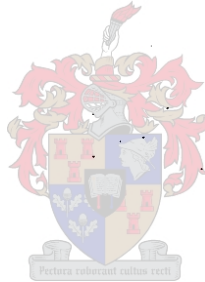


**The Role Of Competition For Glucose Amongst Oral
Species In The Maintenance Of *Candida albicans*
Homeostasis In A Mixed Microbial Habitat**

by

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

OPSOMMING

Kandidose van die mondholte is een van die algemeenste fungale infeksies wat by die mens voorkom. Dit manifesteer in 'n verskeidenheid van vorms en word in die meeste gevalle deur die gis *Candida albicans* veroorsaak. Die algemene gebruik van antibiotika, immuunonderdrukkende middels, die verhoging van die populasie van bejaardes en die toename in VIGS gevalle is almal faktore wat vir 'n verhoogde bewustheid van mond-kandidose onder klinici verantwoordelik is. Dit het daartoe gelei dat 'n aantal studies oor kandidose en oor die meganismes waardeur die siekte veroorsaak word, gedoen is. Alhoewel dit algemeen bekend is dat *C. albicans* 'n opportunistiese patoëen is, is daar nog weinig bekend oor die meganismes wat die opportunistiese optrede van die gis beheer en die rol wat die outoëtone mondbakterieë in dié verband speel.

Dit het oor die laaste aantal jare duidelik geword dat die outoëtone spesies van die spysverteringskanaal as 'n skans teen koloniserings deur alloëtone spesies optree en sodoende as 'n belangrike verdedigingsmeganisme funksioneer. Daar is ook voorgestel dat 'n kompetisie vir groei-beperkende faktore soos glukose tussen die outoëtone spesies en die alloëtone spesies, een van die primêre meganismes is waarvolgens so 'n skans bewerkstellig word.

Die welbekende teorie oor die werking van die chemostaat tesame met die voordeel van 'n "oop sisteem" maak die chemostaat geskik vir langtermyn kwantitatiewe studies wat betref die gedrag en die samestelling van mikrobiële populasies. In hierdie studie is die chemostaat as 'n *in vitro* model gebruik om die toestande wat nodig is vir die samebestaan van *C. albicans* in 'n gemengde populasie van mondbakterieë wat vir dieselfde gemeenskaplike

substraat kompeteer, te ondersoek. Eerstens is 'n model wat uit 'n verskeidenheid van mondbakterieë bestaan onder toestande soortgelyk aan die van die mondholte gevestig. Die model moes in staat wees om die groei van *C. albicans* te kan onderdruk en is gebruik om vas te stel onder watter toestande die uitwas van die gis plaasvind en onder watter toestande die gis groei. Vervolgens is die groeiparameters van die organismes bepaal om hul teoretiese versadigingskurwes op te stel. Die kurwes is gebruik om te bepaal watter organismes teen die gis kompeteer en om te voorspel wat die uitkoms van kompetisie tussen die spesies by verskillende glukose konsentrasies is.

Sewe mondbakterieë insluitende, *Streptococcus sanguis*, *Streptococcus sobrinus*, *Streptococcus mitis*, *Lactobacillus rhamnosus*, *Veillonella parvula*, *Eubacterium saburreum* en *Fusobacterium nucleatum* het onder toestande van glukose tekort in die chemostaat gevestig. Die gis kon nie onder dié toestande met die bakterieë kompeteer nie. Die gis kon egter wel onder toestande van oormaat glukose, waar daar slegs drie mondbakterieë voorgekom het, in die chemostaat vestig. Die drie spesies *S. sanguis*, *S. mitis* en *S. sobrinus* is geïdentifiseer as die organismes wat die vestiging van die gis onderdruk. 'n Verandering in die glukose konsentrasie (0-20mg/ml) sal geen effek op die uitkoms van die kompetisie tussen hierdie drie bakterieë en die gis uitoefen nie en die gis sal altyd in hul teenwoordigheid geëlimineer word.

Die gevolgtrekking van die studie is dat by glukose konsentrasies soortgelyk aan die van die mondholte, by 'n neutrale pH en anaerobiese toestande, die organismes *S. sanguis*, *S. mitis* en *S. sobrinus* die kolonisering en groei van *C. albicans* in 'n gemengde kultuur sal voorkom. Hierdie onderdrukking van groei is as gevolg van die onvermoë van die gis om in 'n gemengde kultuur met die bakterieë vir glukose te kompeteer.

ABSTRACT

Oral candidosis is one of the commonest fungal infections of man and manifests itself in a variety of ways. The widespread use of antibiotics, immunosuppressive drugs, the increasing proportion of the elderly population and the increasing number of AIDS patients, all resulted in an increased awareness of oral candidosis amongst clinicians. This led to the large number of studies on candidosis and the pathogenic mechanisms of *Candida albicans* that has been done. However, little is known about the mechanisms that control the opportunistic behaviour of *C. albicans* in the oral cavity and especially of the role of the autochthonous bacteria in this regard.

Over recent years it has become clear that the autochthonous species throughout the digestive tract have a barrier effect against allochthonous species and acts as an important defence mechanism against such species. It has been hypothesised that one of the primary mechanisms that affords a colonisation resistance by the indigenous populations is a competition for growth limiting substrates, specifically for glucose, amongst the species.

The well-defined theory of the chemostat, together with its benefits as an open system, allows long-term quantitative studies of the behaviour and composition of microbial communities. The chemostat can be used to study the conditions required for the coexistence of stable populations of more than one species competing for common substrates. With this study the chemostat was used as an *in vitro* model to investigate the role of competition for glucose amongst oral species in preventing the growth of *C. albicans* in a mixed culture of oral bacteria.

A chemostat model comprising a mixture of oral bacteria that suppressed the growth of *C. albicans* under growth conditions similar to that of the oral cavity was first established. This model was used to grow oral bacteria with the yeast under glucose limiting and glucose excess conditions in order to establish under what conditions washout or growth of the yeast occurred. The growth parameters of the organisms were estimated and their theoretical saturation curves plotted in order to identify the bacteria that competed against the yeast. The theoretical saturation curves were used to predict the outcome of competition amongst the species at different glucose concentrations.

Seven bacterial species including *Streptococcus sanguis*, *Streptococcus sobrinus*, *Streptococcus mitis*, *Lactobacillus rhamnosus*, *Veillonella parvula*, *Eubacterium saburreum* and *Fusobacterium nucleatum* were able to establish in the chemostat under glucose limiting conditions. The yeast was unable to compete with these bacteria under glucose limitation but could establish under glucose excess conditions. The three species *S. sanguis*, *S. mitis* and *S. sobrinus* were identified as the organisms able to afford a colonisation resistance. At glucose concentrations below 3 mg/ml a change in glucose concentration would not have an effect on the outcome of competition between the bacteria and the yeast.

In conclusion, at glucose concentrations found in the oral cavity, at a neutral pH and under anaerobic conditions, *S. sanguis*, *S. mitis* and *S. sobrinus* will afford a colonisation resistance or prevent the growth of *C. albicans*. This colonisation resistance will be afforded through competition for glucose amongst the oral species and the yeast when grown in a mixed community of the species.

*Do not ask to have your life's load lightened,
But for courage to endure.*

*Do not ask for fulfillment in all your life,
But for patience to accept frustration.*

*Do not ask for perfection in all you do,
But for the wisdom not to repeat mistakes.*

*And finally, do not ask for more,
Before saying "Thank You"
for what you have already received.*

Brenda Short

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Chapter 1

INTRODUCTION

The first exposure to fungi that most humans experience occurs during birth when they come across yeasts while passing through the vaginal canal. During this process yeasts such as *Candida albicans* colonise the oral cavity and portions of the upper and lower gastrointestinal tract of the new-born where it can maintain a lifelong residence as a commensal (Rosebury, 1962). However *C. albicans* is also an opportunistic pathogen that may cause disease and the most important source for *C. albicans* infections in human disease is endogenous. Fortunately, yeast infection as opposed to yeast carriage, is a comparatively rare event. In normal circumstances an equilibrium between the host and the yeast flora ensures the avirulent commensal status of the yeast. Only when this equilibrium is disturbed does the yeast act as an opportunistic pathogen and cause disease (Odds, 1988).

Candidosis* is the name used to describe infections caused by yeast species of the genus *Candida*. Candidosis of virtually every tissue in the human body have been reported. Superficial lesions, particularly infections of the mucous surfaces of the mouth or vagina are by far the most common manifestations of candidosis and are commonly known as "thrush".

* In pathology the suffix "-iasis" is reserved for parasitic infections. The term candidiasis is therefore imprecise. The term candidosis is correct, as the suffix "-osis" reflects the fungal aetiology of this lesion (Odds, 1988). This term candidosis will be used throughout this dissertation.

Thrush, as a clinical entity, has been recognised since antiquity. Hippocrates (460-377 BC) described two cases of oral aphthae, probably meaning thrush, associated with severe underlying diseases. The initial discovery of the organism involved was made in 1839 when Langenbeck described a fungus in oral aphthae in a case of typhus, but the relationship between the thrush fungus and mouth lesions was only properly described in 1846 by Berg. In 1890 Zopf named the thrush fungus *Monilia albicans* which gave rise to the name "moniliásis" for infections due to the fungus. Meanwhile other forms of candidosis had been discovered. In 1849 Wilkinson described vaginal candidosis and in 1862 Zenker described a case of systemic candidosis (reviewed by Odds, 1988). In 1875 Haussmann demonstrated that the causative organism in both oral and vaginal candidosis was the same (Lynch, 1994). Castellani made extensive investigation of yeast mycoses early in the twentieth century and was the first to have suggested that yeast species other than "*M. albicans*" might be involved in the pathogenesis of candidosis. He made the first descriptions of the yeasts currently known as *Candida guilliermondii*, *Candida kefyr*, *Candida krusei*, and *Candida tropicalis* and frequently placed his new species in the genus *Monilia*. This complicated the taxonomic confusion of the genus at that time. However, Berkhout clarified the taxonomic position of the medically important yeasts in 1923. She proposed the name "Candida" for the medically important anascosporogenous "*Monilia*" yeast species that can develop pseudohyphae. This name was then later formerly adopted internationally for the genus (reviewed by Odds, 1988).

New species associated with human disease continue to be reported from time to time and there are now at least 17 species of *Candida* that have been shown to cause disease in humans (Hazen, 1995). Of these the species *C. albicans*, *C. kefyr*, *Candida parapsilosis*, *C. tropicalis*, *Candida viswanathii*, *Candida glabrata*, *C. guilliermondii* and *C. crusei* satisfied the traditional postulates of Koch. These species are therefore the ones that get greatest attention

as potential pathogens, particularly in patients with severely depressed antimicrobial defences (Odds, 1988).

Many yeast species have also been isolated from the oral cavity. The majority of these isolates were members of the genus *Candida*, and the most prevalent species was *C. albicans*. It is generally accepted that *C. albicans* is commonly found in the mouths of 45 to 75% of healthy persons (Cannon *et al*, 1995; Lynch, 1994; Odds, 1988) where it lives in harmony with other members of the oral microbiota. However, *C. albicans* is an opportunistic pathogen and is the primary etiologic agent of oral candidosis.

There are a variety of factors that can alter the normal oral microbial equilibrium and predispose a person to change from a simple carrier of *C. albicans* to one who has a candidal infection (reviewed by Odds, 1988; Samaranayake, 1990; Scully *et al*, 1994). These include natural factors, such as microbial infections, infancy and old age; dietary factors, such as carbohydrate-rich diets that may alter the composition of the endogenous microbial flora; mechanical factors, such as the wearing of dentures; and iatrogenic medical factors, such as treatment with drugs that alter the composition of the endogenous microbial flora or the host defence mechanisms. Other types of predisposition may be deep-seated and related to the underlying disease status of the individual. AIDS, malignancy, impaired granulocyte function, anti-cancer treatments, and long-term antibiotic therapy are all factors that markedly increase the host's susceptibility to oral candidosis and can cause a variety of oral pathologies. Pseudomembranous candidosis is the most common form of oral thrush. This is seen in from 10 to 15% of debilitated elderly people and in neonates whose immune system is immature and whose oral and gastro-intestinal microbiota has not been established (Odds, 1988). *Candida*-associated denture stomatitis is another common condition and affects 25-65% of denture wearers (Budtz-Jørgensen, 1990).

No single factor appears to be responsible for the pathogenicity of *C. albicans*. In the normal host, the yeast has evolved to become a successful commensal. It expresses variant traits critical for existence on mucosal surfaces where constant interplay occurs between innate and acquired host-defence mechanisms. These traits include factors such as adherence, hyphal production, proteinase production and variability (Cutler, 1991). In the abnormal host these traits may become virulence characteristics accounting for invasive abilities as the delicate balance of *C. albicans* with the host shifts in favour of the fungus. It has been proposed that a combination of different factors contribute at each stage of infection and there is also increasing evidence that some virulence traits may be important at specific tissue sites and not others (Culter, 1991).

A broad array of host defence mechanisms, both immune and non-immune, have evolved in humans to protect them against fungal invasion. Indeed, the majority of serious fungal infections occur in patients who have defects in one or more of these defences. The innate host defences form the first line of protection against invasion by micro-organisms, although this system is often taken for granted. An example of this system is the barrier created by the body's skin and mucous membranes. *Candida* species, particularly *C. albicans*, frequently colonise the skin and certain mucous membranes but will invade these tissues only under special circumstances. An increased prevalence of local and systemic candidosis occurs when these barriers are physically disrupted by trauma such as burns, surgical wounds and the wearing of dentures. The normal indigenous bacterial flora provides humans with protection against candidosis by competing for nutrients, receptor sites for adhesion, and perhaps by local production of antifungal substances (Marsh, 1989; Levitz, 1992). For example, administration of broad-spectrum antibiotics increases the risk of mucocutaneous and invasive candidosis by disrupting the normal microbiota, thereby facilitating local overgrowth of *Candida* (Woodman *et al*, 1985; Lacey *et al*, 1983; Sanders and Sanders, 1984).

Although the effects of antibiotic treatment on the local overgrowth of *C. albicans* and candidosis are well-known, little information regarding the colonisation resistance afforded by the natural flora in the oral cavity is available (Odds, 1988). In theory most antibacterial antibiotics possess the ability to eliminate bacteria that normally compete with the yeast for nutrients (Cormane and Goslings, 1963). Under these conditions the yeasts are able to multiply more readily and their overgrowth may lead to invasive infection (Odds, 1988).

The pathogenesis of *C. albicans* infections is complex, involving both yeast and host factors. Much progress has been made in defining the specific defects in host defence mechanisms that predispose patients to invasion by the yeast. Strategies for the prophylaxis and treatment of yeast infections has directly evolved from research in our understanding of host-yeast interactions (Levitz, 1992). Despite these advances, oral candidosis still remains a problem and ongoing research in the various aspects of *Candida* pathogenesis is needed.

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Chapter 2

RATIONALE FOR THIS STUDY

The widespread use of antibiotics, immunosuppressive drugs, the increasing proportion of the elderly in populations and of AIDS patients, resulted in an increased awareness of oral candidosis amongst clinicians. As a result a large number of studies on candidosis and the pathogenic mechanisms of *C. albicans* has been done. In spite of this, little is known about the mechanisms that control the opportunistic behaviour of *C. albicans* in the oral cavity and especially of the the role of the autochthonous bacteria in this respect (Odds, 1988).

Over recent years it has become clear that the autochthonous species (species that are characteristically found in a particular habitat) throughout the digestive tract have a barrier effect against allochthonous (organisms which originate from without a particular habitat) species and acts as an important defence mechanism against such species (Freter *et al*, 1983; Gorbach *et al*, 1988; Marsh, 1989). The importance of this colonisation resistance afforded by the normal microbiota can be seen in the effect antibiotics have on the homeostatic mechanisms in the oral cavity. Antibiotic treatment causes a rapid reduction in sensitive species in the mouth followed by the emergence of resistant organisms or colonisation by allochthonous species, including opportunistic pathogens such as *C. albicans* (Lacey *et al*, 1983; Odds, 1988; Sanders and Sanders, 1984; Woodman *et al*, 1985).

There are a number of mechanisms that may contribute to the colonisation resistance afforded by the autochthonous species. These include: competition for a limited number of receptor sites for adhesion; the production of inhibitory substances; creation of micro-environments

that discourage the growth of allochthonous species; and competition for essential endogenous nutrients and co-factors (Marsh, 1989). It has been hypothesised that one of the primary mechanisms that affords a colonisation resistance by the indigenous populations is a competition for growth-limiting substrates (Freter, 1983). This means that competition for nutrients will be a primary ecological determinant in dictating the type of species and the proportions of particular species in a mixed population. There is evidence that the growth and proportions of oral species are dependent on the availability of essential endogenous nutrients (which is usually limited) and that this will limit microbial growth (Marsh, 1989). For example, competition for individual amino acids, peptides, and carbohydrates has been shown to influence the final proportions of species in mixed continuous culture studies (McKee *et al*, 1985; Rogers *et al*, 1986) and in experiments with gnotobiotic rats (van der Hoeven, *et al*, 1985).

Glucose is a highly consumable substrate for *C. albicans* (Barnett *et al*, 1990; Cormane and Goslings, 1963; Knight and Fletcher, 1971). It has been suggested that the competition for glucose between the oral bacteria and the yeast may be one of the homeostatic mechanisms that control the growth of the yeast in the oral cavity and that antibiotics eliminate the organisms that compete against *C. albicans* for glucose (Cormane and Goslings, 1963; Kennedy *et al*, 1988; Knight and Fletcher, 1971). Candidosis also occurs more often when there is high availability of glucose, as in persons with diabetes (Knight and Fletcher, 1971; Odds *et al*, 1987). Carbohydrate diets favour the oral carriage of *C. albicans* in rats and monkeys, (Hassan *et al*, 1985; Russell and Jones, 1973) whereas sucrose rinses initiate *Candida* stomatitis in human subjects (Olsen and Birkeland, 1976.). It has also been shown that dietary glucose intake is a key determinant of *C. albicans* growth in the gastrointestinal tract (Vargas *et al*, 1993).

A number of *in vitro* studies on the interactions of *C. albicans* with other micro-organisms were published, mostly in the context of possible microbial interactions in various ecological niches, particularly the gastrointestinal tract (Odds, 1988). The problem with most of these studies however, is that they were done under simplified environmental conditions that are not representative of the growth conditions in the oral cavity. There is little similarity between the inhibitory mechanisms for *C. albicans* in these simple *in vitro* systems and those found in the complex microbiota of the intestinal tract or the oral cavity. This can be seen in the results of these experiments that occasionally conflict quite markedly. For example, *Lactobacillus* species have been claimed both to inhibit (Young *et al*, 1956) and to enhance (Isenberg *et al*, 1960) the growth of *C. albicans*. Liljemark and Gibbons (1973) were able to suppress *C. albicans* growth in germfree mice contaminated with a mixed flora of *Streptococcus salivarius* and *Streptococcus mitior*. On the other hand, *in vitro* experiments could not demonstrate any interaction between *S. salivarius* and *C. albicans* (De Miranda *et al*, 1992). Staphylococci inhibited the growth of *C. albicans* (Cormane and Goslings, 1963; Macfarlane and Makrides, 1982) or its growth was unaffected by their presence in culture (Auger and Joly, 1978).

A limited number of studies were performed that investigated the effect of glucose on the growth of *C. albicans*. Cormane and Goslings (1963) found that the growth of *C. albicans* in the presence and absence of bacteria, and its rate of inhibition in mixed cultures was markedly influenced by the concentration of glucose. Knight and Fletcher (1971) found that the yeast will not grow in saliva in the presence of the oral flora unless excess glucose is available through supplementation, or the oral bacteria are suppressed with antibiotics. These studies indicate that a competition for glucose between the yeast and oral bacteria might exist. However, they do not give a definite answer as to which of different possible mechanisms

could be responsible for the observed growth-inhibition of *C. albicans*, or the organisms involved.

The well-defined theory of the chemostat, together with its benefits as an open system, allow long-term quantitative studies of the behaviour and composition of microbial communities (Herbert *et al*, 1956). Furthermore, the study by McKee *et al* (1985) has shown that the chemostat can be used to produce stable communities of oral bacteria that are reproducible in microbial composition. The chemostat can therefore be used to study the conditions required for the coexistence of stable populations of more than one species competing for common substrates. It can be used to investigate the selection of one population over several initially present. It can be used to determine the basis of its selective power over the other organisms (Powell, 1958). The chemostat has indeed been used to study the interaction of *C. albicans* with other organisms in mixed culture, but these studies were all done in context of microbial interactions in the gastrointestinal tract (Bernhardt *et al*, 1995; Freter *et al*, 1983; Kennedy *et al*, 1988).

There can be no doubt that if the oral flora plays a primary role in the maintenance of a candidal homeostasis in the oral cavity, it would be of incalculable benefit to clinical dentistry if the composition of the indigenous oral microbiota could be manipulated in such a way that bacterial species, which are responsible for the beneficial effects, are enhanced or retained during some of the predisposing conditions to oral candidosis. This could only be possible if the homeostatic mechanisms that afford candidal homeostasis were better understood.

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Chapter 3

AIMS OF THE STUDY

The purpose of this study was to investigate the role of competition for glucose amongst oral species and *C. albicans* in maintaining candidal homeostasis in a mixed population.

To be able to do this, the following aims were identified:

- 1) To review the existing information regarding the biology of *C. albicans*.
- 2) To review the existing information regarding fermentation modelling in order to design a suitable model for the study.
- 3) To establish a chemostat model comprising a mixture of oral bacteria that would suppress the growth of *C. albicans* under growth conditions similar to that of the oral cavity.
- 4) To determine if the availability of glucose influences the competition between the identified oral bacteria and *C. albicans*.
- 5) To determine the growth parameters of the organisms in order to calculate the theoretical saturation curve for each organism.
- 6) To use the theoretical saturation curves to identify the species that compete against the yeast for glucose.
- 7) To predict the outcome of competition for glucose between the different organisms and the yeast under different environmental glucose concentrations.

Chapter 4

BIOLOGY OF *CANDIDA ALBICANS*

Taxonomy

Classification of *Candida* species

Candida species are classified as yeasts, that is, as fungi with a predominantly unicellular mode of development. Usually yeasts occur as single-celled budding structures, although these cells may elongate to form pseudomycelium and sometimes true mycelium under the influence of certain environmental conditions (Rippon, 1988).

Two classes of reproduction are distinguished in the fungi:

1. sexual reproduction (teleomorph state) which are produced by the fusion of two nuclei and the subsequent process of meiosis and;
2. asexual reproduction (anamorph state) which arises following mitosis of the parent nucleus.

Fungi in which a teleomorph state is present, are known as “perfect fungi” and are assigned to the subdivision *Ascomycotina*. The fungi in which only an anamorph state is known, are called the “imperfect fungi” and are placed in the subdivision *Deuteromycotina* (Rippon,

1988). The main common feature of the genus *Candida* is the absence of any teleomorph form and the genus is therefore placed in the subdivision *Deuteromycotina* (see Table 1).

Table 1. Classification of the genus *Candida* (Barnett *et al*, 1990).

Kingdom: Fungi

Division: *Eumycotina*

Subdivision: *Ascomycotina*

Subdivision: *Basidiomycotina*

Subdivision: *Deuteromycotina*

Class: *Blastomycetes*

Family: *Sporobolomycetaceae*

Family: *Cryptococcaceae*

Genus: *Candida*

Species: *albicans*

The genus *Candida* comprises 166 species. A variety of cell shapes occur but the vegetative cells are predominantly budding cells, sometimes forming pseudo- and septate hyphae. No sexual reproduction is known. The species have a range of markedly different fermentation and assimilation reactions and are separated on the basis of their physiological properties such as the fermentation and assimilation of various organic compounds, particularly carbohydrates (Barnett *et al*, 1990).

Only a small number of *Candida* species are of medical significance. These include the species *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* of which *C. albicans* is of foremost importance in dentistry (Odds, 1988).

The genus *Candida* is probably unnatural and includes a number of species that are believed to be imperfect forms of ascomycete species. The discovery of a sexual phase for any of the species is certain to result in major taxonomic changes. Indeed, sexual forms have so far been described for six *Candida* species (see Table 2).

Table 2. Names of teleomorph states of *Candida* species found in clinical material (Odds, 1988).

Anamorph state	Teleomorph state
<i>Candida famata</i>	<i>Debaromyces</i>
<i>Candida guilliermondii</i>	<i>Pichia guilliermondii</i>
<i>Candida kefyr</i>	<i>Kluyveromyces marxianus</i>
<i>Candida krusei</i>	<i>Issatchenkia orientalis</i>
<i>Candida lusitaniae</i>	<i>Clavispora lusitaniae</i>
<i>Candida norvegensis</i>	<i>Pichia norvegensis</i>

Although Van der Walt (1970) has described a basidiomycetous sexual form of *C. albicans*, his findings have not been reproduced by others and are therefore still awaiting to be

confirmed. Other taxonomic revisions for clinically important yeasts include the reclassification of *Candida claussenii* and *Candida stellatoidea* to *C. albicans* (see Table 3) (Odds, 1988).

Serotyping of *C. albicans*

Serological tests have been applied to the taxonomy of pathogenic yeasts but a considerable degree of antigenic similarity exists between *Candida* species and also between *Candida* and other yeast species. For instance a high degree of similarity exists between *C. albicans* and *C. tropicalis*. Nevertheless, they can still be distinguished antigenetically (Bruneau *et al*, 1985; Gordon *et al*, 1967).

Table 3. Taxonomic revisions of clinically important yeasts (Odds, 1988).

Former name	Current name
<i>Candida brumptii</i>	<i>Candida catenulata</i>
<i>Candida claussenii</i>	<i>Candida albicans</i>
<i>Candida parakrusei</i>	<i>Candida parapsilosis</i>
<i>Candida paratropicalis</i>	<i>Candida tropicalis</i>
<i>Candida pseudotropicalis</i>	<i>Candida kefyr</i>
<i>Candida ravautii</i>	<i>Candida catenulata</i>
<i>Candida stellatoidea</i>	<i>Candida albicans</i>
<i>Torulopsis candida</i>	<i>Candida famata</i>
<i>Torulopsis glabrata</i>	<i>Candida glabrata</i>

C. albicans can be divided into two serotypes, A and B, on the basis of agglutination tests (Hansenclever and Mitchell, 1961), precipitin reactions (Stallybrass, 1964a; Stallybrass, 1964b; Sweet and Kaufman, 1970) and by immunofluorescence (Poulain *et al*, 1983). *C. albicans* type A occurs more than twice as commonly as type B in clinical material (Odds, 1988).

The major antigenic component in *Candida* cell walls is the mannan polysaccharide. Serotype A mannan contains all the antigenic determinants of serotype B mannan plus additional determinants. The antigenic differences result from variations in the bonding positions between mannose residues and the numbers of residues in mannan side chains (Summers *et al*, 1964).

Biotyping of *C. albicans*

In addition to serotyping of *C. albicans*, strain differentiation into biotypes A and B can also be obtained through the variable resistance to certain chemicals (Odds and Abbott, 1980; Odds and Abbott, 1983; Warnock *et al*, 1979a), assimilation of carbon and nitrogen sources (Odds and Abbott, 1980; Odds and Abbott, 1983), morphological differences between colonies (Phonpaichits *et al*, 1987) and susceptibility to killer factors (Caprilli *et al*, 1985; Polonelli *et al*, 1983; Polonelli *et al*, 1985). Strain differentiation of *C. albicans* at the genetic level is a relatively new approach to biotyping and can be obtained by DNA restriction fragment length polymorphism or electrophoretic karyotyping (Magee *et al*, 1987; Olivo *et al*, 1987).

Growth and Nutrition of Pathogenic *Candida* Species

The commonly encountered pathogenic *Candida* species grow well in aerobic cultures on richly or poorly nutrient media that have a pH of 2.5-7.5. *Candida* species can grow at temperatures in the range of 20-38°C. However, the optimum growth temperature of the two most virulent species, *C. albicans* and *C. tropicalis*, is closer to 37°C than the less pathogenic species such as *C. guilliermondii* (Lemos-Carolino and Madeira-Lopes, 1984; Madeira-Lopes and Çabeça-Silva, 1984; Straube and Fritsche, 1972). *C. albicans* is one of the few species that can grow at an environmental pH below 2 (Odds and Abbott, 1980).

They all assimilate and ferment glucose as a carbon source and none of them assimilate nitrate as a nitrogen source but they vary in their abilities to utilise other carbon and nitrogen sources (Meyer *et al*, 1984). *C. albicans* can also ferment galactose and maltose and assimilate galactose, sucrose and maltose. The reduced sugar xylitol suppresses *C. albicans* growth (Mäkinen *et al*, 1975). Most of the species, including *C. albicans*, require biotin for growth.

Although *Candida* species grow best under aerobic conditions most of the pathogenic species, including *C. albicans*, will grow under anaerobic conditions. However, they will grow at markedly declined growth rates. The growth rate of *C. albicans* was reduced from 0.99 h⁻¹ in air to 0.65 h⁻¹ under anaerobic conditions (Webster and Odds, 1986). *Candida* species will also grow under elevated concentrations of CO₂ in air. A CO₂ of 10% in air had virtually no effect on the growth rate of *C. albicans* but pure CO₂ will inhibit its growth (Aralu, 1971; Eklund and Jarmund, 1983; Webster and Odds, 1986).

Ecology of *Candida* species

Occurrence of *Candida* species in nature

Yeasts in general are commonly found in nature in association with plants and insects (Barnett *et al*, 1990; Meyer *et al*, 1984). However, the yeasts causing candidosis (*Candida* species) have a comparatively restricted natural distribution. Although there are a few *Candida* species that are occasionally isolated from amphibians, indicating that they are not all exclusively restricted to warm-blooded animals as a habitat, they are primarily associated with man and other warm-blooded animals (Odds, 1988). In the majority of animals the digestive tract, especially the oesophagus and the crop, was the most frequent source of *Candida* isolations. In most cases the yeasts were incriminated as causes of disease. The type of *Candida* infections also resembles that in humans with superficial, deep seated and disseminated forms all recognised. Of all the *Candida* species, *C. albicans* has been recovered from a far wider range of animal hosts than any other yeast species. This also correlates with the high prevalence of *C. albicans* among human yeast isolates and suggests that *C. albicans* is the principal opportunistic yeast pathogen in most warm-blooded animals (Odds, 1988).

C. albicans has also been isolated from soil, plants, the atmosphere and water. However, these isolations are unusual and restricted to sources where human or animal contamination is probable (Clayton, 1963; Clayton, 1966; Gentles and La Touche 1969; Rogers and Beneke, 1964). The fact that *Candida* species and specifically *C. albicans* are frequently found in the hospital environment in foods, in the air, on floors and other surfaces in hospital wards is an indication that carriers of *Candida* often contaminate their immediate environment with the yeast. *C. albicans* survives poorly on dry surfaces such as skin and glass (Kashbur *et al*,

1980). On the other hand, *C. albicans* can survive in or on moist objects such as toothbrushes (Koch and Koch, 1981), it can survive on fabrics laundered at temperatures up to 50°C (Rashid *et al*, 1984), it survives in hand creams (France, 1968) and other oil/water emulsions (Brown *et al*, 1986) and in eye cosmetics (Wilson *et al*, 1971).

Carriage of yeasts in the mouth

Candida species reside in the digestive tracts of many individuals, particularly those seeking medical attention (Odds, 1988). However, there are different distributions of individual *Candida* species between the mouth and other parts of the gut and also other parts of the body.

Odds (1988) summarised data obtained from publications since 1960 on the overall prevalence of yeasts in the mouths of normal subjects. From this information it is apparent that the overall prevalence of yeasts in the mouths of normal subjects is generally lower than those who have sought medical attention and that between 33% to 50% of healthy people harbour *Candida* species in the mouth. The isolation frequency of oral yeasts at different ages indicates a peak in infants up to 18 months of age with a lower prevalence in older children and adults. In healthy subjects that harbour yeasts they occur on average at a concentration of about 300-500 colony forming units per ml of saliva. However, the yeast microbiota is not a static population and considerable diurnal variations in yeast counts occur, often with peaks in the early morning and late afternoon (Williamson, 1972a). There is also day by day variation in the quantity of yeasts recovered from any individual (Gergely and Uri, 1966; Williamson, 1972b).

The tongue (specifically the posterior third) is the oral site most densely populated with yeasts, followed by the palate, and the cheeks (Arendorf and Walker, 1979; Arendorf and Walker, 1980).

C. albicans usually accounts for 60-80% of oral isolates while *C. glabrata* and *C. tropicalis* are both found at much lower frequencies of $\pm 7\%$ (Odds, 1988). Other yeast species occur only rarely (Table 4).

Carriage of yeasts in other sites

Roughly the same proportion of humans that carry *Candida* species in their mouths, carry the yeasts throughout the length of the gastrointestinal tract (40-50%). Most have shown high carriage rates in the stomach and the intestine with frequencies of 51.7% and 45.7% respectively (Odds, 1988). In contrast to the mouth *C. albicans* accounted for fewer than 60% of isolates. In the stomach and lower digestive tract *C. glabrata* and *C. tropicalis* accounted for 16% and 10% respectively. Recoveries of yeasts from the vaginas of putatively normal females show a prevalence of less than 20%. In patients the frequency tends to be somewhat higher (Odds, 1988). Samples show a general trend towards a higher prevalence of isolation of *C. albicans*. It was also observed that vaginal yeast concentrations, like those in the mouth, vary from day to day and diurnally (Odds 1982; Sautter and Brown, 1980).

Isolation rates of yeasts from the skin of healthy individuals varies dramatically with the geographical location and occupation of the study group, as well as with the sample site. Most surveys however, reveal very low prevalences with *C. albicans* isolations. Among hospital patients the recoveries of yeasts and *C. albicans* from the skin were often higher than those for normal subjects. The highest yeast prevalences were recorded in cancer patients who are highly predisposed to *Candida* infections (Odds, 1988).

Table 4. Distribution of yeast species isolated from the oral cavity (Odds, 1988).

Species	Percentage ^a
<i>C. albicans</i>	69.6
<i>C. glabrata</i>	6.6
<i>C. tropicalis</i>	6.9
<i>C. parapsilosis</i>	1.9
<i>C. krusei</i>	1.7
<i>C. kefyr</i>	1.0
<i>C. guilliermondii</i>	0.4
Other yeast species	11.9

^a As percentage of all above isolates described

Distribution of *C. albicans* serotypes and biotypes.

In vivo there is a tendency towards selection of serotype A cells. It could be that environmental pressures associated with *C. albicans* favour expression of type A cells. The distribution of *C. albicans* serotypes from clinical sources show a preponderance of serotype A in the range 74-94% (Odds, 1988). It was also shown that in mice fed mixtures containing 75% of serotype B cells and only 25% serotype A cells, the serotype A strains rose to 70% in the stomach and 50% in the colon (Auger *et al*, 1983).

Although different biotypes may occur at different sites in the same person, most individuals who carry *C. albicans* as a commensal or pathogen, carry the same biotype in different anatomical sites. Furthermore, most retain a particular biotype over a period of time (McCreight *et al*, 1984; McCreight *et al*, 1985; McCourtie *et al*, 1986; O'Connor and Sobel, 1986; Odds, 1982; Odds *et al*, 1983; Skinner, 1947; Warnock *et al*, 1979a; Warnock *et al*, 1979b; Watkinson *et al*, 1985).

Mechanisms involved in *C. albicans* pathogenesis

Virulence factors

Although a great deal of information is available concerning the molecular basis of bacterial pathogenesis, relatively little is known about the mechanisms of fungal pathogenesis (Finlay and Siebers, 1995). Fungi rarely cause disease in healthy, immunocompetent hosts. It is only when fungi accidentally penetrate barriers such as intact skin and mucous membrane linings, or when immunologic defects or other debilitating conditions exist in the host, that conditions favourable for fungal infection occur.

The pathogenesis of an organism and the outcome of infection is dependent on the virulence factors of the organism, i.e. their ability to overcome certain cardinal requirements. In general these include 1) colonisation of mucous or other surfaces, 2) multiplication in the *in vivo* environment, 3) entry into host tissues through these surfaces, 4) interference with host defence mechanisms, 5) and damage to the host (Smith, 1995).

The attributes that confer pathogenicity and virulence to *C. albicans* are poorly characterised. In part these properties involve adherence mechanisms and the production of toxic products and hydrolytic enzymes. In addition, some cell wall glycoproteins and polysaccharides interact with host defence mechanisms both to promote and to impair inflammatory and immune responses. Furthermore, rapid phenotypic variability and cellular dimorphism contribute to the commensal and pathogenic behaviour of this organism (Ray and Payne, 1990). No single factor appears to be responsible for the pathogenicity of *C. albicans* and it has been proposed that a combination of different factors contribute at each stage of infection (Cutler, 1991; Ray and Payne, 1990).

Colonisation of mucous surfaces

Studies performed on adherence of micro-organisms to host cells have shown adherence to be an important first step in both the colonisation and the pathogenesis of microbial infections. Mucosal surfaces are coated with a thick covering of mucus that is composed of many carbohydrates (Finlly and Siebers, 1995). This layer is the first barrier that pathogens encounter when they enter the host. For *C. albicans* to successfully colonise and infect host mucosal surfaces, it has to adhere to these epithelial surfaces, i.e. to the mucus layer or epithelial cells. Without attachment, the growth rate of *C. albicans* (with a doubling time in the order of 1.5 h under optimal *in vitro* conditions) is insufficient to maintain carriage in the mouth and will be washed away (Cannon *et al*, 1995a).

C. albicans adheres to a large number of cells and surfaces, including epithelia (Critchley and Douglas, 1985; Fukayama and Calderone, 1991), endothelia (Edwards *et al*, 1992; Klotz, 1994) and phagocytic cells (Diamond, 1993; Kanbe and Cutler, 1994). Of particular importance to oral candidosis may be the adherence of *Candida* cells to saliva coated

surfaces, including prosthetic devices (Cannon *et al*, 1995b; Nikawa *et al*, 1993; Vasilas *et al*, 1992) and to oral bacteria (Jenkinson *et al*, 1990).

Various adherence mechanisms have been reported and are summarised in Table 5. Factors that enhance adherence include fungal cell surface hydrophobicity of the organism, pH, temperature, pregnancy, diabetes and oral contraceptive usage. Some investigators have suggested that increased virulence also occurs with mycelium formation (Vartivarian, 1992). *C. albicans* has been found to adhere better to epithelial cells under conditions enhancing germ tube formation although it may not be a prerequisite for adherence (Kimura and Pearsall, 1978; Sobel *et al*, 1981).

Compared to the extensive evidence for the role of bacterial adherence to mucosal surfaces in pathogenesis (see Roth *et al*, 1995), such evidence in the case of *Candida* is inconclusive. However, invasive *Candida* strains recovered from patients adhered better than strains from carriers (McCourtie and Douglas, 1984). The adherence of *C. albicans* to epithelial cells is better than that of *C. tropicalis*. This matches the virulence rankings of these species (King *et al*, 1980; Rotrosen *et al*, 1980).

The best evidence linking candidal adherence to pathogenicity comes from a study by Calderone *et al* (1985). Calderone described two spontaneous mutants with decreased ability *in vitro* to adhere to fibrin-platelet clots. The 50% infectious doses in a rabbit model of endocarditis were significantly higher for the mutants relative to the wild-type parent strain.

