Disaccharide fermentation by yeasts
Disaccharide fermentation by yeasts

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. ir. K. F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op donderdag 9 juni 1994 te 16.00 uur

doors

Ruud Alexander WEUSTHUIS

doctorandus biologie

geboren te Oldenzaal
Dit proefschrift is goedgekeurd door de promotor
Prof. dr. J. G. Kuenen

Dr. J. P. van Dijken heeft als begeleider in belangrijke mate aan
het totstandkomen van het proefschrift bijgedragen
Van 'n kalaf kö'j nog 'n moal 'n koo verwoch'n.
Mär 'n ezzel blif 'n ezzel.

Voor Juut
Voorwoord

"Rood en groen is boeren fatsoen" luidt het gezegde en zou van toepassing kunnen zijn op de omslag van dit proefschrift. Ik noem dit om mijn enigszins imperialistisch ingestelde Hollandse collegae voor te zijn die zich nog dagelijks amuseren met mijn Twentse accent en woordkeuze. Collegae, speciaal voor jullie heb ik een Twentse spreuk op de vorige bladzijde gezet. Ik ken ook nog een Hollandse: "Wie de schoen past trekke hem aan".


Hans, alleen cliche's voldoen in deze: bedankt voor je grenzeloze inzet en belangstelling, de heerlijke manier waarop je mensen begeleidt, en je vertrouwen ondanks het "eigenaardige" begin. Ik bewonder je. Gijs, bedankt. Ook je hulp op het persoonlijke vlak wordt zeer door Judith en mij gewaardeerd. Verder moet ik bewondering uiten voor alle mede-AIO's, medewerkers, studenten en stagiaires die zich nooit iets van mijn stekelige opmerkingen aantrokken, voor het keuken medium-preparatie-en-sterilisatie personeel die mij nooit in een labjas hebben kunnen krijgen en de mannen van de werkplaats die alles wat ik kan bedenken kunnen maken.

Juut, fantastisch dat je met me bent mee gegaan naar dit helse oord. Juut, dok, met dit boekje kan ik je weer inhalen in de titelstrijd. Dan kan ik straks weer eens de baas spelen.
# Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Sugar transport in yeasts and its study with chemostat cultures</td>
<td>25</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Maltose/proton co-transport in <em>Saccharomyces cerevisiae</em>: comparative study with cells and plasma membrane vesicles</td>
<td>39</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Energetics and kinetics of maltose transport in <em>Saccharomyces cerevisiae</em>: a continuous culture study</td>
<td>47</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Effect of oxygen limitation on sugar metabolism in yeasts: a continuous culture study of the Kluvyer effect</td>
<td>57</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Is the Kluvyer effect caused by product inhibition?</td>
<td>71</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Future prospects</td>
<td>79</td>
</tr>
</tbody>
</table>

Summary  81

Samenvatting  83

List of publications  85
General Introduction

Introduction

The yeast collection of the Centraalbureau voor Schimmelcultures (CBS, Delft, The Netherlands) harbours all (over 4500) natural yeast isolates described in the literature. These belong to 590 species that have been grouped into 83 genera (Barnett et al., 1990). Due to its 'classical' industrial applications in the leavening of dough and in beer and wine fermentation, *Saccharomyces cerevisiae* is the best-known representative of this group of microbes. In fact, 'yeast' and *S. cerevisiae* are frequently used as synonymous terms. However, *Saccharomyces* yeasts are rather exceptional since they are among the few yeasts that are able to grow anaerobically (Visser et al., 1990). Fermentative yeasts of other genera like *Kluveromyces* and *Candida* require considerable amounts of oxygen for growth. This decreases the ethanol productivity and imposes a need for stringent control of the oxygen supply in processes aiming at alcohol production with these yeasts. Furthermore, *Saccharomyces* yeasts generally have a much higher ethanol tolerance. These differences make representatives of the genus *Saccharomyces* favourable for fermentative production of ethanol.

In the past decade, yeasts other than *S. cerevisiae* have gained considerable industrial interest as hosts for the expression of heterologous genes. Examples are methanol-utilizing yeasts such as *Hansenula polymorpha* and *Pichia pastoris* and the lactose-utilizing species *Kluveromyces lactis* and *Kluveromyces marxianus* (Romanos et al., 1992). Several arguments have been put forward to use 'non-*Saccharomyces* yeasts' as hosts for heterologous gene expression, including broader substrate specificity, availability of strong inducible promoters, and absence of aerobic alcoholic fermentation. A major factor, decisive for the use of alternative yeasts in commercial processes, is correct processing of the heterologous gene product, for example with respect to glycosylation and excretion. In various cases *S. cerevisiae* strains exhibit unwanted traits in this respect. It is therefore expected that application of non-*Saccharomyces* yeasts in large-scale fermentations for the production of heterologous proteins will be common industrial practice before the end of this century (Newmark, 1989). Nevertheless, it remains to be seen whether all of the presumed advantages of non-*Saccharomyces* yeasts will still apply in large scale fermentations.

Studies on the regulation of respiration and alcoholic fermentation in yeasts have generally been performed with glucose as the fermentable sugar. It is, however, well known that the type of sugar exerts a strong influence on the kinetics and energetics of alcoholic fermentation by yeasts. This is well documented for the fermentation of xylose (Toivola et al., 1984) and disaccharides (Kluver & Custers, 1940; Barnett, 1992).

In this chapter, a short overview will be given of those aspects of the kinetics of growth and ethanol production by yeasts that are relevant for biotechnological applications. Special attention will be paid to the effects of disaccharides on the physiology of yeasts.

Yeasts in industry

**Industrial ethanol production**

Bio-ethanol is industrially produced for beverages, for vinegar production and for use as fuel alcohol. Fermentative production of ethanol involves batch reactors, since batch processes are relatively simple to operate and have no stringent requirements for assepsis. Since the biosynthesis of unsaturated fatty acids and sterols in yeasts requires molecular oxygen (Andreasen & Stier, 1953; 1954), trace amounts of oxygen are supplied during the process.

Batch-wise production of ethanol with yeasts is a well-established industrial process. Other options such as the use of bacteria of the genus *Zymomonas* or continuous processes with immobilized cells have been studied extensively (Kosaric, 1982), but so far these have not yet found wide application on an industrial scale.

**Industrial biomass production**

Yeast biomass is produced for leavening of dough and for manufacturing of biomass constituents such
Table 1. Sugar composition of molasses as a percentage of total solids (Reed & Nagodawithana, 1991).

<table>
<thead>
<tr>
<th>Component</th>
<th>Cane molasses</th>
<th>Beet molasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>45.5</td>
<td>63.5</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>Invert sugar</td>
<td>22.1</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>5.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 2. Sugar composition of wort (Renger, 1991)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>14.2</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.6</td>
</tr>
<tr>
<td>Maltose</td>
<td>53.2</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>13.1</td>
</tr>
</tbody>
</table>

as vitamins and enzymes. Large-scale industrial yeast production involves fed-batch cultivation in which high cell densities (over 50 g dry weight⁻¹) are reached. Aerobic, sugar-limited cultivation conditions are employed in order to achieve optimal biomass yields (with respect to carbon substrate and oxygen), and minimal byproduct formation. However, in large reactors, due to imperfect mixing, gradients in substrate and oxygen are difficult to avoid. As a result, alcoholic fermentation is triggered in S. cerevisiae. Hence, detailed knowledge of transient-state responses of this type of yeast is a necessity for their application in large-scale processes, for example with respect to production of heterologous proteins. Byproduct formation as a result of sugar excess is generally less strong with non-Saccharomyces yeasts. However, also with these yeasts formation of, for example, acetic acid may occur when cultivation conditions are not carefully controlled. Large-scale yeast cultivation therefore requires the concerted action of microbial physiologists and bioprocess technologists.

Industrial feedstocks

Nearly always, cheap complex media are used in industry (Greasham, 1993). For bakers' yeast production molasses is commonly used as feedstock (Chen & Chiger, 1985; Hacking, 1986). Ethanol is produced from sugar crops (sugar cane, sugar beets, fodder beets, fruit), industrial wastes (spent sulphite liquor, whey), starch (corn, cassava, sweet potato, sweet sorghum, Jerusalem artichoke) and cellulotic materials (Kosaric et al., 1982). The major sugar constituent of such complex media is usually not glucose but an oligosaccharide. For example, beet and cane molasses contain sucrose (table 1). The sugar composition of starch hydrolysates depends on the enzyme(s) and conditions used for hydrolysis. They may, apart from glucose, contain high concentrations of maltose and maltotriose (table 2). Lactose is the major sugar in whey, hydrolysis of cellulotic materials results in the formation of glucose and xylose and of the disaccharide cellobiose (Kosaric et al., 1982). It is evident, therefore, that studies on glucose metabolism by yeasts are insufficient for an understanding of their behaviour during cultivation in industrial media.

Sugar metabolism and energy generation

The biochemistry of sugar metabolism in yeasts is well documented (cf. Barnett, 1976; Gancedo & Serrano, 1989) and shall therefore be treated only briefly. In view of the scope of this thesis only hexoses and disaccharides will be discussed (cf. Barnett, 1981).

The first step in the metabolism of disaccharides may occur either intracellularly or extracellularly (figure 1). For example, in S. cerevisiae sucrose is hydrolyzed extracellularly by the enzyme invertase and the resulting hexoses are subsequently taken up by hexose carriers. The first step in maltose metabolism, on the other hand, is its translocation over the plasma membrane followed by an intracellular hydro-
Table 3. Physiological classification of yeasts on the basis of the occurrence of alcoholic fermentation of sugars

<table>
<thead>
<tr>
<th>Classification</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fermentative</td>
<td><em>Rhodotorula glutinis</em></td>
</tr>
<tr>
<td></td>
<td>Facultatively fermentative</td>
</tr>
<tr>
<td></td>
<td>a. Crabtree positive</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cervisiae</em></td>
</tr>
<tr>
<td></td>
<td><em>Schizosaccharomyces pombe</em></td>
</tr>
<tr>
<td></td>
<td>b. Crabtree negative</td>
</tr>
<tr>
<td></td>
<td><em>Candida utilis</em></td>
</tr>
<tr>
<td></td>
<td><em>Kluveromyces marxianus</em></td>
</tr>
<tr>
<td>Obligately fermentative</td>
<td><em>Candida slooffii</em></td>
</tr>
</tbody>
</table>

Lysis by maltase (see also chapter 2). Once intracellular, the hexoses are converted into glucose-6-phosphate from which pyruvate is produced by the enzymes of glycolysis. During this process metabolic energy (ATP), and reducing equivalents (NADH) are formed. The ATP is used to fuel biomass production and for maintenance purposes. To replenish the NADH that is necessary for the first stages of metabolism, the NADH formed in glycolysis can be oxidized via the conversion of pyruvate into ethanol, or by oxidation with oxygen via the respiratory chain. If respiration is possible, pyruvate can be converted into carbon dioxide and water by the citric acid cycle, yielding additional redox equivalents for ATP generation by the respiratory chain (figure 1). The energy yield of alcoholic fermentation is 2 ATP per hexose. Respiration yields a maximum of 38 ATP per hexose. This theoretical maximum ATP yield is not encountered in practice. For example in the yeasts *S. cervisiae* and *C. utilis* the ATP yield per hexose during respiratory growth is 15.4 and 20.8, respectively (Verduyn, 1992).

Table 4. Fermentation and respiration rate of sugars by *S. cervisiae* in the presence and absence of oxygen (From R. Lagunas, 1979).

