominant form during the early log phase of growth.

3.1.2.5 Respiratory metabolism during cellular morphogenesis of *K. fragilis* NRRL y 2415.

From the previous findings, we are now in a position to study more closely, from the metabolic viewpoint, the role of oxygen in control of *K. fragilis* dimorphism. Firstly, the effect of oxygen availability on differential development of *K. fragilis* NRRL y 2415 into yeast and filamentous phases was studied in ergosterol supplemented media (see Section 2.4.5). Anaerobic cultivation conditions were maintained by purging the media with nitrogen at a rate of 0.05 v/v/m. The aerobic fermentations were aerated at a rate of 0.05 v/v/m. Agitation was set at 400 rpm for both aerobic and anaerobic fermentations. Four series of cultures were set up in Labroferm fermenters with two different growth media as follows:

Fermenter	Medium	Gaseous Environment
A	cheese whey	aerobic
B	cheese whey	anaerobic
C	defined lactose medium	aerobic
D	defined lactose medium	anaerobic

Still (36 hr) cultures grown in cheese whey and in defined lactose media were used as a source of inocula. Fermenters were inoculated with yeast-phase cells grown in their respective media (see Fig 3.5 A and Fig 3.6 A). Samples were withdrawn throughout the fermentation for morphological evaluation, oxygen uptake measurements and for cytochrome analysis. It is apparent, on examination of photomicrographs (see Fig 3.5 and Fig 3.6), that significant phase conversion occurred in aerobic fermentations while no appreciable morphological change occurred during the anaerobic fermentation. Pseudomycelial development and cell elongation peaked 6 hr after inoculation into aerobic environments (see Fig. 3.5 B, 3.6 B). This compares favourably with results in Fig. 3.4 and confirms that the maximum degree of filamentation coincides with the period of active cell division during the growth cycle. As the growth rate of the cells declined on entering into stationary phase at around 12 hr , pseudomycelial production declined in both complex and defined media. Elongated cells were the predominant form after 12 and 24 hr aerobic growth in defined media, whereas cells from the aerobic cheese whey fermentation at 12 and 24 hrs were of a mixed morphology - cells being mostly oval or slightly elongated. During the anaerobic fermentations in cheese whey and in defined media, cells appeared to become progressively rounded in shape.

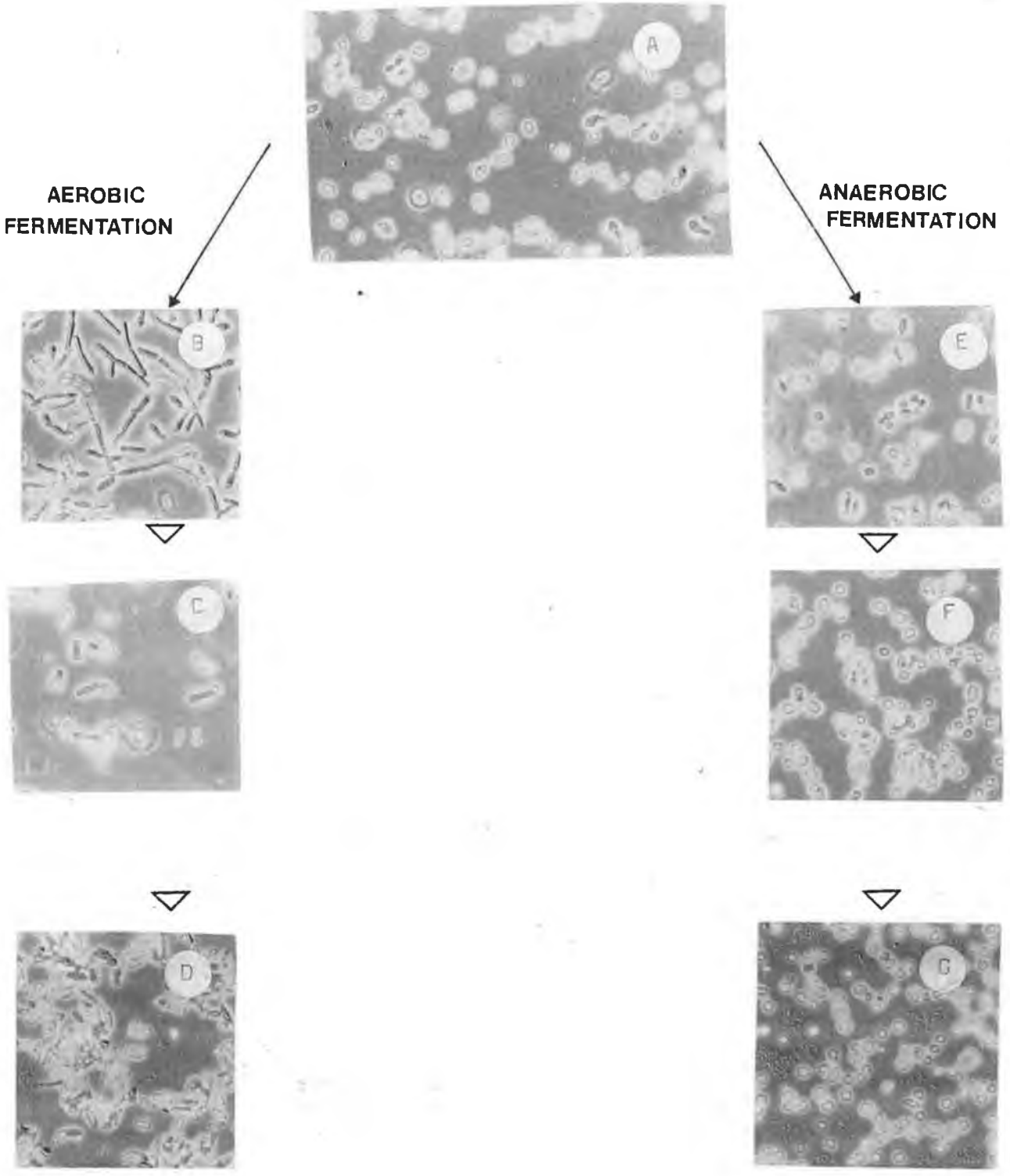

Fig. 3.5 Differential development into yeast (Y) and filamentous cells (F) of K. fragilis NRRL y 2415 during a defined lactose medium fermentation under aerobic and anaerobic conditions. Photomicrographs: (A) still culture inoculum (36 hr); (B), (C), (D) are cultures at 6,12 and 24 hr during the aerobic transition respectively, and (E), (F), (G) represent anaerobically grown cultures at 6, 12 and 24 hr respectively.

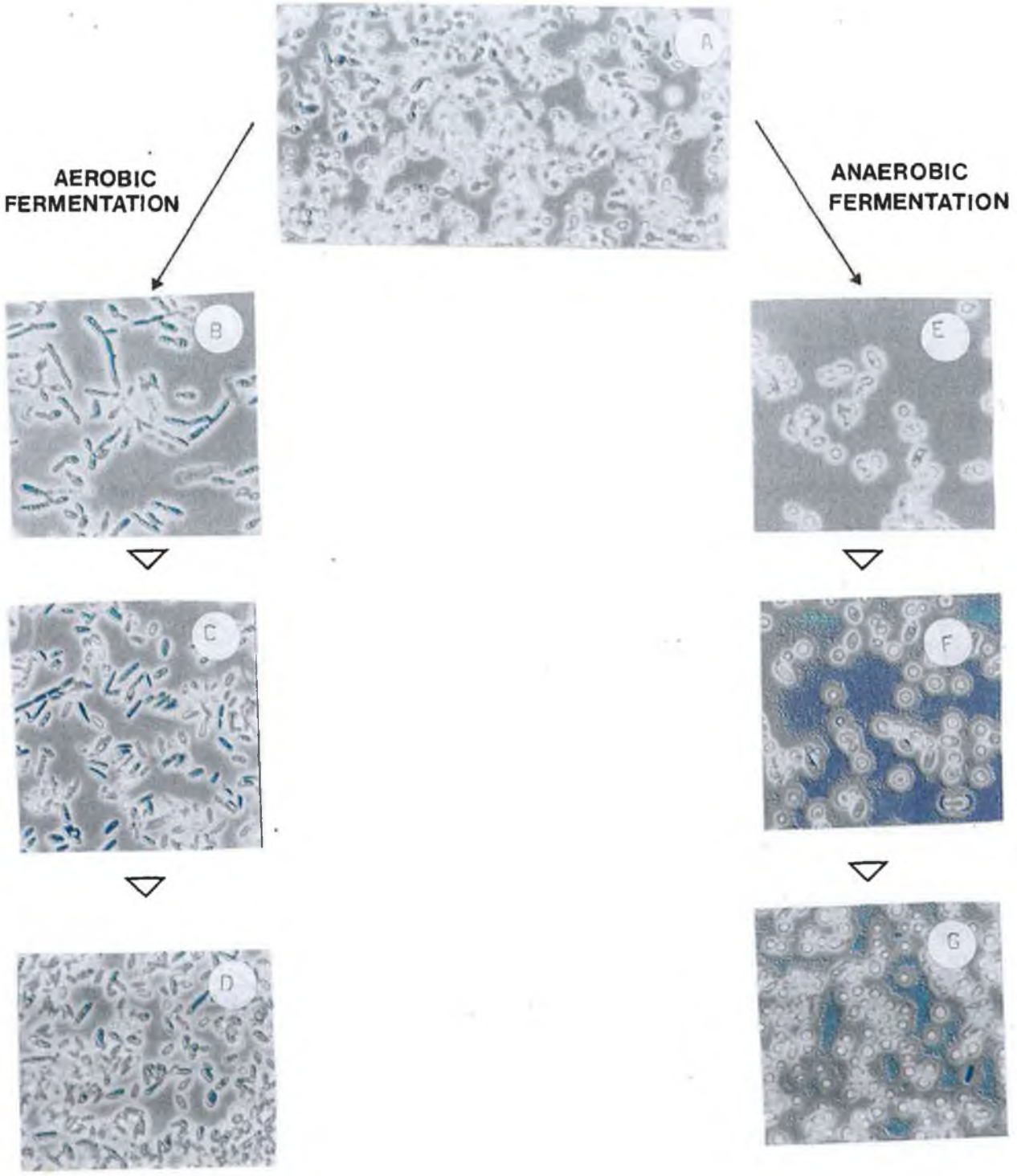

Fig. 3.6 Differential development into yeast (Y) and filamentous cells (F) of K. fragilis NRRL y 2415 during aerobic and anaerobic cheese whey fermentation. Photomicrographs: (A) still culture inoculum (36hr); (B), (C), (D) are cultures during aerobic transition at 6, 12 and 24 hr respectively, and (E), (F), (G) represent anaerobically grown cultures at 6, 12 and 24 hr respectively.

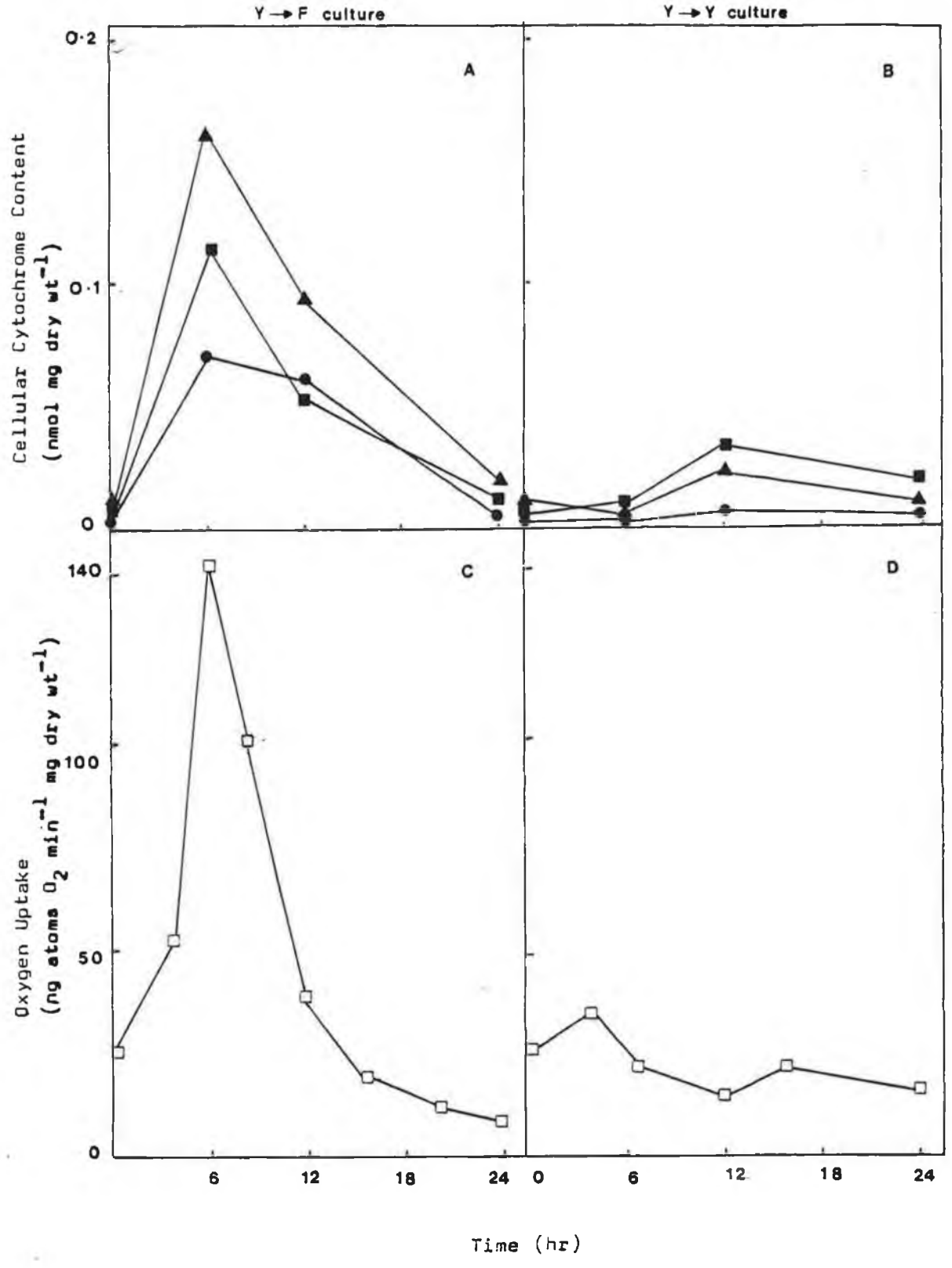

Fig. 3.7 Environmental modifications of mitochondrial composition and activities of K. fragilis NRRL y 2415 in cheese whey culture. Cellular cytochrome contents in aerobic (A) and anaerobic (B) cultures. Oxygen uptake measurements in aerobic (C) and anaerobic (D) cultures. Symbols: (□) oxygen uptake, (▲) cytochrome c, (■) cytochrome b, (●) cytochrome a.