Colonisation resistance and microbial homeostasis

The normal indigenous microbiota is another first line of protection against invasion by micro-organisms. Over the years it has become clear that the autochthonous species present

Table 5. Adherence mechanism of *Candida* species

Adherence mechanism	Candida adhesin	Receptor/ Ligand	Host Tissue/ Surface	Reference
Hydrophobicity	Surface proteins	Hydrophobic surfaces	Epithelial cells, dental materials	Nikawa <i>et al</i> , 1993; Hazen and Glee, 1994
Protein-protein	Surface proteins	Fibronectin, collagen, fibrinogen, laminin	Epithelial cells, extracellular matrix, endothelial cells	Bouchara <i>et al</i> , 1990; Calderone, 1993; Klotz <i>et al</i> , 1994
Lectin-like	Surface proteins	Fucose, or glycosides	Epithelial cells	Critchley and Douglas, 1987; Tosh and Douglas, 1992
	Surface proteins	Oral streptococcal polysaccharides	Colonised epithelial cells, dental plaque?	Holmes <i>et al</i> , 1995
	Fimbriae mannoprotein	Glycosphingolipid receptors	Epithelial cells	Yu <i>et al</i> , 1994

throughout the digestive tract do not only have a passive relationship with the host but have a remarkable beneficial effect. Our knowledge of the beneficial effects of the autochthonous species has come from two main sources: 1) observations of the physiology of germ-free animals, and 2) studies of the consequences following suppression of the natural microflora in animals and humans by antimicrobial agents (Marsh, 1989). The gut of germ-free animals is poorly developed, but when these animals are colonised by components of the normal microbiota, many of the anatomical and physiological deficiencies reverse (Rosebury, 1962). A range of deficiencies in absorption or metabolism of vitamins has also been reported in humans on long-term antibiotic treatment. Antibiotics can also lead to the rapid suppression of the resident oral microbiota and result in overgrowth by drug-resistant, but previously minor, components of the microbiota (Woodman *et al*, 1985). These observations suggest that the autochthonous microbiota not only influences the physiology of the host but also acts as an important defense mechanism. This barrier effect has been termed “colonisation resistance” by van der Waaij *et al* (1971). Competition for essential endogenous nutrients may be one of the mechanisms by which the resident oral microbiota contributes to this colonisation resistance (Marsh, 1989).

The microbial communities of most habitats of man are characterised by a remarkable stability among their component species. This stability is maintained in spite of the host defenses and despite the regular exposure of the community to a variety of moderate environmental stresses. In the oral cavity these include diet, the regular introduction of allochthonous species, the use of dentrifices, and changes in saliva flow (Marsh, 1989). The ability to maintain community stability in such a variable environment has been termed “microbial homeostasis” (Alexander, 1971). This stability results from a balance of dynamic microbial interactions (Sanders and Sanders, 1984). However, despite these microbial interactions, homeostasis can break down in the mouth. This can be caused by a variety of

immune- and by certain non-immune factors which may eventually lead to the emergence or overgrowth of opportunistic pathogens such as *C. albicans* (Odds, 1988). The best-characterised breakdown of homeostatic mechanisms is that caused by antimicrobial factors. A good example is the administration of broad-spectrum antibiotics that increases the risk of mucocutaneous and invasive candidosis by disrupting the normal flora and thereby facilitating local overgrowth of *C. albicans* (Bross *et al*, 1989).

Production of toxins and hydrolytic enzymes

Studies on putative *Candida* toxins have for many years provided contradictory evidence but it is now clear that no *Candida* species secretes into its culture medium any molecule with a biological potency equivalent to that of a bacterial exotoxin (Odds, 1988). However, *C. albicans* cell walls contain endotoxin-like substances such as glycoproteins but these are not true endotoxins and are not nearly as potent as true bacterial endotoxins (Odds, 1988; Vartivarian, 1992; Cannon *et al*, 1995a).

C. albicans secretes several enzymes which include a phospholipase, lipase phosphomonoesterase, hexosaminidase and various proteinases (MacDonald and Odds, 1980; White *et al*, 1993). Of these enzymes the proteinases have been studied the most. Proteinase activity is produced by only the most pathogenic *Candida* species and is also a common characteristic of *C. albicans* (Cannon *et al*, 1995a; Cutler, 1991). Although there is some evidence pointing to a relation between virulence and the production of certain enzymes, there is no definite proof linking fungal enzyme activity to pathogenicity (Vartivarian, 1992).

Dimorphism and phenotypic variability

Dimorphism plays an essential role in the pathogenesis of infections caused by certain systemic dimorphic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis*

(Vartivarian, 1992). *C. albicans* is also a dimorphic fungus and can grow in either a yeast or in a hyphal form. It is true that *C. albicans* germ tubes and hyphae adhere better than yeast cells to human buccal and vaginal epithelia and to vascular endothelia (Anderson and Odds, 1985; Odds, 1988). Furthermore, epithelial penetration by *C. albicans* have shown hyphal forms rather than yeast cells involved in the initial stages (Anderson and Odds, 1985; Cawson and Rajasingham, 1972; Farrel *et al*, 1983; Howlett and Squier, 1980). There is also a common belief that the hyphal form is invasive and pathogenic, while the yeast is the commensal non-pathogenic form (Cannon *et al*, 1995a). However, evidence for this is ambiguous and its pathogenic significance may be overstressed (Sobel *et al*, 1984).

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Chapter 5

MICROBIAL GROWTH

Growth kinetics in batch culture

When microbial cells are inoculated into a nutrient medium and incubated at a suitable temperature, a sequence of changes occur. These changes have been analysed in detail by Monod (1949) and are collectively referred to as the growth cycle (Fig 1). After a variable "lag" period the cells start to grow, increase in mass and then divide. The microbial population then doubles at regular intervals because each of the two daughter cells produced by a division has the same potential for growth as the parent cell. If the population is started from a single cell, the first few divisions are reasonably synchronous, in other words, every cell divides more or less simultaneously, so that the number of cells in the population increases in a stepwise manner.

However, as a result of minor differences in growth rate between the individual members of the population, the times of division soon become random and the number of cells in the population then increases in a continuous fashion. When the time of division is random, both cell mass and cell number increase continuously with time, doubling at fixed intervals. During the latter phase, the so-called "exponential growth phase" nutrients are taken up from the medium and end products of metabolism excreted into it. Thus the process of growth causes the environment to change and eventually it becomes so changed that it is unable to support further growth. At this time the culture enters the so called "stationary phase". The stationary

phase is eventually followed by the "death phase" in which the viable cell population declines as a result of cell death (Stanier *et al*, 1972):

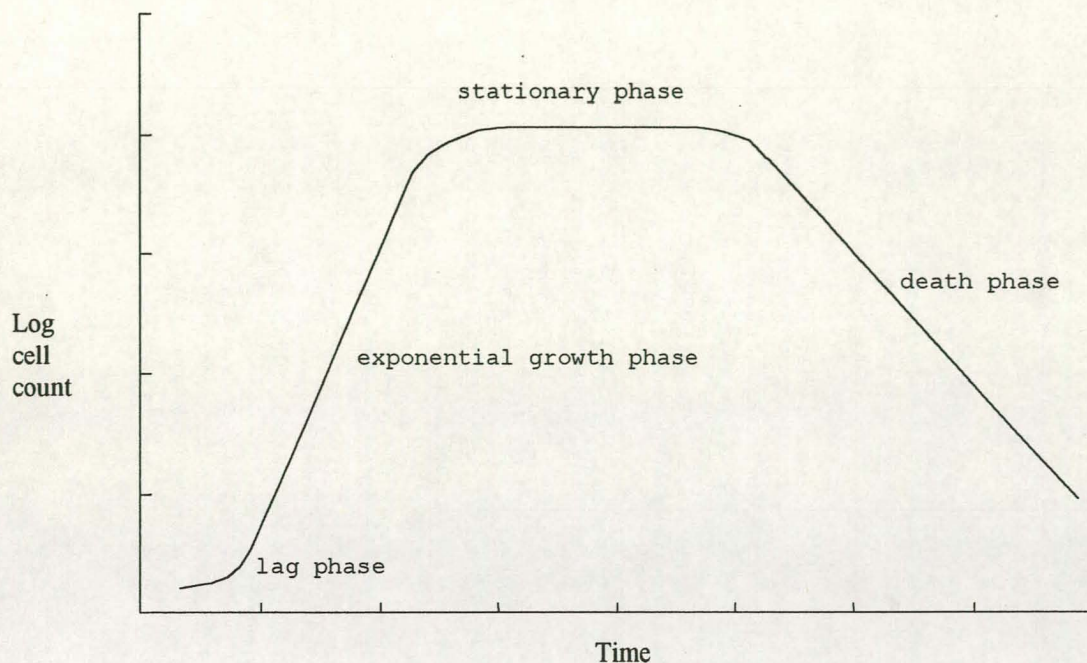


Fig. 1. Generalised growth curve of a bacterial culture

During the exponential growth phase the time for the doubling of the cell mass (or cell number) is a constant known as the mean doubling time. Thus if the initial concentration of the population is x_0 , one can determine the population size in successive generations as follows:

after 1 generation (or 1 doubling time) $x_1 = 2x_0$

after 2 generations $x_2 = 2(2x_0) = 2^2 x_0$

after 3 generations $x_3 = 2(2^2 x_0) = 2^3 x_0$

after n generations $x_n = 2^n x_0$

Or after a time t $x_t = 2^{ct} x_0$ ----- (1)

where x_t is the population size after a time t , and c is the exponential growth-rate constant, defined as the number of doublings per unit-time (n/t , usually expressed as the number of doublings per hour).

Equation (1) is normally written in a form using natural logarithms or Napierian logarithms after Lord Napier their inventor ($\log_e a$ or $\ln a$)*.

Consider the number k such that

$$k = \log_e a \text{ (or } k = \ln a)$$

This may be rearranged to take the form

$$a^n = (e^k)^n = e^{nk}$$

Thus, any function of the type a^n can be converted to an exponential by the use of the above expression.

Therefore, equation (1) can be expressed as

$$x_t = x_0 e^{kt} \text{ -----(2)}$$

the equation for exponential growth.

The exponential growth rate constant (k) can be derived as follows:

$$\frac{x_t}{x_0} = e^{kt}$$

* Mathematicians often use a special notation for natural logarithms – instead of writing $\log_e a$ the shortened form $\ln a$ is used. The $\ln a$ form will be used for \log_e throughout this dissertation.

or

$$kt = \ln \frac{x_t}{x_0}$$

therefore

$$k = \frac{(\ln x_t - \ln x_0)}{t}$$

and the mean doubling time = $\frac{1}{k}$ (Stanier *et al*, 1972).

Growth kinetics in continuous culture

For the calculation of growth kinetics of bacteria in continuous culture a different growth rate constant, i.e. the instantaneous or specific growth rate constant (μ) is used. This constant is derived by differentiation of the equation for exponential growth.

The equation for exponential growth (equation 2) can be regarded as a composite function where $u = kt$.

Then

$$x_t = x_0 e^{kt}$$

becomes

$$x_t = x_0 e^u$$

and when differentiated now becomes

$$\frac{dx_t}{du} = x_0 \frac{d}{du} (e^u) = x_0 e^u$$

and from $u = kt$

$$\frac{du}{dt} = k$$

From the chain rule

$$\frac{dx_t}{dt} = \frac{dx_t}{du} \cdot \frac{du}{dt} = x_0 ke^u$$

When u is replaced with kt

$$\frac{dx_t}{dt} = x_0 ke^{kt}$$

where the constant k is the specific growth rate constant which is usually expressed as μ (Arya and Lardner, 1979).

If

$$x_0 e^{\mu t} = x_t$$

and

$$\frac{dx_t}{dt} = x_0 \mu e^{\mu t}$$

then

$$\frac{dx_t}{dt} = \mu x$$

$$\therefore \mu = \frac{1}{x_t} \left(\frac{dx_t}{dt} \right)$$

For the function $y = \ln x_t$, the derivative $\frac{dy}{dx_t} = \frac{1}{x_t}$ (Arya and Lardner, 1979).

Therefore

$$\frac{1}{x_t} = \frac{dy}{dx_t} = \frac{d(\ln x_t)}{dy}$$

$$\therefore \frac{1}{x_t} = \frac{d(\ln x_t)}{dx_t}$$

and

$$\mu = \frac{dx_t}{dt} \left[\frac{d(\ln x_t)}{dx_t} \right]$$

$$\mu = \frac{d(\ln x_t)}{dt}$$

Let t_d be the time for the culture to double. Then for $t = t_d$ and $x_t = 2$

$$\mu = \frac{d(\ln 2)}{dt_d}$$

$$\therefore \mu = \frac{\ln 2}{t_d} \text{-----(3)}$$

And since the mean doubling time (t_d) = $\frac{1}{k}$

the specific growth rate constant (μ) = $k \ln 2$

$$\therefore \mu = 0.69k$$

The Monod equation

The specific growth rate (μ) and the mean doubling time (t_d) are usually assumed to be constants during microbial growth. However, this assumption is correct only when all substrates necessary for growth are present in excess (Herbert *et al*, 1956). In nature this condition is seldom met and the values of μ and t_d are markedly influenced by the environment and particularly by the concentration of various essential nutrients.

Monod (1942) showed that there is a simple relationship between the specific growth rate and the concentration of an essential growth substrate (see Fig. 2). When one such nutrient is decreased to a low level, then the specific growth rate is lowered correspondingly but at high concentrations it reaches a saturation value.

This relationship can be represented by a Michaelis-Menton type function, i.e.

$$\mu = \mu_{\max} \left(\frac{s}{k_s + s} \right) \text{-----(4)}$$

where s is the substrate concentration, μ_{\max} is the maximum value of the specific growth rate constant (when s is no longer growth-limiting) and k_s is a saturation constant, numerically equal to the growth-limiting substrate concentration at $0.5 \mu_{\max}$ (Herbert *et al*, 1956).

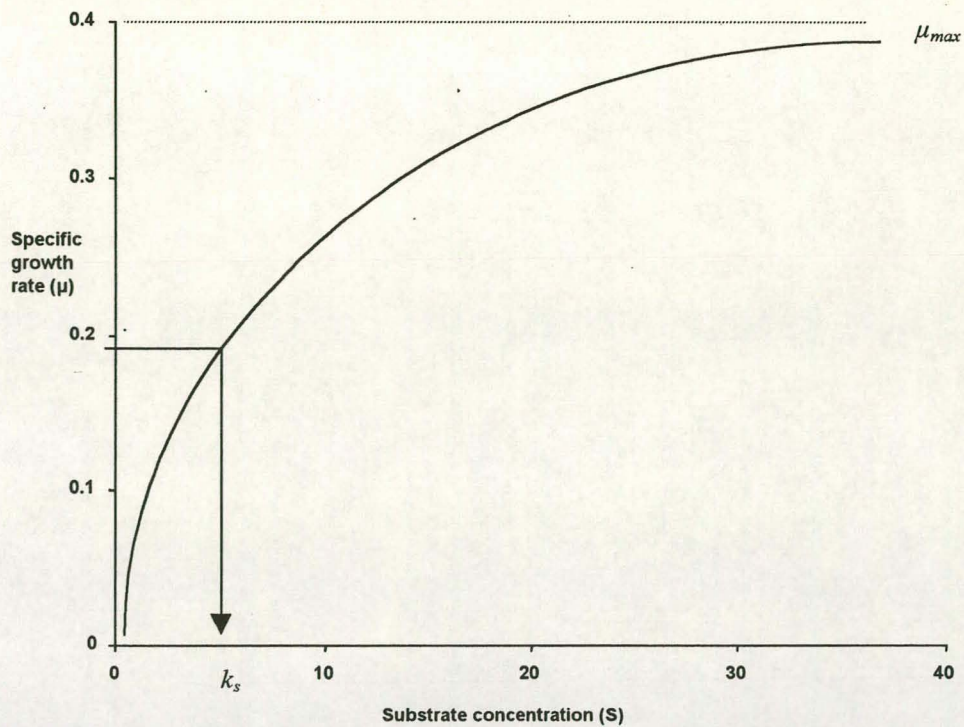


Fig. 2. Specific growth rate (μ) as a function of substrate concentration

Monod (1942) also showed that there is a relationship between the rate of growth and of substrate consumption i.e.

$$\frac{ds}{dt} = -Y \left(\frac{dx}{dt} \right)$$

where Y is the yield constant.

Over a finite period of time, and during the exponential growth phase, the growth yield can be expressed as

$$Y = \frac{\text{mass of bacteria formed}}{\text{mass of substrate used}}$$

Thus, if the values of μ_{\max} , k_s and Y are known, a completely quantitative description of the events during the growth cycle of a batch culture can be made (Monod, 1942). The same equations and constants are equally applicable to the theoretical treatment of continuous culture (Herbert *et al*, 1956).

The chemostat

Medium flow rate

When a growth-limiting nutrient is present in a culture medium at a concentration sufficient to support only a limited amount of growth and the culture is allowed to develop as a batch culture this nutrient would become depleted first, causing growth to cease. In a continuous culture system however, fresh growth medium is introduced continuously into the culture vessel, while spent medium and cells are removed concomitantly and at the same rate, thereby maintaining the culture in the exponential growth phase. However, growth will continue only at a rate proportional to the medium flow rate; that is, at a rate proportional to the rate of supply of the growth-limiting nutrient. The concentration of the growth-limiting nutrient will also depend on the dilution rate (D) of the culture

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

where f = flow-rate of the medium and V = culture volume (Herbert *et al*, 1956).

In the chemostat, substrate concentration and flow rate of the growth medium can be precisely controlled. Variation of these variables affects the growth rate, the concentration of organisms and the concentration of the substrate in the growth vessel.

Dilution rate and the growth rate of organisms

In the chemostat culture the organisms are growing but at the same time they are washed from the fermenter. The net change in concentration of organisms (x) with time therefore will be determined by the relative rates of each process.

$$\text{Increase} = \text{Growth} - \text{Output}$$

$$\frac{dx}{dt} = \mu x - Dx = x(\mu - D) \text{-----(5)}$$

where μ is the specific growth rate constant (see equation 3).

If $\mu > D$, dx/dt will be positive and the concentration of organisms will increase with time.

If $\mu < D$ the organisms will wash out from the growth vessel. Only when $\mu = D$ will dx/dt be zero and the concentration of organisms in the culture will remain constant. The culture will then be in a so called "steady state". Under steady state conditions

$$D = \mu = \mu_{\max} \left(\frac{s}{K_s + s} \right) = \frac{\ln 2}{t_d} \text{-----(6)}$$

and the specific growth rate (μ) is exactly equal to the dilution rate (D)(Herbert *et al*, 1956).

Because the growth rate of the organism in the chemostat is controlled by the dilution rate, the growth rate of the organism can be adjusted, within certain limits, to any value. However, the growth rate cannot be made to exceed μ_{\max} and therefore steady state conditions cannot be obtained at dilution rates above a critical value of the dilution rate (D_c). If the dilution rate is set at a value greater than D_c , the culture will be progressively washed out from the fermenter.

Influence of dilution rate on the growth-limiting substrate and the concentration of organisms in the culture.

For a complete understanding of the behaviour of microbial cultures in a chemostat, one must also consider the effect of dilution rate on the concentration of the growth-limiting substrate (s) and of the organisms (x) in the culture. Substrate enters the growth vessel from the reservoir at a concentration S_r . It is consumed by the organisms and emerges in the overflow at a concentration s . The net change in substrate concentration resulting from passage through the growth vessel can be expressed as:

$$\text{Change} = \text{Input} - (\text{Output} + \text{Consumption})$$

but from the equation for the yield constant

$$\text{Consumption} = \frac{\text{Growth}}{\text{Yield}}$$

Therefore

$$\frac{ds}{dt} = DS_r - \left(Ds + \frac{\text{Growth}}{\text{Yield}} \right)$$

or rearranging

$$\frac{ds}{dt} = DS_r - Ds - \frac{\mu x}{Y}$$

By substituting for μ (equation 4)

$$\frac{ds}{dt} = D(S_r - s) - \frac{\mu_{\max} x}{Y} \left(\frac{s}{K_s + s} \right) \text{-----(7)}$$

The net change in the concentration of organisms x can similarly be obtained by substituting for μ in equation (5)

$$\frac{dx}{dt} = x \left[\mu_{\max} \left(\frac{s}{K_s + s} \right) - D \right] \text{-----(8)}$$

If S_r and D are held constant and D does not exceed the critical value of D_c , unique values of s and x exist when the system is in a steady state (i.e. $ds/dt = 0$ and $dx/dt = 0$). These steady state values of s and x (designated as \bar{x} and \bar{s} by Herbert *et al*, 1956) can be obtained by solving equations 7 and 8 for $ds/dt = 0$ and $dx/dt = 0$.

At equilibrium

$$\mu = D = \mu_{\max} \left(\frac{s}{K_s + s} \right)$$

from equation 7

$$\bar{x} = Y (S_r - \bar{s}) \text{-----(9)}$$

and from equation 8

$$\bar{s} = K_s \left(\frac{D}{\mu_{\max} - D} \right) \text{-----(10)}$$

Substituting \bar{s} in equation

$$\bar{x} = Y \left[S_r - K_s \left(\frac{D}{\mu_{\max} - D} \right) \right] \text{-----(11)}$$

Since μ_{\max} , k_s and Y are constants, the principal effect of varying the dilution rate (equation 10) is to change the growth-limiting substrate concentration in the culture and thereby effecting a change in the specific growth rate and doubling time (equation 6) of the organism. Furthermore, since the growth-limiting substrate concentration in the culture is independent of its concentration in the inflowing medium (equation 11), the sole effect of varying S_r should be to change the steady-state microbial concentration (Herbert *et al*, 1956).

Fig. 3 shows how the mean generation time, the steady state concentration of the organism and substrate concentration in the chemostat culture vary when the dilution rate is varied.

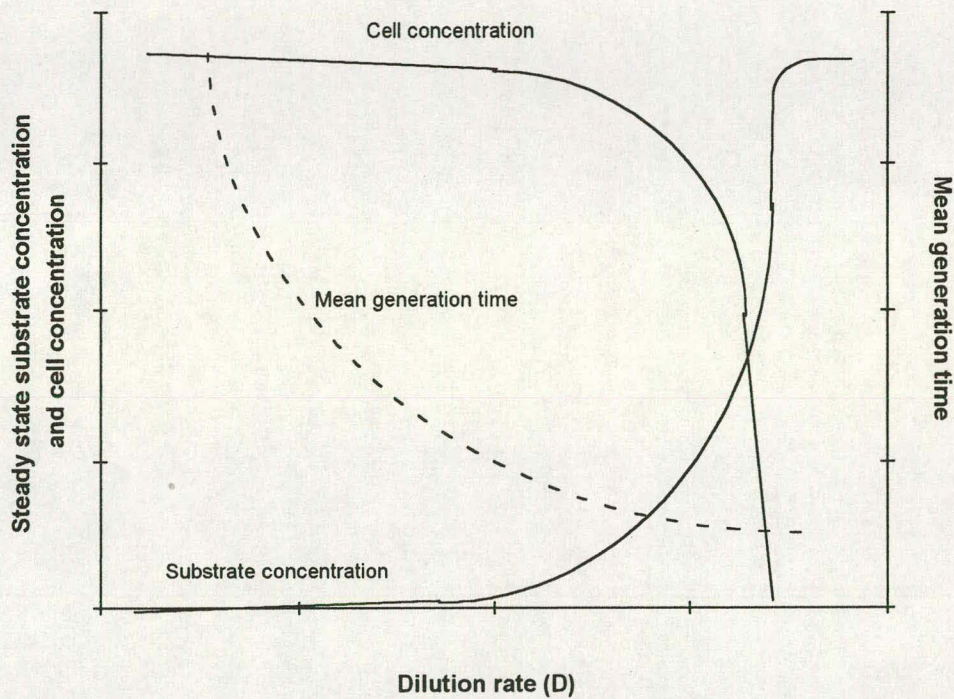


Fig. 3. Steady state relationships in a continuous culture.

Exponential decay (washout) of non-dividing particles

The rate at which non-dividing microbial cells are being lost to the effluent from a sufficiently well stirred culture vessel is proportional to the numbers x , remaining, and to the dilution rate D , and can be given as:

$$\frac{dx}{dt} = -xD \text{ -----(Powell, 1965).}$$

Integration of this expression between the limits x_t and x_0 yields the equation

$$x_t = x_0 e^{-Dt}$$

$$\therefore \frac{x_t}{x_0} = e^{-Dt}$$

or

$$\ln \frac{x_t}{x_0} = -Dt$$

$$\therefore \ln x_t - \ln x_0 = -Dt$$

$$\therefore \ln x_t = \ln x_0 - Dt$$

where x_0 is the number of cells at a time $t = 0$ and x_t is the number of cells after a time t

(h).

Microbial competition

Pure and simple competition

Microbial populations must have chemical compounds and obtain energy in order to grow and proliferate. Among the environmental parameters that commonly influence the properties of microbial cells in nature, the concentration of essential nutrients is of particular importance. Natural systems frequently are virtually depleted of one or more of these nutrients as a consequence of the metabolic activities of indigenous microbial populations. Therefore microbial growth in natural environments is nearly always nutrient limited (Harder and Dijkhuizen, 1983). This gives rise to competition in all but the simplest ecosystems (Frederickson and Stephanopoulos, 1981).

Some microbial populations use several (or many) different chemical compounds to satisfy their needs (the "generalists") whereas other populations are dependent on one or perhaps a few compounds (the "specialist") (Frederickson and Stephanopoulos, 1981). Generalists can gather enough resources from a lean environment to grow, albeit slowly, while the specialists can be regarded as exploiters in the sense that they can exploit an environment rich in the resources they can utilise to grow rapidly. In the competition between two populations for any one resource one can say that populations P1 and P2 compete for resource p if; 1) both P1 and P2 use, but do not necessarily require p and 2) resource p has a dynamic effect (such as the growth rate) on at least one of the populations (and possibly on both). Such a resource must therefore be one of the dependent variables of the system and can also be seen as the growth-limiting substrate of the system. In simple competition only one substance has dynamic effects on the competitors. This substance is a resource and it exerts dynamic effects on both competitors. In pure competition it is the only interaction between two populations. Therefore

in pure and simple competition there is only one nutrient (the growth-limiting nutrient) whose availability affects the growth rates of the populations and competition for this nutrient is the only interaction between the populations (Frederickson and Stephanopoulos, 1981).

Competition for a single growth-limiting substrate

In a highly idealised situation where a system in which the competition occurs is spatially homogenous at all times and where the inputs to the system are constant, theory predicts that under competition for a single growth-limiting substrate, all but one species will be selectively excluded from the system containing a mixed population (Tempest, 1969). This idealised situation can be approximated in the laboratory in a chemostat.

In simple and pure competition, when two organisms are competing for the same growth-limiting substrate while growing in a chemostat under steady state conditions, their behaviour can be predicted from their known relationships between substrate concentration (s) and growth rate (μ) (equation 4) (Veldkamp and Jannasch, 1972). If relationships exist as given in Fig. 4a, organism a will grow faster than b at any value of s , and therefore at any dilution rate in the chemostat b will be selectively excluded. If the saturation curves of both organisms cross, the result of the competition between a and b will depend on the dilution rate applied, which will of course determine the growth rate and substrate concentration (Fig. 4b). There is however one substrate concentration for which the corresponding growth rates of a and b are equal. When this substrate concentration is maintained, the concentrations of both the organisms will be maintained at a constant level and a condition for the coexistence in a steady state is satisfied (Veldkamp and Jannasch, 1972).

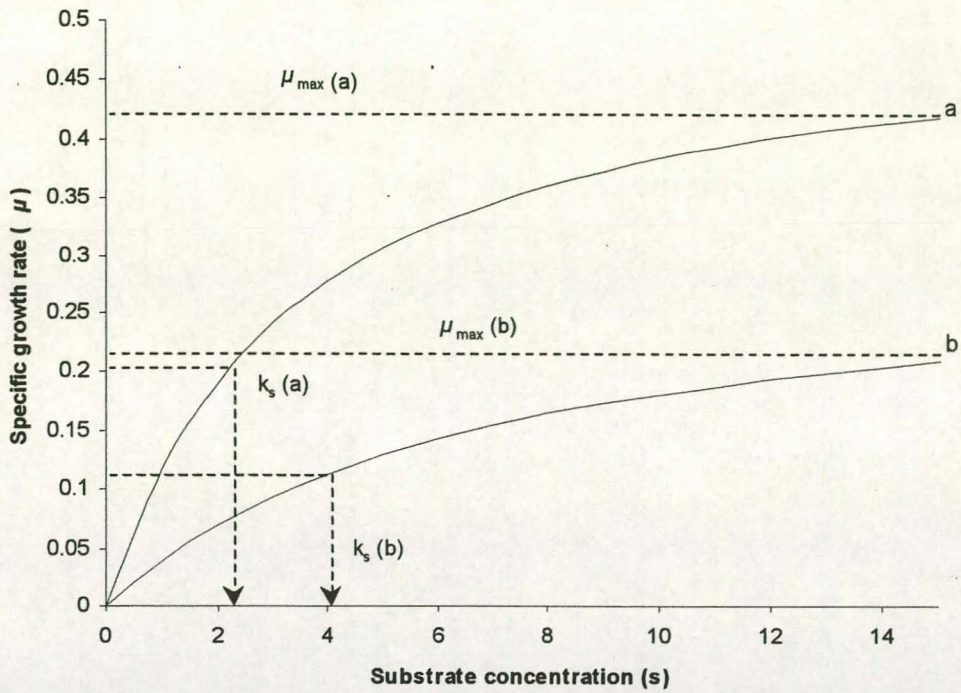


Fig. 4a. Theoretical saturation curves for two organisms (a and b) competing for the same growth-limiting substrate.

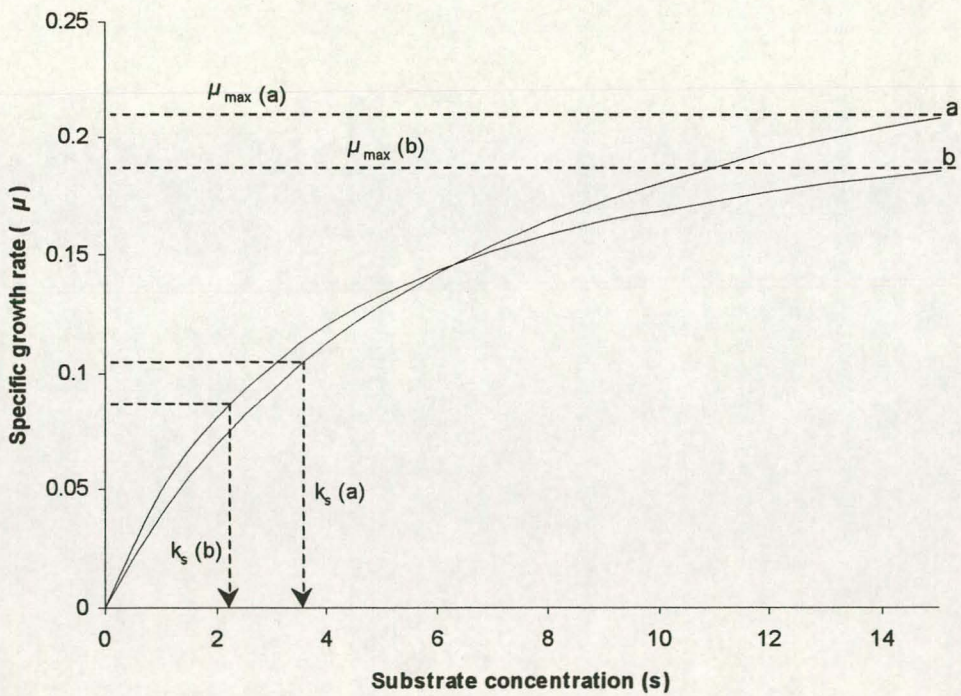


Fig. 4b. Theoretical saturation curves for two organisms (a and b) competing for the same growth-limiting substrate.

However, such a condition will seldom (if ever) be met in practice. A physical parameter such as the chemostat dilution rate, or in nature, a substrate concentration, will always exhibit some random variation with time (Frederickson and Stephanopoulos, 1981). On the other hand, time-varying inputs of some kind in a spatially homogeneous system can allow pure and simple competitors to coexist in a limited cycle, provided that the growth rate properties of the competitors are of the kind shown in Fig. 4b and the changing parameters are kept within certain bounds.

Of particular importance is the effect of k_s on the specific growth rate. The smaller k_s , the more effective the cell is at growing at a high rate at low concentrations of the limiting substrate (Fig. 4b.). In natural environments where the concentrations of nutrients are almost always growth-limiting, the micro-organisms tend to evolve with a k_s value a 100-fold less than the normal limiting substrate concentration of their environment (Sinclair and Cantero, 1990).

Coexistence of populations limited by different substrates

If different growth-limiting substrates are used by different organisms, coexistence will occur since these organisms then occupy different ecological niches (Veldkamp and Jannasch, 1972). This was also proved by the mathematical model developed by Taylor and Williams (1974). They concluded that to sustain a mixed population of a number of species in a chemostat-type continuous-flow system it is necessary that there are at least as many growth-limiting substrates as there are different species.

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Chapter 6

THE ESTABLISHMENT OF A COMMUNITY OF ORAL BACTERIA THAT CONTROLS THE GROWTH OF *CANDIDA ALBICANS* IN A CHEMOSTAT

Materials and Methods

The chemostat

There are several control techniques which can be applied to continuous culture. Substrate limitation, i.e. the chemostat culture technique, is the one control technique most extensively studied (Herbert, 1956; Veldkamp, 1976; Brown, 1990). In this study, substrate limitation was the control technique used. Therefore, the feed medium was designed to contain, in excess, all but one of the nutrients essential for growth, i.e. glucose.

The instrument used was a BIOFLO[®] Model C32 bench top chemostat (New Brunswick Scientific Co., Inc., New York). The apparatus with all its commonly used ancillary equipment is shown in a schematic diagram in Fig. 5. Fresh sterile medium was pumped from a reservoir (A) at a flow rate of f ml/h into the culture vessel (B). This had a working volume (V) of 600 ml. The level of culture in the vessel was regulated by a side overflow tube through

which the culture flowed by gravity into a receiver bottle (C). The flow rate of the sterile medium was regulated with a metering pump (M) and precisely measured with a flow meter (F). The culture was agitated with a stirrer (D) and aerated with gas of which the flow was regulated with a flow meter (G). A sampling port (S) was provided on the culture vessel as well as a point for the addition of inoculum. Temperature was controlled by a thermistor and heater element. The pH was measured and kept constant by the automatic addition of NaOH by means of a pH controller (H).

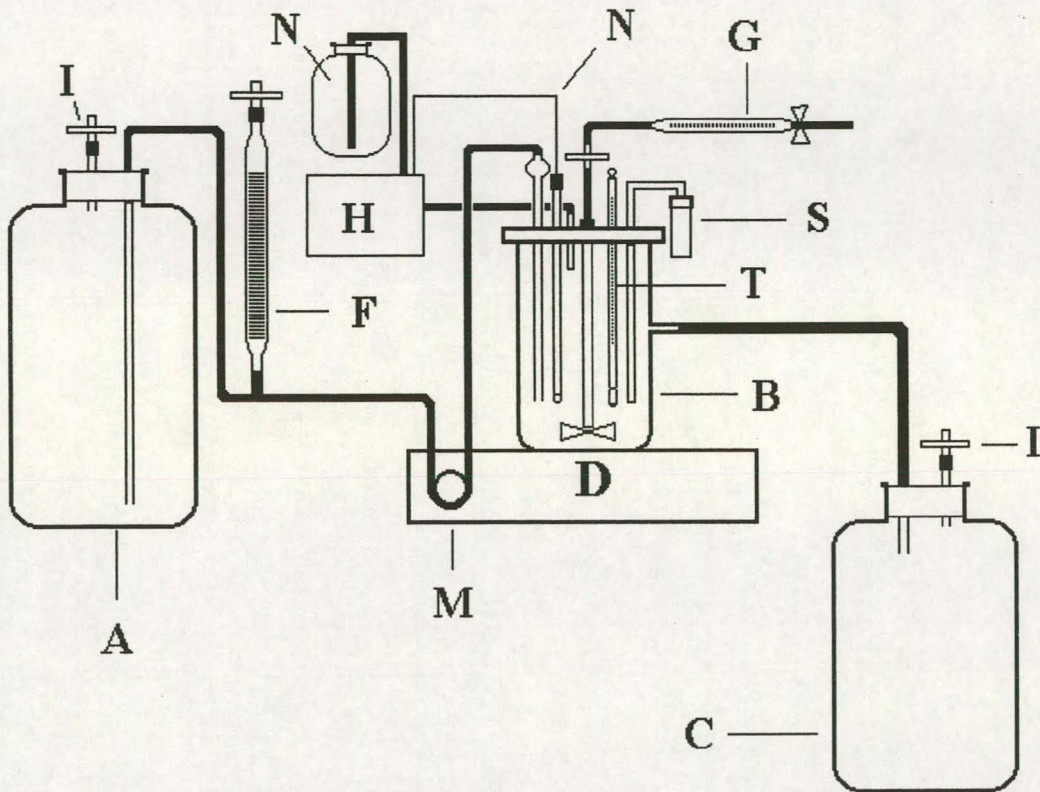


Fig. 5. Schematic diagram of a laboratory chemostat. Medium reservoir (A); culture vessel (B); receiver bottle (C); stirrer (D); pH electrode (E); medium flow meter (F); gas flow meter (G); pH controller (H); filter (I); alkali reservoir (N); metering pump (M); sampling port (S); thermometer (T)

Operation of the chemostat

After thorough cleaning, the components of each section of the chemostat were assembled, the terminal connections wrapped in aluminium foil, and each section was sterilised separately. The components were sterilised in a steam autoclave at a working temperature of 121°C for 30 minutes. The medium reservoir was filled with 10 litres of medium and sterilised separately at 121°C for 80 minutes. All the sections were assembled at the different connection points as shown in Fig. 6.

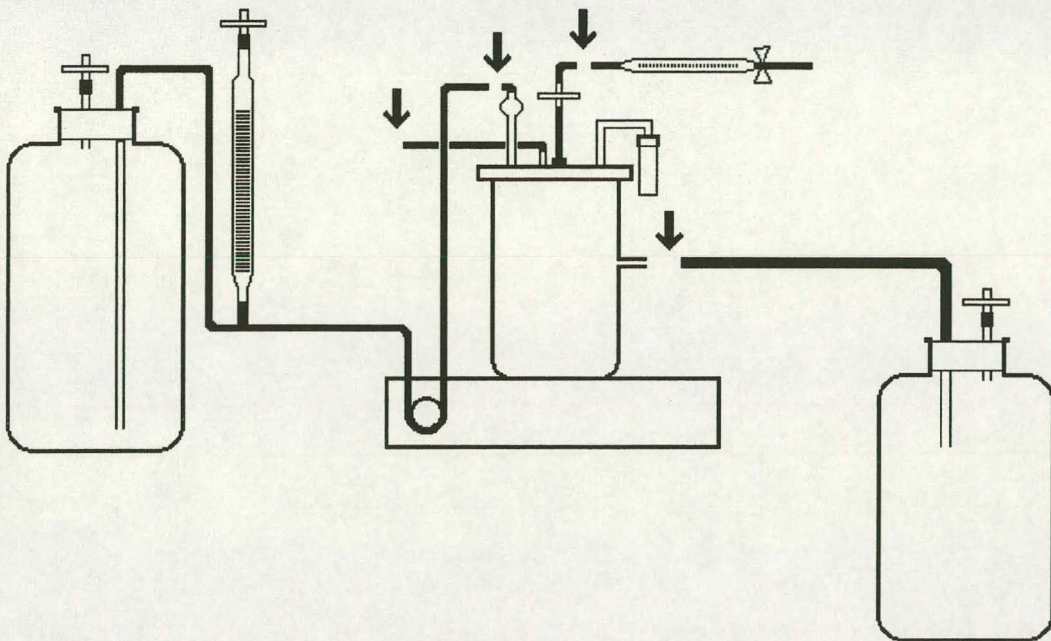


Fig. 6. Disconnection points for sterilisation

The culture vessel was then filled with medium from the medium reservoir, and the stirrer switched on. Good mixing is a prerequisite for proper operation so that the entering growth

medium is instantaneously and uniformly dispersed throughout the vessel. Furthermore, clumping is a source of non-homogeneity and growth conditions within an aggregation of cells are very different from those outside the clump. Therefore homogeneity of the culture must also be maintained at all times. (Veldkamp, 1976). To ensure this an optimum speed of 350 rpm was selected to facilitate good mixing and homogeneity and to prevent foaming of the culture. The temperature and gas flow rate were then adjusted to 37°C and 50 ml/min respectively. The unit was left running for 24 h before it was inoculated to ensure sterility and to ensure that all controls were functioning properly. The chemostat was then inoculated and the medium flow rate was adjusted to a dilution rate of $D = 0.05 \text{ h}^{-1}$ by filling the flow rate tube (F, Fig. 5) and by measuring the withdrawal rate of the medium with a stopwatch. The flow rate was calculated on the basis of the following equation;

$$f = \frac{v}{t} \times 3600$$

where f = medium flow rate (ml/h), v = medium volume in flow rate tube (ml) and t = time in seconds (multiplication with 3600 converts the flow rate to ml/h).