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Aerobic Flux</th>
<th>Anaerobic Flux</th>
<th>Pasteur effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>39</td>
<td>1.1</td>
<td>40</td>
</tr>
<tr>
<td>Galactose</td>
<td>11</td>
<td>4.5</td>
<td>16</td>
</tr>
<tr>
<td>Maltose</td>
<td>28</td>
<td>1.4</td>
<td>29</td>
</tr>
</tbody>
</table>

*Total respiration has been calculated by summation of the rates of sugar fermentation and respiration. *Pasteur effect is expressed as the ratio between glycolytic flux during anaerobiosis and aerobicosis.

* *S. cervisiae* mutants with impaired mitochondrial function, the so-called 'petite' or respiratory-deficient mutants. However, the large majority of the yeast species that have been described so far are facultatively fermentative. They can thrive on either oxidative or substrate-level phosphorylation as a source of ATP for biosynthesis. The group of facultatively fermentative yeasts is larger than is apparent from handbooks on yeast taxonomy: even the yeast *Hansenula nonfermentans* can exhibit alcoholic fermentation at appreciable rates under appropriate cultivation conditions (van Dijken et al., 1986).

Regulation of alcoholic fermentation

The regulation of fermentation and respiration is controlled by environmental conditions, such as the absence or presence of oxygen and the type and concentration of the sugar. The effects of these conditions can be categorized under the headings Pasteur effect, Crabtree effect, Custers effect and Kluver effect.

Pasteur effect

The Pasteur effect has been defined as the inhibition of the sugar consumption rate by aerobicosis (Lagunas, 1986).

This phenomenon was named after Pasteur because of his observations on alcoholic fermentation with brewers' yeast. Pasteur observed that 'Yeast is able to grow in a solution of sugar in the complete absence of oxygen and air. In this case a small amount of yeast is formed and a great amount of sugar disappears, 60 or 80 parts of sugar per 1 part of yeast... If the experiment is done in the presence of air... only 4-10 parts of sugar are transformed per part of yeast formed' (translation taken from Lagunas, 1986). The explanation that has been given in the literature is a preferential utilization of reducing equivalents by respiration. If the fermentative pathway cannot effec-
Figure 2. Specific rate of oxygen uptake (○), carbon dioxide production (●), and cell yield (□) as a function of the dilution rate (= growth rate) in glucose-limited cultures of *S. cerevisiae* CBS 8066. (Acetate is produced at D > 0.3 h⁻¹ and ethanol is produced at D > 0.38 h⁻¹). From Postma et al. (1989).

Figure 3. Typical plots of glucose pulse experiments with cultures of *S. cerevisiae* (A) and *C. utilis* (B) pre-grown under glucose limitation at D = 0.1 h⁻¹. ○, glucose; □, biomass; ●, ethanol. From van Urk et al. (1988).

Tightly compete with mitochondrial respiration this will lead to a reduced rate of alcoholic fermentation. It has been assumed that this is due to the higher ATP yield from respiration, this in turn leading to a higher biomass yield and thus explaining the observations of Pasteur. In her review about the Pasteur effect, Lagunas (1986) has criticized the supposed role of respiration in the inhibition of alcoholic fermentation. As Pasteur had already observed, brewers' yeast also ferments under aerobic conditions and that under the culture conditions he used, the fermentation rate was even higher than under anaerobicism.

As pointed out by Lagunas (1979) the lower fermentation rate under anaerobiosis in the experiments of Pasteur must be explained by the fact that he cultivated the yeast in a medium lacking the extra requirements for growth under anaerobic conditions, namely unsaturated fatty acids and sterols (Andreasen & Stier, 1953; 1954; Lagunas, 1986). The synthesis of these compounds requires molecular oxygen due to the fact that some steps in their synthesis are catalyzed by oxygenases. In the complete absence of oxygen growth ceases because unsaturated fatty acids and sterols are essential compounds for cellular functioning. Alcoholic fermentation, however, can continue although growth has stopped. Under aerobic conditions unsaturated fatty acids and sterols can be synthesized by the yeast and do not have to be present in the growth medium.

*S. cerevisiae* has frequently been used as a model organism in the study of the Pasteur effect. This was possible since, although the Pasteur effect does not occur during growth of *S. cerevisiae* in batch cultures (table 4), it does so under special experimental conditions such as in sugar-limited chemostat cultures, and in resting-cells suspensions. In sugar-limited chemostat cultures the growth rate and the sugar consumption rate are limited by a low sugar concentration. Also in resting cells, the sugar consumption rate is low due to an inactivation of the sugar transport system. Thus, in aerobic sugar-limited chemostat cultures and in resting cells, reducing equivalents can be preferentially channeled into the electron-transport chain.

The Pasteur effect is not specific for *S. cerevisiae* but also occurs in other facultatively fermentative yeasts. For example, in oxygen-limited chemostat cultures of *C. utilis* the rate of sugar consumption is higher than under aerobic conditions (chapter 5).

**Crabtree effect**

The Crabtree effect can be defined as the occurrence of alcoholic fermentation under aerobic conditions (van Dijken & Scheffers, 1986). Two types of this effect can be encountered.

**Long-term Crabtree effect**

The long-term Crabtree effect is the occurrence of aerobic fermentation under fully adapted, steady-
tive yeasts immediately produce ethanol and acetate upon transfer from glucose limitation to glucose excess (figure 3). This has been attributed to the inability of such yeasts to enhance their respiration rate instantaneously (Petrik et al., 1983; Rieger et al., 1983; Kääpeli, 1986). The increased flux of sugar entering the cell as a consequence of the increased extracellular sugar concentration results in an increased production of NADH, which cannot be completely oxidized by the respiratory chain. However, so far the metabolic 'bottlenecks' responsible for such a limited respiratory capacity have not been identified.

Several explanations as to why Crabtree-negative yeasts do not show these short- and long-term effects may be given, including a tighter regulation of sugar transport, the possibility to direct the enhanced glu-cose flux into storage carbohydrate production and, a low pyruvate decarboxylase activity (van Urk, 1989)

Custers effect

Yeast strains from the genera *Brettanomyces*, *Dekkera* and *Energiella* ferment glucose into ethanol and acetate under fully aerobic conditions. However, upon a transition to anaerobic conditions, fermentation and growth are strongly inhibited during a period that can last several hours after which fermentation resumes extremely slowly. (Custers, 1940; Carrascalos et al., 1981; Wijsman et al., 1984; van Dijken & Scheffers, 1986). When oxygen is reintroduced into the culture, alcoholic fermentation recommences immediately (figure 4).

Since the anaerobic inhibition can also be abolished by the addition of H-acceptors such as acetoin, the Custers effect is thought to be caused by redox problems (Scheffers, 1966). Since the conversion of glucose into ethanol involves a closed redox balance, the NADH produced in other processes, like biosynthesis and acetate production, cannot be reoxidized by this process. Under aerobic conditions this NADH is reoxidized by respiration. Under anaerobic conditions glucose is reduced to glycerol at the expense of ATP (figure 5). The yeasts showing the Custers effect are apparently unable to compensate for the overproduction of redox equivalents via the production of glycerol or other highly reduced compounds.

Kluyver effect

The yeast *Candida utilis* is able to ferment glucose and is able to grow on maltose under aerobic conditions. However, this organism is not able to ferment maltose. This phenomenon is an example of the Kluyver effect. Since the yeasts that exhibit a Kluyver effect for a particular sugar are able to ferment glucose, the Kluyver effect must be caused by a difference between the metabolism of this sugar and that of glucose. For clarity, sugars giving rise to the

---

**Figure 4.** The Custers effect in a batch culture of *Brettanomyces intermedius*: upon a shift from aerobic to anaerobic conditions, glucose consumption, alcohol and acetate production and growth stop, but resume upon aeration. From Wijsman et al. (1984).

**Figure 5.** Schematic presentation of redox balances in yeasts under anaerobic conditions. Alcoholic fermentation is a closed redox system. Glycerol is used as an electron sink for NADH from other processes like biosynthesis.

---

state conditions at high growth rates. For example, *S. cerevisiae* CBS 8066, cultivated under glucose-limited conditions, shows fully respiratory metabolism at growth rates below 0.38 h⁻¹. If the growth rate is increased above this value, ethanol is produced (figure 2). The long-term Crabtree effect has been explained in terms of a limited respiratory capacity of the yeast (Fiechter et al., 1981; Kääpeli, 1986), and an uncoupling effect of acetate, formed at high growth rates (Postma et al., 1989).

**Short-term Crabtree effect**

The short-term Crabtree effect is the sudden fermentative response under fully aerobic conditions upon the addition of excess sugar to yeasts that did not ferment before this addition. In this case no adaptation to this fermentative state has occurred (Verduyn et al., 1984; van Urk, 1989). Crabtree-positiveness evidenced by the observed accumulation of ethanol and acetate immediately after the addition of glucose to a glucose-limited culture (Verduyn et al., 1985).
Kluvyer effect in a certain yeast will be denoted as $K^+$ sugars, sugars that do not as $K^-$ sugars.

**Occurrence of the Kluvyer effect**

The occurrence of the Kluvyer effect is widespread among all yeast species and occurs with a variety of sugars. Although the Kluvyer effect was originally described for disaccharides only, the same phenomenon can be observed for galactose. The occurrence of the Kluvyer effect has been catalogued by Sims & Barnett in 1978. Since then the taxonomy of yeasts has been reorganized (Barnett et al., 1990). Table 4 shows the distribution of the Kluvyer effect over the 590 yeast species that have been classified by Barnett et al. (1990). All strains of 215 (36%) species are reported to ferment glucose. Of these, 132 (61%) show the Kluvyer effect for at least one sugar. Included are the yeasts that in addition to glucose ferment at least one other sugar, or show the Kluvyer effect for at least one sugar. Table 5 shows the striking individuality among the yeasts in their responses to different sugars. For example, Candida boleticola shows the Kluvyer effect for galactose whereas it ferments trehalose. On the other hand the yeast Candida buinensis ferments galactose and shows the Kluvyer effect for trehalose. Pichia angophorae exhibits the Kluvyer effect for cellobiose but not for maltose whereas in Pichia euphorbiae the situation is reversed (table 5).

**Table 5.** Sugar fermentation properties of yeast species. Included in the table are yeasts that ferment glucose and either show the Kluvyer effect for at least one other sugar and/or ferment other sugars beside glucose. The data used are from Barnett et al. (1990).