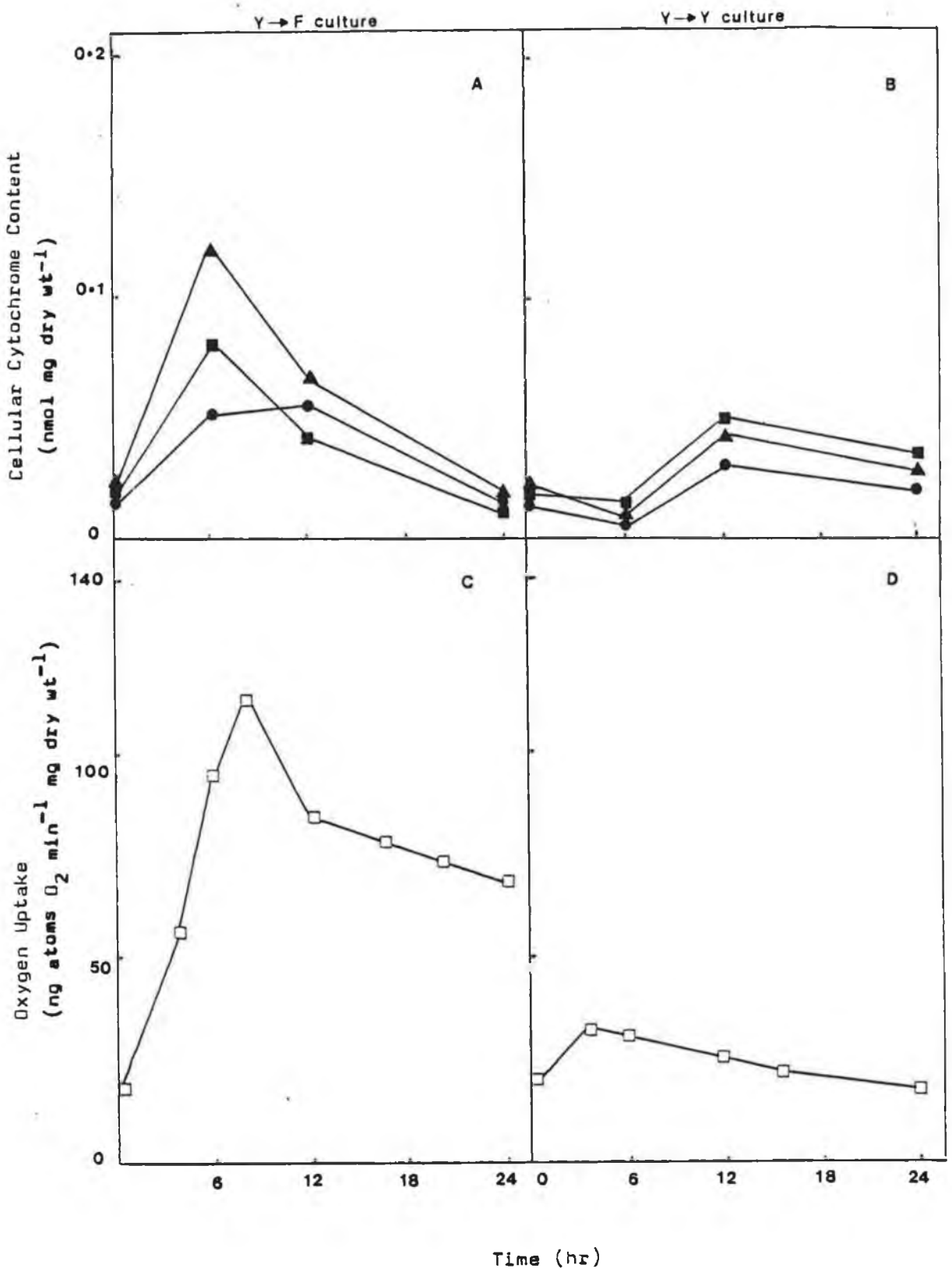

Fig. 3.8 Environmental modifications of mitochondrial composition and activities of K. fragilis NRRL y 2415 in defined lactose medium. Cellular cytochrome contents in aerobic (A) and anaerobic (B) cultures. Oxygen uptake measurements in aerobic (C) and anaerobic (D) cultures. Symbols: (□) oxygen uptake, (▲) cytochrome c, (■) cytochrome b, (●) cytochrome a.

The previous observations suggest that respiratory adaption accompanies Y-F morphological transformation of *K. fragilis.* To investigate this further, oxygen uptake capacities of cells were studied during Y-F transitions in aerobic culture. Fig. 3.7 and 3.8 show that at the period coinciding with maximal Y-F transiton (ie. after 6 hr) there is a dramatic increase in oxygen uptake by the cells in both the cheese whey and defined media. Cellular cytochrome contents were also analysed and Figs. 3.7 and 3.8 show a corresponding increase in cytochromes a,b and c around 6 hr after incubation. As the cells reverted from pseudomycelia to elongated forms, (see Figs. 3.6 B, 3.7 B) oxygen uptake and cytochrome levels declined. In anaerobic fermentations, where cells were maintained in yeast-phase morphological form, decreased levels of cytochromes and lower oxygen uptake rates were observed. It appears from these results that oxygen may act as a trigger for the Y-F dimorphic change in *K. fragilis*. This is discussed further in General Discussion.

Sacchromyces cerevisiae cells cultivated anaerobically with and without ergosterol have been reported to be lacking in cytochromes a,b and c (Slonimski, 1953). However, Morpurgo *et al.*, (1964) have shown that when yeasts grown anaerobically with ergosterol are oxygenated, a normal cytochrome spectrum comparable to that of aerobic cells appears. In the present work it is unclear why levels of *K. fragilis* cytochromes remain detectable during anaerobic fermentation. Perhaps the presence of ergosterol directly influences the appearance of cytochromes during anaerobic fermentation. The additional possibility of samples undergoing respiratory adaptation prior to analysis must also be considered.

The composition of mitochondrial membranes appeared to be influenced by the conditions of aerobiosis and anaerobiosis. The ratios of cytochromes c : b : aa_3 (6hr) under conditions of anaerobiosis in defined lactose medium were 1.0 : 0.66 : 0.375, whereas aerobic conditions gave ratios of 0.83 : 1.0 : 0.625 which indicated that the composition of the mitochondrial membrane was influenced by gaseous environmental conditions. When cheese whey is employed as a fermentation medium, results are qualitatively very similar to those obtained with defined medium although increased levels of pseudomycelia accompanied by increased cytochrome levels and increased rates of oxygen uptake are observed. This finding indicates a possible nutritional influence on oxygen-stimulated dimorphism of K. fragilis.

3.1.3 Reversibility of yeast-filamentous conversion

It has previously been suggested that K. fragilis undergoes a morphological 'life cycle' in which an interchange of different morphologies occurs (see Fig. 3.3). In an effort to examine this model further, two series of cultures were established to examine: (1) yeast to filamentous (Y - F) and (2) filamentous to yeast (F - Y) phase conversion in cheese whey media. In the former case, an overnight still culture was employed as Y-phase inoculum; in the latter case, an overnight shake-flask culture was used for an F- phase inoculum.

Y - F culture:

It is evident from Fig 3.9 that regardless of the degree of Y - F conversion, a certain proportion of normal yeast-phase K. fragilis cells always persists in the culture. Filamentation

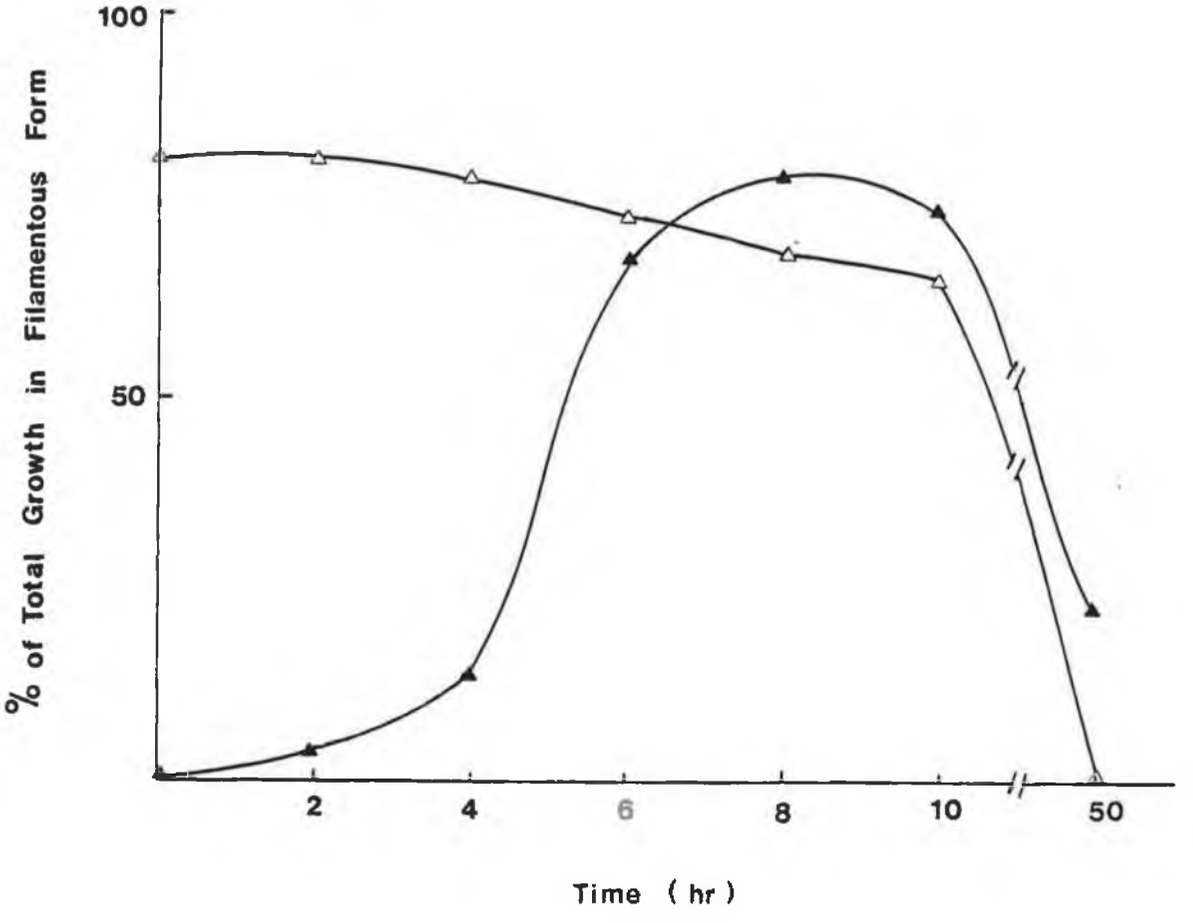

Fig. 3.9 Morphological reversibility: The effect of different culture conditons and inocula sources on the morphology of K. fragilis NRRL y 2415. Open symbols refer to an F → Y culture prepared by inoculating cells from a shake-flask culture into a still culture; closed symbols refer to a Y → F culture prepared by inoculating cells from a still culture into a shake flask condition.

peaks over a 6-10 hr period with yeast-phase cells serving as precursors for the elongated and pseudomycelial forms. After 10 hr of incubation, F - Y conversion is incomplete with 24% of total growth remaining in yeast-phase forms. This may be due to the fragmentation of pseudomycelia (see Fig 3.3). The percentage of Y forms increases significantly on entry into the stationary phase of growth. This observation may be related to the exhaustion of available nutrients or to the accumulation of toxic products of metabolism.

F - Y culture:

The reversion of cells from filamentous form to a yeast form was considerably slower than the Y - F conversion. Nevertheless, after 50 hr of incubation, the filamentous culture had reverted completely to yeast-phase cells (Fig 3.9).

Age of slopes:

A lack of reproducibility in the level of filamentous forms of K. fragilis NRRL y 2415 in pre-cultures prompted a microscopic examination of slopes of varying age. Results, presented in Table 3.5, show that the age of culture appears to be an important factor in the Y - F transformation. For example, cells from a day old slope were predominantly ellipsoid in appearance and appeared to be in a transition phase from an oval to an elongated form. The percentage of filamentous forms of the yeast did not alter significantly between cells aged between 10 days to 3½ months. The Y - F effect in aging cultures has been clearly demonstrated by

many workers. For example, Anderson (1917) reported that yeast cultures incubated over extended periods of time showed marked elongation of cells. Filamentous forms were also observed in old cultures, behaviour which is regarded as being due to the presence of metabolites such as higher alcohols (Segal, 1939). Stelling-Dekker (1931) demonstrated that aging affected yeast cell morphology in different media. Thus, Saccharomyces fragilis (Jorgensen) grown in wort at 25°C for 3 days appeared as oval budding cells, but under similar conditions on wort agar the cells had elongated and formed into filaments.

Table 3.5 Influence of stock-culture age on morphological form. Results are presented as the percentage of filamentous (F) forms.

Age of slope	% (F)
3½ months	92
2 months	95
1½ months	95
10 days	98
1 day	22

Colony morphology:

Studies of the age of cultures was further developed by examining surface colony morphology of K. fragilis. Two strains of yeast were grown on malt extract agar, K. fragilis NRRL y 2415 and K. fragilis CBS 5795. Single colonies were used to study morphological differentiation

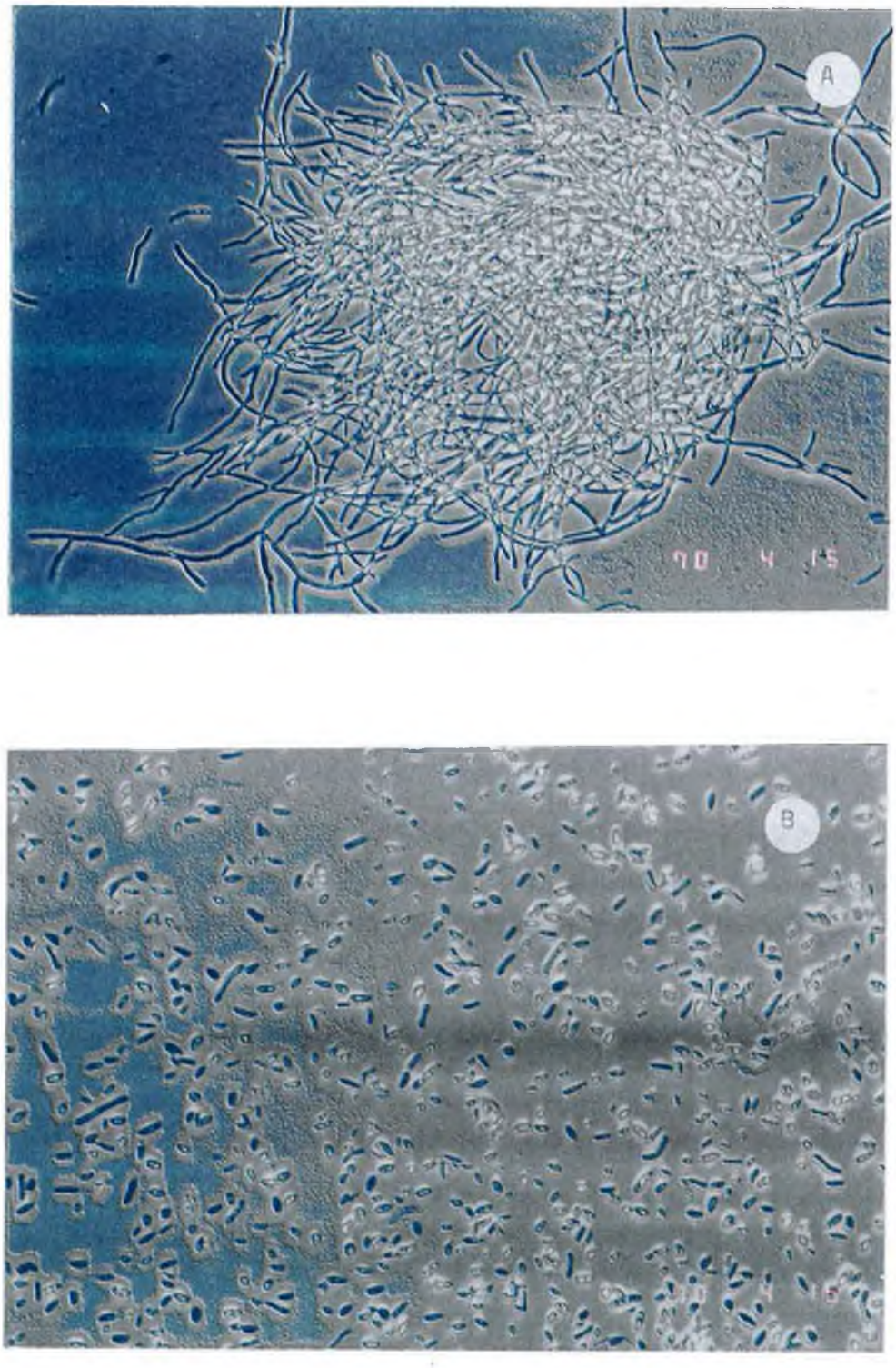

Fig. 3.10 Colony morphology changes in K. fragilis NRRL y 2415. Cells from the edge (A) and centre (B) of single colonies were microscopically examined X 544 (phase 3).

after 7 days of incubation at 30°C.