The dilution rate (D) of the culture was calculated on the basis of the equation;

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

where f = medium flow-rate and V = culture volume in the culture vessel (Herbert, Elsworth and Telling, 1956).

The redox potential (Eh) as well as the pH of the culture in the culture vessel was measured and checked daily by removing a 10 ml sample of the culture. The Eh was measured externally with a redox electrode while the pH was checked against a pH standard.

Chemostat growth medium

The chemostat growth medium (CGM) that was used throughout the study was a modification of the medium used by Shah *et al* (1976). The growth medium contained (per liter of distilled water) 5 g of proteose peptone (Oxoid, Basingstoke, UK), 5 g of tryptone, (Oxoid), 1 g of LabLemco powder (Oxoid), 2 g of yeast extract (Oxoid), 0,5 g of cysteine hydrochloride (Merck, Darmstadt, Germany), 5 g of sodium chloride (Merck), 5 mg of haemin (Sigma, Chemical, Co., Pool, UK), 1 mg of menadione (Sigma), 14.8 g of *di*-sodium hydrogen phosphate dodecahydrate (Merck), 1.2 g of potassium di-hydrogen phosphate (Merck).

Growth conditions

A BIOFLO[®] Model C32 chemostat (New Brunswick Scientific, Edison, NJ) was operated at a dilution rate (D) of 0.05 /h, a temperature of 37°C, and a working volume of 600 ml under a gas phase of 5% (v/v) CO₂ + 95% (v/v) N₂ (flow rate of 50 ml/min). The pH was maintained at 7 ± 0.1 by the automatic addition of 10M NaOH and the Eh measured with a redox electrode. The chemostat growth medium (CGM) was used. To achieve glucose limitation the medium was supplemented with 28 mM glucose. The medium components (without the glucose) were dissolved in 9 liters of distilled water and autoclaved at 121°C for 80 minutes. The glucose was dissolved in one liter of distilled water, autoclaved at 121°C for 30 minutes, and aseptically added to the CGM medium.

Chemostat inoculation

In pure culture studies, chemostats start off as a batch culture. In a study of microbial communities such a start would result in the immediate overgrowth of slow-growing bacteria by those with faster growth rates. In order to prevent this, the first inoculation was made when

the chemostat was only partially filled with medium. Approximately 300 ml of culture medium (half the working volume of the chemostat) was pumped into the chemostat to control the pH and temperature. The dorsal surface of the tongue of a yeast-free individual was scraped with a spatula and the scrapings suspended in 1 ml of reduced transport fluid (RTF) (Syed and Loesche, 1972) containing 1 g of glass beads (2 mm diameter). The suspension was vortexed for 1 minute and 1 ml inoculated into the chemostat medium. Fresh medium was then added at a rate of 31 ml/h (dilution rate of 0.05 /h). This ensured that at no time during this critical period would the bacteria have been growing as in a conventional batch culture. In an attempt to establish a full enrichment culture of oral bacteria, the chemostat was re-inoculated on two subsequent occasions on days 2 and 5 (McKee *et al*, 1985). At least 14 generations were allowed to elapse after the last inoculation before the culture was considered to have reached a steady state.

In order to determine the yeast-free status of the individual, the person was monitored daily for 1 week prior to starting the experiment. This involved spreading 1 ml of a tongue scraping (treated as above) on Sabouraud Dextrose agar (Oxoid) plates. The plates were incubated aerobically for 24 h at 37°C and investigated for yeast colonies.

The chemostat culture was examined daily with Gram's stain to observe the succession of populations during the development of the climax community. After a steady-state had been reached, the total carbohydrate content of the culture effluent was determined by the method of Kingsley and Getchell (1960) to confirm carbohydrate limitation.

Identification of chemostat steady-state community

The microbial composition of the chemostat steady state community was determined by differential colony counts on a variety of selective and non-selective media. One ml aliquots of the chemostat culture were dispersed by vortex mixing for 1 minute with 1 g glass beads.

The sample was serially diluted in RTF and plated on 5% horse blood agar (Blood Agar Base No. 2, Oxoid) supplemented with 5 mg/l haemin (Sigma) and 1 mg/l menadione (Sigma) for total counts; blood agar supplemented with 2.5 µg/ml of vancomycin (Sigma) for enumeration of Gram-negative bacteria; TYC medium (5) for the oral streptococci, and Rogosa SL agar (Difco Laboratories, Detroit, MI) for lactobacilli. All media were incubated anaerobically for 5 days in an atmosphere of 10% CO₂ + 10% H₂ + 80% N₂ (v/v/v), except for one set of vancomycin agar plates and one set of blood agar plates, which were incubated aerobically for 3 days. Each colony type was counted separately and representative colonies were subcultured on blood agar. The organisms were then Gram-stained and tested for their ability to grow aerobically and/or anaerobically. The oral streptococci were identified according to the identification scheme of Beighton *et al* (1991). The lactobacilli were identified by physiological tests described by Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham, 1993). The anaerobes were identified to species level by the analysis of their fatty acid end products produced by the fermentation of glucose (Krieg and Holt, 1984).

The physiological tests used can be summarised as follows: Catalase activity was tested by touching a colony and suspending the cells in a drop of 6% H₂O₂ and examined immediately and after 5 minutes for evolution of gas. Oxidase activity was tested using a fresh solution of a modified Kovács reagent (Barrow and Feltham, 1993). A filter paper disc was wetted with a few drops of the tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) reagent and the test culture (grown on a glucose free medium) was smeared across the moist paper with a glass rod. The appearance of a dark purple colour within 30 seconds denoted a positive reaction. Oxidation or fermentation of glucose was tested by stab-inoculation of duplicate tubes containing Hugh and Leifson's medium (Barrow and Feltham, 1993). To one of the tubes a layer of sterile liquid paraffin was added to seal it from air. The tubes were incubated

at 37°C and examined daily for up to 14 days. Carbohydrate fermentation reactions were determined with a basal medium composed of 24g/l Purple Broth Base (Difco) and 12g/l Thioglycollate medium (Difco) without dextrose or indicator. Filter sterilised stock solutions of the carbohydrates were added aseptically after autoclaving to obtain final carbohydrate concentrations of 1%. The tests were set up in sterile test tubes containing 5 ml medium. Esculin hydrolysis was detected by the presence of dark coloration after 3 days of growth in a broth containing 1% (w/v) Trypticase (Difco), 0.1% (w/v) esculin (Sigma) and 0.05% (w/v) ferric citrate (Sigma). For the carbohydrate fermentation tests and the esculin hydrolysis tests, the facultative anaerobic bacteria were grown aerobically for 48 h in 5 ml volumes of Todd-Hewlett Broth (Oxide) for inoculation into the tubes. Each tube was inoculated with 2 drops of the above cultures, incubated aerobically for 72 h and the results recorded.

The fatty acid profiles of the anaerobes were analysed with a Gas Liquid Chromatograph (GLC) using the method of Salanitro and Muirhead (1975). Each organism to be identified was inoculated into an anaerobe broth that contained (per liter of distilled water) 10 g of proteose peptone (Oxoid), 10 g of tryptone, (Oxoid), 5 g of yeast extract (Oxoid), 0,5 g of cysteine hydrochloride (Merck), 5 g of sodium chloride (Merck), 5 mg of haemin (Sigma), 1 mg of menadione (Sigma) and 5 g of glucose (Merck). The organisms were grown anaerobically at 37°C for 7 days. Cultures were centrifuged to remove the cells, the pH of the supernatants were adjusted to between 9 and 10 with 10M NaOH and 1 ml aliquots were freeze-dried. Butylation of the sodium salts of the acids in the samples were performed by adding 0.8 ml of chloroform (Merck) and 0.2 ml of 1-butanol (Merck) saturated with anhydrous HCl (Merck) to the dry samples. After vortex mixing, the tubes were sealed and the mixture was heated at 80°C for 2 h. The tubes were cooled to room temperature and 0.2 ml of trifluoroacetic anhydride (Merck) was added. The solution was mixed and allowed to react for 1 h. Samples were then washed with 1 ml aliquots of distilled water and the water

layer discarded. Two microlitres of the sample was injected into the GLC. The fatty acid profiles were analysed against a standard mixture containing formic, acetic, propionic, isobutyric, butyric, isovaleric, caproic, heptanoic, lactic, fumaric, and succinic acids.

A Hewlett Packard 5890A gas liquid chromatograph, equipped with a flame ionisation detector and a HP cross-linked methyl silicone fused silica column with a 0.53 mm inside diameter and length of 10 m was used. The operating conditions were; nitrogen carrier gas-flow rate, 3.0 ml/min; injection-port temperature, 250°C; detector temperature, 270°C; initial column temperature, 50°C for one minute and a programmed temperature rate of 20°C/min to a final column temperature of 230°C for one minute.

Inoculation with *C. albicans*

The ability of the steady-state community to limit *C. albicans* growth was tested by inoculating the chemostat with the yeast after a steady-state condition had been reached. *C. albicans* strain NCPF 3118 (Mycological Reference Laboratory, London, UK) was inoculated into 10 ml of the chemostat growth medium and incubated for 24 h at 37°C. The chemostat was inoculated with 1 ml of this suspension and the yeast counted daily to monitor *C. albicans* growth. A sample was removed from the chemostat. One ml of this sample was dispersed by vortex mixing for 1 min with 1 g of 2 mm glass beads, serially diluted in RTF and plated on Sabouraud Dextrose agar (Oxoid). The plates were incubated aerobically for 24 h at 37°C and the yeast colonies were counted. The experiment was repeated 3 times and counts made in duplicate.

The growth rate of *C. albicans* in the chemostat growth medium and under the experimental conditions was determined in batch culture in the chemostat. The medium flask was loaded with 600 ml of sterile basal medium, the gas flow set and the medium brought to 37°C while being stirred. After inoculation with 20 ml of an 18-h seed culture, growth was monitored for

optical density with a Novaspec spectrophotometer (LKB Biochrom, Cambridge, UK) at 560 nm. Yeast dry masses were determined by taking duplicate 10 ml samples, centrifuging and washing the cells twice in distilled water and drying the samples at 105°C for 18 h. A growth curve was constructed and the mean generation time of the yeast in the exponential growth phase was calculated.

Calculation of the theoretical washout rate

The rate of loss of *C. albicans* from the chemostat was calculated and compared with the theoretical exponential decay kinetics of inert (non-dividing) particles by application of chemostat theory. According to Powell (1965), the rate at which non-dividing particles are lost from a well stirred culture vessel can be expressed by the equation

$$\ln x_t = \ln x_0 - Dt \quad (\text{see chapter 3}).$$

When transformed to the logarithm with base 10 this equation becomes

$$\log x_t = \log x_0 - Dt/2.3 \quad (\text{Crowe and Crowe, 1969})$$

where x_t is the number of particles after a time t , x_0 is the initial number of particles and D is the dilution rate.

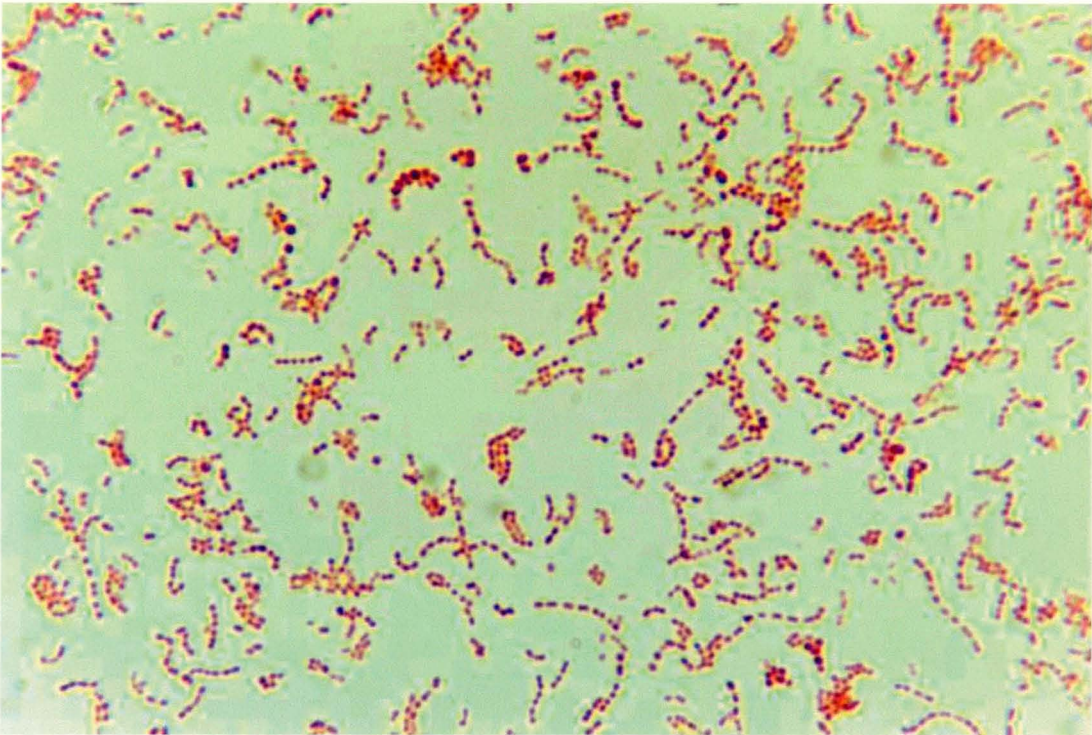
Testing for amensalism amongst the species

Amensal (inhibition of one population by a “toxin” produced by a second population) between members of the climax community and *C. albicans* was tested by cross-streaking each bacterium and the yeast on 5% horse blood agar plates supplemented with 5 mg haemin and 1 mg menadione. The plates were incubated anaerobically at 37°C for 48 h and observed for inhibition of *C. albicans* growth.

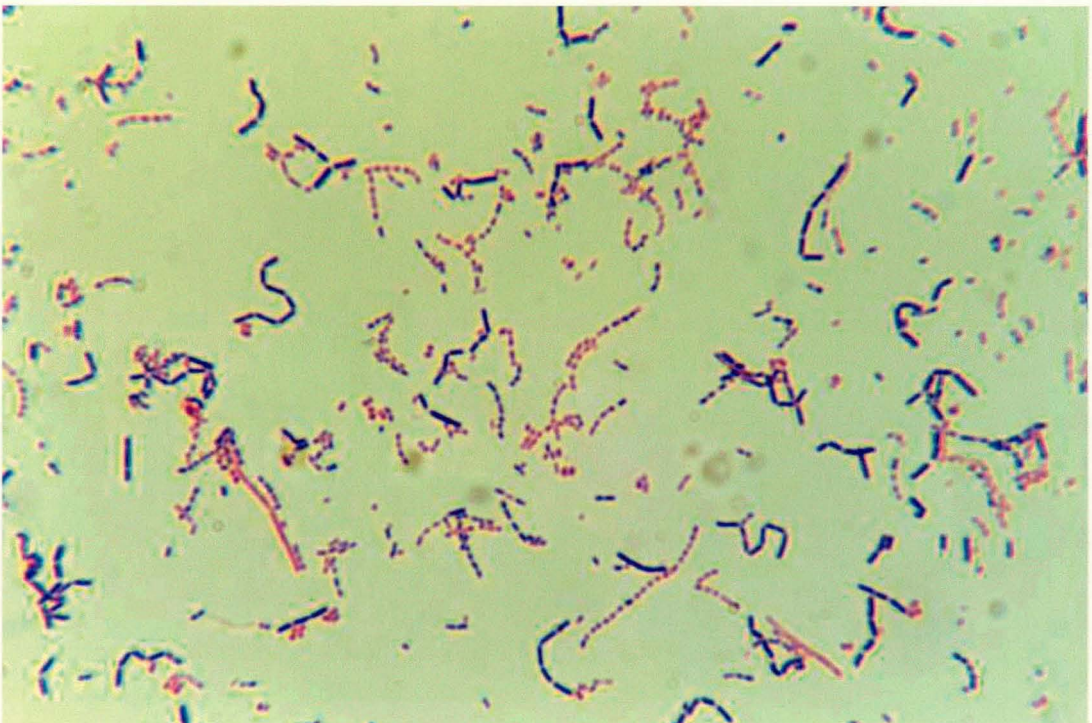
Results

Chemostat inoculation

A steady-state mixed bacterial community comprising of at least 7 oral species established in the chemostat within 10 volume changes of the growth medium (10 generations). A succession of the populations in the development of the community was observed. Gram-positive cocci were the first organisms to establish. Within 5 h (0.25 generations) after inoculation, the cocci reached a high level of growth causing the medium to become turbid and were the only organisms observable in the culture at 24 h (1.2 generations) (Fig. 7a). During this stage the Eh of the medium dropped below -150 mV. Their proportions decreased slightly after the second day, but they remained dominant. Between the second and third day (3.5 generations) Gram-positive rods appeared (Fig. 7b). They increased slowly in number but remained at comparatively low concentrations throughout the experiment. During this period a few small Gram-negative diplococci, whose numbers increased gradually over time, were also seen. After \pm 5 days (6 generations), Gram-negative rods were present but remained at low concentrations (Fig. 8a). The Gram positive cocci decreased to low proportions compared to the initial stages of the community. After 5 days the community was stable, indicating that a climax community had been reached. At this stage microscopic examination showed Gram-positive rods, Gram-negative rods, Gram-negative cocci and a few Gram-positive cocci (Fig. 8b). The Eh of the medium at this stage remained below -200 mV.

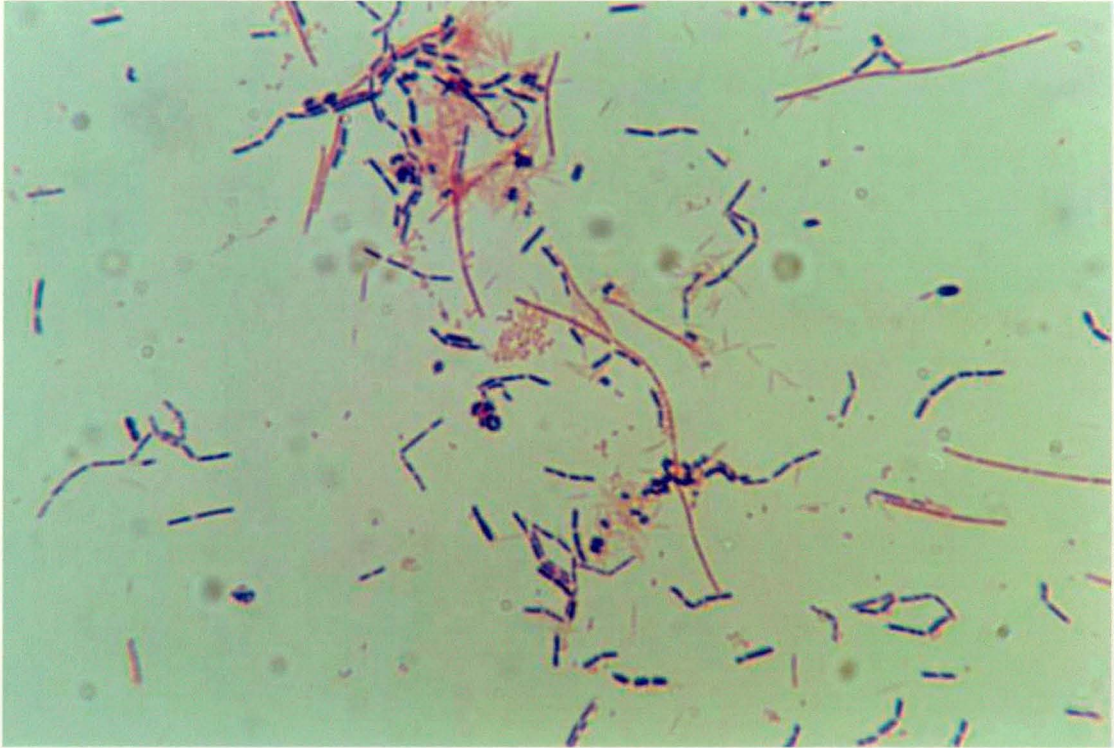


a) the community after 24 hours

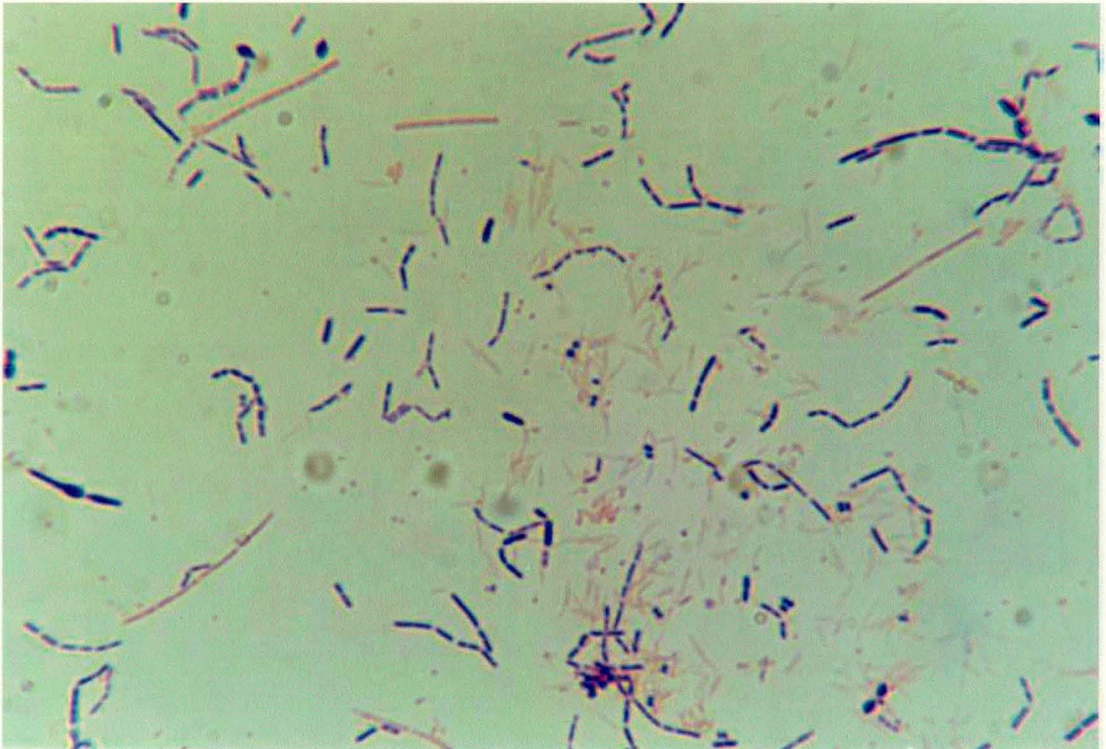


b) the community after 48 hours.

Fig. 7. Microscopic observation of the succession of populations during the development of the chemostat microbial community after 24 and 48 hours.



a) the community after 72 hours



b) the community after 120 hours

Fig. 8. Microscopic observation of the succession of populations during the development of the chemostat microbial community after 72 and 120 hours.

Identification of chemostat steady-state community

In all 3 experiments, 9 different species were isolated from the steady-state climax communities (Table 6). Streptococci accounted for 89-96% of the total anaerobic count. Other populations each were 1% or less of the total anaerobic count.

The species *Streptococcus sanguis*, *Streptococcus sobrinus*, *Streptococcus mitis*, *Lactobacillus rhamnosus*, *Eubacterium saburreum*, *Fusobacterium nucleatum* and *Veillonella parvula* were present in all steady state communities and regarded as a “basic” community. Isolation of the remaining species were infrequent: *Haemophilus segnis* was present in two of the chemostat communities whereas *Bacteroides gracilis* occurred only once.

Inoculation with *C. albicans* and the theoretical washout rate

After inoculation the number of *C. albicans* cells declined rapidly from a mean count of $\log 4,01 \pm 0,21$ colony-forming units (cfu)/ml to $\log 3,27 \pm 0,32$ cfu/ml within 24 h (Table 7, Fig. 9). The mean rate of loss of 0,033 log cfu/ml/h within this period was higher than the theoretical washout rate of 0,022 log particles/ml/h. The decline in the number of yeast cells continued and the rate of loss remained faster than the theoretical washout rate. After 6 days, the yeast counts were below the detection level of 10 cfu/ml. This pattern was consistent in all 3 experiments. In batch culture in the basal medium the yeast had a mean generation time of 2.9 h ($\mu_{\max}=0,237/h$) during the exponential growth phase and reached a final optical density of 0.84.

Testing for amensalism amongst the species

No inhibition of growth of *C. albicans* by any of the bacterial species was noticed on cross-streaked blood agar plates.

Table 6. Steady-state bacterial communities that established in the three different experiments.

Species	Mean log cfu/ml		
	Exp. 1 (n=2)	Exp. 2 (n=2)	exp. 3 (n=2)
<i>Streptococcus sanguis</i>	10.7	10.6	6.3
<i>Streptococcus sobrinus</i>	10.8	7.8	7.9
<i>Streptococcus mitis</i>	5.0	7.6	5.8
<i>Lactobacillus rhamnosus</i>	5.2	3.3	6.5
<i>Veillonella parvula</i>	8.5	9.7	6.2
<i>Eubacterium saburreum</i>	6.2	6.5	5.8
<i>Bacteroides gracilis</i>	7.6	-	-
<i>Haemophilus segnis</i>	6.3	6.5	-
<i>Fusobacterium nucleatum</i>	6.8	4.1	4.9

Table 7. The theoretical washout rate of a non-dividing particle and the true washout rate of *Candida albicans*

Time (days)	log non- dividing particle/ml	<i>C. albicans</i> mean log cfu/ml				Mean all exp. (n=6)	Mean washout rate (log cfu/ml/h)
		exp. 1 (n=2)	exp. 2 (n=2)	exp. 3 (n=2)			
0	4.08	4.15	3.92	3.96	4.01 ± 0.12		
1	3.57	3.46	3.16	3.21	3.27 ± 0.16	-0.03	
2	3.07	2.38	2.51	2.60	2.49 ± 0.11	-0.03	
3	2.56	1.60	1.70	1.48	1.59 ± 0.11	-0.04	
4	2.06	1.00	1.00	1.00	1.00 ± 0.0	-0.02	
5	1.56	<1	<1	<1	<1	-	
6	1.05	<1	<1	<1	<1	-	

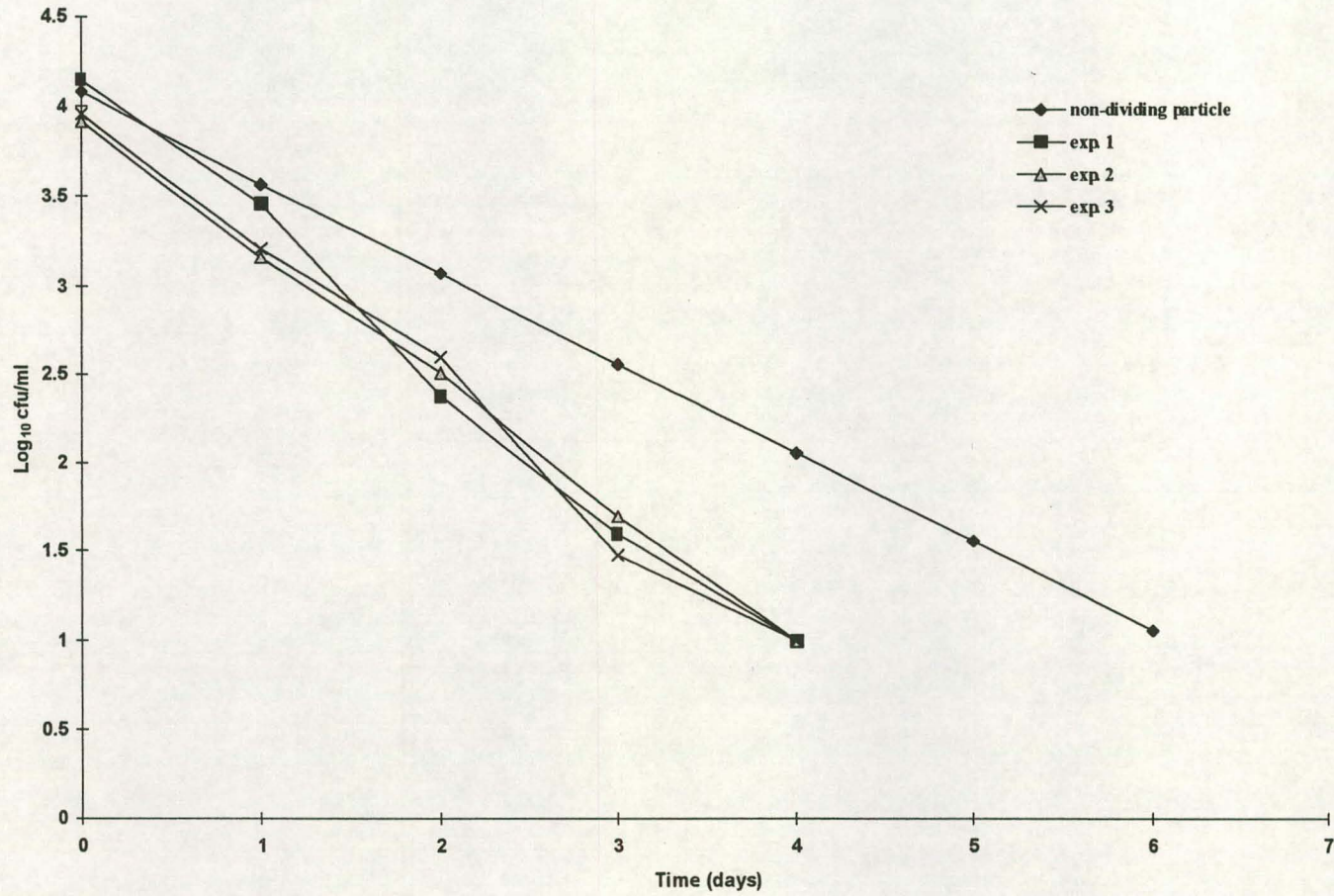


Fig. 9. The rate of loss of *C. albicans* from the chemostat and the theoretical washout rate of a non-dividing particle.

Discussion

The concentration of essential nutrients is of particular importance among the environmental parameters that commonly influence the growth of microbial cells in nature. Natural ecosystems are frequently virtually depleted of one or more of these nutrients because of the metabolic activities of indigenous microbial populations. As a result microbial growth in natural environments is nearly always nutrient-limited (Veldkamp and Jannasch, 1972). In the oral cavity growth is also limited by the availability of essential nutrients as a result of the metabolism of the normal oral flora (de Jong *et al*, 1984; Marsh, 1989). However, these nutrient limitations, particularly of carbohydrates, are often interrupted by periods of substrate excess following food intake by the host (van der Hoeven, 1976; de Jong *et al*, 1984).

Experimentally, the above conditions can be imposed upon microbial populations in a flow-controlled continuous culture. Because it is difficult (if not impossible) to control all the different parameters that are encountered in the oral cavity under *in vivo* conditions, a study into the microbial competition for glucose amongst populations can best be performed under *in vitro* conditions. The chemostat is ideally suited for this purpose. With the chemostat a mixed population of oral bacteria can be grown continuously and under controlled conditions at growth rates, and therefore growth-limiting substrate concentrations, similar to that found in the oral cavity (Marsh *et al*, 1983; McKee *et al*, 1985).

The first experiment was designed to establish and identify a mixed population of oral bacteria that would grow in a steady-state in the chemostat and that would prevent the colonisation and growth of *C. albicans*. To achieve this the chemostat study by McKee *et al* (1985) was used as a method of approach. However, instead of using their defined inoculum, a tongue scraping was used as an inoculum. The dorsum of the tongue is the most common

site of isolation of *C. albicans* (Arendorf and Walker, 1979; Arendorf and Walker, 1980; Marsh and Martin, 1992). Therefore, a tongue scraping would afford the establishment of organisms that are found in the same niche as the yeast.

An inoculation procedure used by McKee *et al* (1985) was followed. Firstly, in order to overcome the problem of the slow-growing bacteria been overgrown by those with faster growth rates, the first inoculation was made when the chemostat was partially filled with medium. Thus, the faster growing organisms were limited to a growth rate of $\mu = 0.05$ (mean generation time = 13,8 h) within the first hours of the experiment. This was within the growth rate limits of 0.03 to 0.08 /h that have been estimated for the growth rate of the oral bacteria *in vivo* (Ellwood, 1976; Gibbons, 1964). Secondly, multiple inoculations were performed to ensure maximum opportunity for bacterial populations to colonise, because a suitable niche for a particular organism may be available only following the interaction of established bacteria with their environment. The succession of the populations that were observed in the development of the steady-state climax community confirmed that this was indeed the case. The initial stages of the chemostat experiment were dominated by Gram positive streptococci. These organisms changed the habitat by reducing the redox potential from an aerobic to an anaerobic environment and made the habitat more suitable for the establishment of the anaerobic species. This was observed as a decrease in the Eh (from +80 mV to -234 mV) at the time of the second inoculation, and also by the appearance of Gram positive rods and Gram negative cocci after the second inoculation (probably lactobacilli, veillonellae and eubacteria according to the composition of the climax community). The more nutritious demanding Gram negative anaerobic bacteria (the genera *Bacteriodes*, *Fusobacterium* and *Haemophilus* were identified amongst the climax community populations) only appeared after the third inoculation at a stage when the pioneer organisms could have established a suitable niche for these species.

An important requisite for the use of the chemostat to study reproducible mixed cultures is for a standardised inoculum to give a similar microbial community in replicate experiments. In this study, repeated runs resulted in microbial communities that were identical with regard to their qualitative bacterial composition while the viable counts of individual populations and their relative proportions to one another were similar and within the bounds of experimental error.

The steady-state community comprised the species *S. sanguis*, *S. sobrinus*, *S. mitis*, *L. rhamnosus*, *V. parvula* and *E. saburreum*. Members of these genera were also present in the majority of samples in a chemostat study performed by Marsh *et al* (1983) and constituted part of the chemostat communities in a study by McKee *et al* (1985). However, in spite of the complexity of the community that was obtained in the chemostat, it is obvious that the composition of the inoculum was not nearly reproduced under the *in vitro* environment. A diverse microbiota with several obligatory anaerobic species can be found on the dorsum of the tongue. The streptococci are the most numerous group of bacteria (approximately 40% of the total cultivable microflora) with *S. salivarius*, *S. anginosus* and *S. mitis* predominating. Other major groups of bacteria include *Veillonella* species, *Actinomyces* species and *Haemophilus* species. *Stomatococcus mucilaginosus* is found almost exclusively on the tongue. Other organisms that can be found in low numbers include anaerobic streptococci, both pigmented and non-pigmented anaerobes, lactobacilli, fusobacteria and some motile bacteria (Marsh and Martin, 1992). Several of these species were absent from the steady-state culture. One possible explanation for the absence of some of the genera can be found from the results obtained from enrichment experiments from river and estuarine sources (Brown *et al*, 1977; Wardell *et al*, 1980; Ellwood *et al*, 1982) and from the results of the composition of the microbial communities of the “spatter-zone” in the chemostat study by Marsh *et al* (1983). The results from these experiments suggest that solid surfaces provide a different environment

and consequently support the growth of different bacterial populations to that of a fluid environment. Another explanation can be found in chemostat theory. Unless microbial interactions or different substrate utilisation occurs, a bacterial population will competitively displace another if a culture is inoculated with a mixture of organisms (Harder and Veldkamp, 1971; Veldkamp and Jannasch, 1972; Taylor and Williams, 1974).

Nevertheless, the seven species that colonised the chemostat were able to prevent the colonisation and growth *C. albicans*. Therefore, although only seven of the multitude of possible organisms were able to establish, the aim of establishing a mixed community of oral bacteria that would prevent the growth of *C. albicans* in the chemostat, was obtained.

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Chapter 7

COMPETITION FOR GLUCOSE BETWEEN *CANDIDA ALBICANS* AND ORAL BACTERIA GROWN IN MIXED CULTURE IN THE CHEMOSTAT.

Materials and Methods

Growth conditions

A BIOFLO[®] Model C32 chemostat (New Brunswick Scientific, Edison, NJ) was operated at a dilution rate of 0.05 / h, a temperature of 37°C, and a working volume of 600 ml under a gas phase of 5% (v/v) CO₂ + 95% (v/v) N₂ (flow rate of 50 ml/min). The pH was maintained at 7 ± 0.1 by the automatic addition of 2 M NaOH and the Eh measured with a redox electrode. The chemostat growth medium (CGM) was used. The medium was supplemented with 28 mM glucose (BDH) to achieve glucose limitation. To obtain glucose-excess conditions the medium was supplemented with 170 mM glucose (BDH). The medium and the glucose were autoclaved separately and the glucose was added aseptically after sterilisation.

Microbial strains

The organisms had been chosen on the basis of their ability to establish and grow in mixed culture in the chemostat in the first experiment (see chapter 6). Type culture strains of the bacteria were obtained from the National Collection of Type Cultures (NCTC), Colindale, UK. The strains were: *Streptococcus sanguis* (NCTC 10904), *Streptococcus sobrinus* (NCTC 10921), *Streptococcus mitis* (NCTC 10712), *Lactobacillus rhamnosus* (*casei* subsp *rhamnosus*)* (NCTC 10302), *Veillonella parvula* (NCTC 11463) and *Fusobacterium nucleatum* (ATCC 25586). A type culture strain of the yeast *C. albicans* strain NCPF 3118 was obtained from The National Collection of Pathogenic Fungi (NCPF), Colindale, UK. The *Eubacterium saburreum* strain (B6I8) was isolated from the chemostat and identified in the first experiment.

Chemostat inoculation

During the first stages of growth after the inoculation of a chemostat with a mixed microbial community, immediate overgrowth of slow-growing bacteria by those with faster growth rates would occur. In order to prevent this, the chemostat was inoculated in a similar way to the first experiment. The first inoculation was made when the chemostat was only partially filled with medium. Approximately 300 ml (half the working volume) of culture medium was pumped into the growth vessel to control the pH and temperature. Each organism was grown separately in anaerobic batch cultures in the chemostat growth medium for 24 h and 10 ml of

* *L. rhamnosus* has been proposed as a new species to replace the species *L. casei* subsp *rhamnosus* Hansen 1968 (EUZÉBY JP. List of bacterial names with standing in nomenclature: a folder available on the Internet (last updated: January 2000, URL: <http://www-sv.cict.fr/bacterio/>). Int. J. Syst. Bacteriol., 1997, 47, 590-592.

each culture (except the yeast) was inoculated into the chemostat. Fresh medium was then added at a rate of 31 ml/h (dilution rate of 0.05 /h). Thus, at no time during this critical period would the bacteria have been growing as in a conventional batch culture. Furthermore, McKee *et al* (1985) found that more than one inoculation was necessary to ensure the establishment of all the organisms contained in an inoculum. Therefore, the chemostat was re-inoculated on two subsequent occasions on days 2 and 5 to establish all strains. Before each re-inoculation a 100 ml of culture was removed. The culture was examined daily with Gram's stain to observe the succession of populations during the development of the community. After a steady state had been reached the chemostat was inoculated with 10 ml of a 12 h culture of *C. albicans* grown in the same medium.

Viable counts of the microbial community

At the time of inoculation with the yeast, and at the termination of the experiment, viable counts of the chemostat communities were made. Samples (1 ml) were removed from the growth vessel, dispersed, serially diluted in RTF (Syed and Loesche, 1972) and plated on selective media. The media used were TYC medium (Carlsson, 1972) for the identification of the streptococci, Rogosa SL agar (Difco) for lactobacilli, 5% horse blood agar (Blood Agar Base No. 2, Oxoid) supplemented with 5 mg/l haemin (Sigma) and 1 mg/l menadione (Sigma) for the enumeration of *E. saburreum*, and blood agar supplemented with 2.5 µg/ml of vancomycin (Sigma) for the enumeration of the Gram-negative bacteria. All media were incubated anaerobically for 5 days in an atmosphere of 10% (v/v) CO₂ + 10% H₂ + 80% N₂ (v/v). After inoculation with *C. albicans*, yeast counts were performed daily to monitor the yeast growth. A sample was removed from the chemostat and 1 ml of this sample was dispersed, serially diluted in RTF and plated on Sabouraud agar (Oxoid). The plates were incubated aerobically for 24 h at 37°C and the yeast colonies were counted.