<table>
<thead>
<tr>
<th>Species</th>
<th>gal</th>
<th>mal</th>
<th>meg</th>
<th>suc</th>
<th>tre</th>
<th>mil</th>
<th>lac</th>
<th>cel</th>
<th>mtz</th>
<th>raf</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ambrosiozyma</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cicatricosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>monospora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>albicans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>auringiensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>boidinii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>boleticola</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bombi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bombicola</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buinensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cacaoi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cantarelli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chilensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coipomensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>conglobata</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dendronema</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diddensiae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>entomaeae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>entomophilosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ergastensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ernobii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fennica</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>floricola</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>freyschussii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>friedrichii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fructus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>haemulonii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homilentoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>humilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insectorum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intermedia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ishiwadae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kruisii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactis-condensi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lodderae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maltosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanolophaga</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanosorbosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>gal</td>
<td>mal</td>
<td>meg</td>
<td>suc</td>
<td>tre</td>
<td>mlb</td>
<td>lac</td>
<td>col</td>
<td>mlz</td>
<td>raf</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>milleri</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>mogii</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>musae</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>naeodendrona</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nanaspora</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>natalensis</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>oleophila</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>ootensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ovais</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>parapsilosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pelita</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pignatae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pseudointermedia</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>quercitrusa</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>ralunensis</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rhagii</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>salmanticensis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sequanensis</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>shehatae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>silvanorum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>silvicultrix</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>+</td>
<td>+</td>
<td>K</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>solani</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sonorenseis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sophiae-reginae</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>spandovensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stellata</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>succiphila</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>terebra</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>tropicalis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tsuchyiæ</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vaccinii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>variovaara</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>veronae</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>versatilis</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>viswanathii</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>wickerhamii</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citeromyces</td>
<td>matriitensia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>Clavispora</td>
<td>lusitaniae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cryptococcus</td>
<td>curiosus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>Debaryomyces</td>
<td>castellii</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pseudopolymerphus</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yamadai</td>
<td>+</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dipodascus</td>
<td>magnusii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Geotrichum</td>
<td>tetrasperma</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hanseniaspora</td>
<td>fermentan</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hansenula</td>
<td>arabbitogenes</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>misumaiensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hasegawaeae</td>
<td>japonica</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kluyveromyces</td>
<td>aestuiæ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>africans</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>blattae</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>lodderae</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>marxianus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Species</td>
<td>gal</td>
<td>mal</td>
<td>meg</td>
<td>suc</td>
<td>tre</td>
<td>mlp</td>
<td>lac</td>
<td>cel</td>
<td>mlz</td>
<td>raf</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>phaffii</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>polysporus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>thermotolerans</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>waltii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>wickerhamii</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yarrowii</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lodderomyces</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>elongisporus</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metschnikowia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pulcherrima</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>reukaulii</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>zobelii</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Nematospora</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coryli</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pachysolen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tannophikus</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pachytichospora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transvaalensis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Pichia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amylophila</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>angophorae</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>angusta</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>anomala</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bovis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>capsulata</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>euphorbiae</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>euphorbiaphila</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>fabianii</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
</tr>
<tr>
<td>hampshirensis</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>heinii</td>
<td>+</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>holstii</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>jadinii*</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>kodamae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>lynterdi</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
</tr>
<tr>
<td>mexicana</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>meyeriae</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mississippiensis</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mucosa</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>muscicola</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>naganishii</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>nakazawai</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>onychis</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
</tr>
<tr>
<td>piperi</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rabaulensis</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
</tr>
<tr>
<td>rhodanensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sargentensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>scolyti</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segobiensis</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>silvicola</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>sorbitophila</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>spartinae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stipitis</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>strasburgensis</td>
<td>-</td>
<td>K</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>subpelliculosa</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>sydowiorum</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
</tr>
<tr>
<td>toletana</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>trehalophila</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>veronae</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td><strong>Saccharomyces</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>castellii</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dairensis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>exigus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5, continued.

<table>
<thead>
<tr>
<th>species</th>
<th>gal</th>
<th>mal</th>
<th>meg</th>
<th>suc</th>
<th>tre</th>
<th>mlb</th>
<th>lac</th>
<th>cel</th>
<th>miz</th>
<th>raf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kluyveri</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>servazzii</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>unisporus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Saccharomyces ludwigii</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
</tr>
<tr>
<td>Schwanniomyces occidentalis</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Torulaspora globosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Wickerhamia pretoriensis</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Wickerhamia fluorescens</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Williopsis beijerinckii</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>+</td>
<td>K</td>
<td></td>
<td></td>
<td>K</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>californica</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>matakii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>pratensis</td>
<td>-</td>
<td>+</td>
<td>K</td>
<td>-</td>
<td>K</td>
<td></td>
<td></td>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>saturnus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>+</td>
</tr>
<tr>
<td>suaveolens</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Wingea robertsiae</td>
<td>K</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>K</td>
</tr>
<tr>
<td>Zygosaccharomyces cidri</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>fermentati</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>florentinus</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>microellipsoides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>matakii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

K, Kluyver effect: fermentation negative and aerobic growth positive; +, fermentation and aerobic growth both positive; -, fermentation and aerobic growth both negative or delayed or variable or unknown.

gal, D-galactose; mal, maltose; meg, methyl α-D-glucopyranoside; suc, sucrose; tre, α, α-trehalose; mlb, melibiose; lac, lactose; cel, cellobiose; miz, melilozite; raf, raffinose. *Current name of Candida utilis.

Possible mechanisms involved in the Kluyver effect

The history of the Kluyver effect has been reviewed recently by Barnett (1992). Nevertheless, in view of the scope of this thesis, a short outline of the literature follows below.

In 1940 Kluvver & Custers confirmed earlier reports that various facultatively-fermentative yeasts were unable to ferment certain disaccharides (figure 6). At that time, the permeability of the plasma membrane was thought to be too inspecific to discriminate between hexoses and disaccharides. It was therefore suggested that the inability to ferment K* disaccharides was caused by an inactivation of the disaccharide hydrolases in absence of oxygen (Kluvver & Custers, 1940).

The term Kluvver effect was introduced by Sims & Barnett (1978). They defined it as "... certain yeasts can utilize particular disaccharides aerobically, but not anaerobically, although these yeasts can use one or more of the component hexoses anaerobically. They determined that glycosidase activity for K* disaccharides was neither inhibited nor inactivated under anaerobic conditions, and suggested that the transport of K* sugars required oxygen. Indeed, the transport activity under anaerobic conditions was considerably lower than under aerobic conditions (table 6). The possible requirement for oxygen to transport K* sugars was then more closely investigated (Barnett & Sims, 1982). From uptake ex-

Figure 6. Anaerobic carbon dioxide production by Torulopsis dattila from glucose (●), maltose (○) and in the absence of substrate (□). From Kluvver & Custers (1940).

periments under aerobic and anaerobic conditions it was concluded that transport under aerobic conditions occurred by proton symport, whereas it proceeded by facilitated diffusion under anaerobic conditions. Barnett & Sims (1982) suggested that the Kluvver effect could be attributed to the inability to accumulate sugars under anaerobic conditions.

The uptake experiments, described in both publications (Sims & Barnett, 1978; Barnett & Sims, 1982), were, however, performed with non-metab-
Table 6. Rates of uptake of D-[1-3H]fructose and 2-deoxy-D-[1-3H]glucose under aerobic and anaerobic conditions by Klyveromyces thermotolerans NCYC 144 after growth on galactose or glucose. *K. thermotolerans* shows the Klyver effect for galactose (from Sims & Barnett, 1978).

<table>
<thead>
<tr>
<th>Carbon source for growth</th>
<th>Condition</th>
<th>Inhibitor</th>
<th>Uptake rate (nmol·min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-galactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aerobic</td>
<td>none</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>none</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>aerobic</td>
<td>D-galactose</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>D-galactose</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>D-glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aerobic</td>
<td>none</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>none</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>D-glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aerobic</td>
<td>none</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>anaerobic</td>
<td>none</td>
<td>0.89</td>
</tr>
</tbody>
</table>

The uptake of both K⁺ and K⁺ sugars under aerobic conditions occurred by proton symport, an energy-dependent transport system. Since energy transduction from the metabolism of the analogues was impossible, it was proposed that the uptake had been energized by an endogenous process (Sims et al., 1984). As the energetic efficiency of fermentation is low compared to respiration, the inability to accumulate non-metabolizable analogues of sugars under anaerobic conditions could have been caused by energy depletion (Sims et al., 1984). This is consistent with the observations of Höfer & Nassar (1987) that also the uptake of the non-metabolizable glucose analogues 2-deoxy-D-glucose and glucosamine in Schizosaccharomyces pombe was severely inhibited under anaerobic conditions. However, when energized by glucose, the accumulation of both sugars was completely restored, indicating that proton symport under anaerobic conditions depends on a sufficient energy supply (Höfer & Nassar, 1987).

These observations led to a series of experiments (Sims et al., 1984; Schulz & Höfer, 1986) in which nitrophenyl glycosides were used as disaccharide analogues. In these experiments it was shown that these analogues were taken up by the specific disaccharide transporter and were hydrolyzed intracellularly. Subsequently, the hexose part could be metabolized to energize transport whereas the nitrophenol, that was expelled from the cells, could be used to determine the transport rate (Sims et al., 1984). Nevertheless, it can be reasoned that also uptake studies with these analogues have pitfalls. Since only one hexose molecule can be metabolized compared to two hexose molecules in the case of disaccharides, the energy yield is only half. Furthermore, with a pKₐ value of 7.15, the 4-nitrophenol partially dissociates and therefore could act as an uncoupler. Uptake experiments performed with these sugar analogues, despite the disadvantages, revealed that the affinity of sugar transport is lower under anaerobiosis than under aerobic conditions (Sims et al., 1984).

The finding that the energy status of the cell can affect sugar proton symport led to the hypothesis that the anaerobic metabolism of K⁺ sugars does not generate sufficient energy for their transport (Schulz & Höfer, 1986). Indeed, in experiments under aerobic conditions the addition of respiration inhibitors and uncouplers almost completely inhibited uptake of lactose. However, also these experiments were performed with a nitrophenyl lactose analogue: 4-nitrophenol-[β-D-galactopyranoside.

Although the transport capacity of the K⁺ sugars and their analogues was severely inhibited under anaerobic conditions, it was not reduced to zero (table 6). Barnett & Sims (1982) concluded that the Klyver effect was not solely caused by a requirement for oxygen of the transport step. Therefore, they directed their investigations to a possible role of the enzymes.
<table>
<thead>
<tr>
<th>Yeast name and strain number</th>
<th>Carbon source(^b) for growth</th>
<th>Pyruvate decarboxylase</th>
<th>Alcohol dehydrogenase</th>
<th>Glycosidase(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida viswanathii</em></td>
<td>D-glucose</td>
<td>0.13</td>
<td>1.04</td>
<td>-</td>
</tr>
<tr>
<td>CBS 4024</td>
<td>maltose</td>
<td>0.11</td>
<td>0.32</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>cellubiose K</td>
<td>0.039</td>
<td>0.23</td>
<td>0.33</td>
</tr>
<tr>
<td><em>Debaryomyces castellii</em></td>
<td>D-glucose</td>
<td>0.17</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>CBS 2923</td>
<td>maltose</td>
<td>0.067</td>
<td>0.088</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>lactose K</td>
<td>&lt;0.001</td>
<td>&lt;0.006</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Debaryomyces polymorphus</em></td>
<td>D-glucose</td>
<td>0.18</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>CBS 4349</td>
<td>maltose</td>
<td>0.35</td>
<td>0.37</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>cellubiose K</td>
<td>0.008</td>
<td>0.038</td>
<td>0.28</td>
</tr>
<tr>
<td><em>Kluyveromyces dobzhanski</em></td>
<td>D-glucose</td>
<td>0.083</td>
<td>0.48</td>
<td>-</td>
</tr>
<tr>
<td>CBS 2104</td>
<td>maltose</td>
<td>0.31</td>
<td>0.070</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>cellubiose K</td>
<td>0.040</td>
<td>1.07</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Kluyveromyces wickerhamii</em></td>
<td>D-glucose</td>
<td>0.16</td>
<td>1.17</td>
<td>-</td>
</tr>
<tr>
<td>CBS 2745</td>
<td>cellubiose K</td>
<td>0.020</td>
<td>0.88</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>lactose K</td>
<td>0.020</td>
<td>0.21</td>
<td>0.34</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>D-glucose</td>
<td>1.62</td>
<td>0.57</td>
<td>-</td>
</tr>
<tr>
<td>CBS 1171</td>
<td>maltose</td>
<td>0.40</td>
<td>0.64</td>
<td>1.01</td>
</tr>
</tbody>
</table>

\(^a\)Means of multiple experiments, expressed as nmol substrate converted min\(^{-1}\) mg protein\(^{-1}\)

\(^b\)Substrates that give the Kluyver effect are indicated by K.