Colonies of K.fragilis NRRL y 2415 had a characteristic serrated edge. Microscopic examination revealed abundant pseudomycelia at the colony peripheral growth zone (see Fig. 3. 10 A). Normal yeast phase cells were found at the centre of the colony (see Fig 3.10 B). The possibility that this F - Y effect may be growth rate related in K. fragilis NRRL y 2415 is discussed further in Section 3.1.5 . K. fragilis CBS 5795, on the other hand, grew in a normal yeast-phase manner with no morphological differentiation of the cells from the edge or centre of the colony.

Lindegren and Hamilton (1944) grew yeast colonies on malt-extract agar and found pseudomycelial growth penetrating the agar, mainly at the edge of the colony, apparently where oxygen and nutrients were most abundant.

Due to the dramatic colony morphology changes observed with K. fragilis NRRL y 2415 it was decided to investigate if yeast phase or filamentous phase cells could be propagated in a singular morphological form. The morphological forms obtained by careful selection of colony centre (Y forms) and periphery (F forms) were used to inoculate cheese whey media under semi-aerobic and semi-anaerobic cultivation conditions (see section 2.4). After 12 hr incubation under semi-aerobic conditions yeast phase cells reverted to cells of a filamentous nature, in a fashion similar to that illustrated in (Fig 3.6.B). Semi-anaerobic cultures on the other hand, displayed no

detectable change in morphology. The suppression of filamentous growth in the colony centre appeared to be relieved on increased availability of (1) nutrients and (2) oxygen. The filamentous inoculum underwent a F - Y conversion under oxygen-limited still culture conditions. Semi-aerobic propagation of the filamentous form yielded a culture of mixed morphology after 12 hr of incubation with approximately 75% of the culture in a filamentous phase.

3.1.4 Effect of mitochondrial inhibitors

Previous studies on the morphological expression of *K. fragilis* NRRL y 2415 have confirmed that aerobiosis promotes filamentation and anaerobiosis promotes yeast-like development. Evidence was also accumulated which suggested that an active respiratory chain was a pre-requisite for Y - F transition in *K. fragilis* (see Section 3.1.2.5). In an attempt to study this relationship between morphology and mitochondrial function further, use was made of various respiratory inhibitors and uncouplers of oxidative phosphorylation.

3.1.4.1 Effect of chloramphenicol on *K. fragilis* morphogenesis

Chloramphenicol has been shown to inhibit *in vivo* mitochondrial protein synthesis in *S. cerevisiae* (Clark-Walker and Linnane, 1967). Chloramphenicol is therefore of value in probing the functional role played by mitochondria in

controlling K. fragilis dimorphism. Initially the effects of chloramphenicol on cytochrome content and morphogenesis in K. fragilis NRRL y 2415 were studied. Cultures were established in shake-flask conditions in defined media in which the inoculum used had approximately 75 per cent of the total growth in the filamentous phase. Fig 3.11A shows that in the absence of chloramphenicol pseudomycelia and elongated cells predominate. After 24 hr incubation Fig 3.9B shows that chloramphenicol inhibits filamentation and encourages the formation of yeast-phase cells. This demonstrates the need to maintain mitochondrial integrity during morphogenesis of K. fragilis NRRL y 2415.

3.1.4.2 Effect of chloramphenicol on growth and respiration of K. fragilis

When K. fragilis was cultured semi-aerobically in defined lactose medium in the presence of $4gl^{-1}$ chloramphenicol, the cells had a growth rate of 0.26 hr^{-1}. This was in agreement with the growth rate obtained during the anaerobic fermentation of cheese whey (see Table 3.4). In the absence of chloramphenicol, oxygen uptake by the cells was 141 ng atoms O_2/min/mg dry weight; whereas cells grown in the presence of the antibiotic exhibited negligable O_2 uptake. The cytochrome content of cells after 24 hr growth in the presence of chloramphenicol was also estimated and the results are presented in Table 3.6.

It is evident that growth in the presence of chloramphenicol results in decreased levels of cytochrome aa_3 and b and

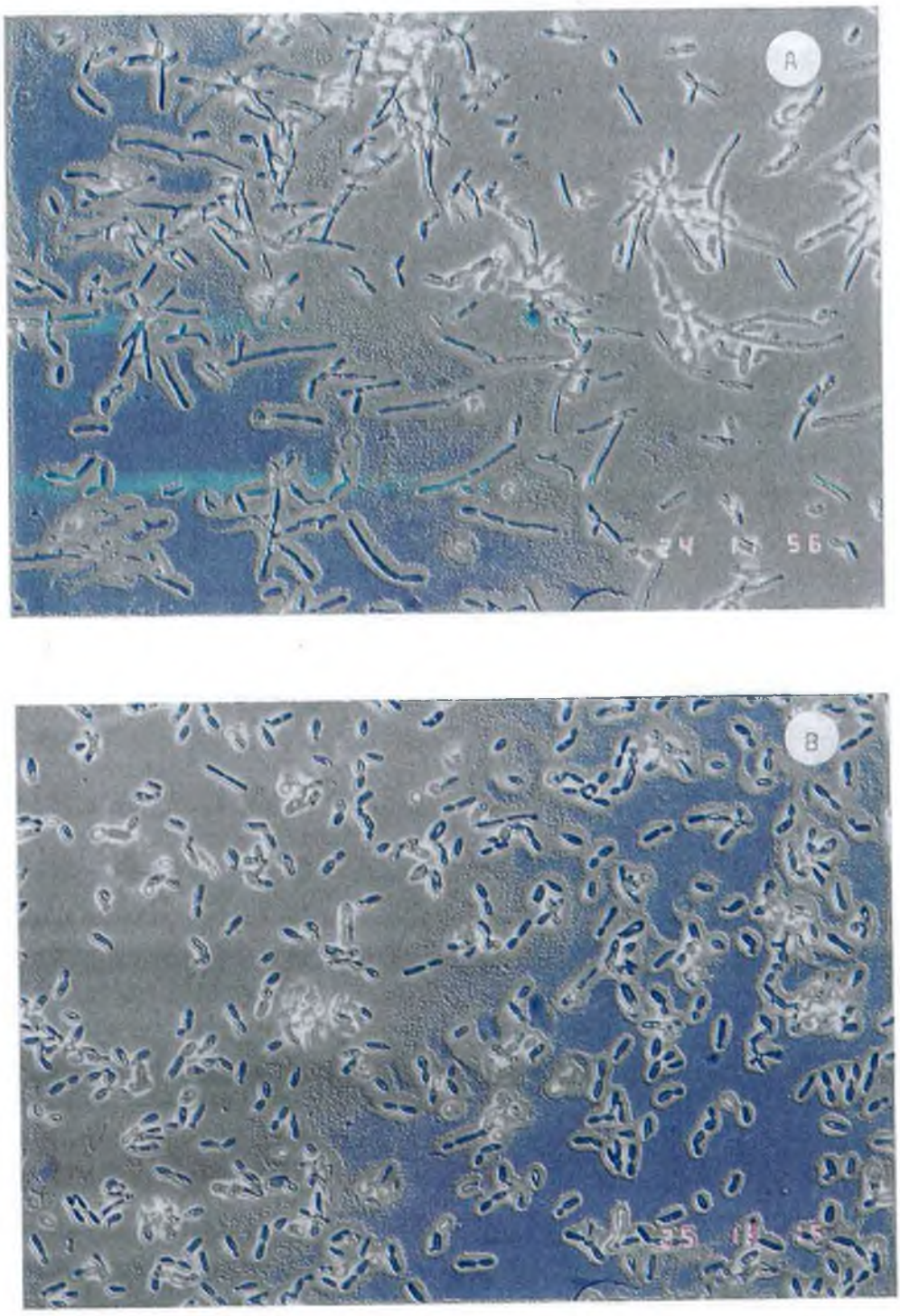

Fig. 3.11 Photomicrographs of K. fragilis NRRL y 2415 grown in semi-aerobic defined lactose medium (24 hr) in the absence (A) and in the presence (B) of 4mg/ml chloramphenicol. Magnification X544, (phase 3).

elevated levels of cytochrome c. These results are in agreement with the findings of Lamb et al., (1968) and Clark-Walker and Linnane, (1967). In Pythium ultimum, Marchant and Smith (1968) have shown that mycelia grown in the presence of 100 µg/ml chloramphenicol contained no detectable cytochromes aa_3 but showed an increase in the content of cytochrome c. The loss of oxidative function by chloramphenicol reflects the degree of inhibition of mitochondrial protein synthesis and of mitochondrial damage. This is substantiated by the finding that mutants of Mucor, introduced by spontaneous mutation, which are unable to transform from the yeast to mycelial phase lack the cytochrome oxidase activity found in wild type strains (Storck and Morrill, 1971).

Table 3.6 Effect of chloramphenicol on cytochrome contents of K. fragilis NRRL y 2415. Cells were propagated semi-aerobically in defined medium for 24 hr.

	cytochromes		
CAP gl^{-1}	aa_3	b	c
	(n moles mg dry wt^{-1})		
0	0.026	0.032	0.125
2	0.006	0.010	0.309
4	0.003	0.007	0.327

3.1.4.3 Effect of various mitochondrial inhibitors on *K. fragilis* morphogenesis

If the enhancement of Y growth caused by chloramphenicol is mediated by an inhibition of respiration, it should also be possible to induce Y forms by blocking the electron transport chain or its energy generation. The following inhibitors and uncouplers were therefore added to defined lactose medium under semi-aerobic conditions : rotenone, potassium cyanide and 2,4, - dinitrophenol. Table 3.7 shows that when electron transport to oxygen is blocked - as in the case of KCN - differentiation into the (F) form is suppressed in favour of Y growth. Furthermore, with a partial inhibition of electron transport - as in the case of rotenone - there is no significant change in morphology. Concerning uncoupling of oxidative phosphorylation - as with 2,4 - dinitrophenol - Y forms are stimulated, but this depends on the concentration of uncoupler used.

The influence of these agents on the dimorphism of *K. fragilis* can be interpreted on the basis of a correlation between fermentation and yeast growth and respiration and filamentous growth, and are thus in accord with the findings of Haidle and Stock (1966) ;Schulz *et al.*; (1974) ; Terenzi and Storck, (1969) and Zorzopulos *et al.*, (1973) in *Mucor.*

Table 3.7

Influence of various respiratory inhibitors on morphology of *K. fragilis* NRRL y 2415

Inhibitors were added at the final concentrations to defined lactose medium under standard conditions and morphology was evaluated after 12 hrs.

Inhibitor (µg/ml)	% Y - Forms
KCN	
0 (control)	37
1	91
10	90
50	90
100	98
2.4 DNP	
0 (control)	30
1	75
10	85
50	87
Rotenone	
0 (control)	20
10	20
50	30
100	25

Inhibitors were dissolved in the following solvents sterilised by membrane filtration. Acetone (Rotenone), Ethanol (2,4-dinitrophenol), Distilled water (KCN). The same amount of solvent, lacking inhibitor, was added to control flasks.

3.1.5 Continuous culture studies

3.1.5.1 Establishment of carbon limiting conditions

In chemostat operations the biomass formed should be directly proportional to the concentration of the 'growth-limiting' nutrient. Therefore, any particular growth medium sould be compounded in a way such that all other nutrients are present in excess. In the following series of experiments, carbon limitiation was established in defined lactose medium by showing that at a fixed dilution rate, yeast biomass was directly proportional to the concentration of lactose (growth limiting nutrient) in the reservoir. Results are presented in Fig 3.12. From this data, a growth limiting concentration of $1.2gl^{-1}$ was chosen for future continuous culture studies.

3.1.5.2 Growth characteristics of K. fragilis in continuous culture.

Morphological examination of K. fragilis NRRL y 2415 cells growing in the centre and periphery of c single colonies revealed two different morphological forms (see Figs 3.10A,B). This filamentous to yeast transition phenomenon suggested that dimorphism in K. fragilis may be directly influenced by nutrient availability and growth rates of individual cells.