Calculation of the theoretical washout rate

The rate of loss of *C. albicans* from the chemostat was calculated and compared with the theoretical exponential decay kinetics of inert (non-dividing) particles by application of chemostat theory. The equation

$$\log x_t = \log x_0 - Dt/2.3$$

where \log is the common logarithm to the base 10*, x_t is the number of particles after a time t , x_0 is the initial number of particles and D is the dilution rate, was used (Crowe and Crowe, 1969).

* Where the term \log is used in this dissertation, it always refers to common \log_{10}

Results

Viable counts of the microbial communities

The glucose content of the growth medium affected the microbial composition of the steady state communities (Table 8). The seven bacterial species that were inoculated into the glucose-limited chemostat all became established. A total population level of $\log 10.4 \pm 2.1$ cfu/ml (total anaerobic count) was reached. *S. sanguis* was the dominant species while *L. casei* had the lowest count. In contrast to the glucose-limited chemostat, the glucose-excess culture had a lower species diversity. Only three of the seven bacterial species inoculated into the glucose-excess chemostat, i.e. *L. casei*, *S. sobrinus* and *E. saburreum* were able to establish (Table 8). The absence of the species *V. parvula* and *F. nucleatum* were not due to an oxidized environment as anaerobe *E. saburreum* grew to population levels of $\log 4$ cfu/ml and the Eh of the medium was below -200 mV. A total population level of 10.5 ± 0.5 log cfu/ml was reached. Both *L. casei* and *S. sobrinus* cell counts were higher than those in the glucose-limited cultures and *S. sanguis* was replaced by *S. sobrinus* as the dominant species.

Washout rate of *C. albicans* and the theoretical washout rate

C. albicans was unable to establish and compete for a niche in the glucose-limited chemostat. Directly after inoculation the mean yeast count was $\log 3.97 \pm 0.06$ cfu per ml (Table 9) and immediately started to decline (Fig. 10). After 4 days the yeast cell counts were below the detection level of 10 cfu/ml. Initially the yeast cells were lost at a mean rate of $\log 0.03$ cfu/ml/h. After 2 days the mean rate of loss increased to 0.05 cfu/ml/h. This was faster than the theoretical washout rate of $\log 0.02$ cfu/ml/h, indicating that cell death also occurred.

There was no change in the bacterial composition of the chemostat culture after inoculation with the yeast.

On the other hand, *C. albicans* was able to establish and grow to a steady state in the glucose-excess chemostat. During the first 2 days after inoculation the yeast cell counts declined at a mean rate of $\log 0.02$ cfu/ml/h (Table 10), equal to the theoretical washout rate. However, after 2 days the yeast growth rate stabilised at the dilution rate and maintained a steady state with a mean yeast count of $\log 2.6 \pm 0.6$ cfu/ml (Fig. 11). As with the glucose-limited culture, no change in the bacterial composition occurred after inoculation with the yeast.

Table 8. The composition of the steady-state bacterial communities under conditions of glucose limitation and excess.

Species	Mean log viable counts/ml (n=2)	
	Glucose limitation	Glucose-excess
<i>S. sanguis</i>	9.2	nd
<i>S. sobrinus</i>	8.8	10
<i>S. mitis</i>	6.1	nd
<i>L. casei</i>	5.0	9.7
<i>V. parvula</i>	8.1	nd
<i>E. saburreum</i>	6.1	4.4
<i>F. nucleatum</i>	5.2	nd
<i>C. albicans</i>	nd	2.9

nd = not detected

Table 9. The theoretical washout rate of a non-dividing particle and the true washout rate of *Candida albicans* under conditions of glucose limitation

Time (days)	Log non- dividing particle/ml	<i>C. albicans</i> mean log cfu/ml					Mean washout rate (log cfu/ml/h)
		exp. 1 (n=2)	Exp. 2 (n=2)	exp. 3 (n=2)	Mean all exp. (n=6)		
0	4.08	3.97	3.92	4.03	3.97 ± 0.06		
1	3.57	3.26	3.16	3.36	3.26 ± 0.1	-0.03	
2	3.07	2.56	2.51	2.53	2.53 ± 0.03	-0.03	
3	2.56	1.2	1	1.6	1.26 ± 0.3	-0.05	
4	2.06	<1	<1	<1	<1	-	
5	1.56	<1	<1	<1	<1	-	
6	1.05	<1	<1	<1	<1	-	

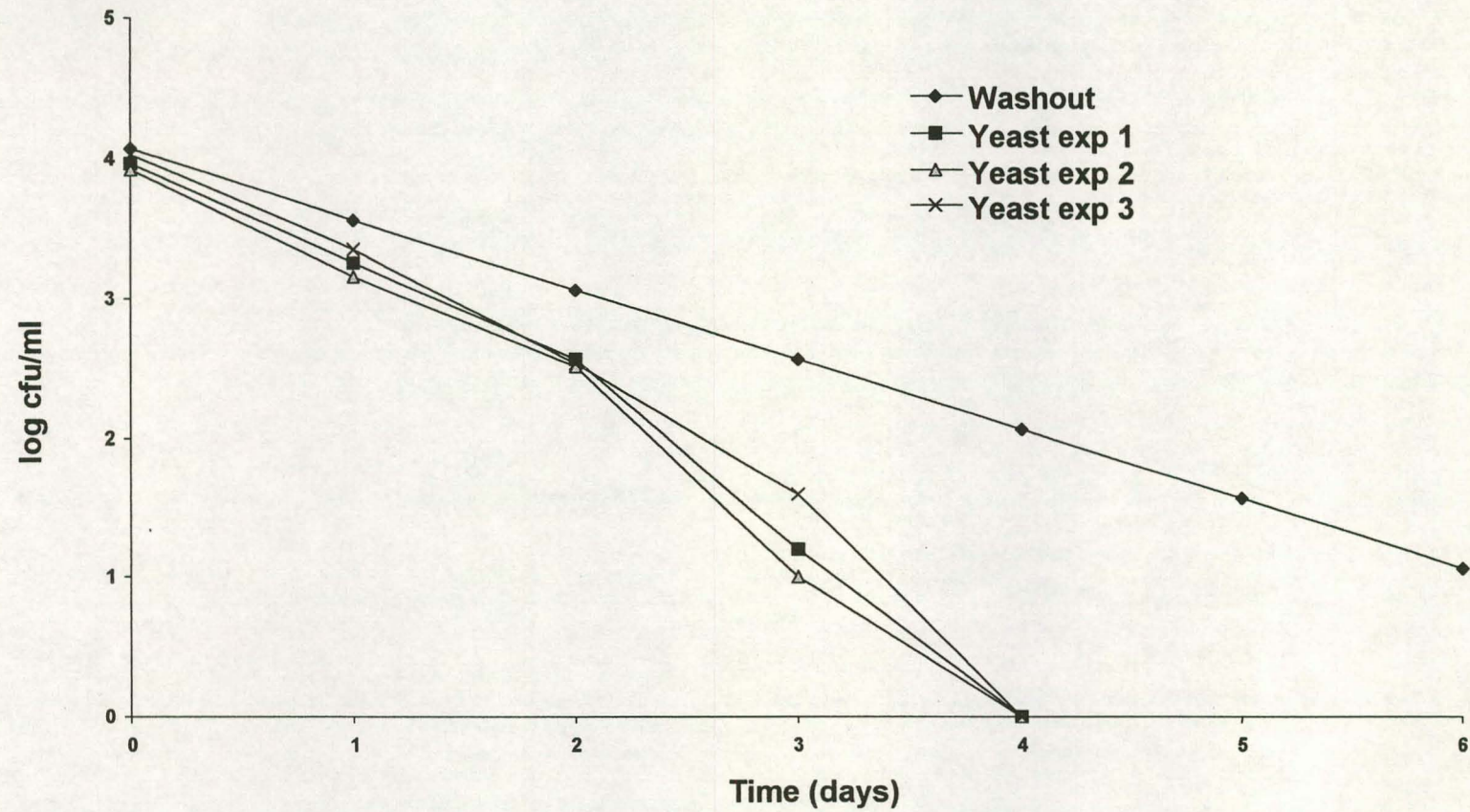


Fig. 10. The washout rate of an inert particle and the loss of *C. albicans* cells under glucose-limiting conditions.

Table 10. The theoretical washout rate of a non-dividing particle and the true washout rate of *Candida albicans* under conditions of glucose-excess

Time (days)	log non- dividing particle	<i>C. albicans</i> mean log cfu/ml				Mean all exp. (n=6)	Mean washout rate (log cfu/ml/h)
		exp. 1 (n=2)	exp. 2 (n=2)	exp. 3 (n=2)			
0	4.08	3.97	3.78	3.83	3.86 ± 0.1		
1	3.57	3.26	2.94	3.59	3.26 ± 0.33	-0.025	
2	3.07	2.9	2.7	2.94	2.84 ± 0.13	-0.017	
3	2.56	2.92	2.51	2.58	2.67 ± 0.22	-0.007	
4	2.06	2.95	2.28	2.56	2.59 ± 0.34	-0.003	
5	1.56	2.8	2.3	2.56	2.55 ± 0.25	-0.001	
6	1.05	2.8	2.6	2.56	2.65 ± 0.13	+0.004	

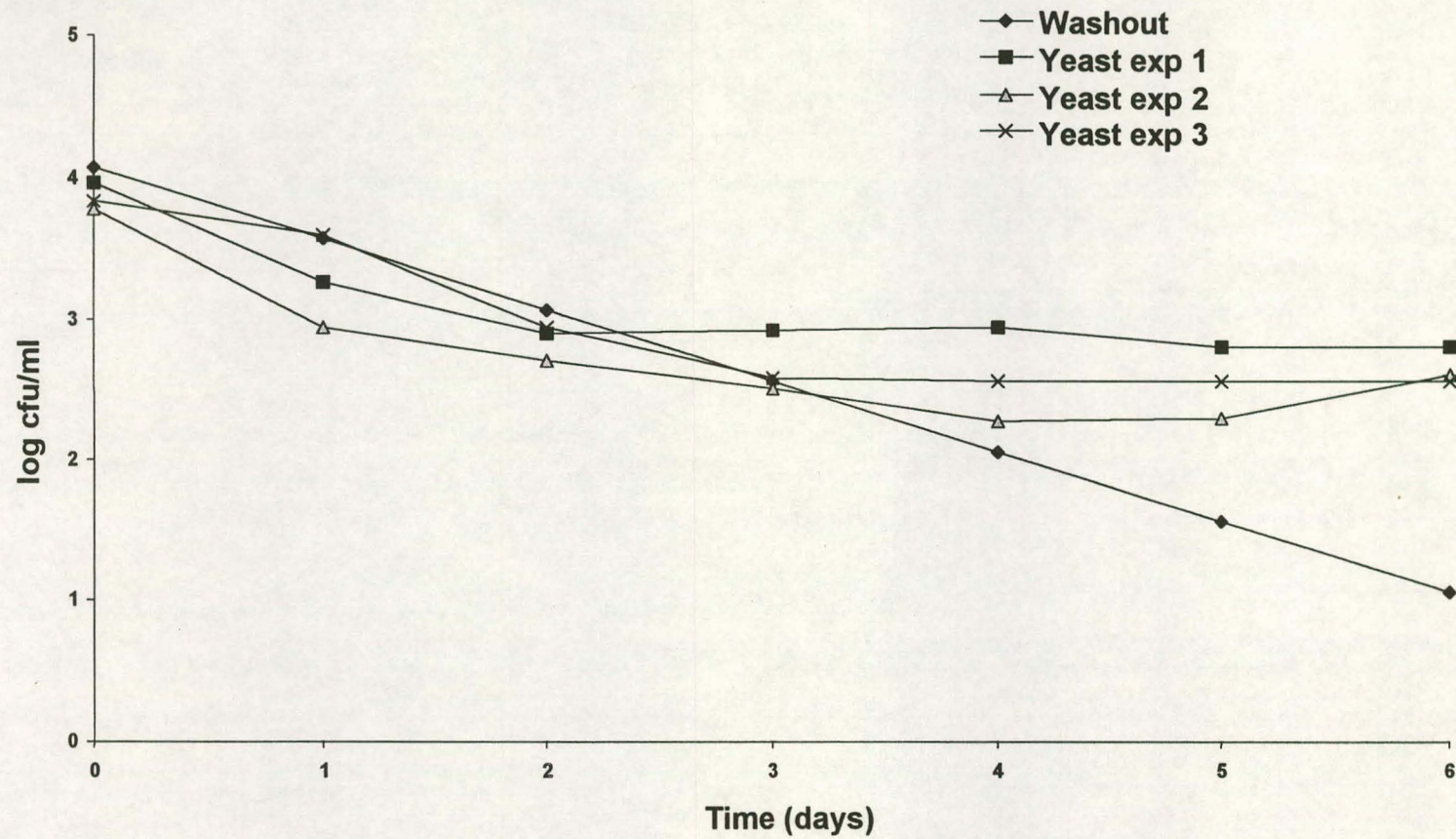


Fig. 11. The washout rate of an inert particle and the growth of *C. albicans* under glucose-excess conditions.

Discussion

In the glucose-limited chemostat the experimental conditions were such that the dilution rate and hence the growth rate was constant, the culture was in a steady state, growth rate was limited by the deficiency of a single nutrient, and the culture was perfectly mixed. Chemostat theory predicts that under these conditions, unless microbial interactions or mixed substrate utilisation occurs, a single bacterial population will competitively displace all others if a culture is inoculated with a mixture of organisms (Fredrickson and Stephanopoulos, 1981; Harder and Veldkamp, 1971; Veldkamp and Jannasch, 1972; Tempest 1969). The theoretical study on the coexistence of competing species by Taylor and Williams (1974) also dictates that to sustain a mixed population of species under these conditions, there must be at least as many growth-limiting substrates as there are different species in the continuous-flow system if no other interaction between the organisms exists. In other words, if different growth-limiting substrates were used by the different organisms, coexistence would occur since the organisms then occupied different ecological niches.

A mixed microbial population was able to establish and maintain a steady state in the glucose-limited chemostat culture. This implies that the populations that established themselves were not all dependent on glucose as a carbon substrate but made use of mixed substrate utilisation to develop into a complex community. This can be due to the interaction between primary feeders (those that use substrates present in the original growth medium) with secondary feeders (those that use metabolic by-products) (Fredrickson and Stephanopoulos, 1981). It can also be related to the ability of some of the populations to avoid direct competition for the overall growth-limiting nutrient (glucose) of the community by using amino acids for their growth and energy requirements. For example, *Veillonella* species use lactate produced by

other organisms (Mikx and van der Hoeven, 1975). *Veillonella* species have also been found to be associated with *E. saburreum* (Mashimo *et al*, 1981). The co-existence of *S. sanguis* with other oral bacteria in glucose-limited chemostat cultures can be explained by the utilisation of arginine by this organism as a source of energy when glucose is unavailable (Carlsson, 1972; Rogers *et al*, 1986). *F. nucleatum* is also known to be able to use amino acids as carbon and energy supplies (Rogers *et al*, 1991). Strong evidence for mixed substrate utilisation also comes from the results of the study by McKee *et al* (1985). They found that in their glucose-limited chemostat several amino acids were depleted under steady-state conditions and their results suggest that competition for amino acids might be an important factor in the establishment of some oral populations and that mixed substrate utilisation is necessary for the development of complex communities.

However, the bacterial composition of the community was markedly influenced by the glucose concentration. The glucose-excess culture had a lower species diversity. Four of the seven bacterial species in the inoculum were unable to colonise the chemostat, even after repeated inoculations. A lower species diversity in the presence of excess glucose was also observed by Marsh *et al* (1983) and McKee *et al* (1985). This can possibly be ascribed to a shortage of nitrogen supply in the form of a limitation of certain amino acids under the glucose-excess conditions. In the presence of an excess of a carbon source, a shortage of a nitrogen supply may develop as a result of the metabolism of the organisms. Under conditions of glucose-excess, at least the three amino acids serine, histidine and arginine, were depleted from the culture medium in a study by McKee *et al* (1985).

The four bacteria that were unable to grow in the glucose-excess chemostat most probably had to compete for a nitrogen source as a result of the depletion of amino acids. *V. parvula* was unable to grow under glucose-excess (amino acid limiting) conditions. Since this organism cannot use glucose but needs amino acids for growth (Delwiche *et al*, 1985) it

probably had to compete with *L. casei*, *S. sobrinus* and *E. saburreum* for essential amino acids. Similarly, the absence of *S. sanguis*, *S. mitis* and *F. nucleatum* in the glucose-excess culture indicates that amino acids acted as essential growth factors for these bacteria. Furthermore, the growth of *L. casei*, *S. sobrinus* and *E. saburreum* under both glucose-limited and glucose-excess conditions, indicates that they competed neither for glucose nor for the same amino acids.

The dilution rate of the chemostat was less than the maximum specific growth rate of all the organisms involved (see Table 37). Therefore no washout of any of the organisms as a result of a faster dilution rate could have occurred. In spite of this, *C. albicans* was the only organism that was unable to colonise and compete for a niche in the mixed community of oral bacteria in the glucose-limited chemostat. In contrast to this it could establish itself and grow in a steady state under glucose-excess conditions.

Except for the availability of glucose, all other growth conditions were favourable for the growth of the yeast in the glucose-limited chemostat. Although *Candida* species grow best under aerobic conditions, the yeast will also grow under anaerobic conditions but at reduced growth rates. Webster and Odds (1986) found that the growth rate of the yeast will be reduced by about 16% when grown anaerobically in an atmosphere containing 5% CO₂. With an aerobic growth rate in the chemostat growth medium of 0.237 h⁻¹, a reduction in growth rate of 16% would give a growth rate of 0.198, much faster than the dilution rate of the chemostat.

No antifungal agent was produced by the bacterial species when tested individually against the yeast. Also no mention of any antifungal agent, produced by the oral bacteria, could be found in the literature. Furthermore, when the glucose-limited chemostat effluent was filter-sterilised and supplemented with glucose, the yeast could grow in this medium. This suggests that the only reason why the yeast could not establish in the glucose-limited chemostat, was

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because it could not compete with the bacteria for glucose under the glucose-limiting conditions.

In “pure and simple” competition there is only one nutrient whose availability affects the growth of an organism and competition for this nutrient is the only interaction between the organism and other populations (Fredrickson and Stephanopoulos 1981). The results of this study suggest that the competition between *C. albicans* and the oral bacteria that was observed, was a “pure and simple” competition for glucose.

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Chapter 8

ESTIMATION OF THE GROWTH PARAMETERS OF *C. ALBICANS*, *S. SANGUIS*, *L. CASEI*, *S. MITIS*, *S. SOBRINUS*, *F. NUCLEATUM*, *E. SABURREUM*, AND *V. PARVULA*.

Materials and Methods

Growth conditions

Each organism was grown as a batch culture in a BIOFLO[®] Model C32 chemostat (New Brunswick Scientific, Edison, NJ) with a working volume of 600 ml. The organisms were grown under a gas phase of 5% (v/v) CO₂ + 95% (v/v) N₂ (flow rate of 50 ml/min). The temperature was maintained at 37°C and the pH at 7 ± 0.1 by the automatic addition of 2 M NaOH. The chemostat growth medium was used. The medium was supplemented with 55 mM of glucose (BDH) for the growth of the oral bacteria and 138 mM of glucose for the growth of *C. albicans*. The medium and the glucose were autoclaved separately and the glucose was added aseptically after sterilisation.

Microbial strains

Type culture strains of the bacteria were obtained from the National Collection of Type Cultures (NCTC), Colindale, UK. The strains were: *Streptococcus sanguis* (NCTC 10904), *Streptococcus sobrinus* (NCTC 10921), *Streptococcus mitis* (NCTC 10712), *Lactobacillus casei* (NCTC 10302), *Veillonella parvula* (NCTC 11463) and *Fusobacterium nucleatum* (ATCC 25586). A type culture strain of the yeast *C. albicans* strain NCPF 3118 was obtained from The National Collection of Pathogenic Fungi (NCPF), Colindale, UK. The *Eubacterium saburreum* strain (B6I8) was isolated and identified from the chemostat in experiment I.

Chemostat inoculation

The chemostat growth vessel was loaded with 600 ml of culture medium and brought to its working temperature of 37°C, the gas flow rate adjusted to 50ml/min and the stirrer adjusted to 4000 rpm. The chemostat was left overnight to stabilise and to be checked for sterility. The growth medium was inoculated with 10 ml of a 24 h seed culture and growth was allowed while being stirred as a batch culture (i.e. medium was not being pumped in). After 16 h of incubation the culture was checked for purity and the culture replaced with fresh medium. Immediately thereafter, and at hourly intervals, a sample was withdrawn for analysis.

Analytical procedures

To monitor the growth cycle, the optical density of a 1:1 dilution of the sample in sterile medium was measured every hour in a Novaspec 4049 spectrophotometer (LKB Biochrom Ltd, Cambridge, England) at a wavelength of 590 nm. Bacterial dry masses were determined by taking duplicate 5 ml samples of killed culture, centrifuging, washing the cells once in distilled water, and drying the washed deposit of cells at 105° C for 48 h. The culture was killed immediately after sampling (within 15 sec after sampling) with 10% (v/v) formalin for

1 h. The supernatant of the centrifuged culture was used for determining the residual glucose concentration.

Glucose was determined by using the method of Kingsley and Getchell (1960). Ten microlitres (μl) of the supernatant were mixed with 2.25 ml peroxidase buffer and 250 μl glucose oxidase reagent. The solution was kept at 37° C for 30 minutes, cooled, and 750 μl of a 50% H_2SO_4 solution was slowly added with continuous mixing. The resulting colour was read against a blank in a spectrophotometer at a wavelength of 530 nm.

The peroxidase buffer was prepared by mixing 5 mg peroxidase (Sigma), 59 ml 0.1 N NaOH, 125 ml 0.1 M KH_2PO_4 and 5 ml of a 1% (w/v) o-dianisidine in methanol and then diluted to 500 ml with distilled water. The glucose oxidase reagent contained 250 mg glucose oxidase (Sigma) in 25 ml of distilled water. The glucose concentration in the supernatant was measured against a glucose (Sigma) control solution (10 mg/ml) diluted to give glucose concentrations of 0.5, 1, 1.5, 2 and 3 mg/ml.

Calculations

The maximum specific growth rate (μ_{max}) was obtained by calculating the slope of the straight line obtained by plotting the log of the cell mass against time. The best line fit was obtained by the method of the least squares. The saturation constant (k_s) for glucose was determined by using the mathematical model of Monod (1949) for microbial growth. According to this model, growth can be described (for a small time lapse) by the equation

$$r_x = \frac{dx_v}{dt} = \mu_{\text{max}} \frac{Sx_v}{(k_s + S)} \text{-----(12)}$$

where r_x (g cells/ml/h) is the rate for cell growth, S (g carbon source/ml) is the substrate concentration, x_v (mg cells/ml) is the viable cell concentration and t is the time interval (Sinclair and Cantero, 1990).

The experimental data was organised as sets of data points. Each set corresponded to one particular value of elapsed time. To determine the rate of growth, the experimental data was first processed by transforming the data into derivatives of r_x (i.e. dx_v/dt). The values of the derivatives were calculated by fitting the mathematical equation for exponential growth ($x_v = a e^{bt}$) to three data points around the point being analysed and the required slope was mathematically computed. This means that the equation

$$x_v = a e^{bt} \text{-----(13)}$$

was differentiated to give

$$\frac{dx_v}{dt} = a b e^{bt} = b(a e^{bt}) = b x_v$$

To find the value of $\frac{dx_v}{dt}$, the value of b was needed.

The logarithmic form of equation (13) can be given as

$$\ln(x_v) = \ln(a) + bt$$

or

$$bt = \ln(x_v) - \ln(a)$$

For the three data points (x_1 , x_2 , and x_3) around the point (x_2) being analysed

$$b(t_3 - t_1) = \ln x_3 - \ln x_1 - (\ln a - \ln a)$$

thus for 1h intervals

$$b = \frac{\ln(x_3) - \ln(x_1)}{2}$$

and

$$\frac{dx_v}{dt} = \frac{\ln(x_3) - \ln(x_1)}{2} \times x_v$$

or

$$r_x = \frac{\ln(x_3) - \ln(x_1)}{2} \times x_2 \quad (\text{Sinclair and Cantero, 1990}).$$

In order to obtain the values of μ_{\max} and k_s , equation (12) was transformed to the form

$$\frac{1}{\mu} = \frac{k_s}{\mu_{\max}} \frac{1}{S} + \frac{1}{\mu_{\max}}$$

The reciprocal values of μ were plotted against S in a Lineweaver-Burke plot. The values of μ were obtained from the intercept ($1/\mu_{\max}$) and the values of k_s were obtained from the slope (k_s/μ_{\max}) of the plot (Sinclair and Cantero, 1990).

The yield factor Y and the maintenance coefficient m_s , for cells on glucose as carbon substrate were found from the equation for substrate consumption rate

$$\frac{ds}{dt} = -r_s = -\left(\frac{r_x}{Y} + m_s x_v\right) \text{-----(14)}$$

where r_s is the rate of substrate consumption (g carbon source/ml/h), Y is the yield factor (g cells/g carbon source) and m_s the maintenance constant (g carbon source/g cells/h) (Sinclair and Cantero, 1990). The values of the variables x_v , and S , were obtained from the experimental data and the derivatives r_x (i.e. dx_v/dt) and r_s (i.e. dS/dt) were calculated as described above.

Equation (14) was transformed to obtain a linear relationship of the form

$$\frac{r_s}{x_v} = \frac{1}{Y} \frac{r_x}{x_v} + m_s$$

The transformed variables r_x/x_v and r_s/x_v were plotted and the value of Y obtained from the reciprocal of the slope and the value of m_s from the intercept (Sinclair and Cantero, 1990).

Best-fit lines were plotted by regression analysis ($r > 0.9$) for all plots.

Results

Growth parameters of *C. albicans*

The experimental data obtained from the growth of *C. albicans* in batch culture, with glucose as carbon substrate, is summarised in Table 11 and Table 12. A plot of the experimental data showed that the yeast produced a typical growth pattern in relation to the glucose concentration in the growth medium (Fig. 12). It can be seen that the glucose acted as a growth-limiting substrate. Growth proceeded exponentially for ± 15 h while the glucose concentration declined to about half the initial concentration. During this stage the maximum specific growth rate of the yeast was $\mu_{\max} = 0.209 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 13), and remained unaffected by the concentration of the glucose. Thereafter, the growth rate slowed down as the glucose concentration decreased. After ± 20 h the culture slowly went into a stationary phase, reaching a maximum stationary phase by the time the glucose had been exhausted. A total growth of 2.02 mg cells/ml (dry mass) was obtained (Table 11).

The processed data points for the estimation of the growth parameters μ_{\max} and k_s are listed in Table 13. A Lineweaver-Burke plot of the reciprocal growth rate *versus* the reciprocal glucose concentration is represented in Fig. 14. The value of μ_{\max} was obtained from the intercept ($1/\mu_{\max}$) and the value of k_s was calculated from the slope (k_s/μ_{\max}). These values were: $\mu_{\max} = 0.197 \text{ h}^{-1}$ and $k_s = 3.3 \text{ mg glucose/ml}$ respectively.

The processed data points for the estimation of the yield factor (Y) and the maintenance coefficient (m_s) are listed in Table 14. A straight line plot of the transformed variables r_x/x_v *versus* r_s/x_v is presented in Fig. 15. The value of the yield factor, obtained from the slope of

the straight line, was $Y = 0.141$ mg cells/mg glucose and the maintenance coefficient obtained from the intercept was $m_s = 0.07$ mg glucose/mg cells/h.

Table 11. Dry cell mass of *C. albicans* against time during growth in batch culture with glucose as carbon substrate

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	0.04	0.11	0.06	0.07 \pm 0.04
2	0.09	0.10	0.06	0.08 \pm 0.03
4	0.15	0.13	0.10	0.13 \pm 0.02
6	0.16	0.14	0.12	0.15 \pm 0.01
8	0.22	0.39	0.18	0.26 \pm 0.09
10	0.54	0.59	0.39	0.51 \pm 0.13
12	0.63	0.75	0.56	0.65 \pm 0.16
14	0.80	1.00	1.04	0.95 \pm 0.25
24	1.80	1.90	1.98	1.89 \pm 0.07
26	2.00	2.10	2.03	2.04 \pm 0.16
28	1.87	2.11	2.03	2.00 \pm 0.24
30	1.92	2.11	2.03	2.02 \pm 0.22
32	1.91	2.11	2.03	2.02 \pm 0.23

Table 12. Glucose concentration towards time in the growth medium of *C. albicans* during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	26.10	24.35	25.8	25.42 \pm 0.94
2	25.89	24.35	25.86	25.37 \pm 0.88
4	25.35	24.35	25.80	25.17 \pm 0.74
6	25.71	24.35	25.86	25.31 \pm 0.83
8	25.47	23.92	23.71	24.36 \pm 0.96
10	23.58	22.84	22.20	22.87 \pm 0.69
12	19.75	21.01	22.24	21.00 \pm 1.24
14	17.00	17.67	17.20	17.29 \pm 0.34
24	1.89	3.02	3.28	2.73 \pm 0.74
26	0.00	0.43	1.11	0.51 \pm 0.56
28	0.00	0.00	0.28	0.09 \pm 0.16
30	0.00	0.00	0.00	0.00

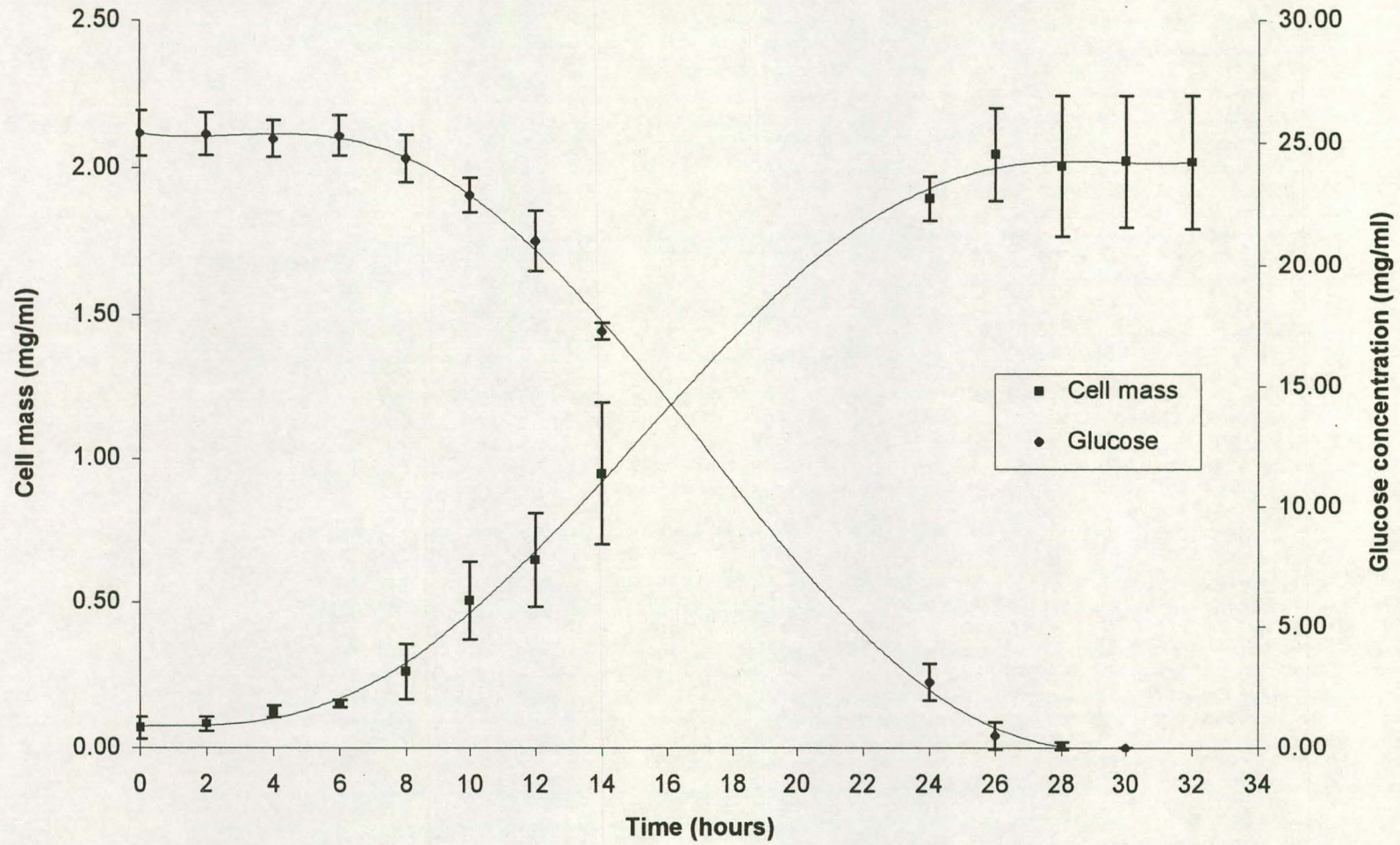


Fig. 12. Growth of *C. albicans* and the utilisation of glucose.

Table 13. Processed data points for the determination of the maximum specific growth rate and the saturation constant of *C. albicans* grown on glucose as carbon substrate.

Time (h)	x_v (mg/ml)	$\ln x_v$	$rx=dx_v/dt$	$\mu=r_x/x_v$	$1/\mu$	S (mg/ml)	$1/S$
0	0.07	-2.65	-	-	-	25.75	0.04
2	0.08	-2.48	0.02	0.195	5.13	25.62	0.04
4	0.15	-1.88	0.02	0.130	7.71	25.19	0.04
6	0.14	-1.97	0.02	0.135	7.40	25.07	0.04
8	0.26	-1.33	0.08	0.322	3.11	25.08	0.04
10	0.51	-0.68	0.11	0.225	4.45	23.38	0.04
12	0.65	-0.44	0.10	0.156	6.40	20.99	0.05
14	0.95	-0.05	0.10	0.109	9.18	16.26	0.06
24	1.89	0.64	0.06	0.030	32.84	5.25	0.19
26	1.98	0.69	0.03	0.014	70.83	2.75	0.36
28	2.00	0.69	0.01	0.005	184.45	1.16	0.86
30	2.02	0.70	0.00	0.002	603.00	0.37	2.70
32	2.02	0.70	-	-	-	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

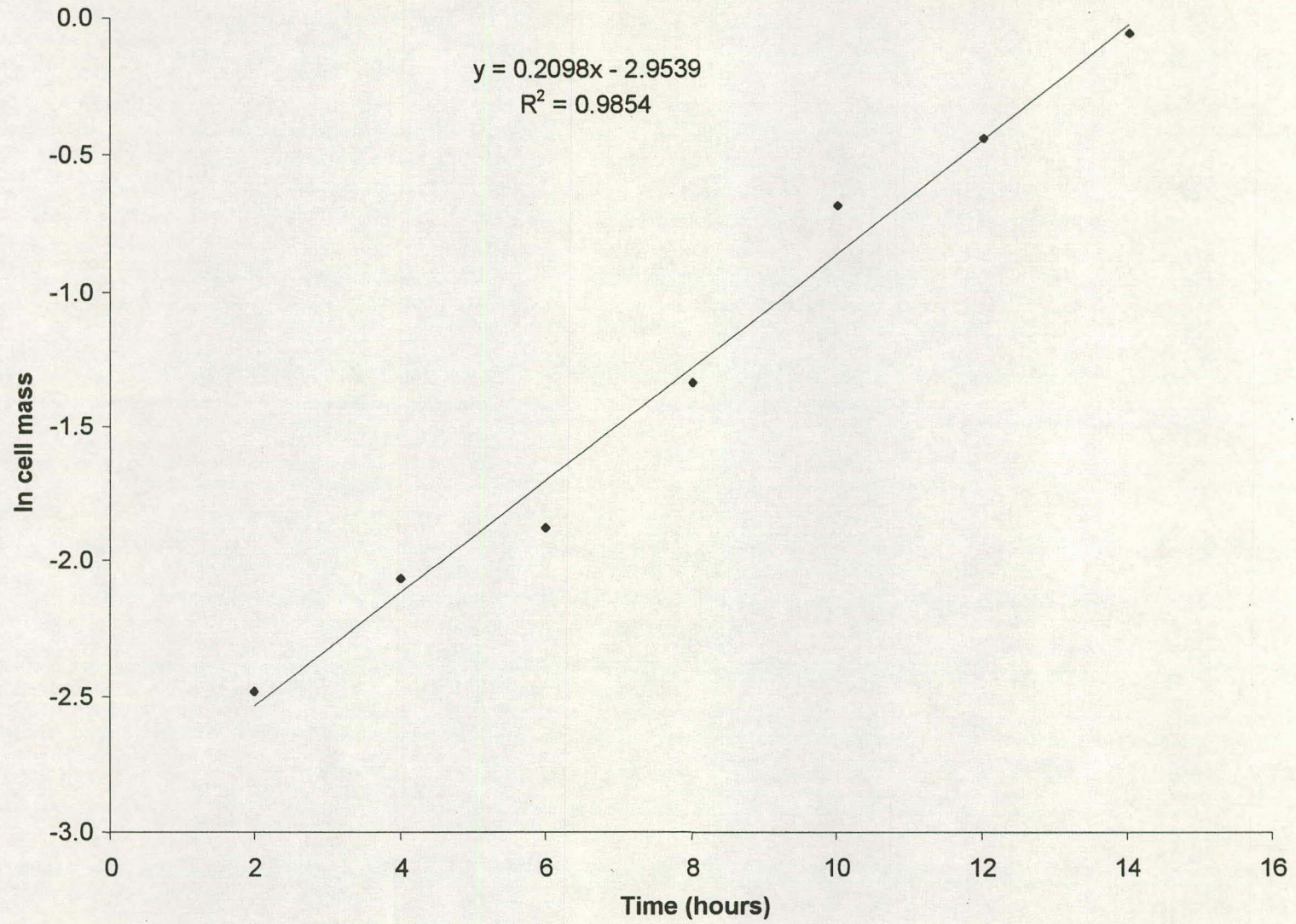


Fig. 13. Logarithmic curve of the exponential phase of the growth curve of *C. albicans*.