\(^c\)Yeast grown on maltose were tested for \(\alpha\)-glucosidase activity, yeasts grown on cellubiose for \(\beta\)-glucosidase activity, yeasts grown on lactose for \(\beta\)-galactosidase activity.

responsible for the conversion of pyruvate into ethanol. It appeared that a number of yeasts had a much lower pyruvate decarboxylase activity when grown on a K\(^+\) sugar than during growth on other sugars. Alcohol dehydrogenase and hydrolase activities were unaffected (Sims & Barnett, 1991; table 7). The pyruvate decarboxylase decreased rapidly under conditions at which the glycolytic activity was low, for example in the absence of glucose or under aerobic conditions (Sims et al., 1991; figure 7).

Summarizing, at the beginning of the work presented in this thesis, the Kluyver effect was thought to be caused by a combination of three factors (Barnett, 1992):

1) Transport activity and its affinity are lower under anaerobic conditions than under aerobic conditions.

2) The affinity of glycosidases for their substrate is very low. Therefore, the intracellular concentration of disaccharides, achieved by the transport carrier, is of great importance.

3) Factors 1 and 2 may lead to a decrease of glycolytic activity, causing an inactivation of pyruvate decarboxylase and preventing ethanol production.

**Genetics of the Kluyver effect**

Another way to study the mechanism of the Kluyver effect is by mutagenesis (Entian & Barnett, 1983). However, the attempts to obtain mutants of *K. lactis* that show the Kluyver effect from strains that did not, and to make mutants not showing the Kluyver effect from a strain that did, were unsuccessful. Nevertheless, it has been described by several authors that some 'petite'-mutations in the yeast *S. cerevisiae* give rise to an inability to ferment certain sugars, including maltose, galactose and \(\alpha\)-methyl D-glucopyranoside (Donnini et al., 1992; Khan, 1982; 1985; Khan & Greener, 1977; Puglisi & Algeri, 1974). Mutations in the nuclear genes designated *PMU1* (petite maltose utilization), *IMP* and *IMP2* (independent of the mitochondrial particle) were responsible for this phenotype.

The *IMP2* gene has been cloned on a 2-kb DNA segment, sequenced and disrupted (Donnini et al., 1992). Disruption mutants showed the same phenotype as the original mutants, namely the inability to ferment maltose, galactose and raffinose. Transformation of these mutants with a 1.6 kb fragment containing the entire coding region restored galactose and raffinose, but not maltose fermentation.

Clearly, these mutations invoke a physiological phenomenon identical to the Kluyver effect as occurring in other yeast species. This has, however, not been recognized as a Kluyver effect so far.

**Outline of this thesis**

The metabolism of disaccharides may differ in only a few steps from monosaccharide metabolism,
including transport and hydrolysis. This similarity probably forms the rationale for the fact that almost invariably fundamental studies on the regulation of glycolysis in yeasts have been performed with glucose, as is evident from the publications concerning the Pasteur, Crabtree and Custers effects. Nevertheless, the occurrence of the Kluiver effect shows that differences between monosaccharide and disaccharide metabolism in yeasts can be significant. The studies presented in this thesis were initiated to investigate the differences in growth parameters on monosaccharides and disaccharides.

In chapter 2, the methodology of sugar transport studies with yeasts is discussed, particularly with respect to the cultivation methods.

In chapter 3, maltose transport in \textit{S. cerevisiae} is characterized. Since uptake experiments with intact cells are disturbed by subsequent metabolism of the transported substrate, transport of maltose was also studied under \textit{in vitro} conditions with plasma membrane vesicles.

The effects of the different transport mechanisms for glucose and maltose on the energetics of \textit{S. cerevisiae} are investigated in chapter 4. These studies were performed with anaerobic chemostat cultures on mixtures of glucose and maltose as sources of carbon and energy. Particular attention is paid to the ethanol yield on both sugars.

All studies described in the literature concerning the Kluiver effect were performed with cells cultivated in batch systems. This cultivation method offers no control over growth rate and oxygenation. These drawbacks make it difficult to interpret results and, though oxygen seems to play a key role, give no possibility to study the effect of oxygen supply on the Kluiver effect. In chapter 5, the effect of oxygen limitation on the metabolism of glucose and maltose is investigated with chemostat cultures of two yeasts: \textit{S. cerevisiae} and \textit{C. utilis}. \textit{C. utilis} is known to show the Kluiver effect for maltose whereas \textit{S. cerevisiae} does not exhibit a Kluiver effect.

Finally, chapter 6 describes a closer study of the Kluiver effect by offering \textit{C. utilis}, cultivated in chemostat cultures under oxygen limitation, glucose and maltose simultaneously. The fate of both sugars, being fermented and/or respired, is followed. From the data presented in chapter 6, a new explanation of the Kluiver effect is proposed.

\textbf{References}


\textbf{Custers MTJ} (1940) Onderzoekingen over het gistgeslacht \textit{Brettanomyces}. PhD Thesis, Delft University of Technology, The Netherlands


\textbf{Donnini C, Lodi T, Ferrero I & Puglisi PP} (1992) IMP2, a nuclear gene controlling the mitochondrial dependence of galactose, maltose and raffinose utilization in \textit{S. cerevisiae}. Yeast 8: 83-93


Kluyster AJ & Custers MTH (1940) The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. Antonie van Leeuwenhoek J. Microbiol. Serol. 6: 121-162


Lagunas R (1986) Misconceptions about the energy metabolism of Saccharomyces cerevisiae. Yeast 2: 221-228


Chemostat studies of sugar transport in yeasts

by R. A. Weusthuis, J. T. Pronk, P. J. A. van den Broek and J. P. van Dijken.

Abstract

The kinetics and energetics of sugar transport in yeasts strongly depend on environmental conditions. Chemostat cultivation is the most suitable method to study sugar-transport kinetics in relation to the metabolic fluxes in growing yeast cells. The specific rate of sugar consumption ($q_s$) in growing cultures is given by $q_s = \text{specific growth rate} \times \text{biomass yield}^1$. Therefore, $q_s$ can be manipulated either by variation of the growth rate or by variation, at a constant growth rate, of the growth yield on the sugar (amount of biomass formed per amount of sugar consumed). The latter can be accomplished, for example, by increasing the maintenance-energy requirement of yeast cultures by the inclusion of non-metabolizable weak acids in the growth medium, by manipulation of the oxygen supply to cultures, or growth on mixtures of sugars. This type of controlled variation of metabolic fluxes cannot be achieved in batch cultures, where various parameters that are decisive for the kinetics of sugar transport cannot be fixed.

A key factor with respect to transport kinetics is the sugar concentration in the cultures. In chemostat cultures, yeasts can be grown continuously under sugar limitation. As a result, cells adapt their uptake systems to cope with low sugar concentrations, often in the micromolar range. At low environmental sugar concentrations, yeasts that possess high-affinity proton-symport sugar-uptake systems have a competitive advantage over yeasts that transport sugars via facilitated diffusion.

Manipulation of growth conditions in chemostat cultures can be used to vary the contribution of various transport mechanisms to the overall sugar-uptake capacity in a controlled and reproducible manner.

Chemostat cultivation also offers unique possibilities to study the energetic consequences of sugar transport. For example, by using anaerobic, sugar-limited chemostat cultures of *Saccharomyces cerevisiae*, it has been established that the biomass yield on maltose is 25% lower than on glucose. This is due to the requirement of 1 ATP-equivalent for the uptake of maltose via proton symport.

Introduction

All yeasts that are presently known are able to utilize one or more sugars as their principal source of carbon and energy (Barnett, 1976; Barnett et al., 1983). Many yeast strains that are commonly employed in biotechnological processes have been obtained from natural habitats with high sugar concentrations, in which they rapidly convert the available sugars to ethanol. In these ecosystems, the growth rate of yeasts is like *S. cerevisiae* (bakers' yeast, brewers' yeast) may be limited by the availability of nutrients other than the sugar carbon source. Therefore, their competitiveness is probably determined by their maximum sugar consumption rate rather than by affinity for the sugar substrate or the energetic efficiency of sugar utilization. This is reflected by the characteristics of their sugar-uptake systems, which generally have a rather poor substrate-saturation constant for the sugar substrate: $K_s^v$ values are usually in the $10^{-2}$-$10^{-2}$ M range (van Urk et al., 1989).

Yet, at saturating sugar concentrations, glycolytic fluxes in these yeasts can attain very high values. This is a bonus in the classical biotechnological applications of *S. cerevisiae*, i.e. during the leavening of dough or the production of alcoholic beverages, for which a high specific rate of alcohol production is desired.

The Crabtree-positive yeasts, which include *S. cerevisiae*, have a strong tendency towards alcoholic fermentation. In these yeasts, high rates of sugar uptake result in alcoholic fermentation, even when oxygen is present in excess (Käppeli, 1986; Petrik et al., 1985; van Dijken & Scheffers, 1988). In modern large-scale production processes, e.g. for the production of bakers' yeast, single-cell protein or heterologous proteins, alcoholic fermentation is not desired, because it inhibits growth and reduces the biomass yield (Kalina, 1993). In these processes, alcoholic fermentation is avoided by feeding the sugar feedstock to the cultures at a low rate in a fed-batch mode. This results in a low concentration of sugar in the
culture and, consequently, in a low rate of sugar uptake. At these low uptake rates, sugar metabolism is fully respiratory (Kappeli, 1986; van Dijken & Schef-
fers, 1986). Consequently, high biomass yields are obtained and accumulation of toxic products is prevented.

The high $K_m$ values for sugar transport that are characteristically found in $S$. cerevisiae strains are not typical for the majority of yeasts, which appear to be well-equipped for growth at low sugar concentrations and are able to synthesize transport systems with $K_m$ values of $10^{-5}$-$10^{-4}$ M (van Urk et al., 1989).

Transport constitutes the first step in the metabolism of a large number of sugars (a notable exception being the metabolism of some oligosaccharides that are hydrolysed outside the cell). As such, sugar transport is likely to have a substantial impact on the regulation of the glycolytic flux as a whole. In this respect, it has been suggested that sugar uptake is a rate-limiting step in glycolysis (van Uden, 1967; Gan-
cedo & Serrano, 1989; Lagunas et al., 1982; Cortassa & Aon, 1993). Because of its impact on the ecology and biotechnological applications of yeasts, sugar transport by yeasts has been the subject of a large number of studies. The majority of these have been performed with samples from shake-flask cultures grown in the presence of excess sugar. Unfortunately, such cultures are poor model systems, since they exhibit a number of inherent drawbacks for quantitative studies on sugar transport in yeasts. The aim of this paper is to review the applicability of chemostat cultivation as a tool for studies on quantitative aspects of sugar transport in yeasts. Particular attention will be paid to the relation between the kinetics of sugar transport in cell suspensions, as determined in experiments with radiolabelled sugars, and the kinetics observed in growing cultures.

### Mechanisms of sugar transport

Since sugars are highly polar molecules, free diffusion across the membrane lipid bilayer probably does not significantly contribute to their rate of entrance into the cell under physiological conditions (Lengeler, 1993). Facilitated-diffusion systems, in which the lipid solubility of the sugar is increased by the presence of a carrier protein, are widespread among yeasts (Barnett, 1976; Barnett, 1981; Lagunas, 1993). In the case of facilitated diffusion, the driving force for solute translocation is provided exclusively by the concentration gradient of the solute over the membrane. Therefore, uptake of sugars by facilitated diffusion does not require metabolic energy.