In order to examine further the effect of growth rate and substrate limitation on the morphology of K. fragilis NRRL y 2415 the yeast was grown under lactose-limited chemostat conditions at different dilution rates (D), which

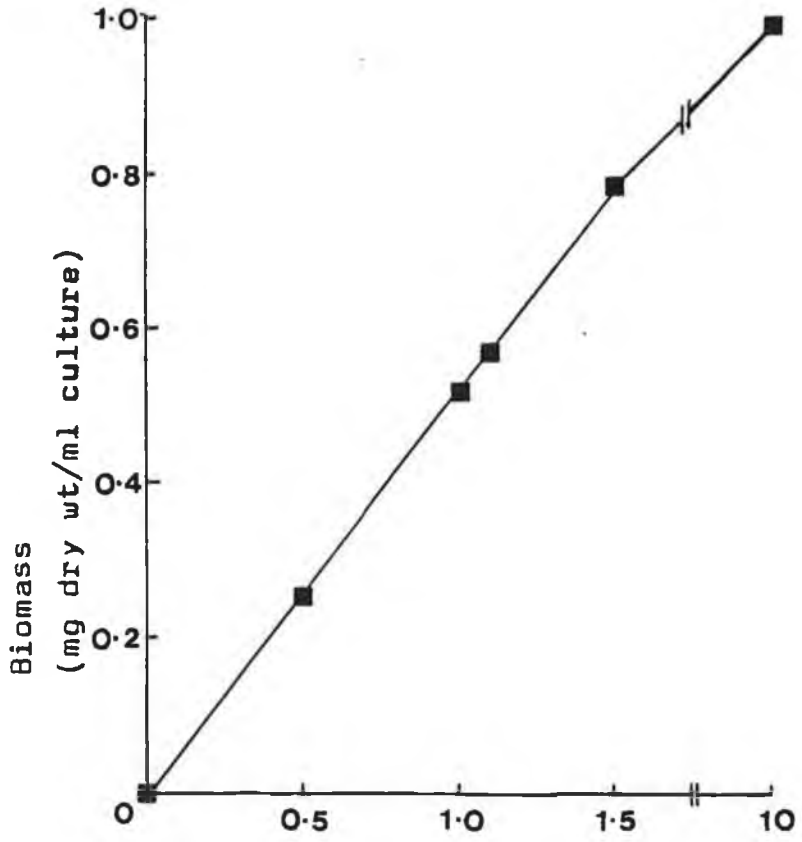

Fig. 3.12 Plot of relationship between growth-limiting lactose concentration and the steady state biomass level of K. fragilis growing in a chemostat at a fixed dilution rate of 0.2 hr^{-1} at 30°C.

under steady-state conditions equals the specific growth rate, μ). It has long been known that cultivation of bakers yeast results in oscillatory growth behaviour at dilution rates lower than the critical dilution rate (Fiechter and von Meyenburg,1966; von Meyenburg, 1969, 1973). In the present study, continuous cultures of K. fragilis exhibited growth rate "oscillations" during transition from stationary phase (batch culture) to steady-state conditions (chemostat at D =0.1 hr^{-1}). Similar oscillations were also observed during shifts between different dilution rates. During these oscillations the culture was characterised by a predominance of cells in a pseudomycelial mode of growth. Oscillations were eliminated once steady-state conditions were established. This coincided with a reversion of cells to a predominantly budding mode of growth. Although the mechanism for triggering these growth rate oscillations and changes in K. fragilis is not understood, it is possible that altered metabolic flux may be involved.

Culturing cells at different fixed dilution rates also resulted in altered morphology of K. fragilis NRRL y 2415. For example, in cultures maintained at 30°C cylindrical-type cells were produced at dilution rates above 0.2 hr^{-1}. However, at lower dilution rates (D = 0.1 hr^{-1}), the cells appeared round to oval in shape (see Fig 3.13). Both morphological forms could be maintained indefinitely by holding the dilution rate at an appropriate level. The transformation of cells from oval-round shape to cylindrical

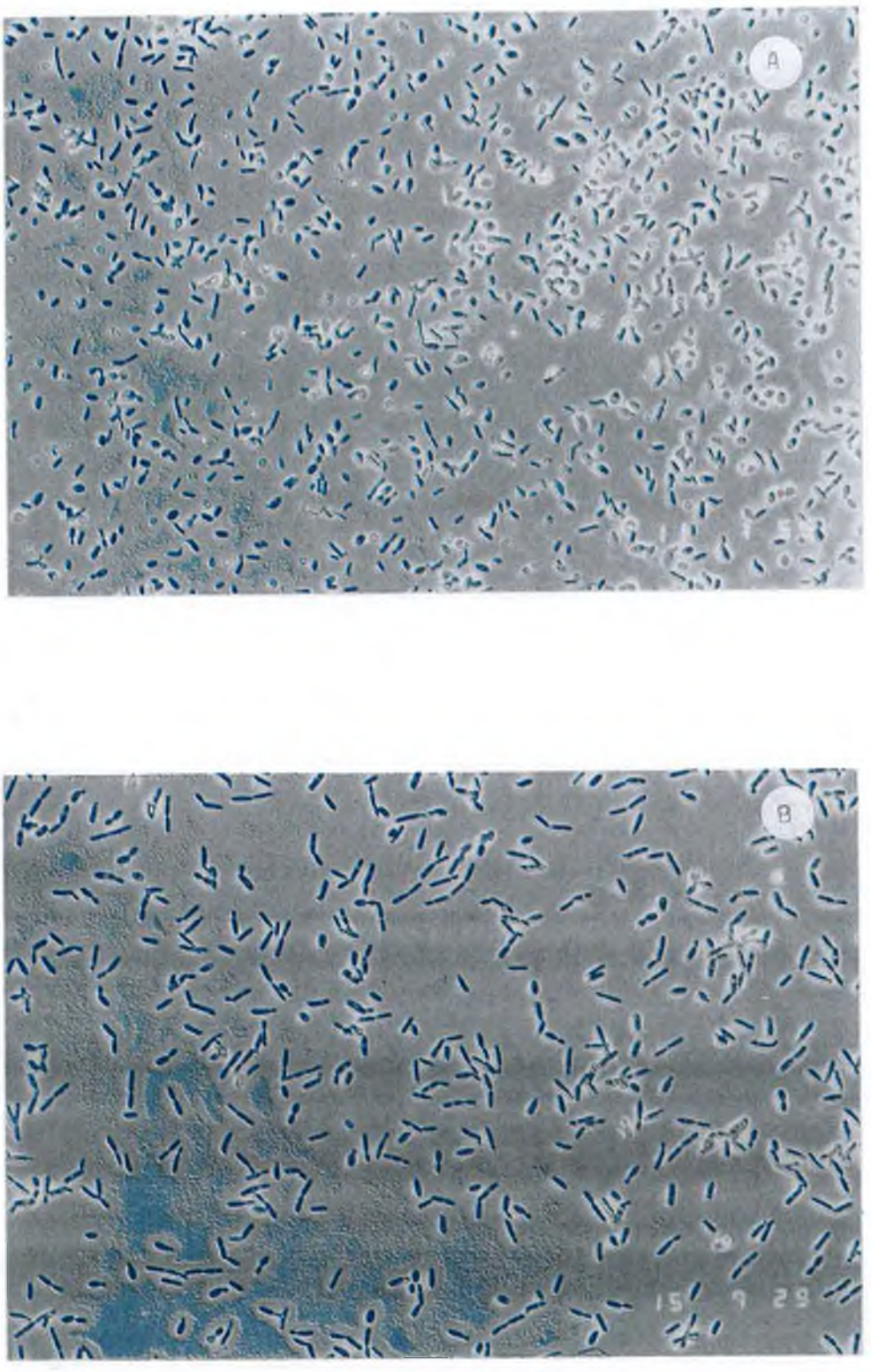

Fig 3.13 Relation of growth rate to cellular morphology in K. fragilis NRRL y 2415. Steady-state populations grown in a chemostat under conditions of lactose limitation examined at (A) $D = 0.1\ hr^{-1}$ and (B) $D = 0.4\ hr^{-1}$. Magnification x 544 (phase 3).

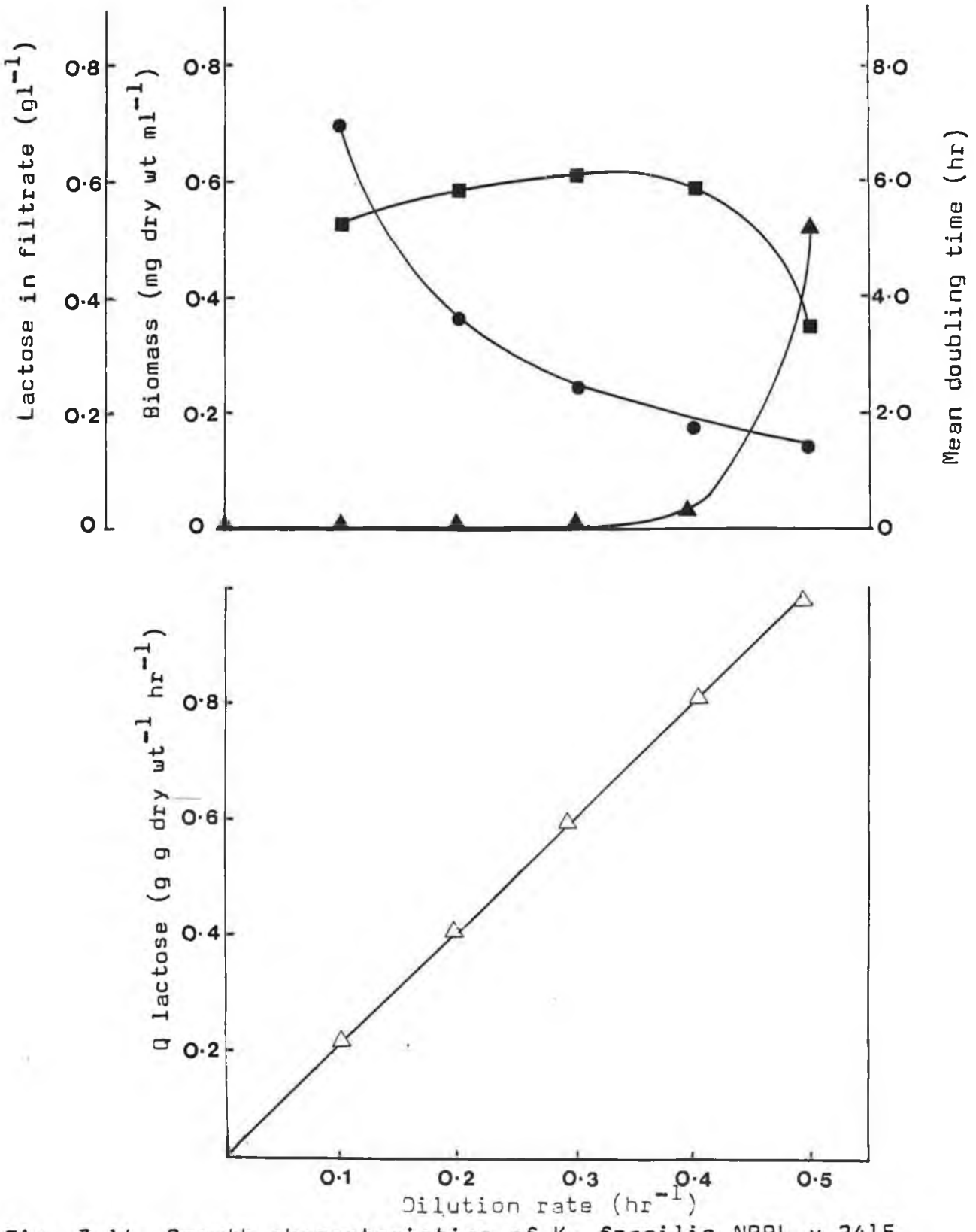

Fig. 3.14 Growth characteristics of K. fragilis NRRL y 2415 in continuous culture. The figure shows the effect of growth rate on A : (●) mean doubling time, (■) biomass, (▲) lactose in filtrate.
B (△) specific rate of lactose utilisation under conditions of lactose limitation. Details of medium composition and cultural conditions are described in Methods Section.

forms was accompanied by changes in oxygen uptake and in cellular cytochrome content, and the findings are discussed in Section 3.1.5.3. This suggests a direct correlation between morphogenesis and specific growth rate. This link is corroborated by previous observations in batch culture in which elongated cells and pseudomycelia appear during the exponential growth (where the specific growth rate is high), whilst oval to round cells appear in the lag and stationary phases (where the growth rate is low). This finding also correlates well with the previous findings on surface colony morphology where elongated and filamentous forms were found at the peripheral growth zone (high growth rate area ?) whilst more rounded cells were located at the colony centre (reduced growth rate area ?).

In steady state cultures of S. cerevisiae it has been shown that the mean cell volume of populations is dependent on the specific growth rate of the cultures. In addition, fast growing yeast cultures contain more RNA and less reserve carbohydrate material than slow growing cultures (McMurrough and Rose, 1967). Using glucose limited chemostats, Luscombe and Gray (1971) reported that cell shape was growth rate-dependent. In Arthobacter spp. Luscombe and Gray (1974) have shown that the coccus/rod transition was a growth rate-determined feature in all species studied, but the actual "μ transition" was species dependent. The effect of growth rate on cell length has been noted in other bacteria (Herbert, 1959) although growth rate appears to have no effect on the diameter of true cocci.

Returning to the present studies with K. fragilis, the growth characteristics of strain NRRL y 2415 (cultivated on lactose-limited continuous culture) are summarised in Table 3.8 and Fig 3.14. The μ max value of 0.5 hr^{-1} obtained in previous batch culture experiments was similar to that observed in continuous culture on examination of the wash-out curve (Fig 3.14A). Chemostat theory predicts that organism concentration should remain constant unless the dilution rate is so low that maintenance energy requirement becomes a relatively important part of the energy used (Veldkamp, 1968). It is believed that the decrease in biomass yield (Y in Table 3.8) at low dilution rates is because endogenous metabolism becomes a higher proportion of total metabolism (Mor and Fiechter,1968). As a consequence, less of the carbon energy source is used to synthesise new cell mass, thus leading to a decrease in cell yield. From Table 3.8, a growth yield of 0.5 $\pm$ 0.02 was obtained. This value compares well with values of 0.5 for growth of Candida utilis on glucose (Johnson,1969) and agrees with the value of 0.50 for glucose limited growth of S. cerevisiae (von Meyenburg, 1969). However, growth yields of only 0.25 for lactose limited growth of Candida albicans has been observed (Shepherd and Sullivan, 1976). On approaching the critical dilution rate there was a noticable decrease in cell yield. This behaviour is typical of yeast cultures (Aiba et al.1973) and demonstrates the invalidity of the generally held view that yield coefficient is independent of the specific growth-rate. The rate of

Table 3.8

Growth characteristics of K. fragilis NRRL y 2415 in continuous culture.

Steady-state values for: mean doubling time, td (hr); final pH; dry wt, X (gl^{-1}); lactose concentration, S (gl^{-1}); Yield coefficient, Y; specific rate of lactose utilisation, Q lactose (g. (g dry weight)$^{-1}$ hr^{-1}); and the specific rate of oxygen uptake, QO_2 (ng atoms O_2 min^{-1} mg dry wt^{-1}) are given as a function of the dilution rate, D (hr^{-1}).

D	td	Final pH	X	S	Y	Q lactose	QO_2
.1	6.93	3.1	.53	.001	.44	0.226	14.7
.2	3.46	3.1	.58	.001	.48	0.413	92.3
.3	2.31	3.5	.61	.001	.51	0.590	136.0
.4	1.73	3.0	.59	.002	.49	0.812	231.0
.5	1.39	3.4	.35	.52	.51	0.971	305.0

Note The specific rate of substrate utilisation (Q lactose) was calculated as μ (So-S)/X where: μ is the specific growth rate (hr^{-1}), S_o is the original substrate concentration in the feed solution (gl^{-1}) and S and X are the steady-state values of the substrate level in the culture (gl^{-1}) and cell dry wt (g/l) respectively. The yield coefficient (Y) was calculated as X/(So-S).

transfer of carbon substrate to yeast cells could be of importance in explaining this phenomenon.

An interesting aspect of Table 3.8 shows that under carbon limited conditions, K. fragilis acidifies its growth medium to a pH value far below that of the reservoir medium (pH 5.5). It is evident from Fig 3.14 that at low growth rates, lactose was barely detectable in the culture vessel indicating near complete exhaustion of that nutrient under carbon limited conditions. In addition, the rate of lactose utilisation increased linearly in relation to the increase in growth rate. Finally, trace amounts of ethanol were found in the culture filtrates (data not shown).