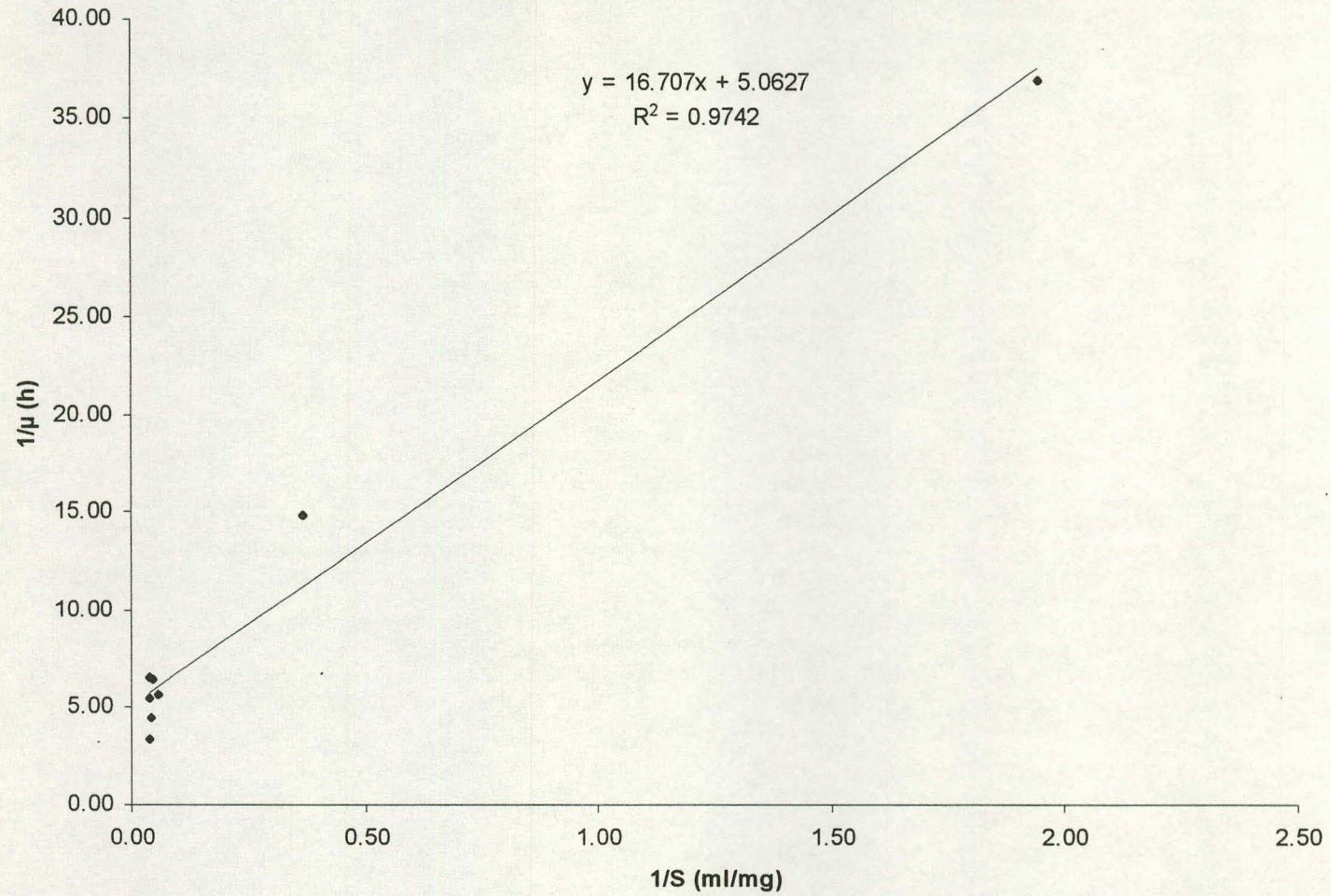


Fig. 14. A Lineweaver – Burke plot of the reciprocal growth rate (μ) versus the reciprocal glucose concentration (S) for the determination of the maximum growth rate (μ_{\max}) and the saturation constant (k_s) of *C. albicans*.

Table 14. Processed data points for the estimation of the growth yield factor and the maintenance coefficient for the growth of *C. albicans* on glucose.

Time (h)	x_v (mg/ml)	$\ln x_v$	$rx=dx_v/dt$	$\mu=r_x/x_v$	S (mg/ml)	r_s	r_s/x_v
0	0.07	-2.65	-	-	-	-	-
2	0.08	-2.48	0.01	0.15	25.37	0.06	0.75
4	0.13	-2.07	0.02	0.15	25.17	0.02	0.12
6	0.15	-1.88	0.03	0.18	25.31	0.20	1.31
8	0.26	-1.33	0.08	0.30	24.36	0.61	2.31
10	0.51	-0.68	0.11	0.22	22.87	0.84	1.66
12	0.65	-0.44	0.10	0.16	21.00	1.40	2.16
14	0.95	-0.05	0.17	0.18	17.29	1.52	1.61
24	1.89	0.64	0.13	0.07	2.73	1.40	0.74
26	2.12	0.75	0.06	0.03	0.51	0.66	0.31
28	2.11	0.75	-	-	0.09	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

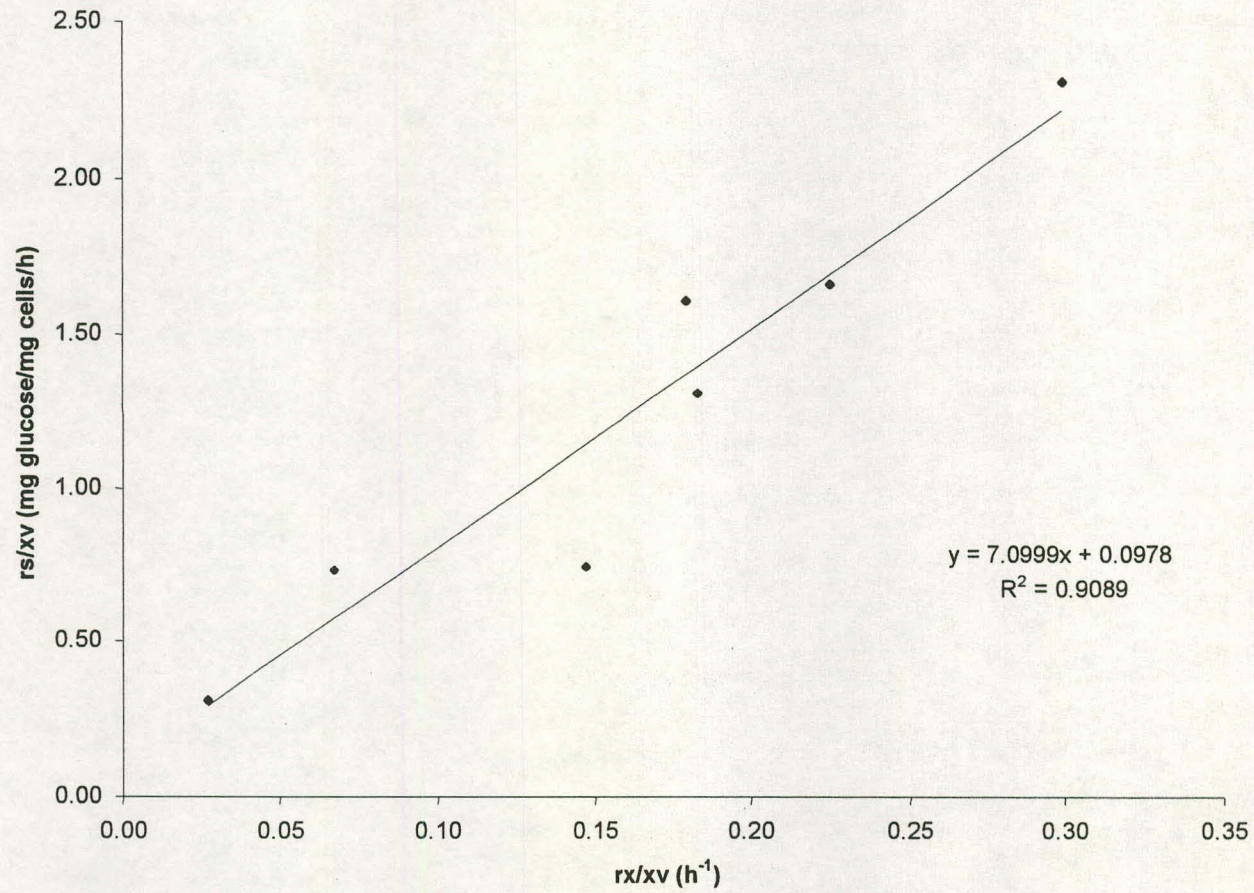


Fig. 15. Plot of glucose utilisation (r_s/x_v) versus growth rate (r_x/x_v) to determine the growth yield (Y) and the maintenance coefficient (m_s) of *C. albicans*.

Growth parameters of *S. sanguis*

The experimental data obtained from the growth of *S. sanguis* in batch culture with glucose as carbon source, is summarised in Table 15 and Table 16. A plot of the experimental data is represented in Fig. 16. The glucose in the growth medium acted as a growth-limiting substrate and the growth of *S. sanguis* produced the typical growth curve with an inverse relationship to the glucose concentration of the growth medium. Growth proceeded exponentially for ± 8 h while the glucose concentration declined to ± 4 mg/ml. During this phase the growth rate remained unaffected by the glucose concentration in the growth medium. The growth rate as determined by the slope of the ln plot of the exponential phase of the growth curve was $\mu = 0.422 \text{ h}^{-1}$ (Fig. 17). After ± 8 h the *S. sanguis* culture went into a stationary phase reaching a maximum stationary phase after 10.5 h. By this time the glucose had been exhausted. The total growth obtained was 1.6 mg of cells (dry mass)/ml.

The processed data points for the estimation of the growth parameters μ_{\max} and k_s are listed in Table 17. A Lineweaver-Burke plot of the reciprocal growth rate *versus* the reciprocal glucose concentration is represented in Fig. 18. The value of μ_{\max} was obtained from the intercept ($1/\mu_{\max}$) and the value of k_s was calculated from the slope (k_s/μ_{\max}). These values were: $\mu_{\max} = 0.514 \text{ h}^{-1}$ and $k_s = 3.4 \text{ mg glucose/ml}$ respectively.

The processed data points for the estimation of the yield factor (Y) and the maintenance coefficient (m_s) are listed in Table 18. A straight line plot of the transformed variables r_x/x_v (glucose utilisation) *versus* r_s/x_v (growth rate) is presented in Fig. 19. The value of the yield factor, obtained from the slope of the straight line, was $Y = 0.193 \text{ mg cells/mg glucose}$ and the maintenance coefficient obtained from the intercept was $m_s = 0.085 \text{ mg glucose/mg cells/h}$.

Table 15. Dry cell mass of *S. sanguis* versus time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)	ln of the Mean
0	0.09	0.10	0.08	0.09 \pm 0.01	-2.41
1	0.11	0.10	0.08	0.10 \pm 0.02	-2.34
2	0.1	0.10	0.09	0.10 \pm 0.01	-2.34
3	0.16	0.13	0.1	0.13 \pm 0.03	-2.04
4	0.2	0.19	0.22	0.20 \pm 0.02	-1.60
5	0.32	0.25	0.28	0.28 \pm 0.04	-1.26
6	0.41	0.39	0.43	0.41 \pm 0.02	-0.89
7	0.8	0.80	0.86	0.82 \pm 0.03	-0.20
8	1.16	1.14	1.17	1.16 \pm 0.01	0.15
9	1.35	1.34	1.43	1.37 \pm 0.05	0.32
10	1.45	1.56	1.59	1.53 \pm 0.07	0.43
11	1.56	1.60	1.59	1.58 \pm 0.02	0.46
12	1.56	1.60	1.58	1.58 \pm 0.02	0.46
13	1.6	1.6	1.6	1.60 \pm 0.0	0.47

Table 16. Glucose concentration in the growth medium of *S. sanguis* during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	10.97	10.14	9.9	10.34 \pm 0.56
1	10.97	10.25	9.80	10.34 \pm 0.59
2	10.97	10.14	9.80	10.30 \pm 0.60
3	10.97	10.14	9.70	10.27 \pm 0.65
4	10.35	10.14	8.97	9.82 \pm 0.74
5	10.35	10.06	8.69	9.70 \pm 0.89
6	8.83	9.58	8.09	8.83 \pm 0.74
7	6.90	7.40	6.89	7.07 \pm 0.29
8	3.45	4.75	4.18	4.13 \pm 0.65
9	1.77	1.86	1.95	1.86 \pm 0.09
10	0.35	0.56	0.18	0.36 \pm .19
11	0.00	0.00	0.00	0.00
12	0.00	0.00	0.00	0.00
13	0.00	0.00	0.00	0.00

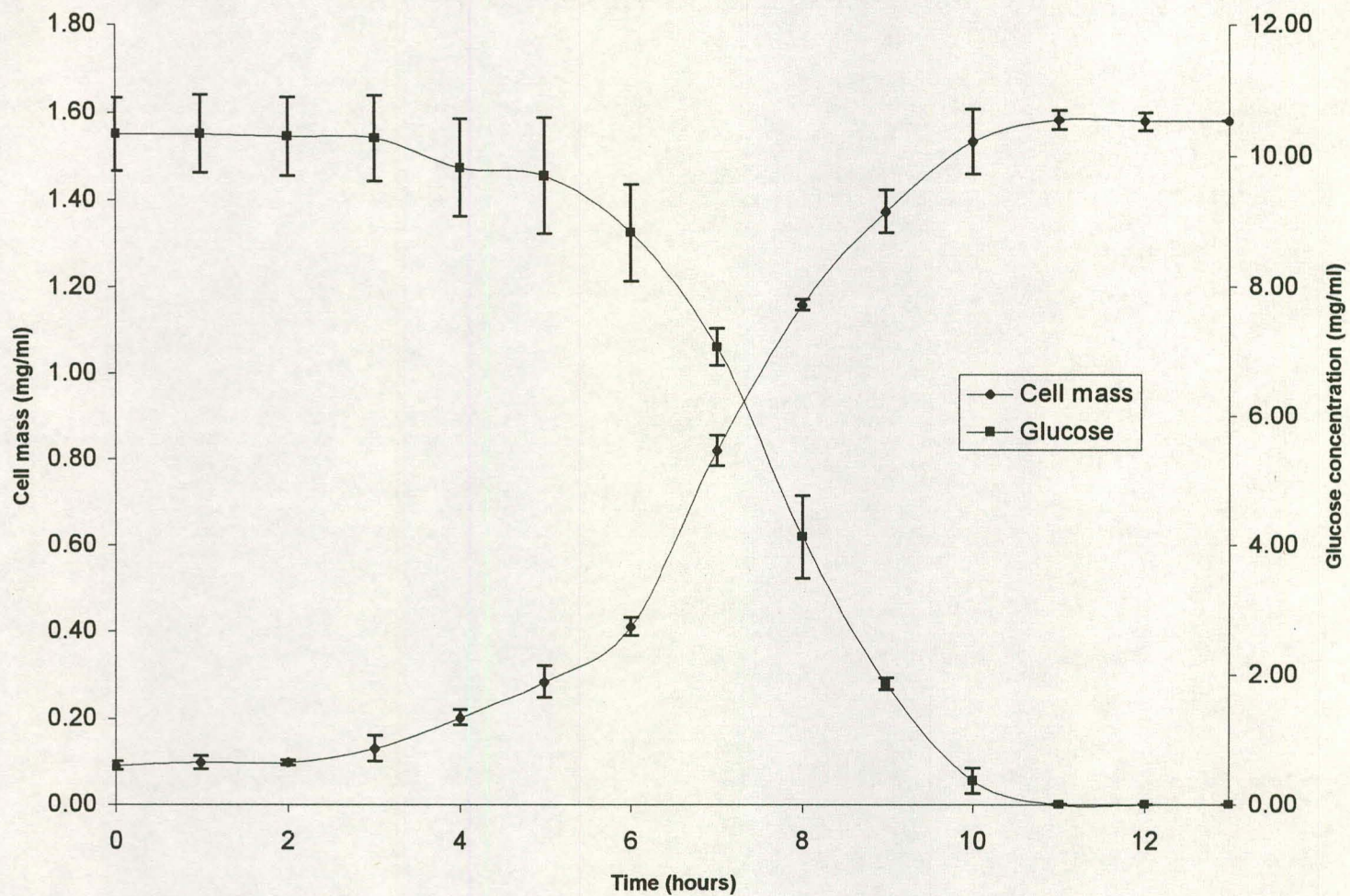


Fig. 16. Growth curve of *S. sanguis* and the utilisation of glucose.

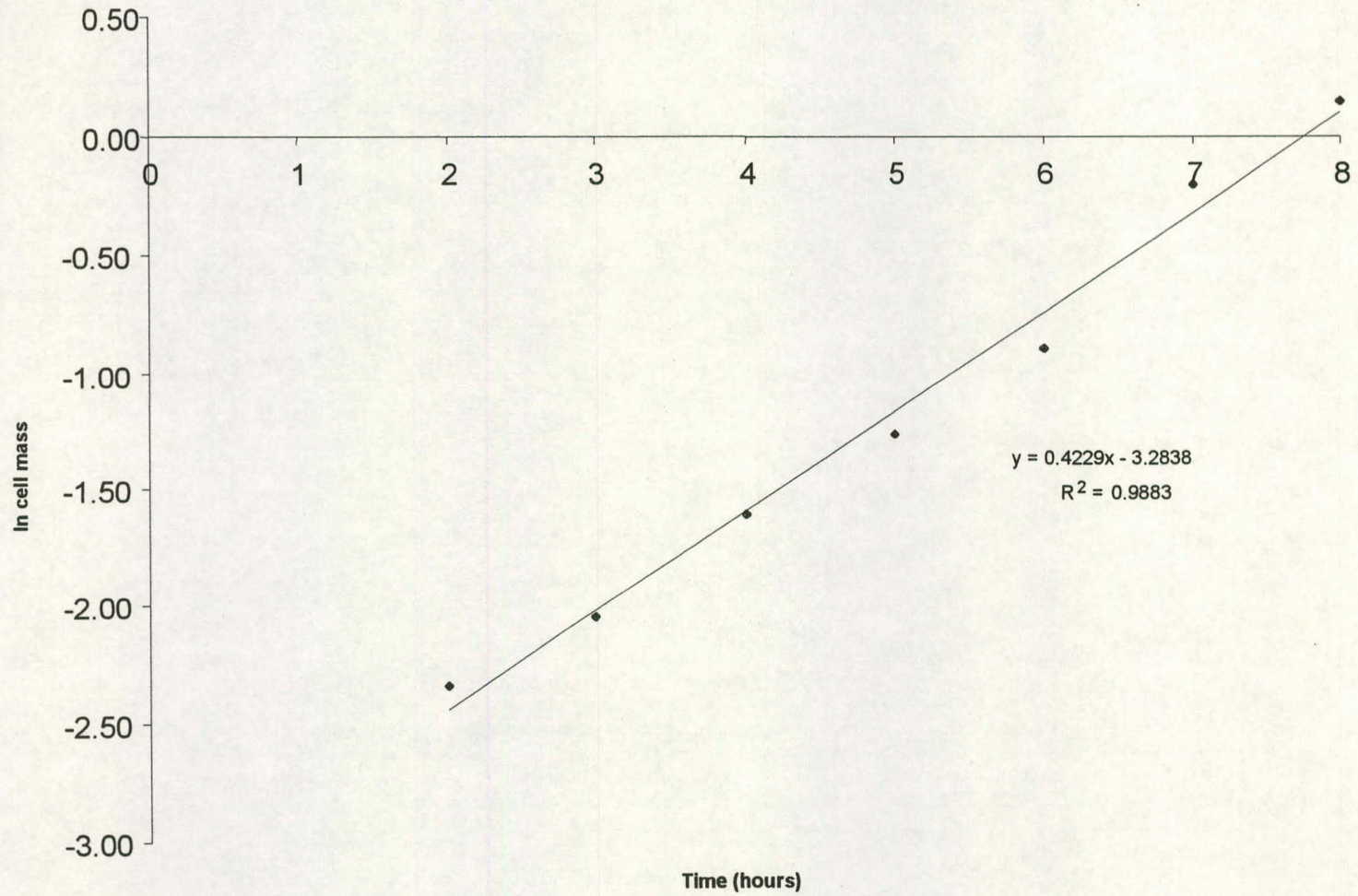


Fig. 17. Logarithmic curve of the exponential phase of the growth curve of *S. sanguis*

Table 17. Processed data points for the determination of the maximum specific growth rate and the saturation constant of *S. sanguis* grown on glucose as carbon substrate.

Time (h)	x_v (mg/ml)	$\ln x_v$	$rx=dx_v/dt$	$\mu=r_x/x_v$	$1/\mu$	S (mg/ml)	$1/S$
0	0.09	-2.41	-	-	-	10.34	0.10
1	0.10	-2.34	0.00	0.04	27.99	10.34	0.10
2	0.10	-2.34	0.01	0.15	6.75	10.30	0.10
3	0.13	-2.04	0.05	0.37	2.72	10.27	0.10
4	0.20	-1.60	0.08	0.39	2.57	9.82	0.10
5	0.28	-1.26	0.10	0.35	2.83	9.70	0.10
6	0.41	-0.89	0.23	0.56	1.78	8.83	0.11
7	0.87	-0.14	0.45	0.52	1.92	7.07	0.14
8	1.16	0.15	0.29	0.25	4.02	4.13	0.24
9	1.43	0.36	0.21	0.15	6.72	1.86	0.54
10	1.56	0.44	0.08	0.05	19.64	0.36	2.74
11	1.58	0.46	0.01	0.01	157.00	0.00	-
12	1.58	0.46	0.01	0.01	191.00	0.00	-
13	1.60	0.47	-	-	-	-	-

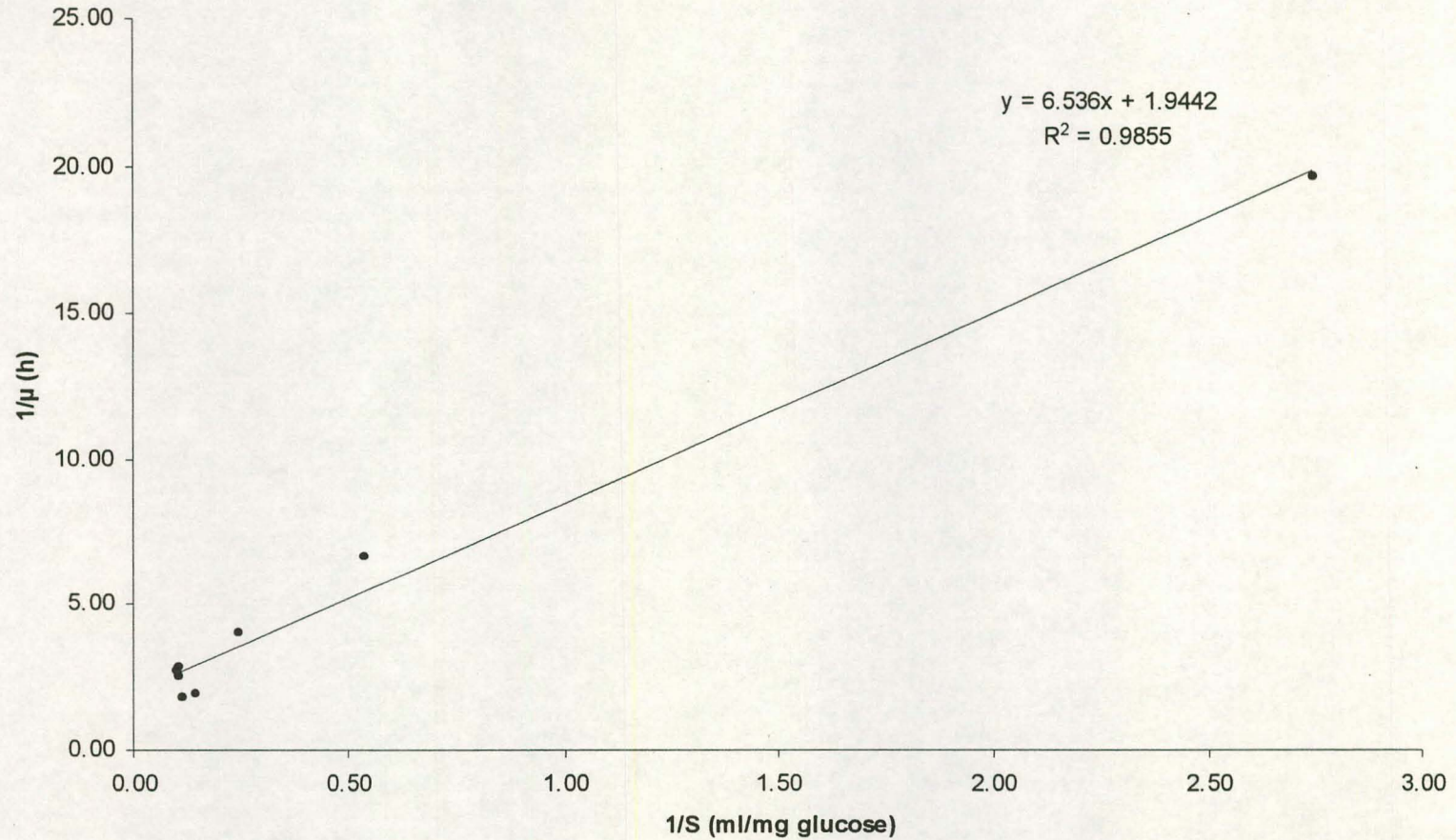


Fig. 18. A Lineweaver – Burke plot of the reciprocal growth rate ($1/\mu$) versus the reciprocal glucose concentration ($1/S$) for the determination of the maximum growth rate (μ_{\max}) and the saturation constant (k_s) *S. sanguis*.

Table 18. Processed data points for the estimation of the growth yield factor and the maintenance coefficient for the growth of *S. sanguis* on glucose.

Time (h)	x_v (mg/ml)	$\ln x_v$	$rx=dx_v/dt$	$\mu=r_x/x_v$	S (mg/ml)	r_s	r_s/x_v
0	0.09	-2.41			10.34		
1	0.10	-2.34	0.00	0.04	10.34	0.02	0.17
2	0.10	-2.34	0.01	0.15	10.30	0.04	0.36
3	0.13	-2.04	0.05	0.37	10.27	0.24	1.86
4	0.20	-1.60	0.08	0.39	9.82	0.28	1.41
5	0.28	-1.26	0.10	0.35	9.70	0.50	1.75
6	0.41	-0.89	0.23	0.56	8.83	1.32	3.22
7	0.87	-0.14	0.45	0.52	7.07	2.35	2.71
8	1.16	0.15	0.29	0.25	4.13	2.60	2.25
9	1.43	0.36	0.21	0.15	1.86	1.88	1.31
10	1.56	0.44	0.08	0.05	0.36	0.93	0.60
11	1.58	0.46	0.01	0.01	0.00	0.18	0.12
12	1.58	0.46	0.01	0.01	0.00	-	-
13	1.60	0.47	-	-	-	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

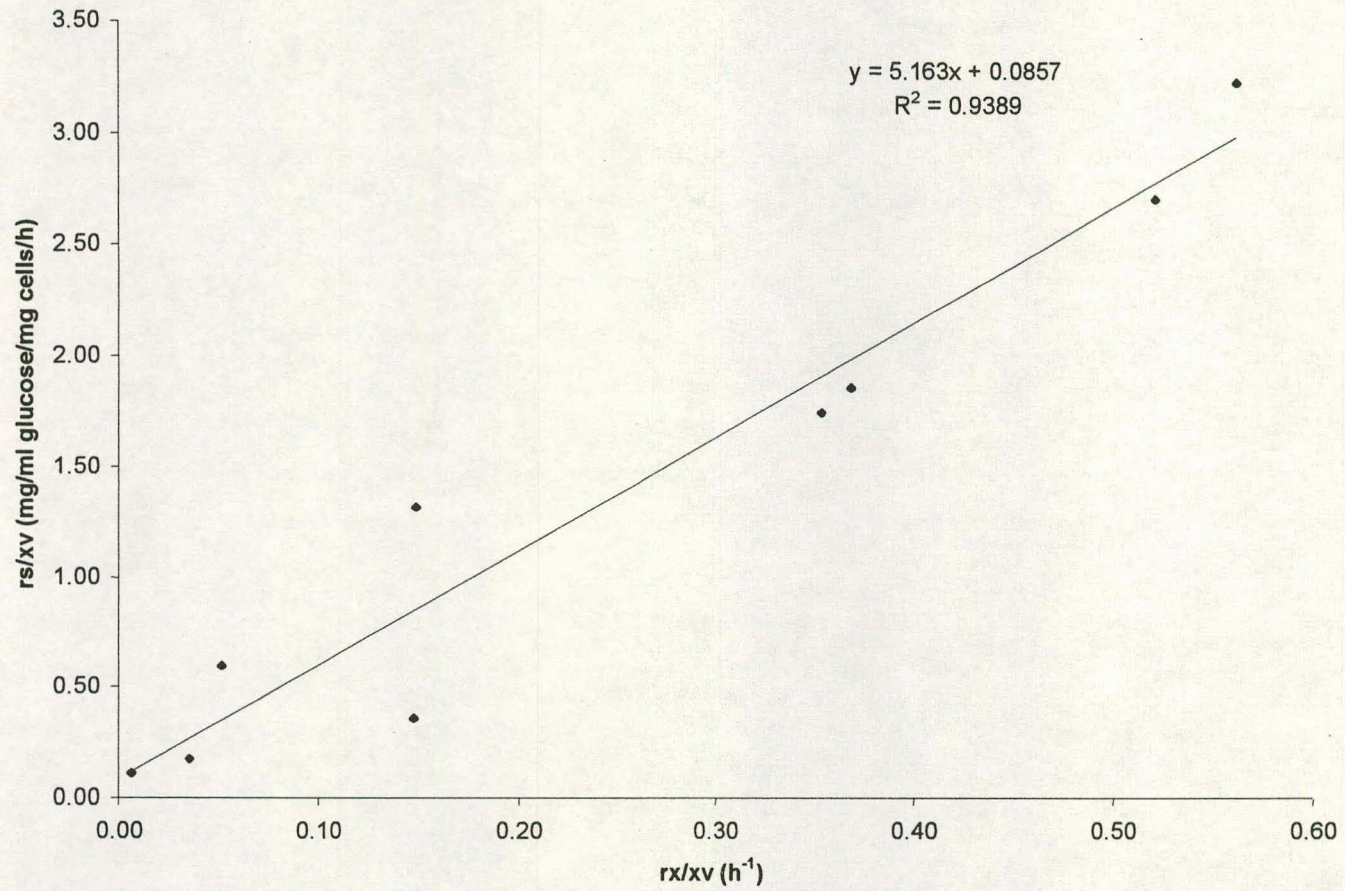


Fig. 19. Plot of glucose utilization (r_s/x_v) versus growth rate (r_x/x_v) to determine the growth yield (Y) and maintenance coefficient (m_s) of *S. sanguis*.

Growth parameters of *L. casei*

The experimental data obtained from the growth of *L. casei* in batch culture, with glucose as carbon substrate, is summarised in Table 19 and Table 20. A plot of the experimental data shows that the bacterium produced a typical growth pattern in relation to the glucose concentration in the growth medium (Fig. 20). It can be seen that the glucose acted as a growth-limiting substrate. Growth proceeded exponentially for ± 13 h while the glucose concentration declined to about half the initial concentration. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.300 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 21), and remained unaffected by the concentration of the glucose. Thereafter, the growth rate slowed down as the glucose concentration decreased. After ± 15 h the culture slowly went into a stationary phase, reaching a maximum stationary phase by the time the glucose had been exhausted. A total growth of 0.59 mg cells/ml (dry mass) was obtained (Table 19).

The processed data points for the estimation of the growth parameters μ_{\max} and k_s are listed in Table 21. A Lineweaver-Burke plot of the reciprocal growth rate against the reciprocal glucose concentration is represented in Fig. 22. The value of μ_{\max} was obtained from the intercept ($1/\mu_{\max}$) and the value of k_s was calculated from the slope (k_s/μ_{\max}). These values were: $\mu_{\max} = 0.303 \text{ h}^{-1}$ and $k_s = 6.77 \text{ mg glucose/ml}$ respectively.

The processed data points for the estimation of the yield factor (Y) and the maintenance coefficient (m_s) are listed in Table 22. A straight line plot of the transformed variables r_x/x_v against r_s/x_v is presented in Fig. 23. The value of the yield factor, obtained from the slope of the straight line, was $Y = 0.099 \text{ mg cells/mg glucose}$ and the maintenance coefficient obtained from the intercept was $m_s = 0.098 \text{ mg glucose/mg cells/h}$.

Table 19. Dry cell mass of *L. casei* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean ± sd (n=6) (mg/ml)
0	0.02	0.01	0.01	0.01 ± 0.00
2	0.03	0.03	0.03	0.03 ± 0.00
4	0.06	0.06	0.06	0.06 ± 0.00
6	0.10	0.11	0.11	0.10 ± 0.01
8	0.17	0.18	0.17	0.17 ± 0.01
10	0.27	0.27	0.26	0.27 ± 0.01
12	0.38	0.33	0.30	0.34 ± 0.04
14	0.45	0.38	0.40	0.41 ± 0.04
16	0.50	0.45	0.50	0.48 ± 0.03
18	0.53	0.48	0.51	0.51 ± 0.03
20	0.56	0.50	0.56	0.54 ± 0.03
22	0.58	0.54	0.58	0.57 ± 0.02
24	0.59	0.55	0.60	0.58 ± 0.03
26	0.60	0.56	0.61	0.59 ± 0.03
28	0.60	0.56	0.61	0.59 ± 0.03

Table 20. Glucose concentration in the growth medium of *L. casei* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	8.62	8.53	8.60	8.58 \pm 0.04
2	8.60	8.55	8.60	8.59 \pm 0.03
4	8.50	8.17	8.18	8.28 \pm 0.19
6	8.00	7.42	7.70	7.71 \pm 0.29
8	7.50	6.77	6.70	6.99 \pm 0.44
10	6.80	6.20	6.0	6.33 \pm 0.42
12	5.90	5.45	5.2	5.52 \pm 0.36
14	4.93	4.55	4.70	4.73 \pm 0.19
24	0.20	0.6	0.4	0.4 \pm 0.20
26	0.00	0.27	0.00	0.09 \pm 0.16
28	0	0	0	0.00

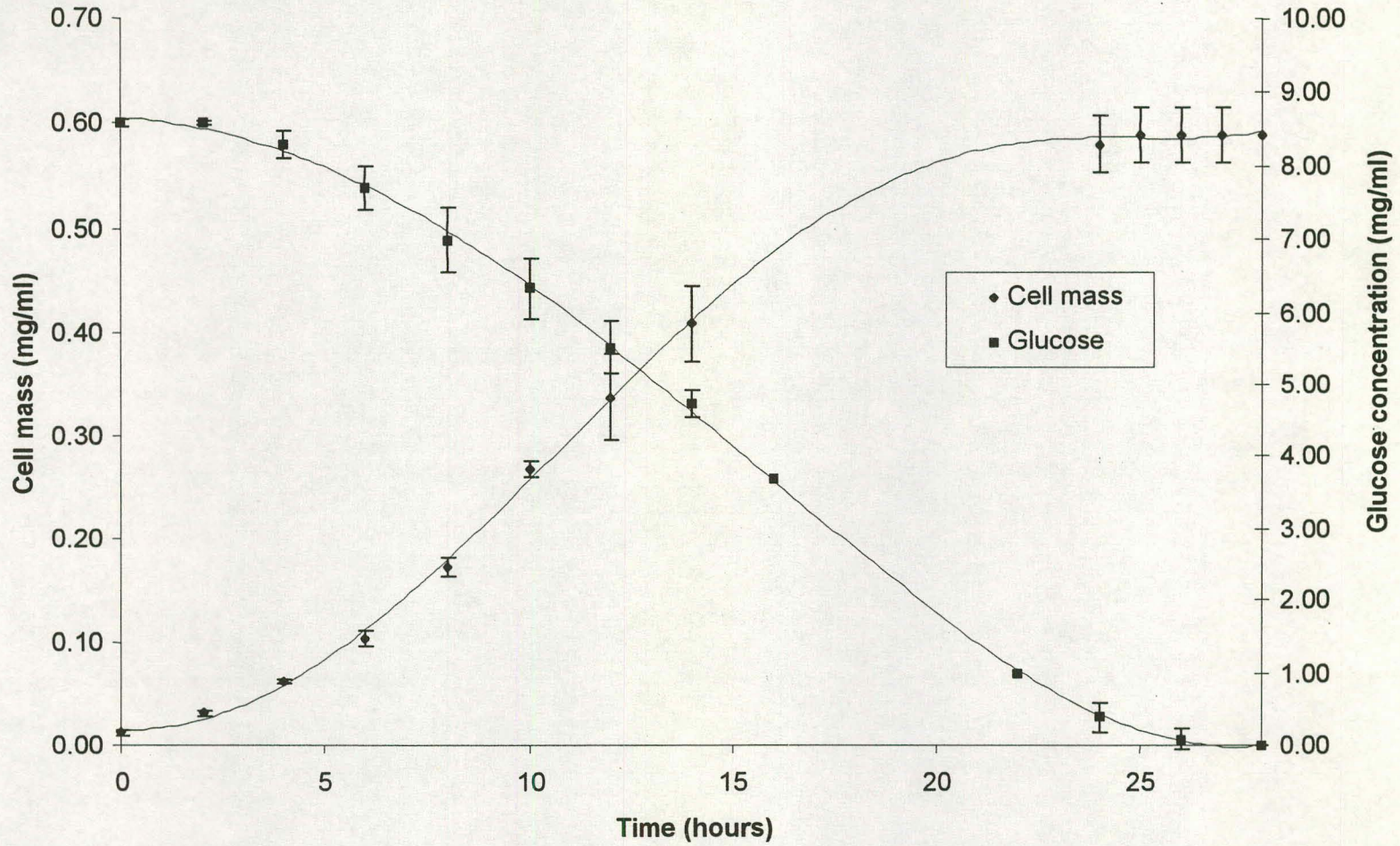


Fig. 20. Growth of *L. casei* and the utilisation of glucose.

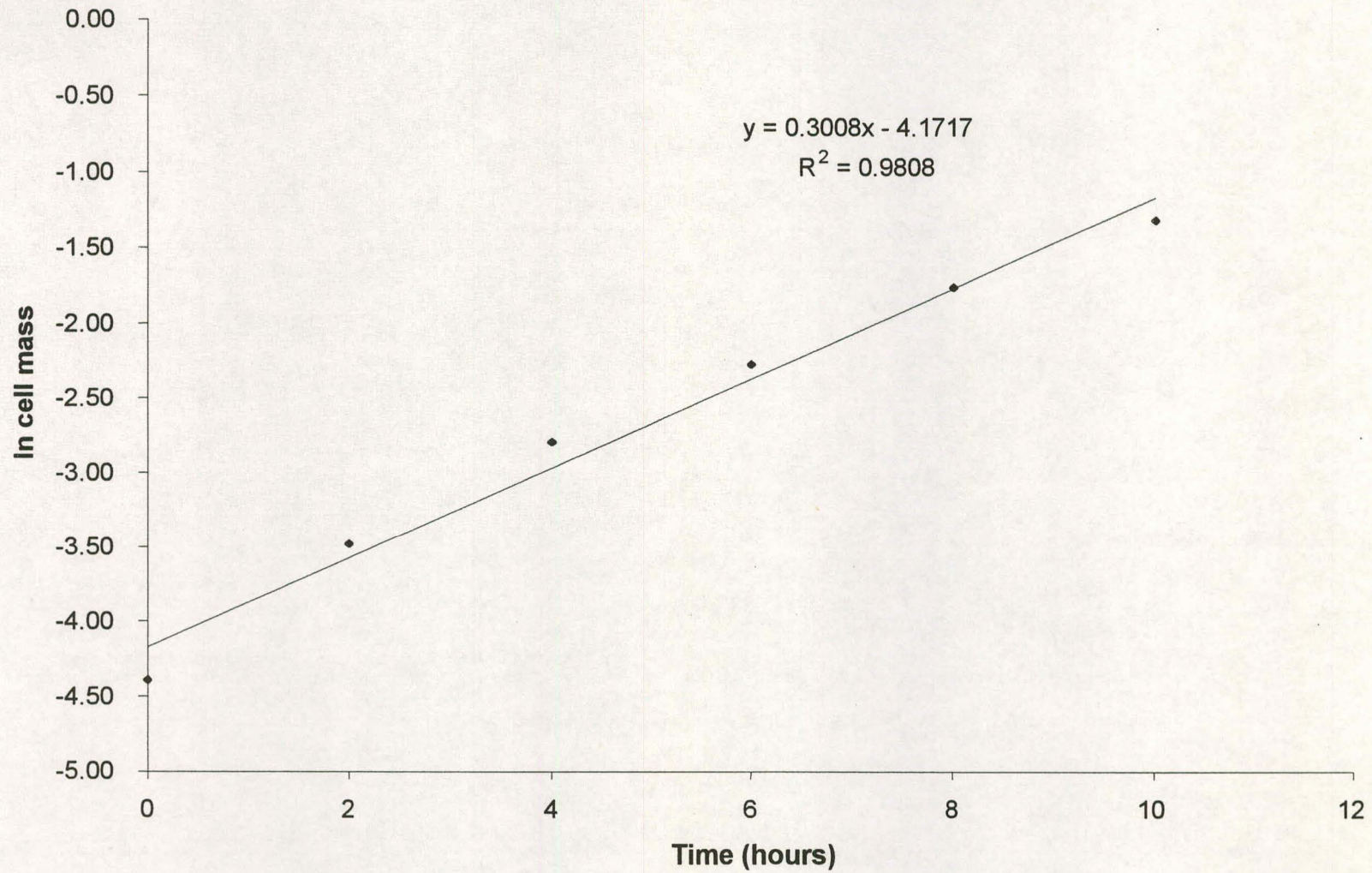


Fig. 21. Logarithmic curve of the exponential phase of the growth curve of *L. casei*.