Since, in the case of facilitated diffusion, the driving force for sugar uptake becomes zero when internal and external solute concentrations are equal, this process does not allow the uptake of sugars against a concentration gradient. In particular during growth at very low extracellular sugar concentrations, intracellular accumulation of sugars may be necessary to allow the cytoplasmic sugar kinases and disaccharide hydrolases to function optimally (Lagunas, 1993). This can be accomplished by coupling the uptake of a sugar molecule to the uptake of one or more protons via proton-symport systems. Thus, in the case of proton symport, the proton-motive force over the plasma membrane is used to drive intracellular accumulation of sugar. Generation of this proton-motive force is accomplished mainly by the plasma membra-
ne H$^+$-ATPase complex which couples the hydrolysis of ATP to ADP and inorganic phosphate to the outward translocation of protons (figure 1).

In many bacteria, sugar uptake and phosphorylation are tightly coupled. In the so-called group-translocation systems (Lengeler, 1993), free intracellular sugar does not occur. The occurrence of a group-translocation mechanism for sugar uptake in yeasts has been a matter of dispute for many years (cf. Lag-
unas, 1993). Recent studies on glucose uptake by $S$. cerevisiae, using an ultra-rapid sampling method for the determination of intracellular metabolite pools, show that the formation of hexose phosphates is a secondary event that follows the appearance of free intracellular glucose (de Koning & van Dam, 1992).

### Methodology of sugar-transport studies

The most widely applied method for studying uptake of sugars by suspensions of yeast cells is the use of radioactive ($^{14}$C or $^3$H) sugars. An inherent problem in transport studies with intact cells is the interference by subsequent metabolism. Nearly always, a significant decrease of the apparent uptake rate is observed within 15 seconds after addition of radiolabelled sugar (Postma et al., 1988). This effect is most likely caused by evolution of $^{14}$CO$_2$ and release of other labelled metabolites such as ethanol and organic acids. In practice, this problem can be reduced by using very short incubation times (5 to 10 seconds). Recent studies using quenched-flow techniques indicate however, that determination of true initial kinetics may require sampling times in the sub-second time scale (Walsh et al., 1993).

Interference of metabolism in uptake studies can be circumvented by the use of non-metabolizable sugar analogues, such as the glucose analogue 6-deoxyglucose (Kotyk et al., 1975; Romano, 1982). Use of these compounds can only provide qualitative information, since kinetic parameters are likely to be different from those for the natural substrates. Nevertheless, when proper controls are included, the use of sugar analogues may yield important information on the uptake mechanism. For example, uptake of radioactive 6-deoxyglucose against a concentration
Gradient can be regarded as a reliable criterion for the presence of an energy-dependent glucose uptake system.

An obstacle inherent to transport studies with cell suspensions is the non-specific binding of labelled substrate to cellular components. Various methods have been employed to determine the contribution of binding to the total amount of radioactivity retained by the cells. These rely on inactivation of cells, e.g. heat treatment (Serrano, 1977) or on control experiments performed at 0 °C (Bisson & Fraenkel, 1983). Correction for non-specific binding is a necessity, since even in short-term uptake studies, it may contribute significantly to the cell-associated radioactivity. Another pitfall in transport studies with radioactive sugars is the chemical impurity of many commercially available radiolabelled sugars. For example, commercially available preparations of D-(U-14C) maltose may contain up to 2% glucose. Uptake of such impurities can contribute to a large extent to the total amount of radioactivity that is transported, in particular when the K_m for uptake of the impurity is much lower than that for the sugar of interest.

In the case of H^+-symport mechanisms, sugar transport can also be determined indirectly by measuring the alkalinization of weakly-buffered cell suspensions upon addition of sugars, using a sensitive pH electrode (Seaston et al., 1973). A major advantage of this method is that non-specific binding does not interfere with the uptake assay. A disadvantage of the measurement of sugar-dependent pH changes is that these assays cannot be performed in standard growth media, which are usually strongly buffered. Furthermore, also in this case, interference of metabolism with the transport assay can be problematic since proton movements may be caused by various other metabolic processes as well, including ATPase activity and production of acidic metabolites. As mentioned for radioactive uptake studies, interference of metabolism with alkalinization studies is reduced by measuring initial rates. If radioactive transport studies and alkalinization assays are performed under identical conditions, the fluxes obtained by these two methods can in principle be used to calculate sugar-proton stoichiometries.

Interference of sugar metabolism in transport studies is avoided by studying uptake in vitro with isolated membrane vesicles. Since cytoplasmic enzymes are absent in membrane vesicles, the transported sugar cannot be metabolized, thus facilitating calculations on uptake kinetics and eventually accumulation ratios. Isolated yeast plasma membranes however do not form well-sealed vesicles. In order to decrease the leakiness of membrane vesicles, membranes have to be fused with artificial liposomes. In this way, membrane vesicles can be obtained that have transport parameters similar to that of intact cells (Ramos et al., 1987; Ongioco et al., 1987). Studies on energy-dependent transport processes in yeast plasma-membrane vesicles are complicated by the absence of a proton-translocating respiratory chain. The physiological membrane-energizing system, the plasma-membrane ATPase complex, cannot be used to energize transport in right-side-out vesicles.
Table 1. Accumulation of \((^4\text{H})\)-6-deoxyglucose, apparent substrate-saturation constants \((K_m)\) for glucose and capacities \((V_{max})\) of glucose uptake by cell suspensions of various yeasts, pregrown in aerobic, glucose-limited chemostat cultures at \(D = 0.10\) h\(^{-1}\), and the residual-glucose concentration \((C_s)\) in these cultures. Data from van Urk et al. (1989).

<table>
<thead>
<tr>
<th>Crabtree-positive yeasts</th>
<th>Accumulation of 6-deoxyglucose</th>
<th>(K_m^1) (mm)</th>
<th>(V_{max}^1) (mmol·g(^{-1})·h(^{-1}))</th>
<th>(C_s^2) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0.5x</td>
<td>1.0</td>
<td>12</td>
<td>110</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>1.1x</td>
<td>1.5</td>
<td>9.0</td>
<td>160</td>
</tr>
<tr>
<td>Torulopsis glabrata</td>
<td>0.5x</td>
<td>1.2</td>
<td>31</td>
<td>100</td>
</tr>
<tr>
<td>Crabtree-negative yeasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida utilis</td>
<td>70x</td>
<td>0.025</td>
<td>8.4</td>
<td>5</td>
</tr>
<tr>
<td>Pichia stipitis</td>
<td>190x</td>
<td>0.015</td>
<td>6.0</td>
<td>5</td>
</tr>
<tr>
<td>Kluyveromyces marxianus</td>
<td>110x</td>
<td>0.025</td>
<td>1.2</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^1\) Determined in 10 sec incubations with D-(U-\(^{14}\)C)-glucose. In many cases, biphasic Hanes plots were obtained.

\(^2\) Determined by rapid sampling of cultures into liquid nitrogen.

Glucose transport in yeasts

The most intensively studied case of sugar transport in yeasts is that of glucose transport in *S. cerevisiae* (for a comprehensive review see Lagunas, 1993). Uptake of radiolabelled glucose by cell suspensions of this yeast shows biphasic kinetics. The two putative glucose-uptake systems have \(K_m\) values of ca. 1 mM (high-affinity uptake) and ca. 20 mM (low-affinity uptake) (Bisson & Fraenkel, 1983b; Postma et al., 1989b). It is as yet unclear which carrier proteins are responsible for each of these two systems. According to Fuhrmann et al. (1990), the low-affinity part of the biphasic glucose-uptake kinetics may reflect passive diffusion of glucose through the plasma-membrane lipid bilayer.

High-affinity glucose uptake in *S. cerevisiae* is mediated by a facilitated-diffusion carrier, possibly in close association with a hexokinase (Clifton et al., 1993). Several mutants have been isolated impaired in glucose transport activity. Since none of them totally lacks the capacity to transport glucose, several genes are expected to code for high-affinity glucose carriers. Based on sequence homology with mammalian glucose transporters, a number of candidates have been identified, including *SNF3* and *HXT1-4* (Lewis & Bisson, 1991; Kruckenberg & Bisson, 1990; Ko et al., 1993; Neigeborn & Carlson, 1984) and it is to be expected that there is at least one other gene coding for a glucose carrier. It is at present not clear whether all of these genes indeed code for a sugar transporter, or that they code for a regulatory protein as has recently been suggested for *SNF3* (Coons et al., 1993). Identification of the structural genes encoding high-affinity glucose carriers will probably have to await purification of their gene products and reconstitution into in vitro systems.

A different type of glucose transport, mediated by a glucose-proton symporter, has been demonstrated in a number of non-*Saccharomyces* yeasts, including *Candida sp.* (Spencer Martins & van Uden, 1985; Postma et al., 1988), *Rhodotorula sp.* (Hauer & Höfer, 1978; Höfer & Misra, 1978), and *K. marxianus* (de Bruijne et al., 1988; Gasnier, 1987). The apparent
Disaccharide transport in yeasts

Glucose is by far the most commonly used substrate for fundamental physiological studies on sugar metabolism in yeasts. However, only few industrial applications are based on glucose as a feedstock. Industrial substrates such as molasses, whey, starch hydrolysates and wort all contain disaccharides (sucrose, maltose or lactose) as the major sugar sources.

In contrast to glucose metabolism, disaccharide metabolism in yeasts is not necessarily initiated by uptake of the sugar molecule. For example, in the yeast *K. marxianus*, sucrose is initially hydrolysed to glucose and fructose by the extracellular enzyme inulinase (Rouwenhorst et al., 1988; 1991). Subsequently, the component hexoses are transported into the cell (figure 3). Conversely, disaccharides can also be transported over the plasma membrane prior to hydrolysis by an intracellular hydrolase (figure 3). This is for instance the case for maltose utilization by *S. cerevisiae*. In *S. cerevisiae*, hydrolysis of sucrose can occur either intracellularly or extracellularly (Mwesigye & Barford, 1993; Santos et al., 1982). The general view however is that extracellular hydrolysis is the predominant route by which *S. cerevisiae* utilizes sucrose (Barnett, 1976; 1981; Barnett, 1981; DeLaFuente & Sols, 1962; Tkacz & Lampen, 1973) and that the presence of a sucrose-proton-symport system may be a strain-dependent property.

In all cases studied so far, uptake of disaccharides by yeasts has been reported to occur via proton symport. Recent studies have shown that a putative facilitated maltose transport can be attributed to an experimental artifact (Benito & Laguna, 1992). Disaccharide-proton symport systems in yeasts characteristically have a relatively high affinity constant of 2-6 mM (Schulz & Höfer, 1986; Serrano, 1977; Cavalo-Silva & Spencer-Martins, 1990; Dickson & Barr, 1983; Santos et al., 1982).

The most studied disaccharide-uptake system in yeasts is the *S. cerevisiae* maltose carrier. In this yeast, three gene products are required for maltose utilization: a maltose-specific transporter, the maltose-hydrolysing enzyme α-glucosidase and an activator of transcription (Needleman et al., 1984; Cheng & Michels, 1991; Cohen et al., 1985; Charron et al., 1986). Clusters of the three genes encoding these proteins occur in 5 different loci named MAL1-MAL4 and MAL6, which exhibit high sequence homology. Recently, van den Broek et al. (1994) compared profiles of membrane proteins in *S. cerevisiae* grown in maltose- and glucose-limited chemostat cultures. Polyaclrylamide-gel electrophoresis of isolated plasma membranes revealed that growth on maltose induced two membrane-associated polypeptides, not present in glucose-grown cells, with apparent molecular masses of 59 and 53 kDa. Partial amino-acid sequencing of both peptides revealed complete identity with amino-acid sequences predicted from the DNA sequence of the MAL61 gene.