3.1.5.3 Effect of growth rate on mitochondrial activity and cytochrome content of K. fragilis:

The influence of specific growth rate on oxygen uptake and cytochrome content of K. fragilis NRRL y 2415 was studied to see if any correlation existed between respiratory metabolsim and cell morphology in continuous culture. Fig. 3.15 shows the effect of dilution rate on the specific oxygen uptake rate (A) and on the cellular cytochrome content of K. fragilis NRRL y 2415 (B). It is evident that the amount of cytochromes present are dependent on the expressed growth rate. For example, from a dilution rate of 0.2 to 0.4 hr^{-1} the levels of cytochromes a and c increased, whereas there was no significant increase in the level of cytochrome b. In addition, although oxygen uptake rate increased with increased growth rate, there was seemingly no direct correlation between oxygen uptake rate and the cytochrome content of cells.

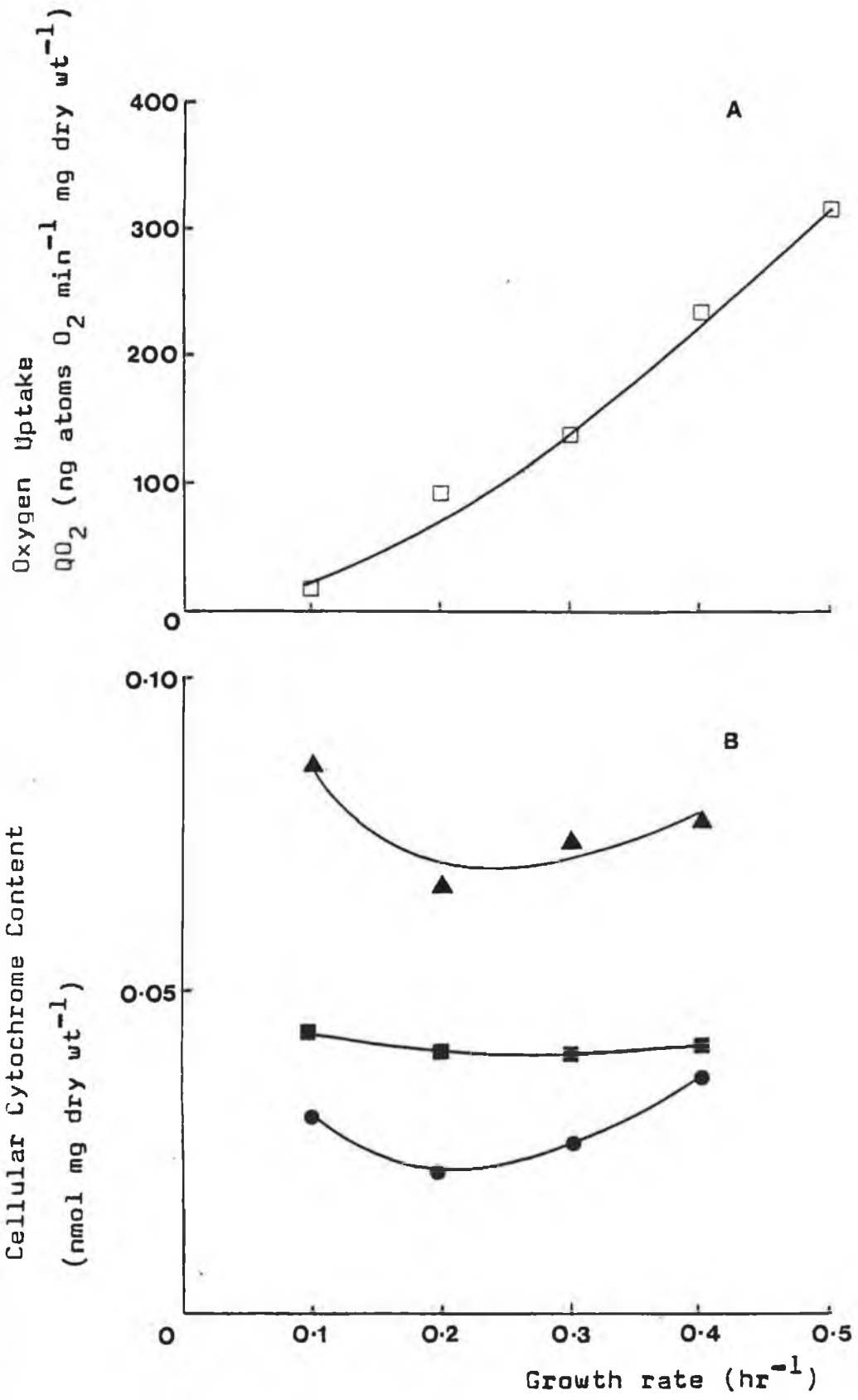

Fig. 3.15 Effect of the specfic growth rate (μ) of K. fragilis NRRL y 2415 on the oxygen uptake rate (□) and cellular cytochrome content; cytochrome a (▲), cytochrome b (■) and cytochrome c (●)

3.1.5.4 Effect of temperature on the morphology of continuously-cultured cells of *K. fragilis*.

The induction of morphological transition in fungi by temperature changes has been reported in the literature. For example, conversion from the mycelial to the yeast phase in *Blastomyces dermatitidis* occurs only at temperatures above 37°C (Levine and Ordal, 1946). The complex effect of temperature on morphogenetic determination has also been shown in *Mycotypha* where the proportion of yeast-like forms increased with increased incubation temperatures (Schulz *et al.*, 1974).

In order to investigate if dimorphism in *K. fragilis* was a temperature dependent phenomenon, the continuous culture technique was used. The object of using continuous culture was to obtain a highly homogeneous population of yeast cells whose physiological properties and morphological forms depended solely on the temperature imposed in the chemostat. Results are presented in Table 3.9 and Fig. 3.16.

Table 3.9 Effect of temperature and dilution rate on the morphology of *K. fragilis* NRRL y 2415

Dilution rate (hr^{-1})	Temperature °C	Morphology
0.3	39	Y
0.3	30	Y
0.3	25	Y
0.3	19	Y
0.1	30	Y
0.1	24	F

Fig. 3.16 Filamentation in K. fragilis NRRL y 2415 grown under conditions of nutrient limitation, $D = 0.1\ hr^{-1}$, temperature, 24°C; Magnification, X 272 (phase 2).

At a dilution rate of 0.3 hr^{-1}, cylindrical budding yeast-phase cells were obtained at steady state conditions. The filamentous form of K. fragilis NRRL y 2415 was only apparent at a dilution rate of 0.1 hr^{-1} at 24°C. Samples for morphological evaluation were taken 96 hr after the shift from batch cultivation to continuous culture operation (see Fig 3.16).

At a dilution rate of 0.1 hr^{-1}, the mean doubling time of the yeast in a steady-state condition is 6.93 hr^{-1}. Hence, it would appear that as the growth rate of cells is progressively lowered, the chemostat culture would be composed mainly of single phase cells in their pre-division phase. This was not the case at $D = 0.1\ hr^{-1}$, 24°C and the possibility should be considered that cells were in a "transition phase" from a stationary phase batch culture condition to a steady state condition. Alternatively, changes in the genotype or in the composition of the growth medium may account for the morphological change. However, in the absence of more definitive experiments, no firm conclusion can be drawn at the present time concerning the influence of temperature on K. fragilis morphogenesis.

3.2 CHAPTER 2 : FERMENTATION STUDIES

3.2.1 Kinetic study of semi-aerobic and semi-anaerobic lactose fermentation by K. fragilis NRRL y 2415.

Unsupplemented cheese whey permeate was the culture medium used in this study. An overnight (24 hr) shake flask cheese whey culture was used as the source of inoculum. Stationary cultures equipped with fermentation locks were used to maintain semi-anaerobic conditions whereas baffled shake-flasks were used to increase oxygen availability. Cultivation conditions were as described in Section 2.4.

A kinetic study of the alcoholic fermentation of whey permeate by Kluyveromyces fragilis NRRL y 2415 was undertaken. Results are presented in Table 3.10. Semi-anaerobic fermentations were characterised by cells in the normal yeast phase whereas filamentous forms predominated in shake flask cultures. Fermentation times varied from 24 hr to 60 hr for semi-aerobic and semi-anaerobic fermentations respectively.

3.2.2 Strictly aerobic and anaerobic lactose fermentations

Previous studies have confirmed the role of oxygen in the morphogenetic expression of K. fragilis NRRL y 2415 (see Section 3.1.2.5). Aerobiosis promotes filamentation and anaerobiosis promotes yeast like development. Studies on K. fragilis NRRL y 2415 were extended so as to evaluate the fermentative capabilities of both morphological forms during fermentations of cheese whey and defined media. The fermentations described are a direct continuation of metabolic

Table 3.10

Kinetic aspects of whey-lactose fermentation by *Kluyveromyces fragilis* under semi-aerobic and semi-anaerobic conditions.

Environment	Initial Lactose (gl^{-1})	Final cell No. cell No. ml^{-1}	μ max (hr^{-1})	Max. Ethanol Conc. (gl^{-1})	Conversion Efficiency %
Semi-aerobic	47	2.1×10^{8}	.507	21.71	85.54
Semi-anaerobic	47	2.5×10^{7}	.255	22.4	88.2

The conversion efficiency was calculated by dividing the actual yield of alcohol by the theoretical yield : The theoretical yield was estimated from the theoretical conversion of 100% lactose to ethanol according to the Gay-Lussac equation; (1 gram of lactose yields 0.54 g ethanol).

studies reported in Section 3.1.2.5 Both aerobic and anaerobic fermentations were carried out in Labroferm fermenters with two different growth media under the following cultivation conditions:

Temp 30°C

Agitation 400 rpm

aerobic cultures - aeration (0.05 v/v/m)

anaerobic cultures - nitrogenation (0.05 v/v/m)

Media-ergosterol supplemented : cheese whey and defined media.

Results are presented in Figs.3.17, 3.18 and Table 3.11.

Table 3.11 presents a comparison between the kinetic data of both aerobic and anaerobic fermentations. It is interesting to note that the ergosterol supplemented anaerobic cultures sustained a higher specific growth rate than that reported in semi-anaerobic shake flask conditions (see Table 3.10). This finding is in agreement with the observations of Janssens et al., (1983) who not only found a higher specific growth rate and an increase in biomass under anaerobic lipid supplemented conditions but also an increase in ethanol production rates during whey fermentations. Kinetic data was accumulated after 48 hr incubation period. Hence, maximum ethanol concentrations, conversion efficiencies and biomass yields may not yet have achieved their maximum value for an anaerobic fermentation.

Table 3.11 Fermentation Parameters of *K. fragilis* NRRL y 2415 grown under strictly aerobic and anaerobic conditions.

	Fermentation			
	CW aerobic	CW anaerobic	DLM aerobic	DLM anaerobic
Final cell No. (cells ml^{-1})	2.75×10^8	4.17×10^7	1.5×10^8	7.6×10^7
μ max (hr^{-1})	0.502	0.287	0.501	0.298
Ethanol yield, Y gl^{-1}	0.420	0.447	0.416	0.450
Biomass yield g_x/g_s	0.069	0.046	0.073	0.049
Maximum ethanol conc. (gl^{-1})	21.0	21.0	20.0	22.5
[a] Conversion efficiency (%)	77.7	77.7	74.1	83.3

a $\frac{\text{actual yield}}{\text{theoretical yield}} \times \frac{100}{1}$

The theoretical yield was calculated on the basis of the theoretical conversion of 100% lactose to ethanol according to the Gay-Lussac equation; (1 gram of lactose theoretically yields 0.54g ethanol).

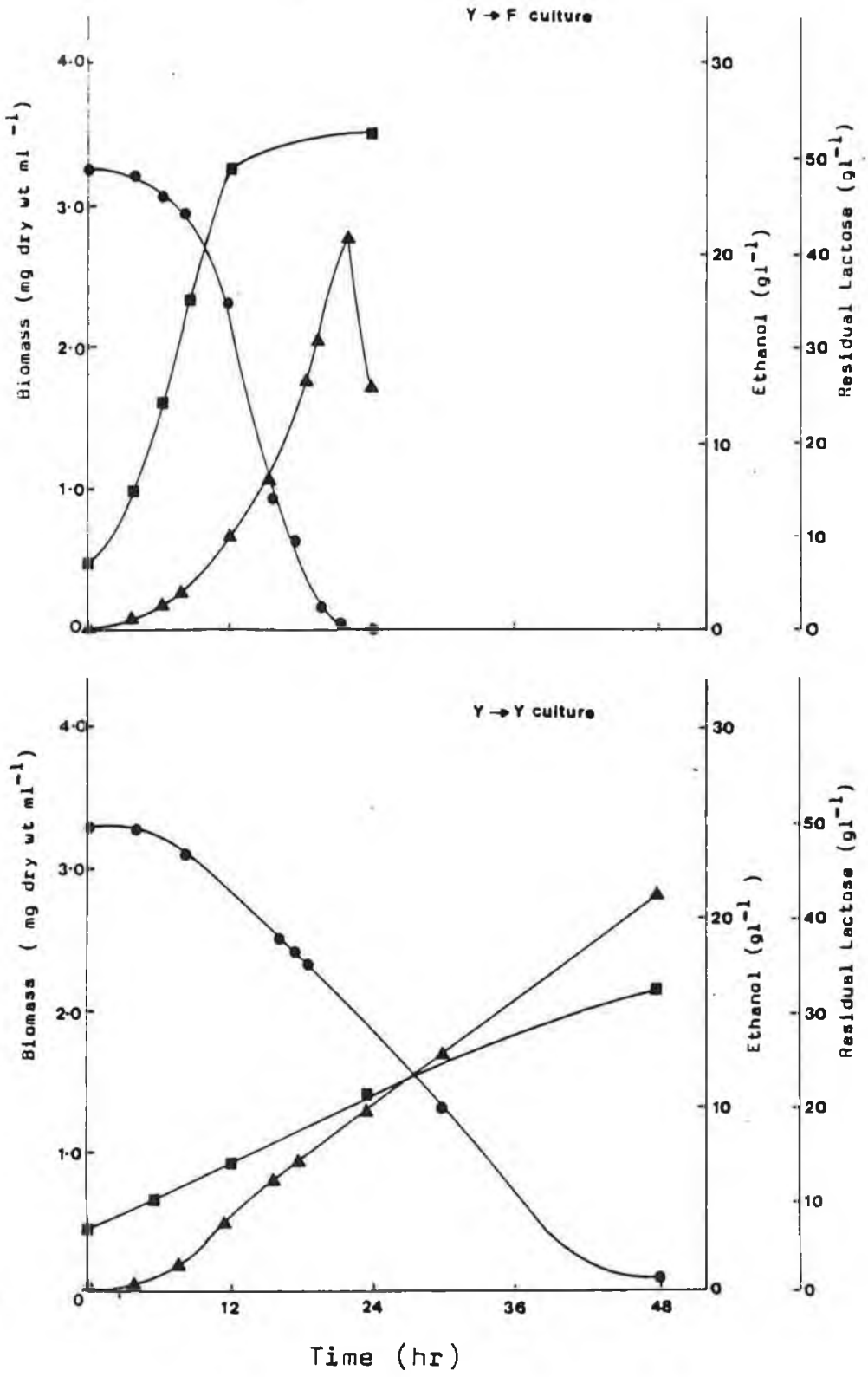

Fig 3.17 Kinetic study of strictly aerobic and anaerobic fermentation of ergosterol supplemented cheese whey by Kluyveromyces fragilis NRRL y 2415. Cultivation conditions are described in Section 2.4.3. Symbols: (●) Residual Lactose, (▲) Ethanol, (■) Biomass.