Table 21. Processed data points for the determination of the maximum specific growth rate and the saturation constant of *L. casei* grown on glucose as carbon substrate.

Time (h)	x_v (mg/ml)	$\ln x_v$	$rx=dx_v/dt$	$\mu=r_x/x_v$	$1/\mu$	S (mg/ml)	$1/S$
0	0.01	-4.40	-	-	-	8.58	0.12
2	0.03	-3.47	0.01	0.40	2.49	8.59	0.12
4	0.06	-2.79	0.02	0.30	3.32	8.28	0.12
6	0.10	-2.27	0.03	0.26	3.87	7.71	0.13
8	0.17	-1.76	0.04	0.24	4.20	6.99	0.14
10	0.27	-1.32	0.05	0.17	5.95	6.33	0.16
12	0.34	-1.09	0.04	0.11	9.40	5.52	0.18
14	0.41	-0.89	0.04	0.09	11.24	4.73	0.21
24	0.58	-0.54	0.01	0.01	67.21	0.40	2.50
26	0.60	-0.51	0.00	0.00	250.85	0.09	11.15
28	0.59	-0.53	-	-	-	-	-

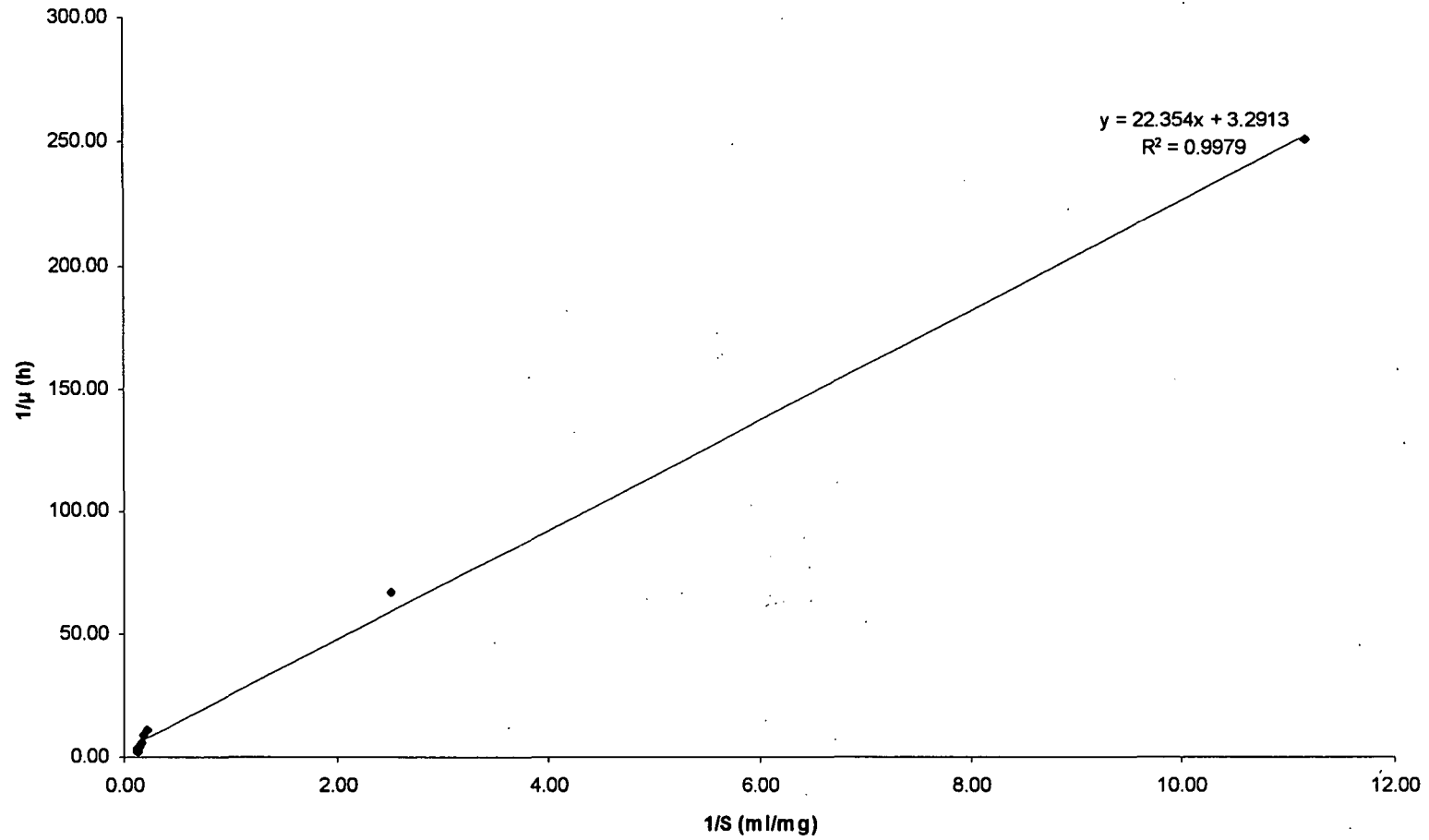


Fig. 22. A Lineweaver – Burke plot of the reciprocal growth rate ($1/\mu$) against the reciprocal glucose concentration ($1/S$) for the determination of the maximum growth rate (μ_{max}) and the saturation constant (k_s) of *L. casei*.

Table 22. Processed data points for the estimation of the growth yield factor and the maintenance coefficient for the growth of *L. casei* on glucose.

Time (h)	x_v (mg/ml)	$\ln x_v$	$r_x = dx_v/dt$	$\mu = r_x/x_v$	S (mg/ml)	r_s	r_s/x_v
0	0.01	-4.40	-	-	8.62	-	-
2	0.03	-3.47	0.01	0.40	8.59	0.13	4.19
4	0.06	-2.79	0.02	0.30	8.10	0.22	3.60
6	0.10	-2.27	0.03	0.26	7.71	0.28	2.68
8	0.17	-1.76	0.04	0.24	6.99	0.34	1.99
10	0.27	-1.32	0.05	0.17	6.33	0.37	1.37
12	0.34	-1.09	0.04	0.11	5.52	0.40	1.19
14	0.41	-0.89	0.04	0.09	4.73	0.45	1.11
24	0.58	-0.54	0.01	0.01	0.40	0.23	0.39
26	0.59	-0.53	0.00	0.00	0.09	0.10	0.17
28	0.59	-0.53	-	-	0	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

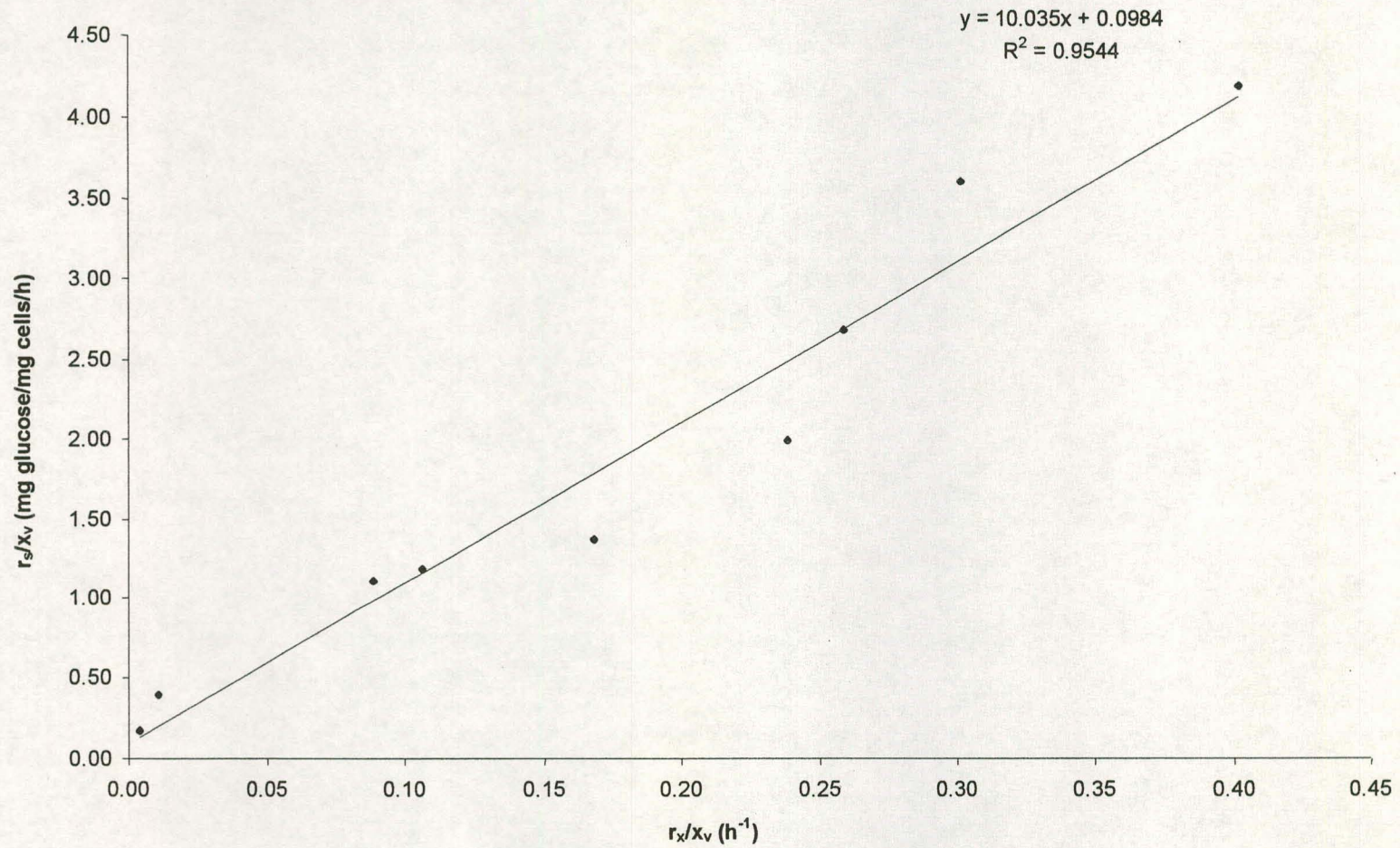


Fig. 23. Plot of glucose utilisation (r_s/x_v) towards growth rate (r_x/x_v) to determine the growth yield (Y) and the maintenance coefficient (m_s) of *L. casei*.

Growth parameters of *S. mitis*

The experimental data obtained from the growth of *S. mitis* in batch culture, with glucose as carbon substrate, is summarised in Table 23 and Table 24. The bacterium produced a typical growth pattern in relation to the glucose concentration in the growth medium (Fig. 24) and it can be seen that the glucose acted as a growth-limiting substrate. Growth proceeded exponentially for ± 8 h while the glucose concentration declined to about half the initial concentration. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.227 \text{ h}^{-1}$ (as determined by the slope of the logarithmic plot of the exponential phase of the growth curve, Fig. 25) and remained unaffected by the concentration of the glucose. Thereafter, the growth rate slowed down as the glucose concentration decreased. After ± 12 h the culture slowly went into a stationary phase, reaching a maximum stationary phase by the time the glucose had been exhausted. A total growth of 0.99 mg cells/ml (dry mass) was obtained (Table 23).

The processed data points for the estimation of the growth parameters μ_{\max} and k_s are listed in Table 25. A Lineweaver-Burke plot of the reciprocal growth rate against the reciprocal glucose concentration is represented in Fig. 26. The value of μ_{\max} was obtained from the intercept ($1/\mu_{\max}$) and the value of k_s was calculated from the slope (k_s/μ_{\max}). These values were: $\mu_{\max} = 0.232 \text{ h}^{-1}$ and $k_s = 3.73 \text{ mg glucose/ml}$ respectively.

The processed data points for the estimation of the yield factor (Y) and the maintenance coefficient (m_s) are listed in Table 26. A straight line plot of the transformed variables r_x/x_v against r_s/x_v is presented in Fig. 27. The value of the yield factor, obtained from the slope of the straight line, was $Y = 0.062 \text{ mg cells/mg glucose}$ and the maintenance coefficient obtained from the intercept was $m_s = 0.090 \text{ mg glucose/mg cells/h}$.

Table 23. Dry cell mass of *S. mitis* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean ± sd (n=6) (mg/ml)
0	0.14	0.17	0.16	0.16 ± 0.02
1	0.16	0.17	0.16	0.16 ± 0.01
2	0.17	0.18	0.16	0.17 ± 0.01
3	0.21	0.20	0.21	0.21 ± 0.01
4	0.28	0.24	0.29	0.27 ± 0.02
5	0.32	0.38	0.35	0.35 ± 0.03
6	0.42	0.40	0.45	0.42 ± 0.03
7	0.53	0.47	0.54	0.51 ± 0.04
8	0.65	0.54	0.60	0.60 ± 0.05
9	0.75	0.63	0.75	0.71 ± 0.07
10	0.83	0.73	0.78	0.78 ± 0.05
11	0.87	0.80	0.86	0.84 ± 0.04
12	0.90	0.90	0.90	0.90 ± 0.00
13	0.89	0.90	0.91	0.90 ± 0.01
14	0.9	0.9	0.9	0.9 ± 0.00
15	0.9	0.9	0.9	0.9 ± 0.00

Table 24. Glucose concentration in the growth medium of *S. mitis* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	11.90	11.90	11.9	11.90 \pm 0.0
1	11.90	11.9	11.9	11.90 \pm 0.0
2	11.22	11.90	11.56	11.56 \pm 0.34
3	11.00	11.90	11.45	11.45 \pm 0.45
4	9.60	10.37	9.985	9.99 \pm 0.38
5	8.67	8.67	8.67	8.67 \pm 0.0
6	7.14	7.14	7.14	7.14 \pm 0.0
7	6.12	5.78	5.95	5.95 \pm 0.17
8	4.42	4.42	4.42	4.42 \pm 0.0
9	3.40	2.89	3.145	3.15 \pm 0.26
10	2.55	1.70	2.125	2.13 \pm 0.42
11	1.40	1.26	1.125	1.26 \pm 0.14
12	0.55	0.85	0.7	0.70 \pm 0.15
13	0.14	0.14	0.14	0.14 \pm 0.0
14	0	0	0	0

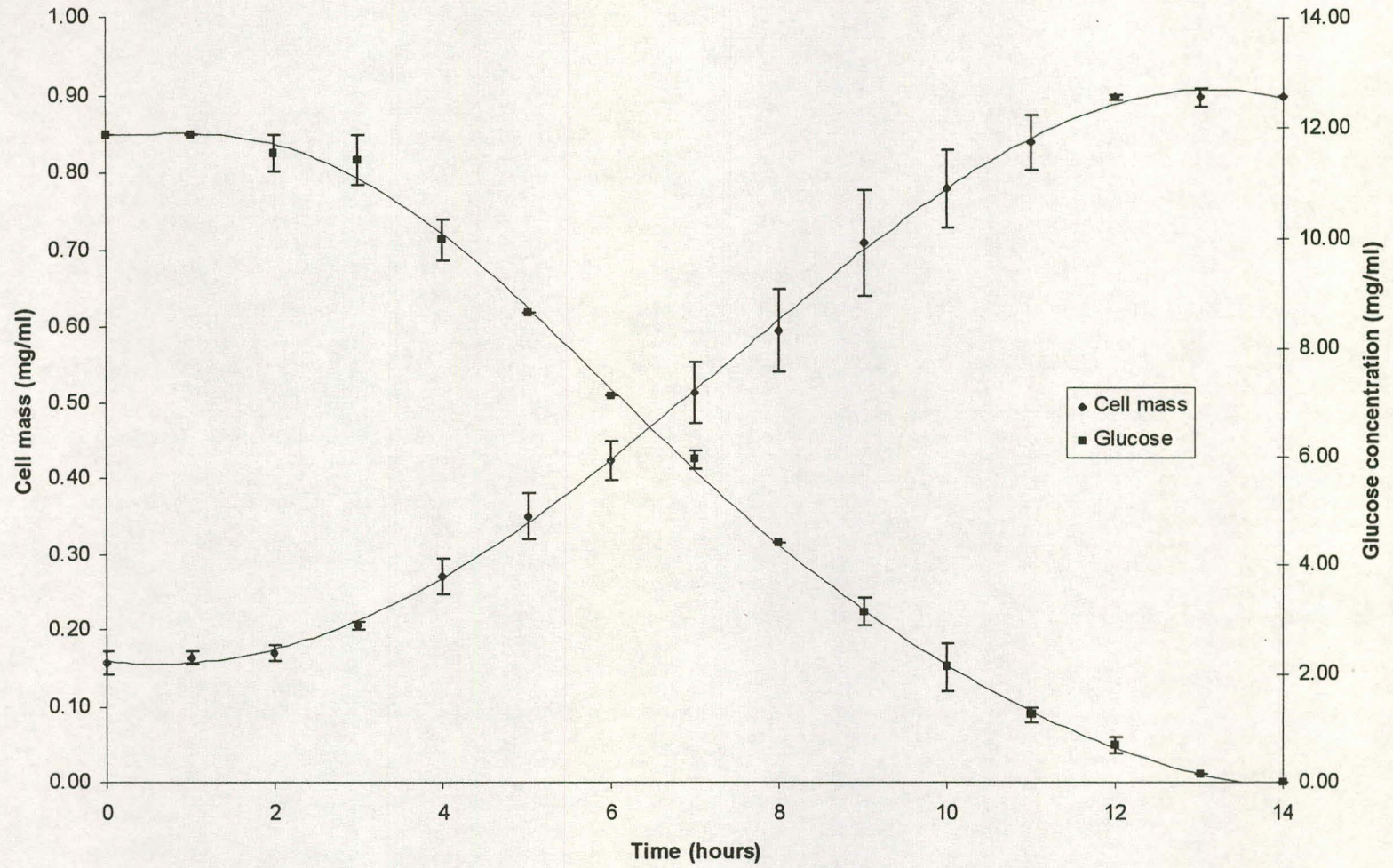


Fig. 24. Growth of *S. mitis* and the utilisation of glucose.

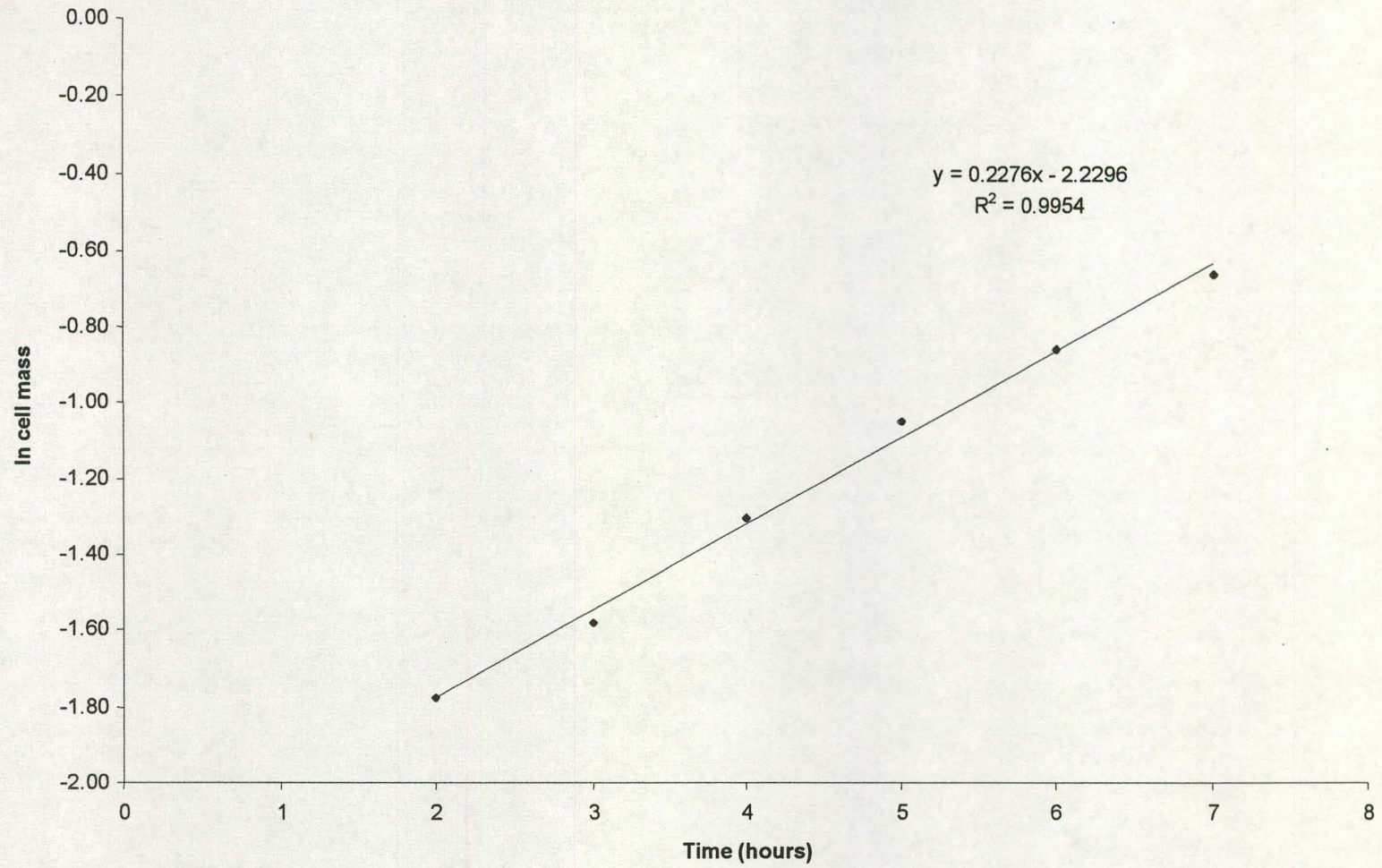


Fig. 25. Logarithmic curve of the exponential phase of the growth curve of *S. mitis*.

Table 25. Processed data points for the determination of the maximum specific growth rate and the saturation constant of *S. mitis* grown on glucose as carbon substrate.

Time (h)	x_v (mg/ml)	$\ln x_v$	$r_x = dx_v/dt$	$\mu = r_x/x_v$	$1/\mu$	S (mg/ml)	$1/S$
0	0.16	-1.85	-	-	-	11.90	0.08
1	0.16	-1.82	0.01	0.04	25.09	11.90	0.08
2	0.17	-1.77	0.02	0.12	8.53	11.56	0.09
3	0.21	-1.58	0.05	0.23	4.26	11.45	0.09
4	0.27	-1.30	0.07	0.27	3.75	9.99	0.10
5	0.35	-1.05	0.08	0.22	4.49	8.67	0.12
6	0.42	-0.86	0.08	0.19	5.23	7.14	0.14
7	0.51	-0.67	0.09	0.17	5.88	5.95	0.17
8	0.60	-0.52	0.10	0.16	6.20	4.42	0.23
9	0.71	-0.34	0.10	0.14	7.39	3.15	0.32
10	0.78	-0.25	0.07	0.08	11.81	2.13	0.47
11	0.84	-0.17	0.06	0.07	14.20	1.26	0.79
12	0.90	-0.11	0.03	0.03	29.98	0.70	1.43
13	0.90	-0.11	0.00	0.00	1079.00	0.14	7.21
14	0.90	-0.11	-	-	-	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

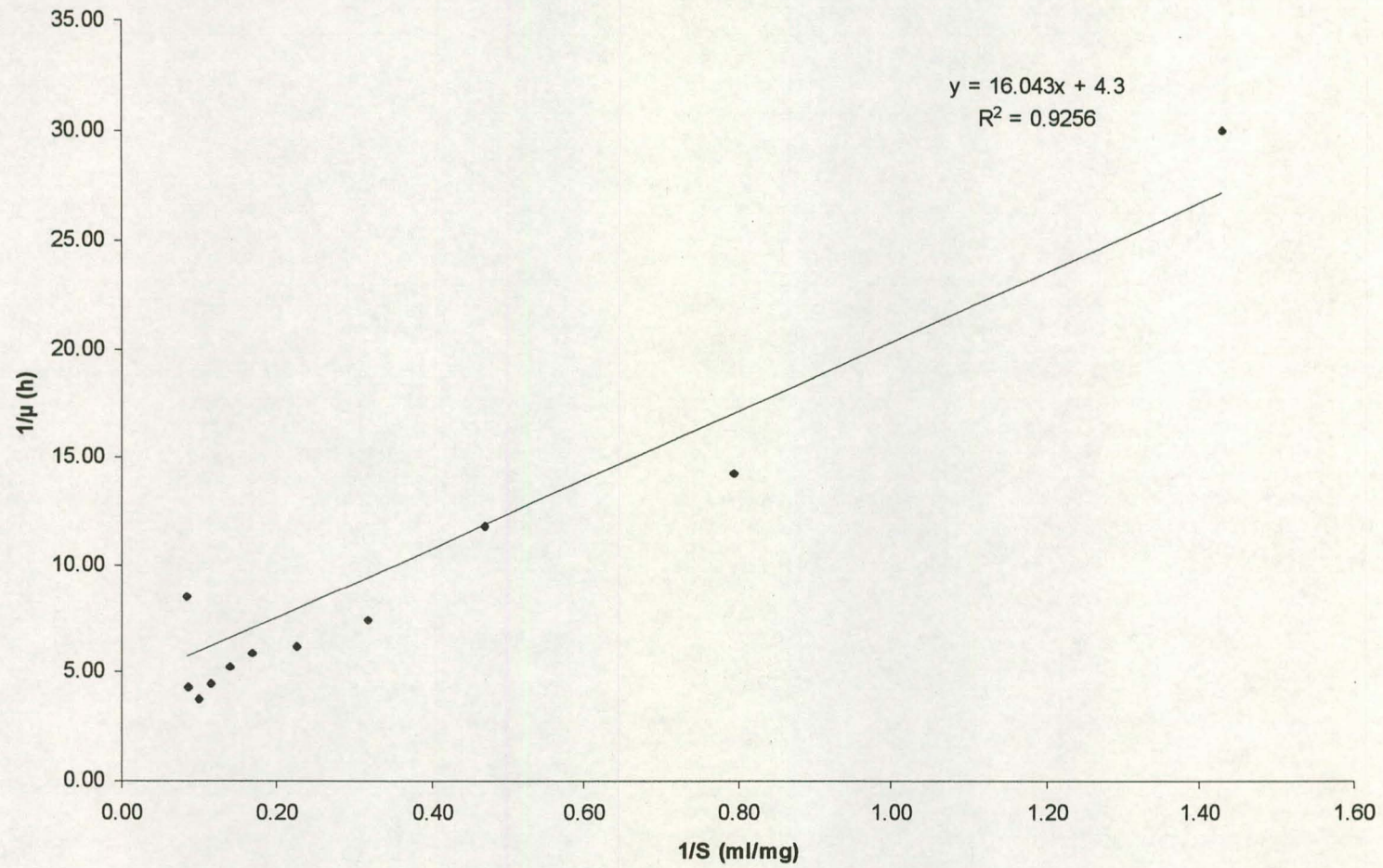


Fig. 26. A Lineweaver – Burke plot of the reciprocal growth rate (μ) against the reciprocal glucose concentration (S) for the determination of the maximum growth rate (μ_{max}) and the saturation constant (k_s) of *S. mitis*.

Table 26. Processed data points for the estimation of the growth yield factor and the maintenance coefficient for the growth of *S. mitis* on glucose.

Time (h)	x_v (mg/ml)	$\ln x_v$	$r_x=dx_v/dt$	$\mu=r_x/x_v$	S (mg/ml)	r_s	r_s/x_v
0	0.16	-1.85	-	-	11.90	-	-
1	0.16	-1.82	0.01	0.04	11.90	0.17	1.05
2	0.17	-1.77	0.02	0.12	11.56	0.23	1.33
3	0.21	-1.58	0.05	0.23	11.45	0.79	3.83
4	0.27	-1.30	0.07	0.27	9.99	1.39	5.12
5	0.35	-1.05	0.08	0.22	8.67	1.42	4.06
6	0.42	-0.86	0.08	0.19	7.14	1.36	3.21
7	0.51	-0.67	0.09	0.17	5.95	1.36	2.65
8	0.60	-0.52	0.10	0.16	4.42	1.40	2.36
9	0.71	-0.34	0.10	0.14	3.15	1.15	1.62
10	0.78	-0.25	0.07	0.08	2.13	0.94	1.21
11	0.84	-0.17	0.06	0.07	1.26	0.71	0.85
12	0.90	-0.11	0.03	0.03	0.70	0.56	0.63
13	0.90	-0.11	0.00	0.00	0.14	0.35	0.39
14	0.9	-0.11	-	-	0	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

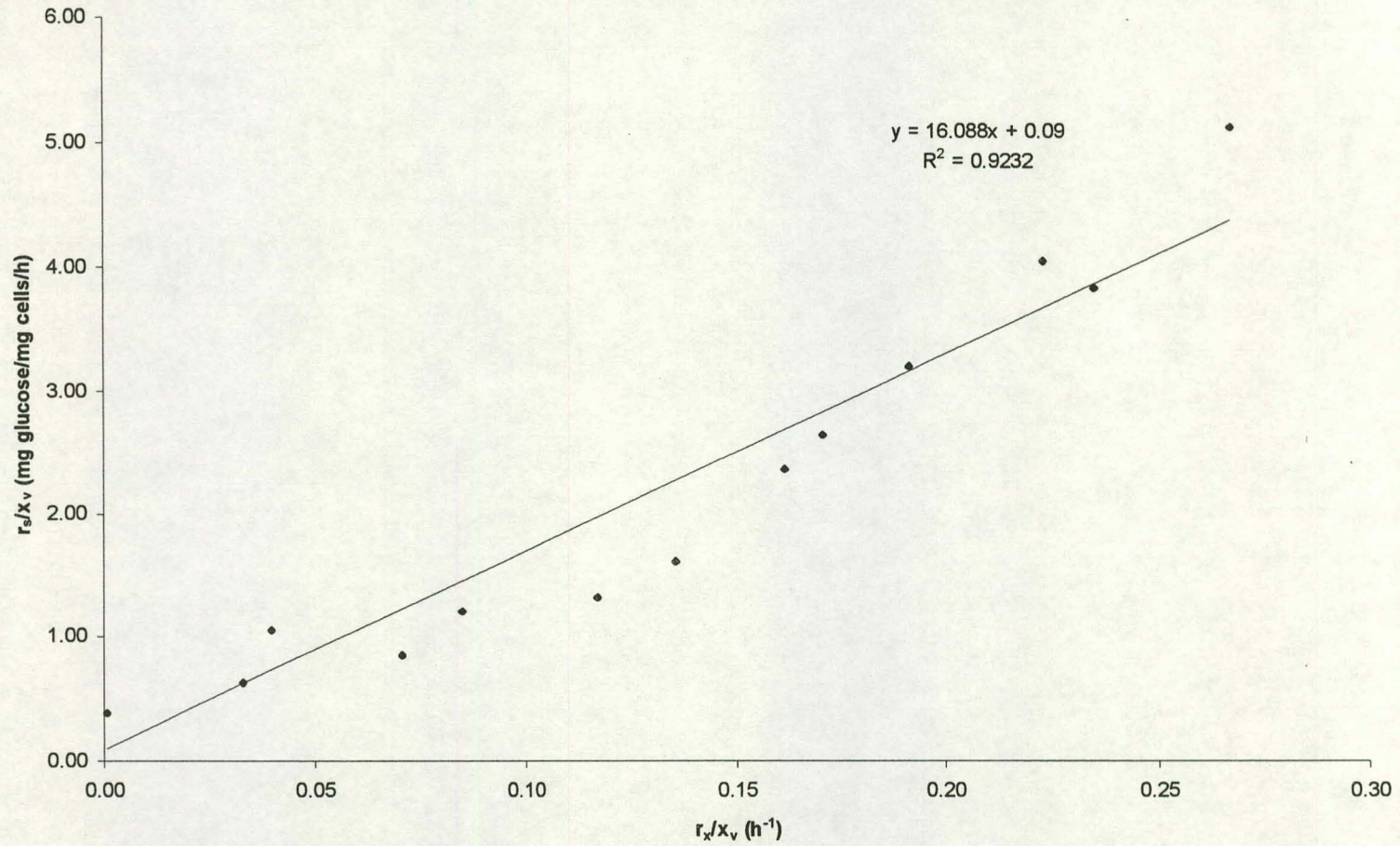


Fig. 27. Plot of glucose utilisation (r_s/x_v) towards growth rate (r_x/x_v) to determine the growth yield (Y) and the maintenance coefficient (m_s) of *S. mitis*.

Growth parameters of *S. sobrinus*

The experimental data obtained from the growth of *S. sobrinus* in batch culture, with glucose as carbon substrate, is summarised in Table 27 and Table 28. A plot of the experimental data shows that the bacterium produced a typical growth pattern in relation to the glucose concentration in the growth medium (Fig. 28). It can be seen that the glucose acted as a growth-limiting substrate. Growth proceeded exponentially for ± 8 h while the glucose concentration declined to about 25% of the initial concentration. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.622 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 29), and remained unaffected by the concentration of the glucose. Thereafter, the growth rate slowed down as the glucose concentration decreased. After ± 8 h the culture slowly went into a stationary phase, reaching a maximum stationary phase by the time the glucose had been exhausted. A total growth of 0.62 mg cells/ml (dry mass) was obtained (Table 27).

The processed data points for the estimation of the growth parameters μ_{\max} and k_s are listed in Table 29. A Lineweaver-Burke plot of the reciprocal growth rate against the reciprocal glucose concentration is represented in Fig. 30. The value of μ_{\max} was obtained from the intercept ($1/\mu_{\max}$) and the value of k_s was calculated from the slope (k_s/μ_{\max}). These values were $\mu_{\max} = 0.610 \text{ h}^{-1}$ and $k_s = 6.49 \text{ mg glucose/ml}$ respectively.

The processed data points for the estimation of the yield factor (Y) and the maintenance coefficient (m_s) are listed in Table 30. A straight line plot of the transformed variables r_x/x_v against r_s/x_v is presented in Fig. 31. The value of the yield factor, obtained from the reciprocal of the slope of the straight line, was $Y = 0.128 \text{ mg cells/mg glucose}$ and the maintenance coefficient obtained from the intercept was $m_s = 0.232 \text{ mg glucose/mg cells/h}$.

Table 27. Dry cell mass of *S. sobrinus* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	0.07	0.11	0.11	0.10 \pm 0.02
1	0.06	0.10	0.10	0.09 \pm 0.02
2	0.06	0.10	0.12	0.09 \pm 0.03
3	0.07	0.11	0.13	0.10 \pm 0.03
4	0.08	0.12	0.14	0.11 \pm 0.03
5	0.15	0.18	0.20	0.18 \pm 0.03
6	0.33	0.36	0.42	0.37 \pm 0.05
7	0.77	0.82	0.73	0.77 \pm 0.05
8	1.15	1.24	1.28	1.22 \pm 0.07
9	1.40	1.30	1.42	1.37 \pm 0.06
10	1.51	1.38	1.51	1.46 \pm 0.07
11	1.50	1.49	1.52	1.50 \pm 0.02
12	1.51	1.45	1.58	1.51 \pm 0.06

Table 28. Glucose concentration in the growth medium of *S. sobrinus* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	11.33	13.69	11.42	12.14 \pm 1.34
1	11.30	13.60	11.40	12.10 \pm 1.30
2	11.33	13.60	11.42	12.11 \pm 1.29
3	11.33	13.50	11.30	12.04 \pm 1.26
4	11.33	13.31	11.00	11.88 \pm 1.25
5	10.80	11.93	10.83	11.19 \pm 0.65
6	10.09	10.23	9.27	9.86 \pm 0.52
7	7.80	6.35	6.54	6.90 \pm 0.79
8	4.77	3.89	3.03	3.89 \pm 0.87
9	2.90	2.00	1.23	2.04 \pm 0.84
10	0.68	0.50	0.40	0.53 \pm 0.14
11	0.00	0.00	0.30	0.10 \pm 0.17
12	0.00	0.00	0.00	0.00 \pm 0.00

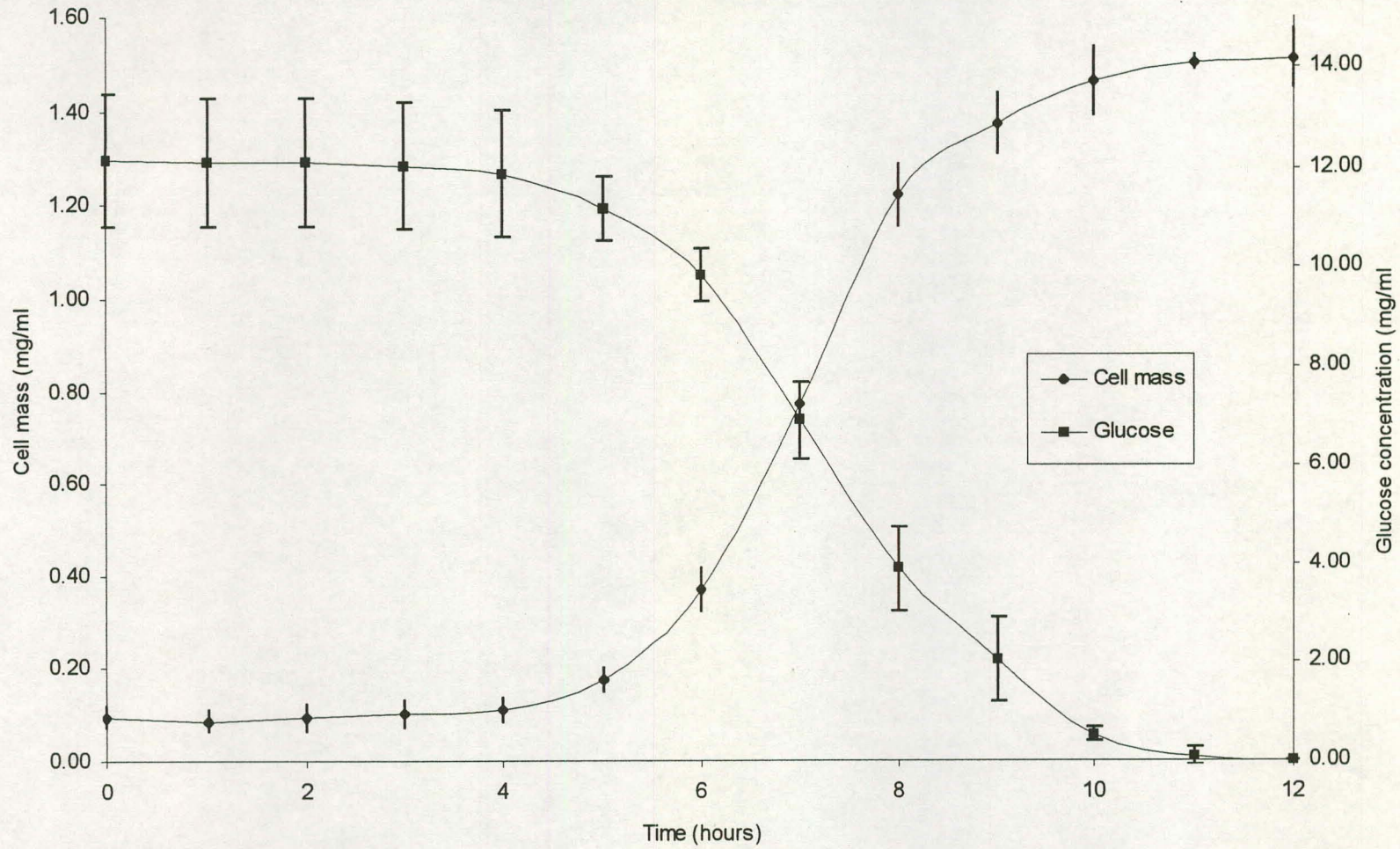


Fig. 28. Growth of *S. sobrinus* and the utilisation of glucose.