Regulation of sugar transport

Sugar uptake by yeasts is strongly regulated by environmental conditions, both at the level of enzyme synthesis and at the level of enzyme activity. When a yeast possesses multiple transport systems for a given sugar, its concentration in the environment is often a key factor in the regulation of the synthesis of the individual carriers. At high sugar concentrations, high-affinity carriers are generally repressed, but repression is relieved when the sugar concentration in the environment decreases. This type of regulation is well-documented for glucose transport in yeasts (Lagunas, 1993; Postma et al., 1988; Bisson, 1988; Postma & van den Broek, 1990). Also the synthesis of disaccharide carriers such as those for maltose and lactose is governed by induction-repression mechanisms (Barnett, 1981; Schulz & Höfer, 1986; Peinado et al., 1987; de Bruine et al., 1988) in a variety of yeasts (Barnett, 1976).

Existing transport capacity for a sugar can also be controlled by post-translational modification of the carrier protein. For example, in the absence of a nitrogen source for growth, the glucose-transport activi-
ty in *S. cerevisiae* rapidly declines (Lagunas et al., 1982; Busturia & Lagunas, 1986; Gö ort, 1969). Catalytic inactivation has also been observed for galactose transport (Matern & Holzer, 1977; DeJuan & Lagunas, 1986) and maltose transport (Busturia and Lagunas, 1985; Gö ort, 1969). In the case of maltose, studies with antibodies have indicated that the disappearance of activity is associated with proteolysis of the maltose carrier (Lucero et al., 1993). Apart from irreversible inactivation maltose transport can also be reversibly inactivated, depending on the growth conditions (Peinado & Loureiro-Dias, 1986). The physiological necessity for short-term regulation of transport activity is illustrated by studies on maltose transport with *S. cerevisiae* mutants defective in glucose repression (Entian, 1980; Entian & Loureiro-Dias, 1990) and with wild-type cells exposed to a sudden change in the maltose concentration (Postma et al., 1990). In these studies it was shown that uncontrolled maltose uptake leads to substrate-accelerated death, a phenomenon also known to occur in bacteria (Postgate & Hunter, 1964).

Evidently, the sugar concentration in the environment can strongly influence the kinetics of sugar uptake. As a consequence, cultivation methods employed for transport studies should involve control of the sugar concentration.

**Cultivation methods**

**Batch cultivation**

For studies on transport kinetics in growing cells, it is of crucial importance to use controlled cultivation conditions. This allows manipulation of growth parameters that affect transport. Shake-flask cultures do not meet this requirement, since pH and dissolved-oxygen tension (DOT) cannot be regulated. Both these parameters exert a strong influence on the rate of sugar transport: culture pH mainly affects transport at the level of enzyme activity (Hauer & Höfer, 1978; van Leeuwen et al., 1992), whereas oxygen availability may affect the expression of sugar-transport systems at the level of gene expression.

Batch cultivation in fermenters in which pH, DOT and temperature are controlled may at first glance seem a suitable cultivation method for studying sugar transport *in situ*. The sugar concentration in the environment, although continuously decreasing (figure 4), can be chosen in such a way that it does not limit the rate of transport, and the specific growth rate is constant ($\mu = \mu_{max}$). During exponential growth, the rate of sugar transport ($q_s$) is constant and described by equation 1:

$$q_s = \frac{\mu}{Y_{sx}}$$  \hspace{1cm} (1)

in which $Y_{sx}$ is the biomass yield, expressed as amount of biomass formed per amount of sugar consumed. Only when the sugar concentration in the culture is no longer sufficient to saturate the existing transport capacity, the growth rate decreases as a result of a decrease in the rate of sugar transport. This only holds if the biomass concentration that is obtained at the end of the exponential phase is solely determined by the initial sugar concentration in the growth medium. If other nutrients (for example the nitrogen source) are limiting, sugar transport and growth may become uncoupled due to changes in biomass composition and production of overflow metabolites, thus making the relation between uptake
and growth much more complicated. When the amount of sugar does limit the biomass concentration, a rapid transition to zero growth occurs upon exhaustion of the sugar. In batch cultures, the expression and properties of sugar-transport systems in yeasts depend strongly on the time of harvesting (Bisson & Fraenkel, 1984; de Bruijne et al., 1988).

The situation depicted in figure 4 with respect to the kinetics of growth and sugar consumption is more complicated when byproducts are formed that affect cellular physiology. This for example occurs in aerobic batch cultures of S. cerevisiae and other Crabtree-positive yeasts, which exhibit alcoholic fermentation in the presence of sugar concentrations above 1 mM (Verduyn et al., 1984). Apart from ethanol, also other products that negatively affect biomass yield, such as acetic acid, may be formed.

**Fed-batch cultivation**

An intrinsic disadvantage of batch cultivation is that the data it provides on sugar uptake can only be extrapolated to situations where sugar is present in excess ('in excess' is used here to indicate concentrations that do not limit the growth rate). Such studies bear little relevance for the industrial cultivation of yeasts in sugar-limited fed-batch cultures (figure 4). In the latter system, often started with a batch phase, sugar is supplied to the culture at a growth-limiting rate (i.e. the growth rate that is allowed by the rate of sugar addition is lower than \( \mu_{\text{max}} \)).

In fed-batch systems, a constant specific growth rate can be accomplished by an exponentially increasing feed rate that takes into account the increase in culture volume and biomass concentration. In practice, fed-batch cultivations are not operated at a constant growth rate but involve a feed profile that leads to a continuous decrease in growth rate. This profile is governed by a variety of factors, among others the oxygen-transfer and cooling capacities of industrial bioreactors (Beudeker et al., 1990; de Hollander, 1993). In addition to its experimental complexity, the use of fed-batch cultivation for fundamental studies on sugar transport suffers from the potential danger of a change in growth conditions related to the increase in biomass. For example, the formation of many toxic byproducts is linearly proportional to the amount of biomass, whereas adverse effects may be exponentially related to byproduct concentration. Even in the absence of byproduct formation, growth conditions that result in a constant (submaximal) growth rate can only be maintained for a relatively short period (hours rather than days). After this period, accumulation of biomass leads to a situation where oxygen-transfer properties of the fermenter set the limits for the medium feed.

**Chemostat cultivation**

Chemostat cultivation does not suffer from the disadvantages of batch and fed-batch cultivation and is therefore better suited for transport studies. In a chemostat, nutrients are continuously fed to the culture. The culture volume is kept constant by continuous removal, at the same rate, of culture fluid containing biomass, products and non-depleted nutrients (figure 4). The medium that is fed into the culture is designed in such a way that one nutrient of choice (for example the sugar) determines the biomass concentration in the culture. As a result, this limiting nutrient is almost completely consumed and its residual concentration in the culture is very low. The biomass yield \( Y_{sx} \) on the limiting nutrient is given by equation 2,

\[
Y_{sx} = \frac{C_x}{C_{s0} - C_s}
\]

in which \( C_{s0} \) is the reservoir concentration of the limiting nutrient and \( C_s \) and \( C_x \) are the residual substrate concentration and the biomass concentration in the culture, respectively. The flow rate at which the medium is fed to the culture (h⁻¹) divided by the volume of the culture (l) equals the dilution rate \( D \) (h⁻¹) (equation 3).

\[
D = \frac{\text{flow rate}}{\text{culture volume}}
\]

The reciprocal value of the dilution rate equals the time required for one volume change. Usually, after approximately 5 volume changes a steady-state situation is reached, in which the growth rate \( \mu \) equals the dilution rate \( D \), according to equation 4,

\[
\mu = D = \frac{\mu_{\text{max}} \cdot C_s}{K_s + C_s}
\]

in which \( C_s \) is the residual concentration of the growth-limiting nutrient and \( K_s \) the affinity constant for growth on this nutrient (Monod, 1949). In this steady state, the concentration of all nutrients, including the growth-limiting substrate, is constant in time. Since the rate of sugar consumption \( q_s \) equals \( \mu \times Y_{sx} \), this parameter can be manipulated by varying the dilution rate (figure 5). In practice, \( q_s \) is often a linear function of \( \mu \) since the biomass yield is usually growth-rate independent over a broad range of growth rates (Pirt, 1975).

**Methodology of chemostat cultivation in relation to sugar-transport studies**

Since the uptake of solutes by growing cultures is strongly dependent on environmental conditions, these conditions must not only be kept constant, but also defined. Using balanced mineral media and ade-
Figure 5. Residual glucose concentration ($C_r$, $q_s$), specific glucose-consumption rate ($D$, $q_s$) and biomass yield ($Y_{sx}$) as a function of the dilution rate in aerobic glucose-limited chemostat cultures of *Candida utilis* CBS 621. The glucose concentration in the cultures was determined after rapid sampling in liquid nitrogen. Data from Postma et al., (1988).

Figure 6. Schematic representation of sugar metabolism in yeasts. The ATP required for assimilatory purposes is generated by respiration and/or alcoholic fermentation. Part of the ATP is required for maintenance purposes (Pirt, 1975). This maintenance requirement can be artificially increased by the inclusion of non-metabolizable weak acids in the growth medium (Verduyn et al., 1990; 1992). As a result, the dissimilatory flux increases at the expense of the assimilatory flux, resulting in a lower biomass yield. When the growth rate is kept constant, this results in a higher specific rate of sugar consumption.

A parameter of obvious importance for studies on sugar transport in chemostat cultures is $C_s$, the residual sugar concentration in the culture. In comparison with studies of the kinetics of intracellular enzymes, studies of the relation between $C_s$ and in situ carrier activity appear to be relatively straightforward. For measurement of $C_s$, sample preparation only encompases the separation of culture supernatant from the cells and permeabilization of cells is not required. However, in sugar-limited chemostat cultures, and in particular those of Crabtree-negative yeasts, the residual sugar concentration is generally very low (usually well below 1 nM, table 1). Therefore, rapid sampling and separation of biomass from the growth medium is required to prevent consumption of the sugar during sampling. The following example may serve to illustrate this.

In an aerobic, glucose-limited chemostat culture of *Candida utilis* growing at $D = 0.3$ h$^{-1}$ with a reservoir concentration of 5 g glucose l$^{-1}$, the biomass yield and the residual glucose concentration are 0.5 g biomass g glucose$^{-1}$ and 18 µM, respectively (Postma et al., 1988). From these data, it follows that the specific rate of glucose consumption ($q_s$, see equation 1) equals 3.33 mmol glucose g biomass$^{-1}$h$^{-1}$. Since the reservoir concentration is 5 g l$^{-1}$ and $Y_{sx}$ = 0.5 g biomass g glucose$^{-1}$, the volumetric rate of sugar consumption equals 0.5 x 5 x 3.33 = 8.33 mmol h$^{-1}$ l$^{-1}$ = 2.3 µM s$^{-1}$. If this rate of sugar consumption were to continue during sampling, a sampling time of 8 seconds would result in complete depletion of the sugar initially present in the sample.

Minimization of substrate consumption during sampling for analyses of residual sugar concentrations should not only encompass ultra-short sampling times, but also the use of low steady-state biomass concentrations. This approach has been followed in the elegant studies of Egli et al. (1993) on the growth kinetics of chemostat cultures of *Escherichia coli* in which residual-sugar concentrations were in the nMolar range.