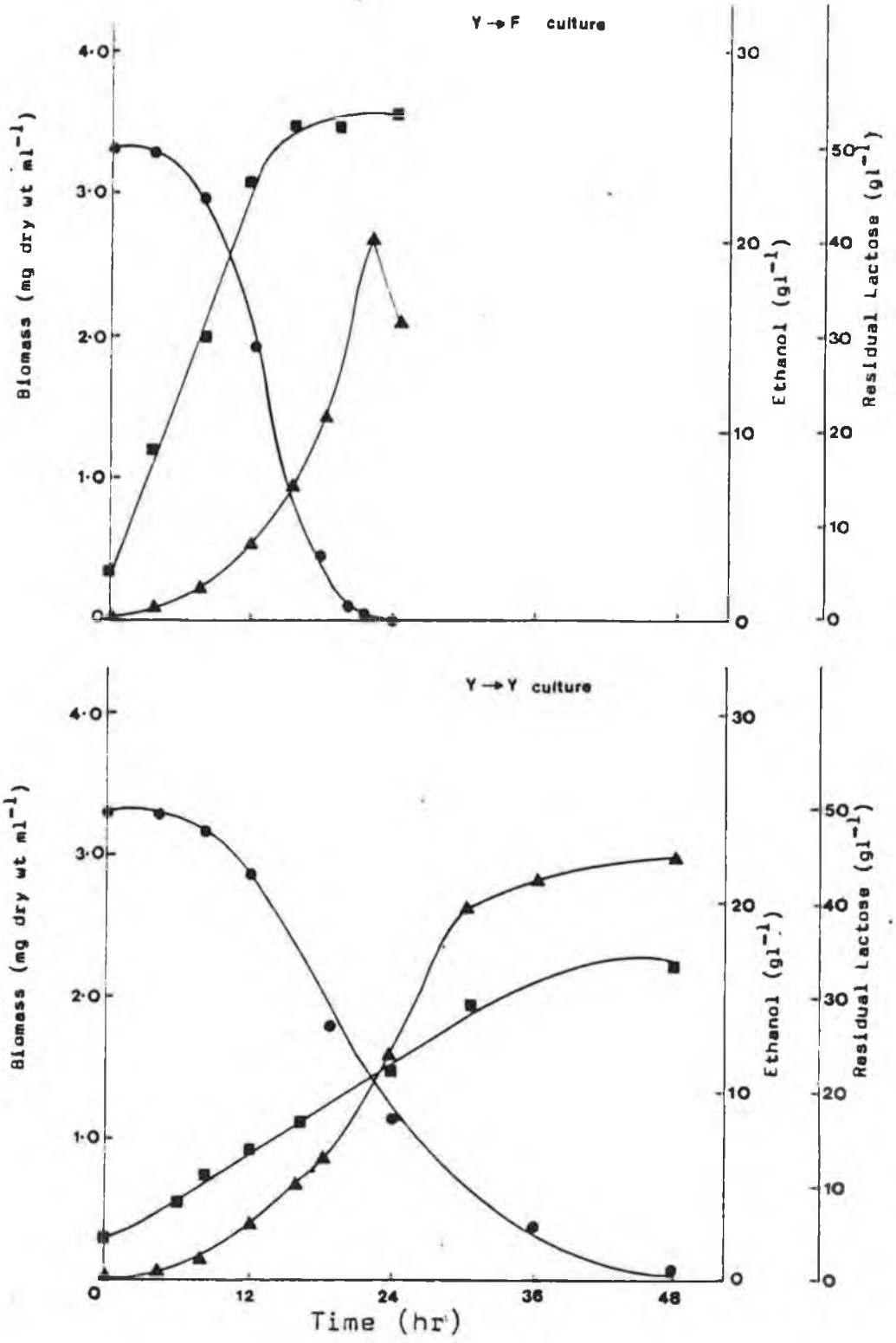

Fig 3.18 Kinetic study of strictly aerobic and anaerobic fermentations of ergosterol supplemented defined lactose medium. Cultivation conditions are as described in Section 2.4.3. Symbols: (●) Residual Lactose, (▲) Ethanol, (■) Biomass.

Aerobic fermentation resulted in slightly increased biomass yields over anaerobic cultures. Moulin et al., (1983) discussed the importance of residual aerobic fermentation in aerated media for the production of biomass from glucidic substrates. Ethanol conversion efficiencies were relatively low compared to conversion efficiencies in shake flask cultures (see Table 3.10). This may be due to sparging of the fermentation broth with air or with nitrogen resulting in the volatilisation of ethanol thus reducing yields.

Lactose metabolism and respiration of K. fragilis were compared under conditions which permitted maximum filamentous or maximum yeast growth. Changes in metabolism were monitored by comparing the rate of oxygen consumption (see Section 3.1.2.5) and the rates of ethanol and biomass production. During the initial 6 hrs of aerobic fermentation, ethanol production and lactose consumption were low. With reference to Figs. 3.7 C, 3.8 C, it can be seen that there was a dramatic increase in the rate of oxygen consumption upon respiratory adaptation and in filamentation (see Fig. 3.5 B, 3.6B). Increased lactose utilisation and ethanol production rates (see Fig. 3.17A, and 3.18B) occurred between 12 and 24 hr with a concomitant decrease in the percentage of total growth in the filamentous form. It appeared that morphological changes were expressed metabolically as a shift from aerobic to anaerobic metabolism. The results are consistent with the hypotheses correlating filament production with changes in carbohydrate metabolism with the cell.

In Candida albicans, filamentation under conditions of low phosphate, high glucose and increased cellular redox potential, accompanied by a decrease in Krebs' cycle activity, suggests a correlation of morphogenesis with Crabtree-like repression of mitochondrial function (Land et al., 1975a). Filamentous forms of this yeast produced more ethanol, evolved less CO_2, and consumed less oxygen that the yeast form which suggested that an abrupt change from aerobic to fermentative metabolism had occurred (Land et al., 1975b). Results obtained from these studies were consistent with the earlier observations predicting changes in glucose metabolism (Nickerson, 1954) during Y-M conversion.

Little is known about the facultative anaerobic species of Mucor, except from a study by Terenzi and Storck (1969), who suggested that yeast-like morphology and fermentation are linked and that phenylethyl alcohol promotes yeast-like growth due to its uncoupling of oxidative phosphorylation.

4. CONCLUDING DISCUSSION

Dimorphism in K. fragilis is a strain dependent phenomenon. Strain dependent morphological changes have been reported previously for Mycotypha (Schulz et al., 1974). In K. fragilis, the present study indicates that morphology is related to nutritional factors; for example, growth on poor carbon sources such as glycerol resulted in increased levels of normal-yeast phase cells. This finding is in contrast with the findings of Nisbet (1979). Furthermore, the defined lactose medium did not favour filamentation to the same extent as cheese whey or complex media, indicating a nutritional influence in dimorphism in K. fragilis (see Section 3.1.1). This is not a particularly novel result since nutritional factors have long been known to influence dimorphism in yeast (eg. Scherr and Weaver, 1953; Nickerson and Mankowski, 1953).

Microscopic examination of single colonies revealed striking morphological differentiation of cells. Pseudomycelia were abundant at the colony periphery whereas true-budding yeasts were found at the colony centre. In batch culture studies (see Section 3.1.2.3), filamentous to yeast transition coincided with nutrient depletion and lag phase conditions. The possibility was raised that nutrient deprivation or reduced growth rates in the colony centre resulted in development of true-yeast forms. This was investigated in lactose-limited continuous cultures (Section 3.15).

Microscopic examination of aerobically grown cells at 30°C and under lactose limited conditions yielded budding yeast forms under steady-state conditions irrespective of dilution rate. This seemed to indicate a nutrient-limited and growth rate-related influence on dimorphism. Cell shape, which is the gross manifestation of chemical composition, is well known to be environmentally determined (Bull and Brown, 1979). A shift from low dilution rates ($D = 0.1\ hr^{-1}$) to higher dilution rates ($D = 0.4\ hr^{-1}$) was accompanied by a transition from oval-round cells to cylindrical shaped cells. This supported the argument that morphology in K. fragilis was growth rate-related (see Section 3.1.5.2). Many studies have investigated the relationship between cell size, temperature and growth rate in yeasts (eg. Luscombe and Gray, 1971, 1974; Brown and Hough, 1965). Concerning growth rate McMurrough and Rose (1967) reported that the rate at which S. cerevisiae was grown, under conditions of glucose and NH_4^+ limitation, affected the relative proportions of the cell wall components, the dimensions of the cell and the synthesis of the periplasmic enzyme, β-fructofuranosidase.

With regard to nutritional status and growth rate, therefore, morphogenesis in K. fragilis appears to behave qualitatively similar to other yeasts. However, the extent of filamentation observed under certain cultivation systems, especially surface growth on agar, dramatically demonstrates the true dimorphic nature of strain NRRL y 2415. S. cerevisiae on the other hand, is not generally regarded as being a dimorphic organism.

Oxygen availability is another key factor in the morphogenetic expression of K. fragilis NRRL y 2415. This is clearly demonstrated by growing cells anaerobically (conditions which generate true-budding yeast forms) or aerobically (conditions which favour filamentation - see Section 3.1.2.4). In Mucor rouxii, mycelial transformation is readily observable when yeast-like forms of the organism are exposed to the air. This conversion is accompanied by increased mitochondrial activity, indicating that respiratory adaption accompanies the transformation (Haidle and Storck, 1966). These findings are in agreement with the present observations in K. fragilis NRRL y 2415. That is, when cells of this strain are shifted from an anaerobic to aerobic environment, filamentation correlates with increased rates of oxygen consumption (see Section 3.1.2.5). The aerobic fermentation of filamentous form of K. fragilis NRRL y 2415 was also characterised by rapid lactose utilisation and rapid ethanol production. Conversely, anaerobic fermentation of budding yeast-phase cells was characterised by lower rates of lactose consumption and ethanol production, with concomitant increased fermentation times. The current practice of aerobic fermentation of cheese whey would seem justified by these studies. These observations are consistent with the hypothesis correlating changes in cellular carbohydrate metabolism with changes in morphology. Further studies are required to determine the nature of the relationship between the patterns of lactose metabolism and the morphology of K. fragilis NRRL y 2415. Changes in the distribution of flux of carbon into lactose catabolic pathways could reveal changes in metabolic

intermediates, enzymes or end products important to the regulation of morphogenesis. The decreased rates of lactose utilisation in true-budding yeast forms compared to filamentous cultures may indicate an increased incorporation of glucose into the latter, perhaps as a response to the need of increasing cell wall components in the mycelial forms.

Preliminary findings show that temperature may have an important effect in the influence of dimorphism in *K. fragilis* NRRL y 2415 in continuous culture. Further studies are required to elucidate the precise effect of temperature on dimorphism.

In any future work on dimorphism in *K. fragilis*, it is imperative that conditions be established for the propagation of both morphological forms under continuous cultivation conditions. Any examination of the physiological regulation of differentiation in this yeast would be most meaningful when carried out under chemostat conditions where environmental parameters are controlled and maintained over long periods of time.

Considering comparable studies in *Mucor* it has been shown by Inderleid and Sypherd (1978) that aerobically grown mycelial cells displayed significantly different patterns of glucose metabolism from that of anaerobic yeast cells. Thus, aerobic mycelia were shown to catabolise glucose respiratively whilst anaerobic yeast cells fermented this sugar. In contrast to those studies in *Mucor*, and those presented here in *K. fragilis*, Land *et al.*, (1975a) suggest that in *C. albicans*, a Crabtree-like repression of mitochondrial function favours

filamentation. Clearly, there are no general rules concerning respiratory metabolism and differentiation in all dimorphic fungi.

Investigations of the underlying biochemical mechanism of yeast-filamentous dimorphism in K. fragilis NRRL y 2415 indicate that, in addition to being promoted by anaerobiosis, yeast forms of the organism are also favoured by the action of certain inhibitors of the respiratory chain or of mitochondrial protein synthesis (see Section 3.1.4). This statement could be made a different way: respiratory inhibitors inhibit filamentation. The results with mitochondrial inhibitors indicate that both the electron transport chain and a generation of respiratory energy are pre-requisites for the development of mycelial growth. Similar results have been obtained for Mycotypha (Schulz et al., 1974).

Inhibition of respiration and enhancement of fermentation appear to restrict morphological differentiation in a number of filamentous fungi and higher eukaryotic cells (Crabtree, 1929; Gupta et al.,1971; Houston et al., 1969; Koobs, 1972; Nickerson, 1949). Guillermond (1940) suggested that Y-F morphological transformation may be interpreted as a dedifferentiation to a more "primitive cell". Nickerson and Mankowski (1953) considered filamentous growth in yeasts as an uncoupling between cellular growth and division. This concept is in agreement with the views of Warburg (1968) regarding cellular differentiation in oncogenic cells, and is applicable to fungal morphogenesis. To quote Warburg: "Respiration energy creates and maintains a high differentiation

of body cells. Fermentation energy can only maintain a low differentiation. It follows that if respiration is replaced by fermentation in body cells, high differentiation must disappear". Although this thesis has concerned itself with studies of a lower eukaryote, some of the findings display corollaries with more highly "differentiated" cells.

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6. REFERENCES

AIBA, S., HUMPHREY, A.E. and MILLIS, N.F. (1973) Biochemical Engineering. London: Academic Press.

AIKING, H., STERKENBERG, A. and TEMPEST, D.W. (1977) Influence of specific growth rate limitation and dilution rate on the phosphorylation efficiency and cytochrome content of mitochondria of Candida utilis NCYC 321. ARCH. MICROBIOL. 113, 65 - 72.

ALUM, L., (1982) Effect of fermentation of lactose, glucose and galactose content in milk and suitability of fermented milk products for lactose intolerant individuals. J. DAIRY SCI. 65, 346 - 352.

ANDERSON, H.W., (1917) Yeast-like fungi in the human intestinal tract. J. INFECTIOUS DISEASES 21, 341 - 385.

BAILEY, R.B., BENITEZ, T. and WOODWARD, A. (1982) Saccharomyces cerevisiae mutants resistant to catabolite repression: use in cheese whey hydrolysate fermentation. APPL. ENV. MICROBIOL. 44, 631 - 639.

BARTNICKI-GARCIA, A. (1963) Symposium on the biochemical basis of morphogenesis in fungi 111. Mould-Yeast dimorphism in Mucor. BACTERIOL. REV. 27, 293 - 304.

BARTNICKI-GARCIA, S. (1968) Control of dimorphism in Mucor by hexoses. Inhibition of hyphal morphogenesis. J. BACTERIOL. 96, 1586 - 1594.

BARTNICKI-GARCIA, and LIPPMAN, E.(1972) The bursting tendency of hyphal tips of fungi: presumptive evidence for a delicate balance between cell wall synthesis and wall lysis in apical growth. J. GEN.MICROBIOL. 73, 487 - 500.

BARTNICKI-GARCIA, S. and McMURROUGH, I. (1971) Biochemistry of morphogenesis in yeasts. In The Yeasts, Vol 2, pp 440- 491. Edited by A.H. Rose and J.S. Harrison. London : Academic Press.