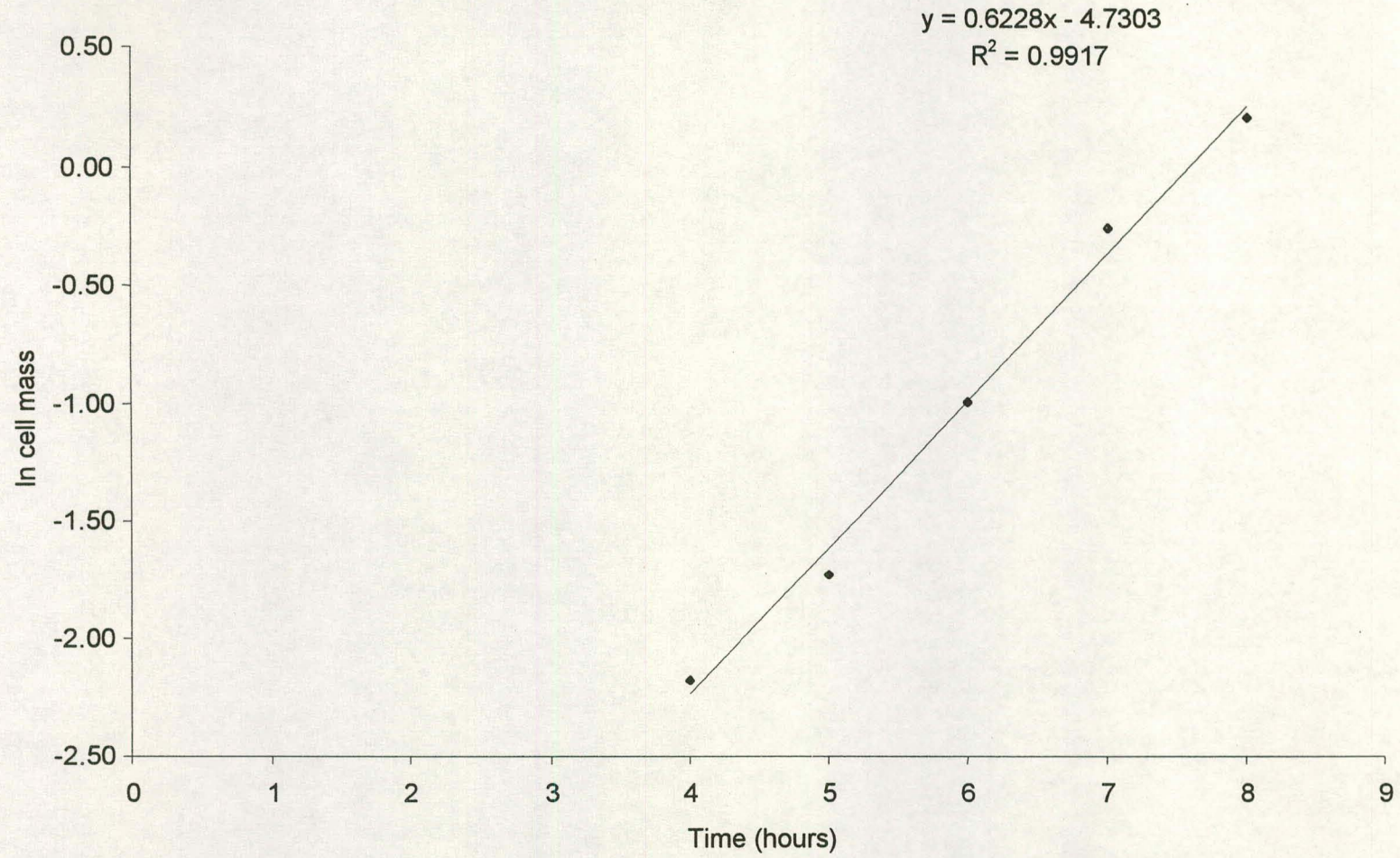


Fig. 29. Logarithmic curve of the exponential phase of the growth curve of *S. sobrinus*.

Table 29. Processed data points for the determination of the maximum specific growth rate and the saturation constant of *S. sobrinus* grown on glucose as carbon substrate.

Time (h)	x_v (mg/ml)	$\ln x_v$	$r_x = dx_v/dt$	$\mu = r_x/x_v$	$1/\mu$	S (mg/ml)	1/S
0	0.10	-2.34	-	-	-	12.14	0.08
1	0.09	-2.45	0.00	-0.01	-143.00	12.10	0.08
2	0.09	-2.36	0.01	0.09	11.37	12.11	0.08
3	0.10	-2.27	0.01	0.09	11.11	12.04	0.08
4	0.11	-2.18	0.03	0.27	3.73	11.88	0.08
5	0.18	-1.73	0.10	0.59	1.69	11.19	0.09
6	0.37	-1.00	0.27	0.74	1.36	9.86	0.10
7	0.77	-0.26	0.46	0.60	1.67	6.90	0.14
8	1.22	0.20	0.35	0.29	3.46	3.89	0.26
9	1.37	0.32	0.12	0.09	11.06	2.04	0.49
10	1.46	0.38	0.07	0.05	22.17	0.50	2.00
11	1.50	0.41	0.02	0.02	62.39	0.10	10.00
12	1.51	0.41	0.02	0.02	63.62	0.00	-
13	1.55	0.44	-	-	-	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

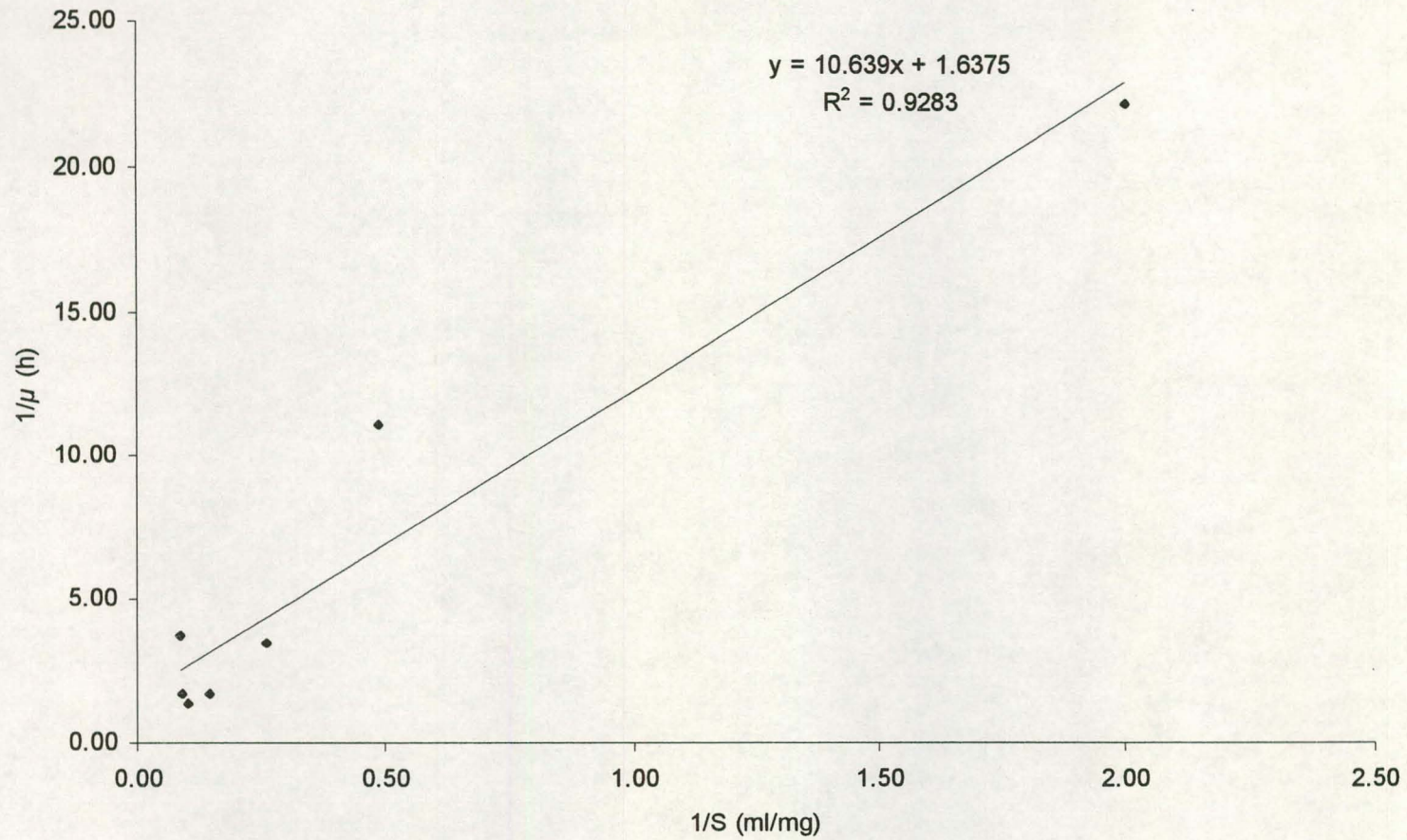


Fig. 30. A Lineweaver - Burke plot of the reciprocal growth rate (μ) against the reciprocal glucose concentration (S) for the determination of the maximum growth rate (μ_{\max}) and the saturation constant (k_s) of *S. sobrinus*.

Table 30. Processed data points for the estimation of the growth yield factor and the maintenance coefficient for the growth of *S. sobrinus* on glucose.

Time (h)	x_v (mg/ml)	$\ln x_v$	$r_x = dx_v/dt$	$\mu = r_x/x_v$	S (mg/ml)	r_s	r_s/x_v
0	0.10	-2.34	-	-	12.14	-	-
1	0.09	-2.45	0.00	0.00	12.10	0.02	0.17
2	0.09	-2.36	0.01	0.09	12.11	0.03	0.30
3	0.10	-2.27	0.01	0.09	12.04	0.12	1.13
4	0.11	-2.18	0.03	0.27	11.88	0.43	3.78
5	0.18	-1.73	0.10	0.59	11.19	1.01	5.71
6	0.37	-1.00	0.27	0.74	9.86	2.14	5.81
7	0.77	-0.26	0.46	0.60	6.90	2.98	3.87
8	1.22	0.20	0.35	0.29	3.89	2.43	1.99
9	1.37	0.32	0.12	0.09	2.04	1.70	1.24
10	1.46	0.38	0.07	0.05	0.50	0.97	0.66
11	1.50	0.41	0.02	0.02	0.10	0.25	0.17
12	1.51	0.41	0.02	0.02	0.00	0.05	0.03
13	1.55	0.44	-	-	0	-	-

x_v = mean (n=6) cell mass (mg/ml)

μ = specific growth rate

S = mean (n=6) glucose concentration (mg/ml)

r_s = rate of carbon substrate consumption

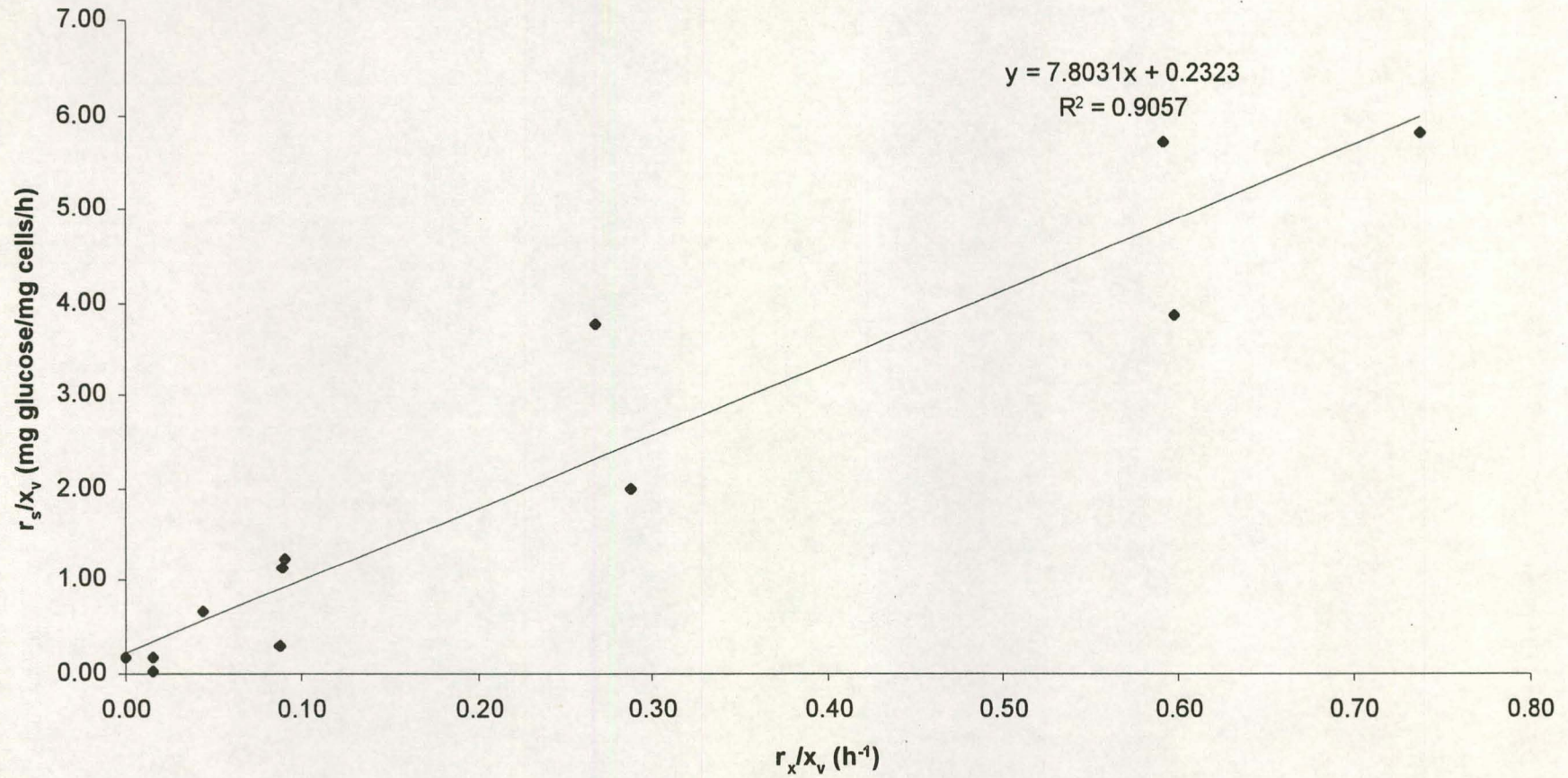


Fig. 31. Plot of glucose utilisation (r_s/x_v) against growth rate (r_x/x_v) to determine the growth yield (Y) and the maintenance coefficient (m_s) of *S. sobrinus*.

Growth parameters of *F. nucleatum*

The experimental data obtained from the growth of *F. nucleatum* in batch culture, with glucose as carbon substrate, is summarised in Table 31 and Table 32. A plot of the experimental data shows that the bacterium used some of the glucose for growth, however, glucose did not act as a growth-limiting substrate for the organism (Fig. 32). Growth proceeded exponentially for ± 14 h while the glucose concentration declined to 8 mg/ml. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.153 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 33), and remained unaffected by the concentration of the glucose. Thereafter, the growth rate slowed down as the glucose concentration decreased by another 1.31 mg/ml. After ± 15 h the culture slowly went into a stationary phase although more than 92% of the original glucose in the medium remained. A total growth of 0.65 mg cells/ml (dry mass) was obtained (Table 31).

Glucose did not act as a growth-limiting substrate for *F. nucleatum*. Therefore the estimation of the growth parameter k_s , the yield factor Y and the maintenance coefficient m_s for glucose could not be calculated.

Table 31. Dry cell mass of *F. nucleatum* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean ± sd (n=6) (mg/ml)
0	0.09	0.11	0.09	0.10 ± 0.01
1	0.09	0.11	0.09	0.10 ± 0.01
2	0.09	0.11	0.09	0.10 ± 0.01
3	0.11	0.13	0.10	0.11 ± 0.01
4	0.12	0.14	0.12	0.13 ± 0.01
5	0.13	0.14	0.14	0.14 ± 0.01
6	0.15	0.16	0.15	0.15 ± 0.01
7	0.18	0.20	0.18	0.19 ± 0.01
8	0.21	0.23	0.22	0.22 ± 0.01
9	0.25	0.26	0.25	0.25 ± 0.01
10	0.27	0.28	0.26	0.27 ± 0.01
11	0.32	0.33	0.32	0.32 ± 0.01
12	0.34	0.36	0.34	0.35 ± 0.01
13	0.38	0.40	0.38	0.39 ± 0.01
14	0.41	0.43	0.41	0.42 ± 0.01
15	0.44	0.46	0.44	0.45 ± 0.01
16	0.47	0.49	0.47	0.48 ± 0.01
17	0.49	0.51	0.49	0.50 ± 0.01
18	0.53	0.55	0.53	0.54 ± 0.01
19	0.57	0.59	0.57	0.58 ± 0.01
20	0.59	0.61	0.59	0.60 ± 0.01
21	0.60	0.62	0.60	0.61 ± 0.01
22	0.61	0.63	0.62	0.62 ± 0.01
23	0.64	0.65	0.64	0.64 ± 0.01
24	0.64	0.66	0.61	0.64 ± 0.02
25	0.64	0.66	0.65	0.65 ± 0.01

Table 32. Glucose concentration in the growth medium of *F. nucleatum* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	8.45	8.32	8.65	8.48 \pm 0.17
1	8.45	8.30	8.65	8.47 \pm 0.18
2	8.45	8.32	8.65	8.48 \pm 0.17
3	8.45	8.25	8.65	8.45 \pm 0.20
4	8.45	8.30	8.65	8.47 \pm 0.18
5	8.45	8.32	8.65	8.48 \pm 0.17
6	8.45	8.40	8.65	8.50 \pm 0.13
7	8.45	8.32	8.65	8.48 \pm 0.17
8	8.30	8.17	8.50	8.32 \pm 0.17
9	8.20	8.00	8.40	8.20 \pm 0.20
10	8.20	8.07	8.40	8.22 \pm 0.17
11	8.07	7.94	8.27	8.10 \pm 0.17
12	8.20	8.07	8.40	8.22 \pm 0.17
13	7.90	7.90	8.10	7.97 \pm 0.12
14	8.00	7.87	8.20	8.02 \pm 0.17
15	7.80	7.67	8.00	7.82 \pm 0.17
16	7.60	7.47	7.80	7.62 \pm 0.17
17	7.60	7.50	7.80	7.63 \pm 0.15
18	7.40	7.27	7.60	7.42 \pm 0.17
19	7.35	7.22	7.55	7.37 \pm 0.17
20	7.30	7.17	7.50	7.32 \pm 0.17
21	7.00	6.87	7.20	7.02 \pm 0.17
22	6.90	6.77	7.10	6.92 \pm 0.17
23	7.00	6.87	7.20	7.02 \pm 0.17
24	6.81	6.68	7.01	6.84 \pm 0.17
25	6.69	6.56	6.89	6.71 \pm 0.17

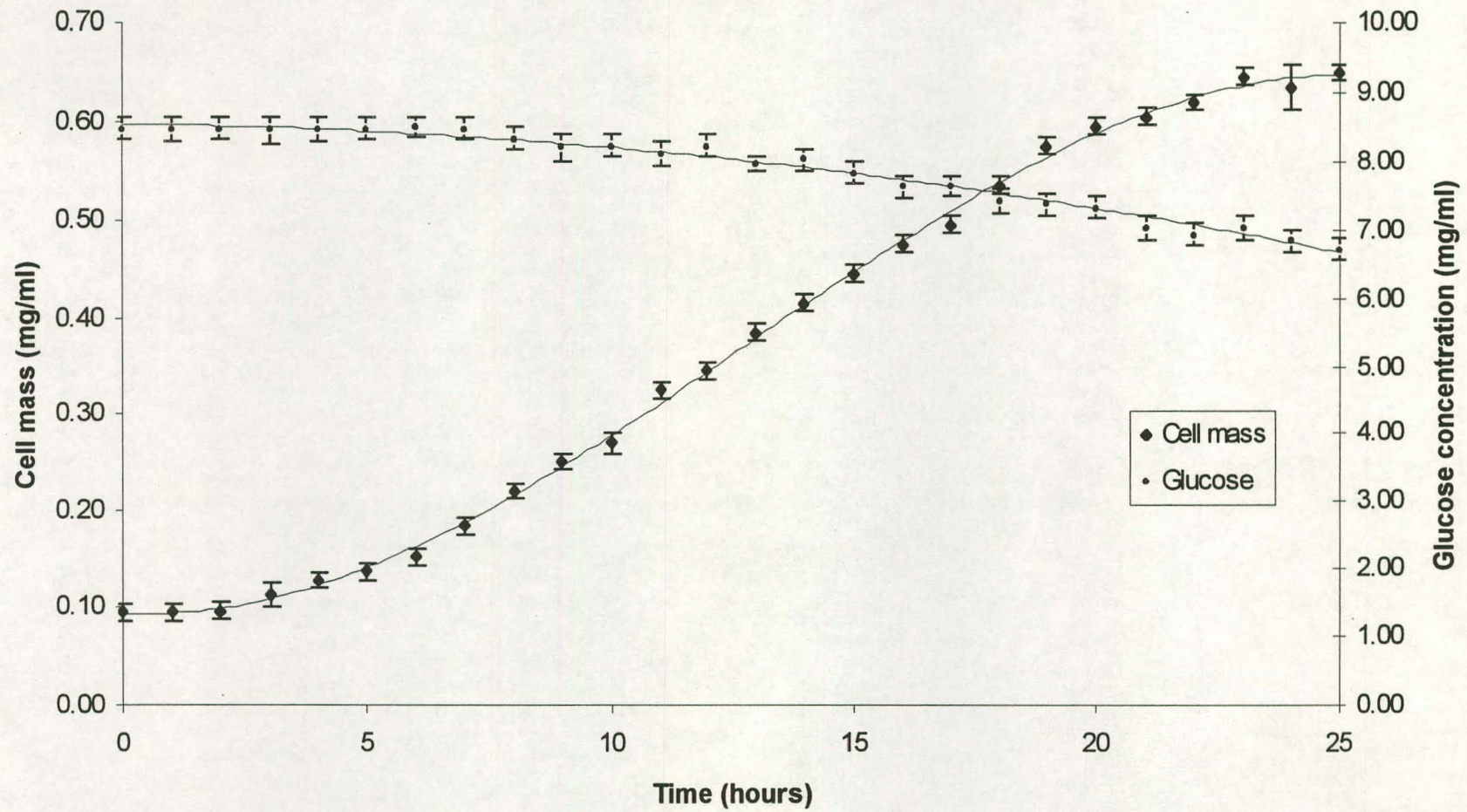


Fig. 32. Growth of *F. nucleatum* and the utilisation of glucose.

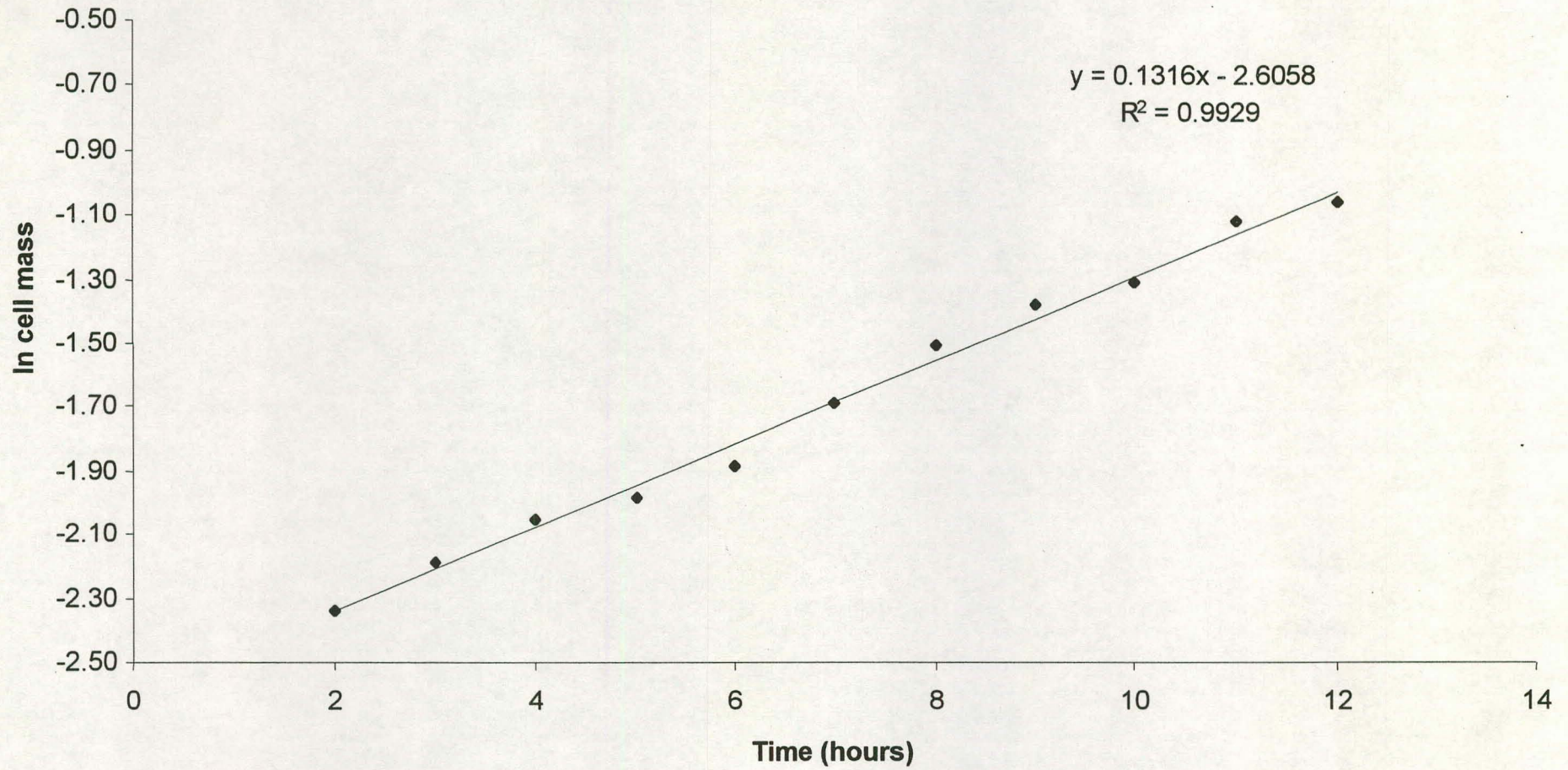


Fig. 33. Logarithmic curve of the exponential phase of the growth curve of *F. nucleatum*.

Growth parameters of *E. saburreum*

The experimental data obtained from the growth of *E. saburreum* in batch culture, with glucose as carbon substrate, is summarised in Table 33 and Table 34. A plot of the experimental data shows that, although the bacterium used some of the glucose for growth, glucose did not act as a growth-limiting substrate (Fig. 34). Growth proceeded exponentially for ± 8 h while the glucose concentration remained constant at 8 ± 0.11 mg/ml. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.143 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 35), and remained unaffected by the concentration of the glucose. Eventually, the growth rate slowed down and after ± 9 h the culture slowly went into a stationary phase without the loss of any of the glucose in the medium. A total growth of 0.095 mg cells/ml (dry mass) was obtained (Table 33).

Glucose did not act as a growth-limiting substrate for *E. saburreum*. Therefore the estimation of the growth parameter k_s , the yield factor Y and the maintenance coefficient m_s for glucose could not be calculated.

Table 33. Dry cell mass of *E. saburreum* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean ± sd (n=6) (mg/ml)
0	0.023	0.023	0.024	0.023 ± 0.001
1	0.023	0.023	0.023	0.023 ± 0.000
2	0.023	0.023	0.028	0.025 ± 0.003
3	0.024	0.024	0.035	0.028 ± 0.006
4	0.028	0.028	0.036	0.031 ± 0.005
5	0.035	0.034	0.040	0.036 ± 0.004
6	0.039	0.039	0.044	0.041 ± 0.003
7	0.051	0.050	0.046	0.049 ± 0.003
8	0.055	0.056	0.055	0.056 ± 0.001
9	0.067	0.067	0.055	0.063 ± 0.007
10	0.077	0.074	0.058	0.070 ± 0.010
11	0.082	0.082	0.070	0.078 ± 0.007
12	0.092	0.092	0.093	0.092 ± 0.001
13	0.093	0.092	0.097	0.094 ± 0.003
14	0.093	0.093	0.098	0.095 ± 0.003

Table 34. Glucose concentration in the growth medium of *E. saburreum* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	8.65	7.74	8.10	8.16 \pm 0.45
1	8.48	7.47	7.80	7.91 \pm 0.52
2	8.65	7.74	7.90	8.10 \pm 0.48
3	8.65	7.74	7.90	8.10 \pm 0.48
4	8.65	7.74	8.00	8.13 \pm 0.47
5	8.65	7.74	8.00	8.13 \pm 0.47
6	8.65	7.74	8.10	8.16 \pm 0.45
7	8.48	7.47	8.00	7.98 \pm 0.51
8	8.31	7.19	7.90	7.80 \pm 0.57
9	8.31	7.19	8.10	7.87 \pm 0.60
10	8.48	7.47	7.90	7.95 \pm 0.51
11	8.45	7.40	7.90	7.92 \pm 0.53
12	8.40	7.43	8.10	7.98 \pm 0.50
13	8.30	7.50	8.00	7.93 \pm 0.40
14	8.35	7.60	7.90	7.95 \pm 0.38

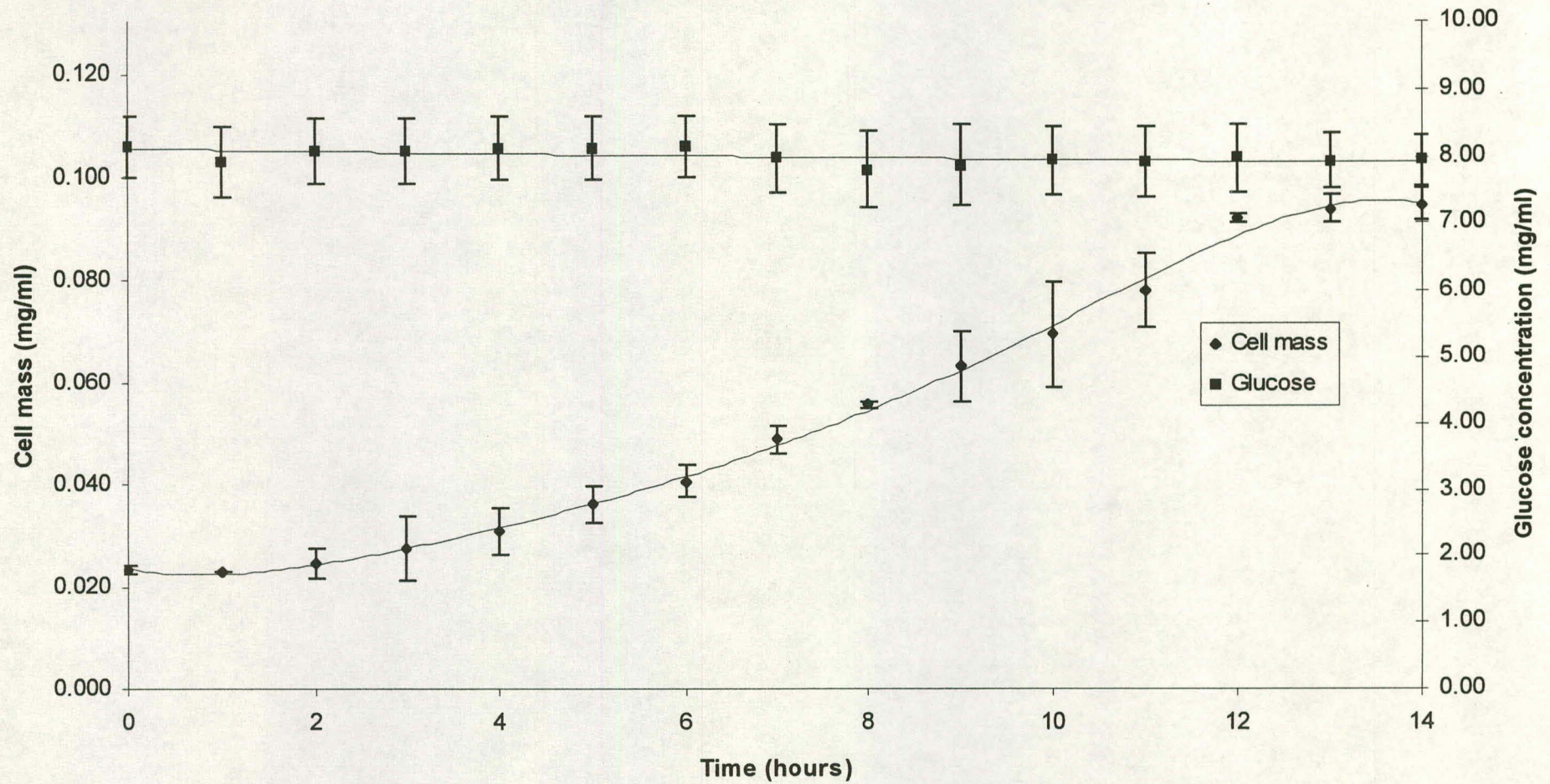


Fig. 34. Growth of *E. saburreum* and the utilisation of glucose.

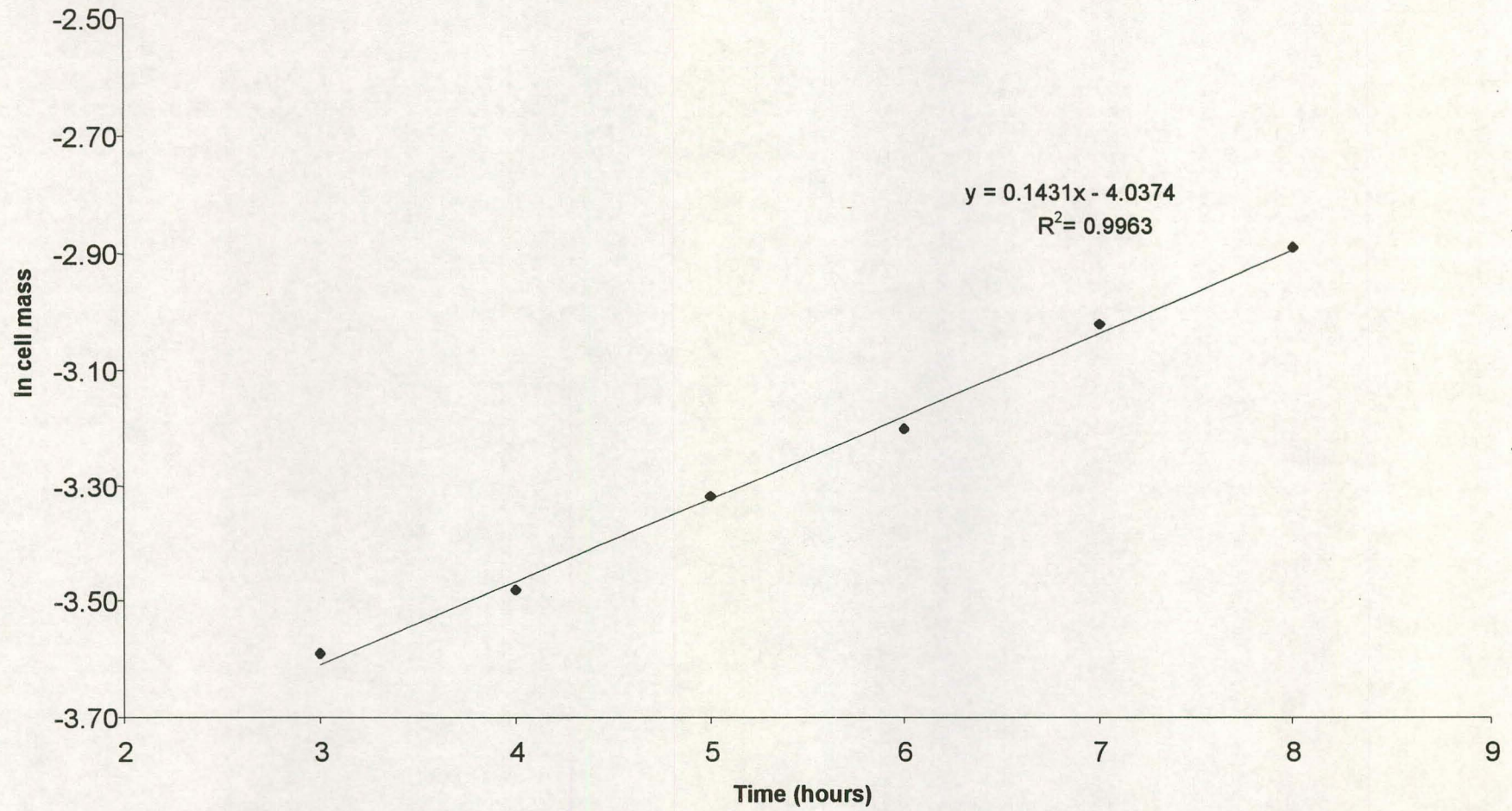


Fig. 35. Logarithmic curve of the exponential phase of the growth curve of *E. saburreum*.

Growth parameters of *V. parvula*

The experimental data obtained from the growth of *V. parvula* in batch culture and with glucose as carbon substrate is summarised in Table 35 and Table 36. A plot of the experimental data shows that, although the bacterium used some of the glucose for growth, glucose did not act as a growth-limiting substrate (Fig. 36). Growth proceeded exponentially for ± 4 h while the glucose concentration remained constant at 9.24 ± 0.07 mg/ml. During this stage the maximum specific growth rate of the bacterium was $\mu_{\max} = 0.355 \text{ h}^{-1}$, as determined by the slope of the logarithmic plot of the exponential phase of the growth curve (Fig. 37), and remained unaffected by the concentration of the glucose. Eventually, the growth rate slowed down and after ± 5 h the culture slowly went into a stationary phase without the loss of any of the glucose in the medium. A total growth of 0.059 ± 0.001 mg cells/ml (dry mass) was obtained (Table 33).

Glucose did not act as a growth-limiting substrate for *V. parvula*. Therefore the estimation of the growth parameter k_s , the yield factor Y and the maintenance coefficient m_s for glucose could not be calculated.

Table 35. Dry cell mass of *V. parvula* against time during growth in batch culture with glucose as carbon substrate.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	0.013	0.013	0.013	0.013 \pm 0.00
1	0.013	0.013	0.013	0.013 \pm 0.00
2	0.019	0.018	0.018	0.019 \pm 0.001
3	0.028	0.028	0.030	0.028 \pm 0.002
4	0.036	0.036	0.037	0.036 \pm 0.001
5	0.042	0.044	0.045	0.044 \pm 0.001
6	0.055	0.057	0.056	0.056 \pm 0.001
7	0.057	0.059	0.059	0.058 \pm 0.001
8	0.059	0.059	0.060	0.059 \pm 0.001

Table 36. Glucose concentration in the growth medium of *V. parvula* against time during growth of the organism in a batch culture.