**Manipulation of metabolic fluxes via chemostat cultivation**

Since, in chemostat cultures, the specific rate of sugar consumption ($q_s$) obeys equation 1, $q_s$ can be manipulated by varying either $\mu$, $Y_{sx}$, or both. Variation of $\mu$ is easily accomplished by varying the dilution rate (equation 4). For example, using this approach, it is possible to vary the rate of transport in aerobic chemostat cultures of *C. utilis* over one order of magnitude ($\mu_0 = 0.05$ to $\mu_{\text{max}} = 0.59$ h$^{-1}$).

Alternatively, the rate of transport may be varied by manipulating $Y_{sx}$ at a constant growth rate. For example, in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* grown at $D = 0.10$ h$^{-1}$, $Y_{sx} = 0.5$ g g$^{-1}$. In anaerobic cultures grown at the same dilution rate, $Y_{sx} = 0.1$ g g$^{-1}$, which is associated with a five-fold higher glucose-uptake rate (equation 1).

By the addition of non-metabolizable weak organic acids (e.g. benzoic acid) to the growth medium of
Transport studies with chemostat cultures

Figure 7. Specific rates of glucose (O) and maltose (●) consumption ($q_s$) in anaerobic, sugar-limited chemostat cultures of *S. cerevisiae* CBS 8068 grown at a dilution rate of 0.10 h$^{-1}$ on mixtures of glucose and maltose. Specific sugar-consumption rates were calculated from the biomass concentration in the cultures and the amount of sugar consumed. $q_s$ has been plotted as a function of the percentage of the total hexose consumption in the cultures that was consumed as maltose. The curves are slightly bent due to the fact that the biomass yield on maltose is 25% lower than on glucose (see text). Glucose consumption during growth on maltose as a single substrate is due to an approximately 2% (mol/mol) contamination of the maltose with glucose. Data from Weusthuis *et al.* (1993).

sugar-limited chemostat cultures, enhancement of the sugar-transport rate can also be achieved at a fixed dilution rate (Verduyn *et al.*, 1990; Verduyn *et al.*, 1992). These compounds dissipate the trans-membrane pH gradient by diffusing from the acidic extracellular environment into the near-neutral cytosol. Inside the cells, the acid molecules dissociate. To prevent acidification of the cytoplasm, the released protons must be expelled from the cell by the plasma-membrane ATPase complex. As a result of the enhanced ATP requirement for intracellular-pH homeostasis, less ATP is available for biosynthetic purposes (figure 6). This in turn results in a decrease of the biomass yield ($Y_{sx}$) and, consequently, in an increase of the sugar uptake rate (equation 1). Yet another possibility to manipulate the specific rate of sugar transport in chemostat cultures is cultivation on mixed substrates. In batch cultures, utilization of sugar mixtures usually occurs via a sequential (diauxic) pattern (Monod, 1949) due to catabolite-repression phenomena. In contrast, the low residual-substrate concentrations in sugar-limited chemostat cultures allow the simultaneous utilization of sugar mixtures or mixtures of sugars and other substrates (Harder & Dijkhuizen, 1976; Egli *et al.*, 1993). For example, glucose and maltose can be utilized simultaneously in dual substrate-limited chemostat cultures of *S. cerevisiae* (Weusthuis *et al.*, 1993). In such cultures, the specific rates of glucose and maltose consumption can not only be manipulated by varying the dilution rate, but also by changing the relative concentrations of both sugars in the medium feed (figure 7).

A rather complex modulation of sugar-transport rates, caused by simultaneous changes of $\mu$ and $Y_{sx}$, occurs in aerobic, glucose-limited chemostat cultures of *S. cerevisiae*. At low dilution rates, growth of this yeast is fully respiratory and the glucose-uptake rate increases linearly with increasing $D$ (Käppeli *et al.*, 1986; Postma *et al.*, 1989b; Flechert & Seghezzi, 1992). However, above a certain critical dilution rate, respiration and alcoholic fermentation occur simultaneously, resulting in a decrease of $Y_{sx}$. Above the critical dilution rate, the simultaneous increase of $D$ and decrease of $Y_{sx}$ result in a strongly enhanced rate of sugar consumption (figure 8).

Summarizing, it can be concluded that chemostat cultivation offers a unique possibility to manipulate the rate of sugar transport in growing cultures. So far however, little is known about the mechanisms used by yeast cells to adapt their transport kinetics to accommodate these variations in flux. One example is discussed below.

Regulation of glucose transport in chemostat cultures

In most yeasts, more than one system for uptake of glucose may be present during growth on this sugar. In such cases, the specific rate of sugar consumption ($q_s$) is equal to the sum of the transport rates by the individual carriers according to equation 5,
Figure 9. Hanes plots of the kinetics of $^{14}$C-glucose transport by cells of *Candida utilis* CBS 621 grown in an aerobic, glucose-limited chemostat culture at a dilution rate of 0.52 h$^{-1}$. The intercept with the ordinate gives the apparent substrate-saturation constant ($K_m$) of the carrier and the slope equals its maximum capacity ($V_{max}$). Data from Postma et al. (1988).

\[
q_a = \sum_{i=1}^{n} \frac{V_{max,i} \cdot C_s}{K_{m,i} + C_s}
\]

(5)

in which $V_i$ is the in situ rate of transport mediated by carrier $i$, $V_{max,i}$ is the maximum capacity of transport system $i$ and $K_{m,i}$ its Michaelis-Menten constant for glucose.

The kinetic constants $K_m$ and $V_{max}$ for the individual carriers can be determined by measuring initial rates of $^{14}$C-glucose uptake by culture samples at different substrate concentrations. In such cases non-linear kinetic plots are obtained (figure 9). When also the residual glucose concentration in the culture is known, the in situ contribution of the different carriers can be calculated (figure 10). From figure 10 it is clear that in glucose-limited chemostat cultures of *C. utilis*, the contribution of the different carriers varies with the dilution rate. At dilution rates approaching $\mu_{max}$ (0.59 h$^{-1}$), synthesis of the two high-affinity carriers is repressed and a low-affinity carrier plays a predominant role.

The above results illustrate the usefulness of chemostat cultivation: in batch cultures, growth occurs at $\mu_{max}$ and the high affinity systems are not detectable. Only during the very short transition phase between the exponential growth phase and the stationary phase, synthesis of these carriers is derepressed. Nearly all studies on the mechanism and kinetics of high-affinity sugar transport in yeasts have therefore been performed with substrate-deprived cells.

In general, the facilitated-diffusion glucose-transport systems of Crabtree-positive yeasts, such as *S. cerevisiae* and *Schizosaccharomyces pombe* have a much higher $K_m$ for glucose than the high-affinity proton-sympot mechanisms that are common in Crabtree-negative yeasts (table 1). The observed correlation between transport kinetics and residual-sugar concentrations in glucose-limited chemostat cultures (table 1) suggests that the kinetic properties of glucose uptake may to a large extent determine the growth kinetics. The importance of transport kinetics for growth of yeasts under sugar-limited conditions becomes particularly evident when different yeasts compete for a single growth-limiting sugar. As can be predicted from table 1, *S. cerevisiae* is rapidly outcompeted in mixed cultures with the Crabtree-negative yeast *C. utilis* during aerobic, glucose-limited growth (figure 11). The better adaptation of various Crabtree-negative yeasts to growth at low sugar concentrations offers an explanation for the competitive advantage of so-called 'wild yeasts' when these contaminate industrial bakers'-yeast-production processes (Postma et al., 1989a).

**Chemostat cultivation and energetics of sugar transport**

The amount of ATP required for sugar-proton symport mechanisms is determined by two stoichiometries: the sugar-proton stoichiometry of the proton-sympot carrier and the ATP-proton stoichiometry of the plasma-membrane ATPase complex (figure 1). In principle, the overall ATP requirement for sugar uptake can be calculated by independent determination of each of these two stoichiometries, for example in
Figure 11. Competition between *Saccharomyces cerevisiae* CBS 8066 (●) and *Candida utilis* CBS 521 (○) in an aerobic, glucose-limited chemostat culture at a dilution rate of 0.10 h⁻¹. At zero time, a pure culture of *S. cerevisiae* was inoculated with 1% (on a dry-weight basis) of *C. utilis* cells. The number of cfu's of both yeasts are expressed as the percentage of the total cfu's. Numbers are plotted as a function of the number of volume changes. The residual-glucose concentration (□) decreases during the competition to the value that is typical for pure cultures of *C. utilis*. *S. cerevisiae* cannot maintain itself in the mixed culture due to its lower affinity for glucose. Data from Postma et al. (1989a).

References


Hauer R & Höfer M (1978) Evidence for interactions bet-
ween energy-dependent transport of sugars and the membrane potential in the yeast *Rhodotorula gracilis* (Rhodospiridium toruloides). J. Membrane Biol. 43: 335-349


Postma E, Scheffers WA & van Dijken JP (1990b) Kinetics of growth and glucose transport in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Yeast 5: 159-166


Tkeaz J & Lampen JO (1973) Surface distribution of invertase on growing *Saccharomyces* cells. J. Bacteriol. 113: 1073-1075


Maltose/proton co-transport in *Saccharomyces cerevisiae*

Comparative study with cells and plasma membrane vesicles

By C. C. M. van Leeuwen, R. A. Weusthuis, E. Postma, P. J. A. van den Broek and J. P. van Dijken

Abstract

Maltose/proton co-transport was studied in intact cells and in plasma membrane vesicles of the yeast *Saccharomyces cerevisiae*. In order to determine uphill transport in vesicles, plasma membranes were fused with proteoliposomes containing cytochrome c oxidase as a proton-motive force-generating system. Maltose accumulation, dependent on the electrical and pH-gradients, was observed. The initial uptake velocity and accumulation ratio in vesicles proved to be dependent on the external pH. Moreover, kinetic analysis of maltose transport showed that $V_{\text{max}}$ values greatly decreased with increasing pH, whereas the $K_m$ remained virtually constant. These observations were in good agreement with results obtained with intact cells, and suggest that proton binding to the carrier proceeds with an apparent pK of 5.7. The observation with intact cells that maltose is co-transported with protons in a one to one stoichiometry was ascertained in the vesicle system by measuring the balance between proton-motive force and the chemical maltose gradient. These results show that maltose transport in vesicles prepared by fusion of plasma membranes with cytochrome c oxidase-proteoliposomes behaves in a similar way as in intact cells. It is therefore concluded that this vesicle model system offers a wide range of new possibilities for the study of maltose/proton co-transport in more detail.

Introduction

A large number of yeast species possess sugar carriers capable of uphill transport. The energy for the accumulation of these sugars comes from the proton-motive force, which is created by the action of the plasma membrane ATPase. In *S. cerevisiae*, however, almost all sugars are transported by carrier mediated diffusion. A noticeable exception is maltose transport. The uptake and metabolism of maltose is inducible. When the growth medium contains maltose, and no other more favorable sugars, such as glucose, are present, the maltose permease and $\alpha$-glucosidase are synthesized (Harris & Millin, 1964). Controversy exist concerning the number of maltose carriers. High-affinity maltose transport is conceived to be a proton symport with a 1:1 stoichiometry and an affinity constant of 1-5 mM (Görts, 1969; Seaston et al., 1973; Postma et al., 1990). The existence of a low affinity carrier is debatable, but it could be a facilitated transporter with a $K_m$ of 70-80 mM (Busturia & Lagunas, 1985; Cheng & Michels, 1991). The rationale for the presence of an $H^+/maltose$ co-transport mechanism in *S. cerevisiae* is probably the fact that the cytoplasmic maltose-hydrolysing enzyme ($\alpha$-glucosidase; maltase) has a poor affinity for maltose ($K_m = 10-17$ mM, Needleman et al., 1978; Postma et al., 1990). Therefore, internal accumulation of the substrate is required when the external maltose concentration is low.