BARTNICKI-GARCIA, S. and NICKERSON W.J. (1962a) Nutrition growth and morphogenesis in Mucor rouxii J. BACTERIOL. 84, 841 - 848.

BARTNICKI-GARCIA, S. and NICKERSON W.J. (1962b) Isolation, composition and structure of cell walls of filamentous and yeast forms of Mucor rouxii . BIOCHIM. BIOPHYS. ACTA. 58, 102 - 119,

BARTNICKI-GARCIA, S. and NICKERSON W.J. (1962c) Induction of yeast-like development in Mucor by carbon dioxide.
J. BACTERIOL. 84, 829 - 840.

BAJPAI, P. and MARGARITIS, A. (1985) Immobilisation of Kluyveromyces marxianus cells containing inulinase activity in open pore gelatin matrix: 2 application for high fructose syrup production. ENZYME. MICROB. TECHNOL. 7, 459 - 461.

BAJPAI, P. and MARGARITIS, A. (1986). Ethanol production from Jerusalem artichoke juice using flocculent cells of K. marxianus.
BIOTECHNOL. LETT. 8, 361-364.

BEECHEY, R.B. and RIBBONS D.W. (1972) Oxygen uptake measurements. In Methods in Microbiology 6B, Chpt 2, pp 24 - 63. New York: Academic Press .

BIANCHI, D.E. (1968) The lipid content of cell walls obtained from juvenile yeast-like and filamentous cells of Candida albicans. J. MICROBIOL. SEROL. 33, 324 - 332.

BOURGI, J., GUIRAUD J.P. and GALZY, P. (1986) Isolation of a Kluyveromyces depressed mutant.Hyper-producer of inulinase for ethanol production from Jerusalem artichoke. J. FERMENT. TECHNOL. 64, 239 - 243.

BROWNE, C.M. and HOUGH, J.S. (1965) Elongation of yeast cells in continuous culture. NATURE 206, 676 - 678.

BROWNE, S.W. and OLIVER, S.G. (1982) The effect of temperature on the ethanol tolerance of the yeast, Saccharomyces uvarum. BIOTECHNOL. LETT. 2, 269 274.

BULL, A.T. and BROWN, C.M. (1979) Continuous culture applications to microbial biochemistry. In International Review of Biochemistry, chpt 5, pp 177 - 206. Edited by J.R.Quayle. Baltimore : University Park Press.

BULLERMAN L.B and BERRY E.C (1966) Use of cheese whey for vitamin B_{12} production 111. Growth studies and dry weight activity. APPL. MICROBIOL. 14, 358 -360.

BURGESS, K.J. and KELLY J. (1979) Alcohol production by yeast in concentrated whey ultrafiltration permeate from cheddar cheese. IR. J. FOOD SCI. TECHNOL. 3, 1 - 9.

CASSIDA, L.E. (1968) Industrial Microbiology. New York : John Wiley.

CASTILLO, F.J., IZAGUIRRE, M.E., MICHELENA, V. and MORENO, B. (1982) Optimisation of fermentation conditions for ethanol production from whey. BIOTECHNOL. LETT. 4, 567 - 572.

CHATTAWAY, F.W., HOLMES, M.R. and BARLOW,(1968) Cell wall composition of the mycelial and blastospore forms of Candida albicans. J. GEN. MICROBIOL. 51, 367 - 376.

CHATTAWAY, F.W., BISHOP, R., HOLMES, M.M. Odds, F.C. and BARLOW, A.J.E. (1973) Enzyme activities associated with carbohydrate synthesis and breakdown in yeast and mycelial forms. J. GEN. MICROBIOL. 75, 97 - 109.

CHEN, H.C. and ZALL, R.R. (1982) Continuous fermentation of cheese whey into alcohol using an attached film expanded bed reactor. PROCESS BIOCHEM. 17, 20 - 25.

CHERYAN, M. and MEHAIA, M.A. (1983) A high performance membrane bioreactor for continuous fermentation of lactose to ethanol. BIOTECHNOL. LETT. 5, 519 - 524.

CLARKE-WALKER, G.D. and LINNANE, A.W. (1967) The biogenesis of mitochondria in Saccharomyces cerevisiae. A comparison between cytoplasmic respiratory deficient mutant and chloramphenicol-inhibited wild type cells.
J. CELL. BIOL. 34, 1 - 14.

COOPER, T.J. (1977) Potentiometric techniques, in : Tools of Biochemistry pp 30 - 35. Edited by T.G. Cooper. New York : John Wiley.

COTON, S.J. (1980) The utilisation of permeates from the ultrafiltration of whey and skim milk.
J. SOC. DAIRY TECHNOL. 33, 89 - 94.

CORTAT, M., MATILE, P. and WIEMKEN, A. (1972) Isolation of glucanase containing vesicles from budding yeast.
ARCH. MICROBIOL. 82, 189 - 205.

CRABTREE, H.G. (1929) Observations on carbohydrate metabolism of tumours.
BIOCHEM. J. 23, 536 - 545.

CURTIS, N.S. and CLARKE, A.G. (1960) New yeast culture plant.
J. INST. BREW. 66, 287 - 292.

DABROWA, N. TAXER, S.S. and HOWARD, D.H. (1976) Germination of Candida albicans induced by proline.
INFECT. IMMUN. 13, 830 - 835.

DEMOTT, B.J., DRAUGHTON, F.A. and HERALD, P.J. (1981) Fermentation of lactose in direct-acid set cottage cheese whey.
J. FOOD PROTECTION. 44, 588 - 590.

DEMPSEY, R. (1985) Personal communication.
Dept. of Biology, Maynooth College, Kildare.

DIDDENS, H.A. and LODDER, J. (1942) Die Anaskosporogenen Hefen, 11 Halfte. Amsterdam : North Holland. Publishing Company.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A and SMITH, F. (1956) Colorimetric method for the determination of sugars and related substances.
ANAL. CHEM. 28, 350 - 356.

ELMER A.W. and NICKERSON, W.J. (1970) Nutrional requirements for growth and yeast-like development of Mucor rouxii under carbon dioxide.
J. BACTERIOL. 101, 593 - 602.

FIECHTER, A. and von MEYENBURG, H.K. (1966) In Proceedings of Second International Symposium on Yeast, pp 387 - 398. Edited by A. Kockova-Kratochrilova. Bratislava, Stovenskerg, Vied.

FRIEND, B.A. and SHANANI K.M. (1979) Whey fermentation
N.Z.J. DAIRY SCI. TECHNOL. 14, 143 - 152.

GAWEL, J. and KOSIKOWSKI, F.V. (1978) Improving alcohol fermentation in concentrated ultrafiltration permeates of cottage cheese whey.
J. FOOD SCI. TECHNOL. 43, 1717 - 1719.

GEKAS, V. and LÓPEZ - LEIVA, M., (1985) Hydrolysis of lactose: A literature review.
PROCESS BIOCHEM. 20, 2-12.

GUPTA, R.K. and HOWARD, D.H. (1971) Comparative physiological studies on the yeast and mycelial forms of Histoplasma capsulatum : uptake and distribution of L - leucine. J. BACTERIOL. 105, 690 - 700.

GUILLERMOND, A. (1940) Sexuality, developmental cycle and phylogeny of yeasts. BOT. REV. 6, 1 - 24.

HAHN - HAGERDAL, B. (1985) Comparison between immobilised K. fragilis and Saccharomyces cerevisiae co-immobilised with β-Galactosidase with respect to continuous ethanol production from concentrated whey permeate. BIOTECHNOL. BIOENG. 27, 914 -916 .

HAIDLE, C.W. and STORCK, R. (1966) Control of dimorphism in Mucor rouxii. J. BACTERIOL. 92, 1236 - 1244.

HALTER, D.M. (1973) M.Sc. Thesis. Morphology of Saccharomyces fragilis and Rhodotorula gracilis yeasts grown in cottage cheese whey. Oklahoma State University, Stillwater, Oklahoma, U.S.A.

HANLEY, S. and YOCUM, R. (1986) Expression of the lactose utilisation genes of K. lactis in S. cerevisiae, In : Book of abstracts from the thirteenth international conference on yeast genetics and molecular biology, Banff, Alberta, Canada. Edited by R.C. von Borstel and T.G. Cooper. New York : John Wiley. YEAST, 2, S 148 .

HAYASHIDA, S. and OHTA, K. (1980) Effect of phosphatidyl-choline or ergosterol oleate on physiological properties of Saccharomyces saké.
AGRIC. BIOL. CHEM. 44, 2561 - 2567.

HERBERT, D. Some principles of continuous culture, (1959) In Recent Progress in Microbiology, pp 381 - 396 Edited by G.Tunevall. Oxford : Blackwell Scientific Publications.

HOUSTON, M.R., MEYER K.H., THOMAS, N. and WOLF, F.T. (1969) Dimorphism in Cladosporium wernickii.
SABOURAUDIA 7, 195 - 198.

INDERLEID, C.B. and SYPHERD, P.S. (1978) Glucose metabolism and dimorphism in Mucor.
J. BACTERIOL. 133, 1282 - 1286 .

INGRAM, L.O. (1982) Regulation of fatty acid composition in Escherichia coli : a proposed mechanism for changes induced by ethanol, chaotrophic and a reduction of growth temperature.
J. BACTERIOL. 149 , 166 - 172.

IZAGUIRRE, M.E. and CASTILLO,F.J. (1982) Selection of lactose fermenting yeasts for ethanol production from whey.
BIOTECHNOL. LETT. 4, 257 - 262.

JANSSENS J.H., BURRIS, N., WOODWARD, A. and BAILEY, R.B. (1983) Lipid enhanced ethanol production by Kluyveromyces fragilis.
APPL. ENV. MICROBIOL. 45, 598 - 602.

JANSSENS, J.H., BERNARD, A. and BAILEY, R.B. (1984) Ethanol from whey : continuous fermentation with cell recycle. BIOTECHNOL. BIOENG. 26, 1 - 5.

JOHNSON, M.J. (1969) Microbial cell yields from various hydrocarbons. In Fermentation Advances pp 833 - 847. Edited by D. Perlman. London, New York : Academic Press.

JONES, M. and PIERCE, J.S. (1964) Absorption of amino acids from wort by yeast. J. INST. BREW. 70, 307 - 315.

JORGENSEN, A. (1909) Die Mikro organismen der Garung industrie ste Auflage, P. Parey, Berlin.

KIERSTAN, M. and BUCKE, C. (1977) The immobilisation of microbial cells subcellular organelles and enzymes in calcium alginate gels. BIOTECHNOL. BIOENG. 19, 387 - 397.

KING V.A.E. and Zall, R.R. (1983) Ethanol fermentation of whey using calcium alginate entrapped yeasts. PROCESS BIOCHEM. 18, 17 - 20.

KOOBS, D.H. (1972) Phosphate mediation of the Crab tree and Pasteur effects. SCIENCE 178, 127 - 133.

KOSIKOWSKI and WZORECK. (1977) Whey wines from concentrates of reconstituted and whey powder. J. Dairy. SCI. 62, 1982 - 1986.

KREGER-van RIJ, N.J.W. (1984) The yeasts, a taxonomic study. Edited by N.J.W. Kreger-van Rij. Amsterdam : Elsevier Science Publications.

LAHAM-GUILLAUME, M., MOULIN, G. and GALZY, P. (1979) Sélection de Souches de levures en vue de la production d'alcool sur lactosérum.
LE LAIT. 59, 489 - 496.

LAMB, A.J., CLARKE-WALKER, G.D. and LINNANE, A.W. (1968) The biogenesis of mitochondria. The in vitro differentiation of mitochondrial and cytoplasmic protein synthesising systems by antibiotics.
BIOCHEM. BIOPHYS. ACTA. 161, 415 - 442.

LAND, G.A., McDONALD, W.C., STJERNHOLM and FRIEDMAN, L. (1975a) Factors affecting filamentation in Candida albicans. Relationship of the uptake and distribution of proline to morphogenesis.
INFECT. IMMUN. 11, 1014 - 1023.

LAND, G.A., McDONALD, W.C. and STJERNHOLM, R.L. and FRIEDMAN, L. (1975b)
Factors affecting filamentation in Candida albicans changes in respiratory activity during filamentation.
INFECT. IMMUN. 12, 119 - 127.

LANGERON, M. and TALICE, R.V. (1932) Nouvelles méthodes d'étude et essai de classification des champignons levuriformes.
ANN.PARASITOL.HUM. COMP. 10, 1 - 80.

LEE, J.H., WILLIAMSON, D. and ROGERS, P.L. (1980) The effect of temperature on the kinetics of alcohol production by Saccharomyces uvarum.
BIOTECHNOL. LETT. 2, 141 - 146.

LEE, K.J., SKOTNICKI, M.L., TRIBE, D.E. and ROGERS, D.L. (1981) The effect of temperature on the kinetics of ethanol production by strains of Zymomonas mobilis
BIOTECHNOL. LETT. 3, 291 - 296.

LEVINE, S. and ORDAL, Z.J. (1946) Factors influencing the morphology of Blastomyces dermatitidis. J. BACTERIOL. 52, 687 - 694.

LIGHT, P.A. and GARLAND, P.B. (1971) A comparison of mitochondria of Torulopsis utilis grown in continuous culture with glucose, ammonium, magnesium on phosphate as the growth limiting nutrient.
BIOCHEM. J. 124, 127 - 134.

LINDEGREN, C.C. and HAMILTON, E. (1944) Autolysis and sporulation in the yeast colony.
BOTAN. GAZ. 105, 316 - 321.

LINKO, Y.Y. and LINKO, P. (1981) Continuous ethanol production by immobilised yeast reactor.
BIOTECHNOL. LETT. 3, 21-26.

LINKO, Y.Y., JALANKA, H. and LINKO, P. (1981a) Ethanol production from whey with immobilised living yeast.
BIOTECHNOL LETT. 3, 263 - 268.

LINKO, P. SORVARI, M. and LINKO, Y.Y. (1981b) Ethanol production with immobilised cell reactors.
ANN. N.Y. ACAD. SCI. 413, 424 - 434.

LINKO, Y.Y., SORVARI, M. LINKO, P., HARJU, M. and HEIKONEN, M. (1984) Experiences on continuous ethanol production from whey lactose with immobilised Kluyveromyces fragilis cells. In: Conference proceedings from the third European congress on Biotechnology, Munich, W. Germany.
Weinheim : Verlag Chemie GmbH .

LODDER, J. and KREGER-van RIJ, N. J.W. (1952) The Yeasts, a Taxonomic Study.
Amsterdam: North Holland Publishing Company.

LUSCOMBE B.M. and GRAY T.R.G. (1971) Effect of varying growth rate on the morphology of Arthobacter
J. GEN. MICROBIOL. 69, 433 - 434.

LUSCOMBE, B.M. and GRAY T.R.G. (1974) Characteristics of Arthobacter grown in continuous culture.
J. GEN. MICROBIOL. 82, 213 - 222.

MADDOX, I.S. (1980) Production of n-Butanol from whey filtrate using Clostridium acetobutylicum NCIB 2951.
BIOTECHNOL. LETT. 2, 493 - 498.