Time (h)	Exp. 1 (n=2) (mg/ml)	Exp. 2 (n=2) (mg/ml)	Exp. 3 (n=2) (mg/ml)	Mean \pm sd (n=6) (mg/ml)
0	9.65	9.10	8.90	9.22 \pm 0.39
1	9.48	9.00	8.80	9.09 \pm 0.35
2	9.65	9.20	9.00	9.28 \pm 0.33
3	9.65	9.10	9.10	9.28 \pm 0.31
4	9.65	9.50	8.90	9.35 \pm 0.40
5	9.65	9.10	8.80	9.18 \pm 0.43
6	9.65	9.30	8.90	9.28 \pm 0.37
7	9.48	9.20	9.00	9.23 \pm 0.24
8	9.70	9.00	9.10	9.27 \pm 0.38

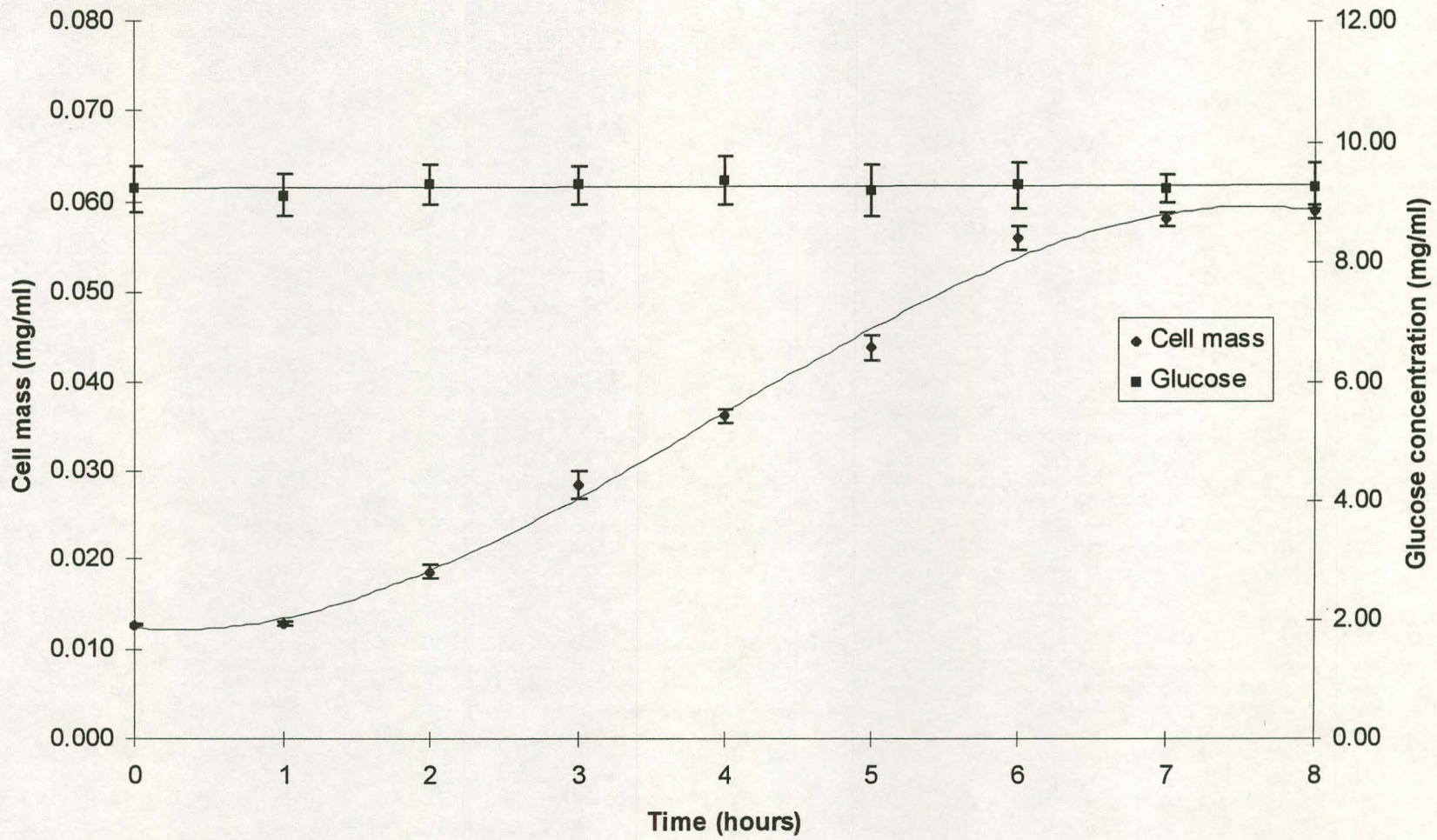


Fig. 36. Growth of *V. parvula* and the utilisation of glucose.

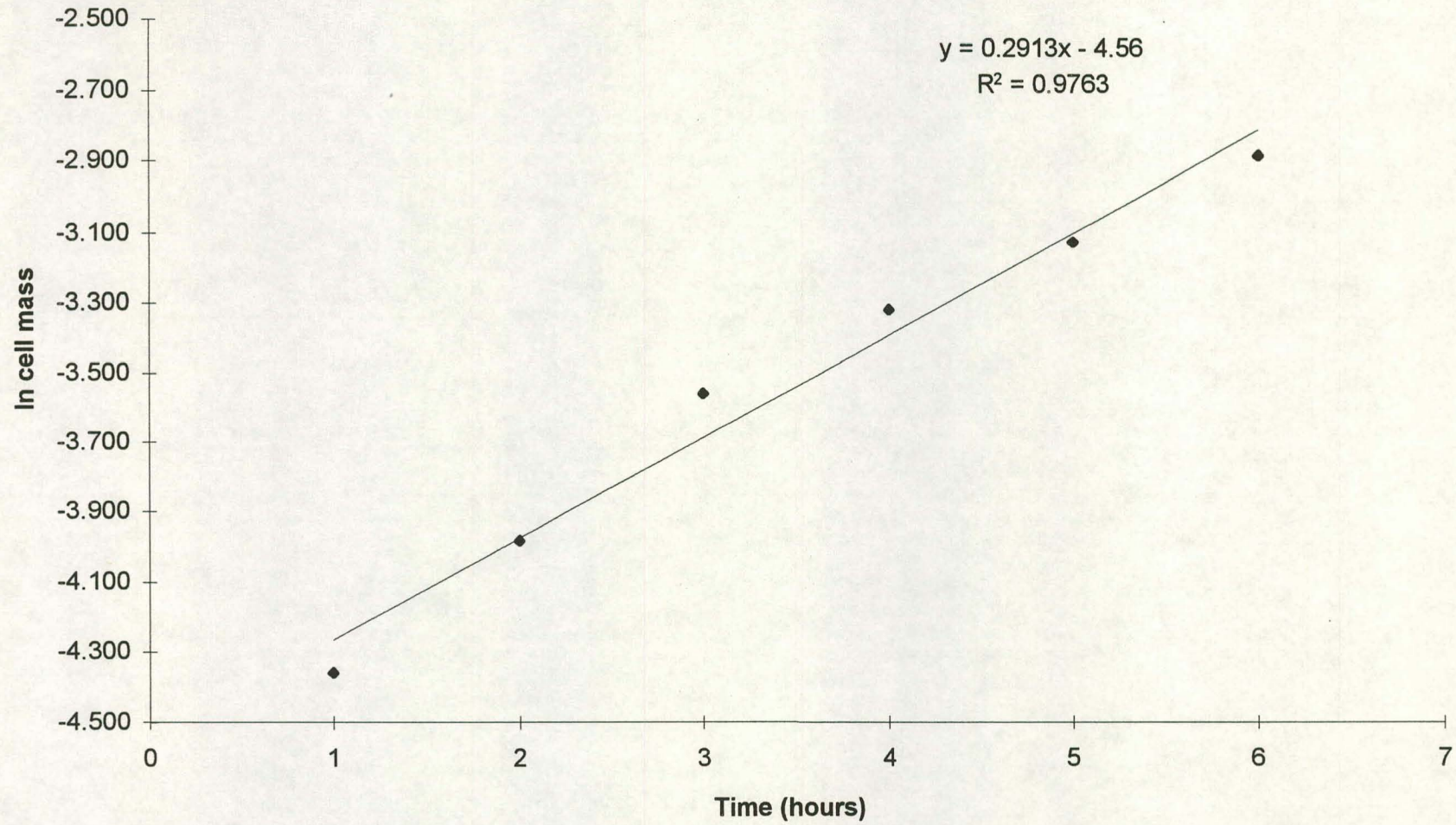


Fig. 37. Logarithmic curve of the exponential phase of the growth curve of *V. parvula*.

**Summary of the growth parameters of *C. albicans*, *S. sanguis*,
L. casei, *S. mitis*, *S. sobrinus*, *F. nucleatum* *E. saburreum*, and
V. parvula.**

The maximum specific growth rates of the organisms ranged from 0.143 to 0.610. The growth rates of *S. sobrinus* and *S. sanguis* stood out above the rest of the bacteria with *S. sobrinus* having the highest growth rate followed by *S. sanguis* (Table 37). The organisms *V. parvula*, *L. casei*, *S. mitis* and *C. albicans* formed an intermediate group with growth rates about half of that of *S. sanguis* and *S. sobrinus*. The anaerobic rod-shaped bacteria *F. nucleatum* and *E. saburreum* had the lowest growth rate. The organisms *S. sobrinus* and *L. casei* had higher substrate affinities (K_s) than the species *S. sanguis*, *S. mitis* and *C. albicans*. *S. sanguis* had the highest growth yield followed by *C. albicans* and *S. sobrinus*. The latter had a much higher maintenance coefficient than the rest of the organisms. All the anaerobic bacteria were asaccharolytic species with the result that their K_s , Y and m_s could not be determined.

Table 37. The growth parameters of the different organisms grown in batch culture under growth conditions similar to that of the chemostat culture with glucose as carbon substrate.

Organism	μ_{\max} (h ⁻¹)	K_s (mg glucose/ml)	Y (mg cells/mg glucose)	m_s (mg glucose/mg cells/h)
<i>S. sobrinus</i>	0.610	6.49	0.128	0.232
<i>S. sanguis</i>	0.514	3.40	0.193	0.085
<i>V. parvula</i>	0.355	-	-	-
<i>L. casei</i>	0.303	6.77	0.099	0.098
<i>S. mitis</i>	0.232	3.73	0.062	0.090
<i>C. albicans</i>	0.197	3.30	0.141	0.070
<i>F. nucleatum</i>	0.153	-	-	-
<i>E. saburreum</i>	0.143	-	-	-

- values could not be determined

Discussion

As far as could be determined, no other interactions, except for the competition for glucose, occurred between the yeast and the different organisms in the chemostat steady state cultures. According to the principle of growth-limiting substrate competition, the outcome of competition for a growth-limiting substrate depends on growth characteristics such as a measure of maximum specific growth rate (μ_{\max}), substrate affinity (k_s), and to a lesser extent, upon the growth yields (Y) and maintenance energy (m_s) of cells on the limiting substrate (Veldkamp, 1977, 1972). It was therefore pertinent to determine the relevant growth parameters of the organisms for glucose in order to understand the outcome of competition of the species.

Theoretically the direct estimation of k_s values are most easily determined from continuous culture experiments as a single measurement of the steady-state substrate concentration at a fixed dilution rate allows k_s to be calculated from the Monod equation (equation 4). This is probably the most accurate method for determining k_s , provided the analytical method for determining the glucose concentration is sufficiently sensitive (Herbert *et al*, 1956). However, it has been found that the residual glucose concentrations were usually too low to be measured with sufficient accuracy (Rogers *et al*, 1986; Rogers *et al*, 1987; van der Hoeven *et al*, 1985; Bowden and Hamilton, 1989). Therefore it was decided to determine the parameters through mathematical modelling of data points obtained from batch culture experiments and the transformation of the μ vs s plots into linear relationships (Sinclair and Cantero, 1990). However, there is a disadvantage in using this type of plot. Because reciprocal values for μ and s are plotted, the points on the line tend to become bunched together nearer to the origin and progressively wider apart on the parts of the line further from the origin. Because linear

regression assumes that the scatter of points around the line follows a Gaussian distribution and that the standard deviation is the same at every value of s , which is not the case with the transformed linear regression, the result of the linear regression may be inaccurate. This may influence the slope and the intercept ($1/\mu_{max}$) of the line and therefore the accuracy of the values of the variables. To overcome this problem the μ_{max} values were also estimated by the usual method in which cell populations are measured during the exponential growth phase in batch culture. These values were used to confirm the intercept of the Lineweaver-Burke plots and to make corrections to the slope where necessary.

The growth parameters obtained could not be compared to those from previous studies because the growth parameters are influenced by the growth conditions such as medium composition, pH, Eh, gas phase, etc. under which the organisms are grown. Furthermore, studies on the growth parameters of oral bacteria are limited and are mainly directed towards plaque bacteria responsible for caries (Rogers *et al*, 1986). The only relevant parameters that could be found were the μ_{max} values of *L. casei* (0.50), *S. sanguis* (0.95) and *C. albicans* (0.70), all under different growth conditions than those of the present study (Bowden and Hamilton, 1989; van der Hoeven *et al*, 1985; Webster and Odds, 1986).

Theoretical considerations and experimental studies by Gottschall and Thingstad (1982) have indicated that the growth yield may determine the proportions of organisms in mixed cultures under multiple-substrate limitation. The growth yields of *S. sobrinus*, *S. sanguis* and *C. albicans* were strikingly higher than that of *L. casei* and *S. mitis* (Table 37). It is noteworthy that these organisms also dominated the steady-state cultures. In the glucose-limited cultures, *S. sanguis*, with the highest yield factor for glucose, dominated the culture with the highest cfu/ml (Table 8). In the glucose-excess cultures, *S. sobrinus* (*S. sanguis* was absent) dominated the culture. However, the growth yield probably only becomes important in natural environments when the growth limitation becomes absolute and no growth is possible

anymore. Under such conditions the population with the highest initial population size would have the best chances of survival.

The maintenance energy represents the collective energy demands for processes that do not produce a net increase in biomass. The significance of the maintenance demands for the competitiveness of a micro-organism is probably expressed in its effect on the growth yield (Rogers *et al*, 1986). The growth yield and the maintenance energy are two interrelated parameters and variations in the yield, when the limiting nutrient is the energy source, are due to energy requirements for maintenance (van Uden, 1969). Except for *S. sobrinus*, the difference in the maintenance rates of the organisms in this study was relatively small and of the same order of magnitude. Therefore, it was unlikely to have had any significant effect on the competition between the organisms under the steady state conditions.

The two most important parameters that determine the outcome of competition for a single growth-limiting substrate between organisms are probably the maximum specific growth rate and the saturation constant (Veldkamp and Jannasch, 1972). If the values of these growth parameters are known, the relationship between the specific growth rate and the substrate concentration can be represented by the Monod equation (Tempest, 1969). A plot of this relationship (the so called saturation curve) can be used to predict the behaviour of two organisms that are competing for the same growth-limiting substrate (see Fig. 4a and b) when no other interactions between the organisms occur (Tempest, 1969; Veldkamp and Jannasch, 1972).

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Chapter 9

THEORETICAL SATURATION CURVES FOR *C. ALBICANS*, *S. SANGUIS*, *L. CASEI*, *S. MITIS*, *S. SOBRINUS*.

Materials and Methods

To order the mass of data obtained from the estimation of the growth parameters, the saturation constant (k_s) and the maximum specific growth rate (μ_{\max}) were used to calculate the theoretical saturation curves for the different organisms. The theoretical saturation curves were used to predict the outcome of competition between the saccharolytic organisms used in this study. The generally accepted expression for cell growth with a single growth-limiting substrate,

$$\mu = \mu_m \left(\frac{S}{k_s + S} \right) \text{ (Herbert, Elsworth and Telling, 1956)}$$

was used to calculate the growth rates at different glucose concentrations in order to plot the theoretical saturation curves for the different microbial species.

Results

The theoretical growth rates calculated for the different organisms at glucose concentrations ranging from 0 – 20 mg/ml glucose are summarised in Table 38. The theoretical saturation curves of the organisms plotted from these values (Fig. 38) show that *S. sobrinus* will out-compete all other organisms at glucose concentrations higher than 13 mg/ml. However, for glucose concentrations lower than 13 mg/ml *S. sanguis* will outgrow all the organisms including *S. sobrinus*. For glucose concentrations between 0 and 8 mg/ml (Fig. 39) the outcome of competition between *L. casei*, *C. albicans* and *S. sanguis* will depend on the glucose concentration. For glucose concentrations lower than 6 mg/ml, *S. mitis* will grow faster than both *L. casei* and *C. albicans*. For concentrations higher than 6 mg/ml *L. casei* will outgrow both *S. mitis* and *C. albicans*. For concentrations between 3 mg/ml and 6 mg/ml *L. casei* will outgrow *C. albicans*. However, below 3 mg/ml the yeast will grow faster than *L. casei*. At glucose concentrations ranging from 0 – 0.01 mg/ml, the glucose concentration found in human saliva, *S. sanguis*, *S. sobrinus* and *S. mitis* would grow faster than *C. albicans* while *L. casei* would grow slower than the yeast (Fig. 40).

These curves indicate that at the dilution rate ($D = 0.05$) used in the chemostat studies, *C. albicans* had to compete only with *S. sanguis*, *S. sobrinus* and *S. mitis*.

The three asaccharolytic species *F. nucleatum*, *E. saburreum* and *V. parvula* did not use glucose as a growth-limiting substrate and theoretical saturation curves could therefore not be constructed for these organisms.

Table 38. The theoretical specific growth rates of the saccharolytic microbial species at different glucose concentrations.

Glucose concentration (mg/ml)	The specific growth rate μ (h ⁻¹) of				
	<i>L. casei</i>	<i>C. albicans</i>	<i>S. sanguis</i>	<i>S. mitis</i>	<i>S. sobrinus</i>
0	0	0	0	0	0
1	0.039	0.046	0.117	0.049	0.081
2	0.069	0.074	0.190	0.081	0.144
3	0.093	0.094	0.241	0.103	0.193
4	0.113	0.108	0.278	0.120	0.233
5	0.129	0.119	0.306	0.133	0.265
6	0.142	0.127	0.328	0.143	0.293
7	0.154	0.134	0.346	0.151	0.317
8	0.164	0.139	0.361	0.158	0.337
9	0.173	0.144	0.373	0.164	0.354
10	0.181	0.148	0.384	0.169	0.370
11	0.188	0.152	0.393	0.173	0.384
12	0.194	0.155	0.401	0.177	0.396
13	0.199	0.157	0.407	0.180	0.407
14	0.204	0.159	0.414	0.183	0.417
15	0.209	0.161	0.419	0.186	0.426
16	0.213	0.163	0.424	0.188	0.434
17	0.217	0.165	0.428	0.190	0.441
18	0.220	0.166	0.432	0.192	0.448
19	0.223	0.168	0.436	0.194	0.455
20	0.226	0.169	0.439	0.196	0.461

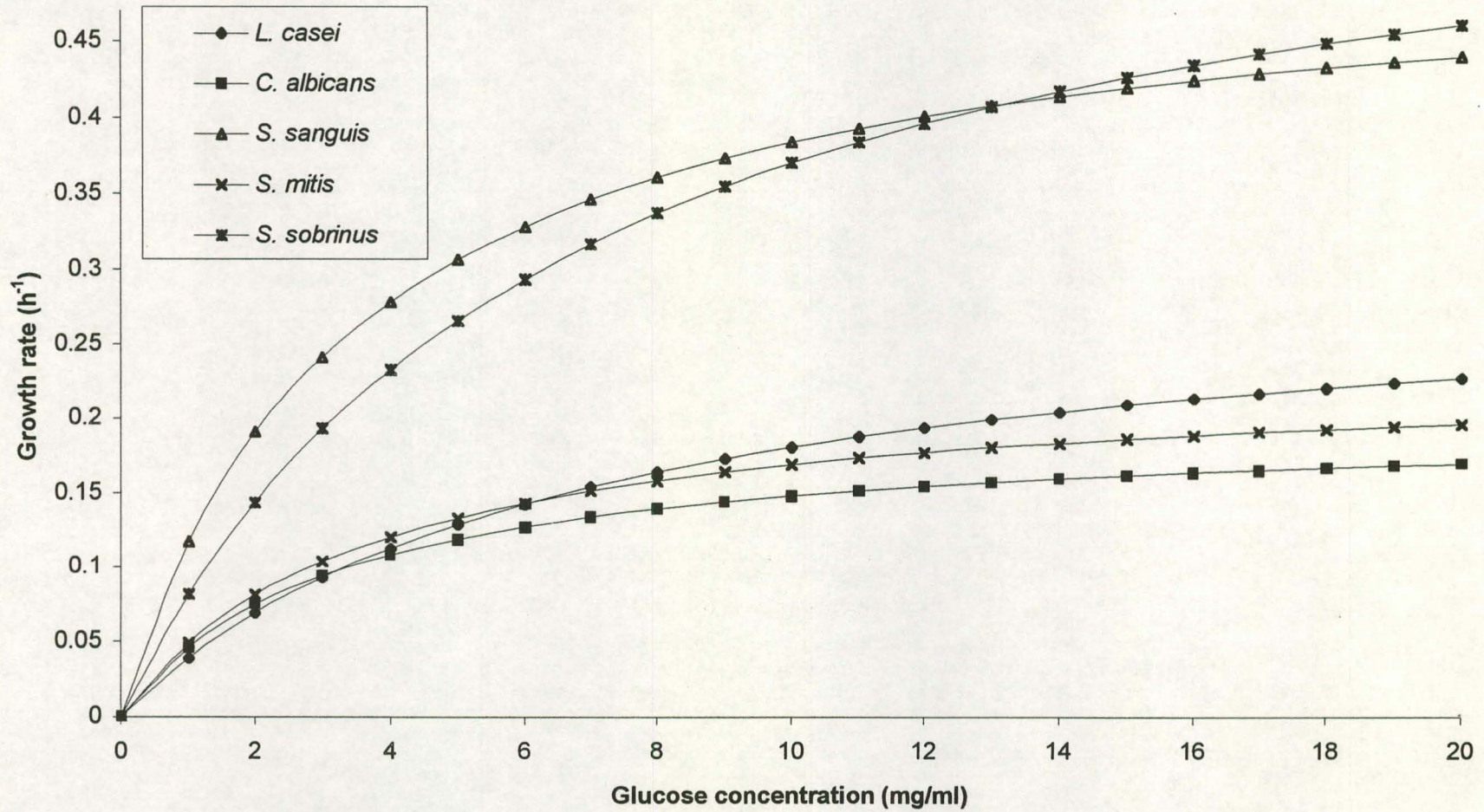


Fig. 38. Theoretical saturation curves of the oral bacteria and the yeast for glucose concentrations between 0 and 15 mg/ml .

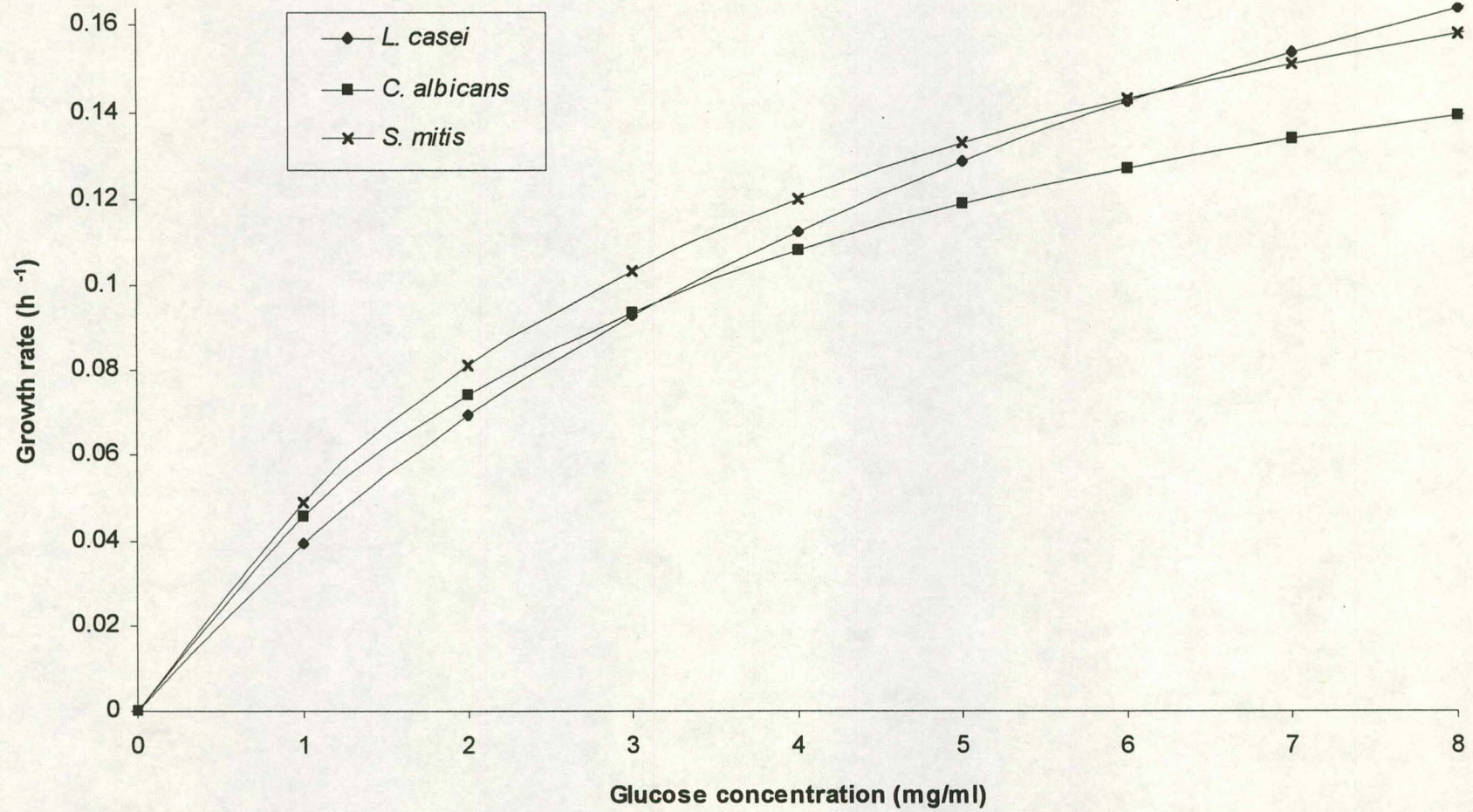


Fig. 39. Theoretical saturation curves of *L. casei*, *C. albicans* and *S. mitis* for glucose concentrations between 0 and 8 mg/ml.

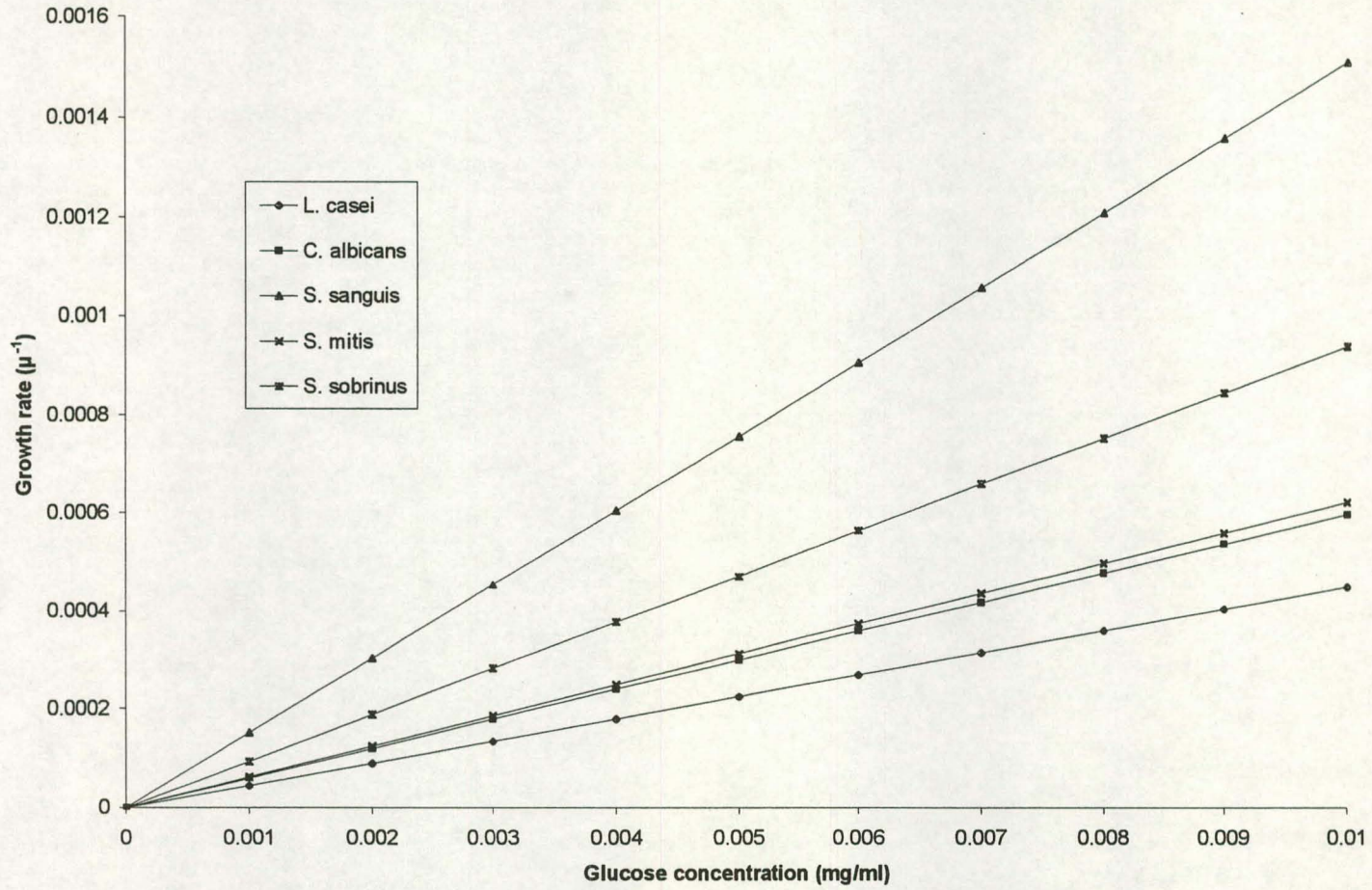


Fig. 40. Theoretical saturation curves of the oral bacteria and the yeast for glucose concentrations between 0 and 0.01 mg/ml.

Discussion

The dilution rate of the chemostat, and therefore the specific growth rate of the steady state culture, was fixed at a rate of $D = 0.05 \text{ h}^{-1}$. According to the theoretical saturation curves, if glucose was the only growth-limiting substrate in the steady-state culture, *L. casei* would be the first organism to be lost from the culture followed by *C. albicans*, *S. mitis* and *S. sobrinus* while *S. sanguis* would eventually outgrow all the organisms (Fig. 38). This, and the fact that *S. sanguis*, *S. sobrinus*, *S. mitis* and *L. casei* were all able to grow in the glucose-limited chemostat (see Chapter 7), is further proof that they did not compete for glucose. However, the yeast was unable to grow in the glucose-limited culture. From the theoretical saturation curves it can be seen that the yeast would not be able to compete against the organisms *S. sanguis*, *S. sobrinus* and *S. mitis* at the dilution rate (or glucose concentration) of the culture. This is a further proof of the role of competition for glucose between the yeast and the three bacteria *S. sanguis*, *S. sobrinus* and *S. mitis*. The yeast would however be able to outgrow *L. casei* which would have had a lower growth rate under these conditions. Under conditions of glucose-excess, the bacterial competition as a result of the faster growth rates would have been eliminated and enough glucose would have been available for the growth of the yeast.

Several of the predisposing factors to oral candidosis suggest that abnormal glucose concentrations in the oral cavity (and mainly in the saliva) may cause a higher oral carriage of the yeast, its overgrowth, or clinical candidosis (Odds, 1988; Samaranayake and Lamey, 1989). For example, candidosis occurs more often when there is a high availability of glucose in the saliva, as in persons with diabetes and in patients receiving total parenteral nutrition (Bodey, 1984; Cormane and Goslings, 1963). Diabetic patients who carry *C. albicans* intra-orally had significantly higher salivary glucose concentrations than those in whom the yeast

could not be isolated (Darwazeh *et al*, 1991). Samaranayake *et al* (1986) also indicated that a higher intake of carbohydrate has been implicated in oral candidosis. Sucrose rinses initiated *Candida* stomatitis in human subjects, whereas carbohydrate-rich diets also favour the oral carriage of *C. albicans* in rats and monkeys (Hassan, 1985; Olsen, 1976).

The micro-organisms growing on the surface of the tongue (the primary habitat of *C. albicans*) are continuously bathed in saliva, which supports the growth of the major oral micro-organisms. Saliva may also act as a selective force by providing different substrates to different oral micro-organisms (De Jong *et al*, 1984; De Jong *et al*, 1986; De Jong and Van der Hoeven, 1987). It has been found that the yeast will not grow in saliva in the presence of the oral flora unless excess glucose is available through supplementation, or the oral bacteria are suppressed with antibiotics (Knight and Fletcher, 1971). This observation, and those obtained from other studies (see Cormane and Goslings, 1963; Paine, 1958; Rosebury *et al*, 1954; Kennedy, *et al*, 1988; Odds, 1988), suggest that competition for glucose amongst the oral flora and the yeast may play an important role in affording colonisation resistance. This may be an important homeostatic mechanism that controls the yeast in the oral cavity. The results of the present study also strongly suggest that certain bacteria and specifically members of the oral streptococci, will successfully compete and outgrow the yeast when a limited amount of glucose is available. It is interesting that Sprunt and Redman (1968) and Sprunt, Leidy and Redman (1971) observed that *in vivo* “viridans streptococci” played a major role in the prevention of bacterial overgrowth of organisms not commonly found in significant proportions in the oropharynx. They suggested that the streptococci are “key inhibitors” in the normal flora.

As was mentioned above, when organisms are competing for the same growth-limiting substrate and no other interactions between these organisms occur, their behaviour can be predicted from their known relationships between substrate concentration and growth rate, as

illustrated in their theoretical saturation curves (Veldkamp and Jannasch, 1972). The glucose concentration of human saliva ranges from 0 - 0.01 mg/ml with 85% of persons being in the range 0 - 0.004 mg/ml (Thorstensson *et al*, 1989). Under an idealistic situation where the dilution rate in the oral cavity is constant and lower than that of the micro-organisms, the relationships as given in Fig. 40 would predict that *S. sanguis*, *S. sobrinus* and *S. mitis* would all outgrow *C. albicans* at the glucose concentrations in saliva. It can also be seen from the theoretical saturation curves that an increase in glucose concentration, within the limits of that found in saliva, would have no effect on the competition between these organisms except for an increased cell mass of the faster growing organism, e.g. *S. sanguis*. This could explain why different workers could not find significant differences in frequency and quantity of oral *Candida* in diabetic and control groups although significantly higher salivary glucose concentrations occurred in the diabetic groups compared to the control groups (Thorstensson *et al*, 1989; Darwazeh *et al*, 1991).

There are however a multitude of organisms that could have different saturation curves and that could react differently. Nevertheless, if the streptococci identified in this study were present, they would always outgrow *C. albicans* under the conditions of this investigation. Only with glucose concentrations maintained above 3 mg/ml would a shift in the dominant organism occur (see Fig. 39). A glucose concentration of 3 mg/ml would not easily be attained in the oral cavity (glucose concentration of human saliva range from 0-0.01 mg/ml). This strongly suggests that the overgrowth of *C. albicans* in the oral cavity of persons with elevated glucose concentrations in their saliva, such as diabetic patients, must be due to other mechanisms. These would include the elimination of certain glucose-utilising bacteria, making this sugar more available to the yeast.

The role of antibiotics in suppressing the normal flora and the resulting overgrowth of *C. albicans* has been suggested by various authors (Zimmerman, 1950; Woods *et al*, 1951;

Seelig, 1966; Odds, 1988) and has also been shown to occur *in vivo* in human and animal studies (Walker *et al*, 1979; Fitzgerald and Topley, 1966; and *in vitro* (Paine, 1952; Paine, 1958; Knight and Fletcher, 1971). The elimination of bacterial competition is almost certainly the important mechanism by which antibiotics affect *Candida* numbers *in vivo*. Therefore it would be possible to change the outcome of competition in favour of *C. albicans* by eliminating the faster growing organisms such as *S. sanguis*, *S. sobrinus* and *S. mitis* with antibiotics. Selection of antibiotics that would ideally not affect these species could possibly prevent the overgrowth of the yeast as a result of antibiotic treatment.

The competition for a nitrogen source amongst the streptococci could also influence the outcome of competition between the bacteria and the yeast. Results from previous studies suggest that amino acids could be a growth-limiting substrate for some oral bacterial populations, specifically oral streptococci (Rogers, 1987; McKee, 1985). The present study also suggests that some species (*S. sanguis* and *S. mitis*) may be eliminated from a mixed microbial habitat under glucose-excess conditions that may cause amino acid limitation. It is therefore possible that a competition for amino acids in the oral cavity can eliminate or reduce the numbers of organisms such as *S. sanguis*, *S. mitis* and *S. sobrinus*, resulting in a loss of competition for glucose between these organisms and the yeast.

The pH of the environment is another parameter that could influence the outcome of competition between the bacteria and the yeast. In patients with xerostomia the acid tolerating species such as *S. mutans*, *L. casei* and *C. albicans* increase. These population shifts have been attributed to a reduction in the buffering capacity of saliva (Marsh, 1989). It has also been found that *S. sanguis* will outgrow *S. mitior* (*S. mitis*), *S. sobrinus* and *L. casei* at a pH value of 7 and under glucose-limiting conditions because of the greater affinity for glucose of the former organism. However, at a pH of 4.1 the latter two species, both aciduric, became predominant (McDermid *et al*, 1986). Other studies have shown that a low pH may cause the

breakdown of microbial homeostasis and lead to the predominance of aciduric species at the expense of acid-sensitive populations such as *S. sanguis* (Svanberg, 1980; Bowden and Hamilton, 1987). Since *C. albicans* is an aciduric organism, a lower pH may favour the growth of the yeast in a similar way by influencing the maximum specific growth rate and substrate affinity of the organism. However, the pH of most surfaces of the mouth is regulated by saliva (dental plaque is an exception to the rule because the pH of plaque can reach values below 5 as a result of plaque metabolism) (Marsh, 1992). The mean pH of unstimulated whole saliva is in the range 6.75-7.25 so that, in general, optimum pH values for microbial growth will be provided at sites bathed by saliva such as the posterior dorsal surface of the tongue, the primary habitat for *C. albicans* in the oral cavity (Marsh, 1992).

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Chapter 10

CONCLUSIONS

Although the chemostat can provide some insight into the significance of microbial competition, which controls ecosystems, it has limitations. For example, the cells in the chemostat are in suspension, whereas in the oral cavity the cells are attached to surfaces and occur as microbial films (biofilms). *In vivo*, cells may cease to metabolise under glucose-limiting conditions, but remain in their habitat, physically protected by other cells in the community. In the chemostat, cells that cannot compete in the environment, and are not able to adhere to a surface, are washed out of the culture. Although this aspect of chemostat cultures is useful, since it allows those parameters which adversely affect a given species to be readily identified on the basis of changes in the population density and will provide valuable data on the response of species to environmental changes, it cannot be used to simulate the oral cavity and the results cannot be directly extrapolated to the oral cavity. However, a number of new and important results were obtained from the present study.

A model was established to investigate the competition for glucose between the oral bacteria and the yeast under controlled *in vitro* conditions similar to that found in the oral cavity. This model is ideal to produce a stable and reproducible mixed community of oral bacteria, that can be used as an environmentally related model, with which the growth relationships between different oral micro-organisms can be investigated. At the same time, the large number of environmental parameters that influence these relationships can be controlled.

The competition for glucose between the bacteria and the yeast was identified as the cause of colonisation resistance against the yeast in a mixed microbial community of oral bacteria. Under the particular environmental conditions, the availability of glucose acted as a growth-limiting substrate for the growth of *C. albicans* and under glucose-limited conditions the yeast had to compete with certain oral bacteria for the available glucose.

The theoretical saturation curves obtained from the study identified the three species *S. sanguis*, *S. mitis* and *S. sobrinus* as the organisms able to afford such a colonisation resistance.

The outcome of a competition between the yeast and these bacteria will depend on the growth parameters, specifically the maximum specific growth rates and saturation constants, of the bacteria present. According to the theoretical saturation curves, the organisms, *S. sanguis*, *S. mitis* and *S. sobrinus* would outgrow the yeast at glucose concentrations ranging from 0 to 20 mg/ml. This suggests that, at the glucose concentrations found in the oral cavity (0-0.01 mg/ml), any of these streptococci would be able to suppress the growth of *C. albicans* as a result of a competition for glucose. Furthermore, an increase in glucose concentration alone would be of little significance in the outcome of competition between these organisms and the yeast. Only when these organisms are eliminated from the community will the yeast be able to grow. It is important to note that the results of this study indicate that the elimination of the competitive species (such as during antibiotic therapy) is probably much more significant in the outcome of a competition for glucose, than a pure increase in glucose concentration within the constraints of the glucose concentrations normally experienced in saliva and the oral cavity.

It is clear that the above-mentioned parameters can be influenced by factors such as pH and redox potentials (McDermid *et al*, 1986; Webster and Odds, 1986) and the effect of these on

the outcome of a competition between these organisms needs to be investigated in future studies. Furthermore, the results obtained have implicated the oral streptococci as major competitors of the yeast and the remaining oral streptococcus species need to be more thoroughly investigated to determine their involvement in the competition for glucose.

References

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