MAHAMOUD, M.M. and KOSIKOWSKI, F.V. (1982) Alcohol and single cell protein production by Kluyveromyces in concentrated whey permeates with reduced ash.
J. DAIRY SCI. 65, 2082 - 2087.

MAHONEY, R.R., NICKERSON, T.A. and WHITTIER, J.R. (1974) Selection of strain growth conditions and extraction procedures for optimum production of lactase from K. fragilis.
J. DAIRY SCI. 58, 1620 - 1629.

MARCHANT, R., and SMITH, D.G. (1968) The effect of chloramphenicol on growth and mitochondrial structure of Pythium ultimum.
J. GEN. MICROBIOL. 50, 391 - 397.

MARDON, D.N., BALISH, E. and PHILLIPS, A.W. (1969) Control of dimorphism in a biochemical variant of Candida albicans.
J. BACTERIOL. 100, 701 - 707.

MARTH, E.H. (1970) Fermentation products from whey.
In By-products from Milk, Chpt. 3, pp 43 - 82
Edited by B.H. Webb and E.P. Whittier.
Westport, Co. : AVI Publishing Company.

MARWAHA, S.S., KENNEDY, J.F. and SETHI,R.P. (1983)
Vitamin B_{12} production from whey and stimulation
of optimal cultural conditions.
PROCESS BIOCHEM. 18, 24 - 27.

MARWAHA, S.S. and KENNEDY, J.F (1984a) Alcohol production
from whey permeate by immobilised and free cells
of Kluyveromyces marxianus NCYC 179
PROCESS BIOCHEM. 19, 79 - 80.

MARWAHA, S.S. and KENNEDY, F.F. (1984b) Ethanol production
from whey permeate by immobilised yeast cells.
ENZ. MICROB. TECHNOL. 6, 18 - 22.

MAYES, P.A. (1981) Metabolism of carbohydrate. In Harper's
Review of Biochemistry. Chpt 15, pp 160 - 185.
Edited by D.W. Martin, P.A. Mayes and V.W. Rodwell.
California: Lange Medical Publications.

McMURROUGH and ROSE, A.H. (1967) Effect of growth rate
and substrate limitation on the composition and
structure of the cell wall of S. cerevisiae.
BIOCHEM. J. 105, 189 - 203.

MEYRATH, J. and BAYER, K. (1979) Biomass from whey.
In Economic Microbiology chpt. 4, pp, 207 - 269.
Edited by A. H. Rose, London: Academic Press.

MILLER, T.L. (1959) The use of dinitrosalicylic acid
reagent for the determination of reducing sugars.
ANAL. CHEM. 31, 426 - 428.

MONOD, J. (1942) Recherches sur la croissance des cultures bacteriennes. Paris: Herman & Cie.

MOR, J.R. and FIECHTER, A. (1968) Continuous cultivation of Saccharomyces cerevisiae 1. Growth on ethanol under steady-state conditions.
BIOTECHNOL. BIOENG. 10, 159 - 176.

MORPURGO, G., SERLUPI-CRESCENZI, G., TECCE, G., VALENTE, F. and VENETTACCI, D. (1964) Influence of ergosterol on the physiology and ultra-structure of Saccharomyces cerevisiae.
NATURE 201, 897 - 899.

MOULIN, G. and Galzy P. (1981) Alcohol production from whey, In Advances in Biotechnology, vol 2 pp 181-189. Edited by M. Moo-Young, New York: Pergamon Press.

MOULIN, G. and GALZY, P. (1984) Whey, a potential substrate for biotechnology. In Biotechnology and Genetic Engineering Reviews, vol. 1 pp 347 - 373. Edited by G.E. Russell, Newcastle Upon Tyne: Intercept.

MOULIN, G., GUILLAUME, M. and GALZY, P. (1980) Alcohol production by yeast in whey ultrafiltrate.
BIOTECHNOL. BIOENG. 22, 1277 - 1281.

MOULIN, G., MALIGE, B. and GALZY, P. (1981) Etude physiologique de Kluyveromyces fragilis: consequence sur la production de levure sur lactoserum.
LE LAIT 61, 323 - 332.

MOULIN, G., LEGRAND, M. and GALZY, P. (1983) The importance of residual aerobic fermentation in aerated medium for the production of yeast from glucidic substrates. PROCESS BIOCHEM. 18, 5 - 8.

NICKERSON, W.J. (1954) Experimental control of morphogenesis in microorganisms. ANN. N.Y. ACAD. SCI. 60, 50 - 57.

NICKERSON, N.J. (1963) Symposium on the biochemical basis of morphogenesis in fungi IV. Molecular basis of form in yeasts. BACTERIOL. REV. 27, 305 - 324.

NICKERSON, W.J. and EDWARDS, G.A. (1949) Studies on the physiological basis of morphogenesis in fungi I. The respiratory metabolism of dimorphic fungi. J. GEN. PHYSIOL. 33. 41 - 55.

NICKERSON, W.J. and FALCONE, G. (1956) Identification of protein disulphide reductase as a cellular division enzyme in yeast. SCIENCE 124, 722 - 723 .

NICKERSON, W.J. and FALCONE, G. (1959) Function of protein disulphide reductase in cellular division in yeasts. In Sulphur in Proteins pp 409 - 424. Edited by R. Benesch. London : Academic Press.

NICKERSON, W.J. and MANKOWSKI, Z.T. (1953) Role of nutrition in the maintenance of yeast shape in Candida albicans. AMERICAN J. BOT. 40, 584 - 592.

NISBET, B. (1979)PhD Thesis ; NH_4^+ assimilation in Kluyveromyces fragilis. Heriot-Watt University, Edinburgh.
EH1 1HX , Scotland.

ODDS, F.C (1979) Morphogenesis in Candida with special reference to C. albicans. In Candida and Candidosis, chpt 4, pp 29-41.
Edited by F.C. Odds. Leicester University Press.

O'LEARY, V.S., GREEN, R., SULLIVAN, B.C. and HOLSINGER, V.H. (1977a). Alcohol production by selected yeast strains in lactose hydrolysed acid whey.
BIOTECHNOL. BIOENG. 19, 1019 - 1036.

O'LEARY, V.S., SUTTON, C., BENCIVENGO, M., SULLIVAN, B. and HOLSINGER. (1977b) Influence of lactose hydrolysis and solids concentration on alcohol production by yeast in whey ultrafiltrate.
BIOTECHNOL. BIOENG. 19, 1689 - 1707.

PANCHAL, C.J. and STEWART, G.G. (1980) The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain.
J. INST. BREW. 86, 207 - 210.

PEPPLER, H.F. (1970) Food Yeasts. In the Yeasts, vol. 3 pp 421 - 462. Edited by A.H. Rose and J.S. Harrison. London : Academic Press.

PHAFF, H.J. (1966) α- 1,4 - Polygalacturonide glycanohydrolase from Saccharomyces fragilis. In Methods in Enzymology, vol 8, pp 636 - 641.
Edited by E.F. Neufeld and V.Gunsburg,
New York: Academic Press.

PIRT, S.J. (1975) Principles of Microbes and Cell Cultivation Oxford Blackwell Scientific Publications.

ROGOSA, M., BROWNE, H.M. and WHITTIER, E.D. (1947) Ethyl alcohol from whey.
J. DAIRY SCI. 30, 263 - 669.

ROMANO, A.H. (1966) Dimorphism. In The Fungi , vol 2, pp 181 - 209. Edited by G.C Ainsworth and A.S. Sussman. New York : Academic Press.

SAN-BLAS, G. and SAN-BLAS, F. (1984) Molecular aspects of fungal dimorphism.
CRC. CRITICAL REV. MICROBIOL. 11, 101 - 127.

SCHERR, G.H. and WEAVER, R.H. (1953) The dimorphism phenomenon in yeasts.
BACTERIOL. REV. 17, 51-92.

SCHULZ, B.E., KRAEPELIN, G. and HINKELMAN, W. (1974) Factors affecting dimorphism in Mycotypha (Mucorales). Correlation with fermentation respiration equilibrium.
J. GEN. MICROBIOL. 82, 1 - 13.

SEGAL, R.B. (1939) The effect of higher alcohols on yeast. (Russian)
MIKROBIOLOGIKA 8, 466 - 470.

SHEPHERD, M.G. and SULLIVAN, P.A. (1976) The production and growth characteristics of yeast and mycelial forms of Candida albicans in continuous culture.
J. GEN. MICROBIOL. 93, 361 - 370.

SLONIMSKI, P. (1953) Formation des enzymes respiratoires chez la levure.
Paris : Masson.

STELLING-DEKKER, N.M. (1931) Die Sporogenen Hefen.
VERHANDL. KONINKL. NED. AKAD.
WETEMSCHAF.; AFDEL. NATUURK., 28, 1 - 574 .

STEWART, R.R. and ROGERS, P.J. (1978) Fungal dimorphism: a particular expression of cell wall morphogenesis. In Filamentous Fungi, vol 3, pp 164 - 196. Edited by J.E. Smith and D.R. Berry. London : Edward Arnold Ltd.

STEWART C.G. and RUSSEL, I. (1983) Aspects of the biochemistry and genetics of sugar and carbohydrate uptake by yeasts, In Yeast Genetics; Fundamental and Applied Aspects,chpt. 15, pp 461 - 484 Edited by J.F.J. Smith, New York : Springer Verlag.

STORCK, R. and MORRILL, R.C. (1971) Respiratory - deficient yeast-like mutant of Mucor. BIOCHEM. GEN. 5, 467 - 479.

TEMPEST, D.W. (1970a) The continuous culture of micro-organisms, I : Theory of the chemostat. In Methods in Microbiology, vol 2, pp 259 - 276. Edited by J.R. Norris and D.W. Ribbons. New York : Academic Press.

TEMPEST, D.W. (1970b) The place of continuous culture in microbiological research, In Advances in Microbial Physiology , 4, 223 - 250.

TEMPEST, D.W. and NEIJSSEL, O.M. (1976) Microbial adaption to low nutrient environments. In continuous culture 6 : Applications and New Fields, Chpt. 22, pp 283 - 296. Edited by A.C.R. Dean, D.E. Ellwood, G.J. Evans and J. Melling. Chichester : Ellis Horwood.

TERENZI, N.F. and STORCK, R. (1969) Stimulation of fermentation and morphogenesis in Mucor rouxii by phenylethyl alcohol.
J. BACTERIOL. 97, 1248 -1261.

TERREL, S.L., BERNARD, A. and BAILEY, R.B. (1984) Ethanol from whey: Continuous fermentation with a catabolite resistant 1. Saccharomyces cerevisiae mutant.
APPL.ENV. MICROBIOL. 48, 577 - 580.

THOMAS, D.S., HOSSAK, J.A. and ROSE, A.H. (1978) Plasma-membrane lipid composition and ethanol tolerance in Saccharomyces cerevisiae.
ARCH. MICROBIOL. 117, 239 - 245.

TYAGI, R.D. (1984) Participation of oxygen in ethanol fermentation.
PROCESS BIOCHEM. 19, 136 - 141.

VAN DER WAALT, J.P. (1970) Kluyveromyces van der Waalt emend van der Waalt. In The Yeasts, pp 316-378.
Edited by J. Lodder.
Amsterdam and London: North Holland Publishing Company.

VAN GELDER, B.F. (1966) The extinction coefficient of cytochrome a and cytochrome a_3.
BIOCHIM. BIOPHYS. ACTA. 118, 36 - 46.

VON MEYENBURG, H.K. (1969) Energetics of the budding cycle of Saccharomyces cerevisiae during glucose limited aerobic growth.
ARCHIV. FUR. MIKROBIOLOGIE. 66, 289 - 303.

VON MEYENBURG, H.K. (1973) In Biological and Biochemical Oscillations, pp 411 - 417.
Edited by B. Chance, E.K. Pye, T.K. Ghosh and B. Hess. New York : Academic Press.

VELDKAMP, H. (1968) Bacterial physiology. In Ecology of soil Bacteria, pp 201 - 219.
Edited by T.R.G. Gray and D. Parkinson.
Liverpool: Liverpool University Press.

VIENNE and VON STOCKAR, U. (1983) Alcohol from whey permeate, strain selection temperature and medium optimisation. BIOTECHNOL. BIOENG. SYMP. 13, 421 - 435.

VIENNE, P. and VON STOCKAR, U. (1985a) An investigation of ethanol inhibition and other limitations occuring during the fermentation of concentrated whey permeate by Kluyveromyces fragilis. BIOTECHNOL. LETT. 7, 521- 526.

VIENNE, P. and VON STOCKAR, U (1985b) Metabolic, physiological and kinetic aspects of the alcoholic fermentation of whey permeate by Kluyveromyces fragilis NRRL y 665 and Kluyveromyces lactis NCYC 571 ENZYME MICROB. TECHNOL 7, 287 - 294 .

WAIN, W.H., PRICE, M.F. and CARSON R.A., (1975) A re-evaluation of the effect of cysteine on Candida albicans. SABOURAUDIA 13, 74 - 82.

WANG, D.I.C and FEWKES, R.C.J. (1977) Effect of operating and geometric parameters on the behaviour of non-newtonian mycelial antibiotic fermentations. DEV. IND. MICROBIOL. 18, 39 - 56.

WANG, D.I.C., COONEY, C.L. DEMAIN, A.L., DUNILL, P., HUMPHREY, A.E. and LILLY, M.D. (1979) Translation of laboratory pilot, plant scale data. In Fermentation and Enzyme Technology, chapter 10, pp 194 - 211. New York : John Wiley.

WARBURG, O., GEISSLER, A.W. and LORENG, S. (1968) Oxygen, the creator of differentiation. pp 327 - 375. In Aspects of Yeast Metabolism. Edited by A.K. Mills. Philadelphia : L.F.A. Davies.

WENDORFF, W.L., AMUNDSON, C.H. and OLSON, N.F. (1970) Nutrient requirements for production of lactase enzyme by Saccharomyces fragilis J. MILK FOOD TECHNOL. 33, 451 - 455.

WICKERHAM, L.J. (1951) Taxonomy of Yeasts. Technical Bulletin No. 1029. United States Department of Agriculture.

WICKERHAM, L.J. and BURTON (1948) Carbon assimilation tests for the classification of yeasts. J.BACTERIOL. 56, 363 - 371.

WICKERHAM L.J. and BURTON, K.A. (1956) Hybridisation studies involving Saccharomyces lactis and Zygosaccharomyces ashbyii. J. BACTERIOL. 71, 290 - 295.

WILHARM, G. and SACK, U. (1947) The properties of several lactose fermenting yeasts. MILCHWISSENSCHAFT. 2, 382 - 389.

WILSON, D. and EPEL, D. (1968) The cytochrome system of sea urchin sperm. ARCH. BIOCHEM. BIOPHYS. 126, 83 - 90.

WINSTEIN, S. and MURRAY, T.S. (1956) Virulence enhancement of a filamentous strain of Candida albicans after growing on media containing cysteine. J. BACTERIOL. 71, 738.

ZERTUCHE, L. and ZALL, R.R. (1985) Optimising alcohol production from whey using computer technology. BIOTECH. BIOENG. 27, 547 - 554.

ZORZOPULOS, J., JOBBAGY, A.J. and TERENZI,H.F. (1973) Effects of chloramphenicol on mitochondrial activity and morphogenesis of Mucor rouxii. J. BACTERIOL. 115, 1198 - 1203.