Studies on the mechanism of active sugar transport across the plasma membrane of yeasts have so far been limited to intact cells, since a system of plasma membrane vesicles, showing carrier-mediated transport, has only recently become available. Studies in such a model system have the advantage over intact cell studies that sugar transport is not influenced by its subsequent metabolism. Moreover, in a cell-free system, the transport parameters can be varied selectively. So far only the glucose and galactose permease of *S. cerevisiae* have been studied in plasma membrane vesicles in some detail (Franzusoff & Cirillo, 1983; Ongjoco et al., 1987; Ramos et al., 1999). These sugars, however, are transported via carrier-mediated diffusion, or possibly via sugar-kinase-dependent transport.

Studies on energy-coupled solute transport in plasma membrane vesicles have been scarce. PMF-driven-uphill-leucine transport was observed in vesicles of *S. cerevisiae*, but the accumulation ratios obtained were low (Opekara et al., 1987; Calahorra et al., 1989). Recently we have developed a procedure to measure energy-coupled uphill galactose transport in isolated plasma membranes of the yeast *Kluyveromyces marxianus* fused with proteo-
liposomes containing the PMF-generating cytochrome c oxidase (van Leeuwen et al., 1991). This method, based on reconstitution of bacterial transporters (Driessen et al., 1985), yielded vesicles capable of giving high sugar accumulations.

In this paper it is shown that our method of preparing membrane vesicles, containing the co-reconstituted cytochrome c oxidase, can also be applied to study active transport of maltose by the yeast S. cerevisiae.

Material and methods

Strain and growth conditions

*S. cerevisiae* CBS 8056 was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and was maintained on malt agar slopes at 4 °C. Chemostat cultivation was performed in a laboratory fermentor (Applikon Dependable Instruments, The Netherlands) with a working volume of 1 litre at 30 °C and a stirrer speed of 1000 rpm. The pH was maintained at 5.0 by the automatic addition of 1 M KOH. The fermentor was flushed with air at a flow rate of 1.0 l/min. The dissolved oxygen tension was above 50% air saturation. For continuous cultivation the dilution rate was set at 0.1 h⁻¹ and the working volume was maintained by efficient pumping when the culture fluid made contact with a sensor.

The mineral growth medium contained per litre: 5.0 g (NH₄)₂SO₄, 3.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 15.0 mg EDTA, 4.5 mg ZnSO₄·7H₂O, 0.3 mg CoCl₂·6H₂O, 1.0 mg MnCl₂·4H₂O, 0.3 mg CuSO₄·5H₂O, 4.5 mg CaCl₂·H₂O, 3.0 mg FeSO₄·7H₂O, 0.4 mg Na₂MoO₄·2H₂O, 1.0 mg H₂BO₃, 0.1 g KI and 0.05 ml siliconeantifoam (BDH). After heat sterilization at 120 °C and cooling, a filter sterilized vitamin solution was added, giving final concentrations per litre of: 0.05 mg biotin, 1.0 mg calcium pantothenate, 1.0 mg nicotinic acid, 25.0 mg inositol, 1.0 mg pyridoxine/ HCl, 1.0 mg thiamin/HCl and 0.2 mg p-aminobenzoic acid. In order to prevent hydrolysis, maltose was heat-sterilized separately (Postma & van den Broek, 1990) at 110 °C during 10 min and was added to the medium to a final concentration of 10 g·l⁻¹. The above described conditions resulted in maltose-limited growth at a cell density of 4.7 g dry weight·l⁻¹.

Isolation of plasma membranes

Membranes were isolated by the procedure described by van Leeuwen et al. (1991), except that the crude membranes were subjected to an additional washing in order to decrease the contamination with soluble cell-constituents, especially α-glucosidase. To this end, the pellet of centrifuged (20 min, 6200 g) crude plasma membranes was suspended in a large volume, e.g. 100 ml, buffer A (0.1 M glycine, 0.3 M KCl at pH 7.0), frozen in liquid nitrogen and defrosted at room temperature. After centrifugation (20 min, 6200 g) the pellet was resuspended in 15 ml of the same buffer and the procedure continued with the titration.

Isolation of cytochrome c oxidase

Cytochrome c oxidase was isolated according to Yu et al. (1975). The activity was determined by the method of Yonetani (1961).

Purification of lipids

Commercially obtained phospholipids were extracted with acetone/ether (Viltane et al., 1986). The lipids were dissolved in chloroform and stored at -20 °C under nitrogen.

Preparation of liposomes with reconstituted cytochrome c oxidase

Cytochrome c oxidase-containing proteoliposomes were prepared according to van Leeuwen et al. (1991), using Escherichia coli lipids (containing 50 % phosphatidyethanolamine).

Fusion of cytochrome c oxidase-containing liposomes with yeast plasma membranes

Cytochrome c oxidase-containing liposomes (5 mg lipid) and plasma membranes (0.25 mg protein) were thawed and mixed in 25 mM KP, 1 mM MgCl₂ or 100 mM KP, 1 mM MgCl₂ at the indicated pH in a volume of 300 µl in an Eppendorf reaction vessel. The mixture was frozen in liquid N₂ thawed at room temperature, and sonicated for 2 x 10 seconds in an ultra K42 sonication bath. During this sonication step the white suspension became clear. The obtained hybrid cytochrome oxidase liposome-plasma membrane vesicles (CL-PMV) were used immediately.

Internal volume

The internal volume, determined according to van Leeuwen et al. (1991), was 0.9 µl·mg phospholipid⁻¹.

Determination of the transmembrane electrical potential

The membrane potential (ΔΨΔΨ) was determined from the distribution of TPP⁺, using a TPP⁺ selective electrode (Shinbo et al., 1978). Fused vesicles (0.42 mg phospholipid) were suspended in 1.5 ml 25 mM KP, 1 mM MgCl₂ or in 100 mM KP, 1 mM MgCl₂ at the indicated pH and 2 µM TPP⁺. 'Energization' was started with 100 µM TMPD, 10 µM cytochrome c and 10 mM ascorbate (adjusted to the indicated pH with KOH). ΔΨΔΨ was calculated according to Loikema et al. (1982) and de Vrij et al. (1986), assuming symmetrical binding.
**Determination of the pH gradient**

The transmembrane pH gradient was measured according to Clement & Gould (1981), using pyranine (pK 7.2) and the same electron donor and buffer system as for the ΔV determination.

**Assay of sugar uptake in CL-PMV**

Uptake of sugar in fused CL-PMV was determined according to van Leeuwen et al. (1991), except that the buffer was 25 mM KPi, 1 mM MgCl₂ for uptake experiments at tracer (15 μM) concentration of sugar and initial sugar uptake experiments, or 100 mM KPi, 1 mM MgCl₂ for the 2.5 mM sugar uptake experiments. For kinetic analysis of uptake, the CL-PMV were energized 1 min before sugar addition and the PMF-driven maltose uptake was determined from the amount of label accumulated during the first 60 s at pH 5.5 and 90 s at pH 6.7. Diffusion and binding were determined with the same method, using lactose instead of maltose. The reaction was stopped by addition of 2 ml 100 mM ice-cold LiCl to the reaction mixture, followed by subsequent filtration on cellulose nitrate filters and washing.

**Kinetic analysis of sugar uptake in intact cells**

Samples of the culture fluid were washed twice in a mineral salts solution (5.0 g l⁻¹ (NH₄)₂SO₄, 3.0 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O) adjusted to the indicated pH with 1 M NaOH or 1 M phosphoric acid. Maltose uptake and binding of [U-¹⁴C] maltose to cells and filter were determined as described by Postma et al. (1988), using 200 μl washed cell suspension (0.9 mg dry weight) per assay.

**Kinetic analysis of proton uptake in intact cells**

Uptake of protons by intact cells was determined from the H⁺-disappearance from the medium, according to van Urk et al. (1989), 5.0 ml of 5.0 g l⁻¹ yeast suspension was used. To calculate H⁺ uptake rates, alkalization curves were calibrated with a standard NaOH solution.

**Protein**

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

**Materials**

Radiolabeled sugars: [U-¹⁴C]maltose (11.3-20 GBq mmol⁻¹), [D-glucose-1-¹⁴C]lactose (2.1 GBq mmol⁻¹), D-[U-¹⁴C]glucose (111 GBq mmol⁻¹), L-[U-¹⁴C]glucose (2.04 GBq mmol⁻¹) were obtained from Amersham (U.K.).

Miscellaneous chemicals were obtained as follows: L-α-phosphatidylethanolamine (type IX from E. coli) (Sigma P6999), maltose monohydrate (Merck art 5912), n-octyl-β-D glucopyranoside, tetraphenylphosphoniumbromide, carbonyl cyanide p-trifluoromethoxy-phenyldrazzone (Fluka); valinomycin, cytochrome c, phenylmethylsulfonyl fluoride and α-glucosidase (Boehringer); N,N,N',N'-tetrathemethyl-p-phenylene diamine (British Drug House); ascorbic acid (J.T. Baker Inc.); Pyranine (Eastman); nigericin (Sigma); bovine serum albumin (A-8022, 5.9 % H₂O; Sigma); Scintillator 299 (Packard Instrument Co.); Ready Safe Liquid Scintillation Cocktail (Beckman); cellulose nitrate filters pore size 0.45 μm (Schleicher & Schuell).

**Results**

In order to determine PMF-driven uphill maltose transport, membrane vesicles were prepared containing a PMF-generating system. Plasma membranes were fused with proteoliposomes containing cytochrome c oxidase by freeze/thaw/sonication, as adopted for the preparation of K. marxianus plasma membrane vesicles (van Leeuwen et al., 1991). Using the detection method for membrane fusion described by Hoekstra et al. (1984) it was established that fusion of plasma membranes with proteoliposomes had indeed taken place (see also Ongjoco et al., 1987; van Leeuwen et al., 1991). Contamination of plasma membranes with the maltose hydrolysing enzyme α-glucosidase (EC 3.2.1.20), which is present in the cell with high specific activity (2.5 μmol min⁻¹·mg total protein⁻¹; Postma et al., 1990), was decreased by introducing an extra freeze/thaw and washing step. This resulted in a 100-fold decrease of the specific enzyme activity, to 26 nmol min⁻¹·mg of protein⁻¹.

A previous study on galactose transport in K. marxianus membrane vesicles had shown that not all vesicles contained an active galactose transporter (van Leeuwen et al., 1991). This was attributed to the fact that maximal galactose transport rates in intact cells were low (35 μmol min⁻¹·g dry weight⁻¹). However, in the present study, the rate of maltose transport was high (260 μmol min⁻¹·g dry weight⁻¹. It might therefore be expected that CL-PMV of S. cerevisiae contained more active maltose carriers. The percentage CL-PMV with an active maltose transport carrier was determined by measuring passive maltose transport over the maltose proton symport carrier in non-energized vesicles. Since S. cerevisiae does not contain a permease for lactose, this sugar was used as control for binding and passive diffusion. Figure 1 shows that maltose was initially taken up faster than lactose. Subsequently there was a slow uptake of maltose occurring at the same rate as passive lactose diffusion. Assuming that the maltose and lactose passive permeability coefficients are identical, the difference between maltose and lactose uptake can be taken as a measure for carrier-mediated trans-
Figure 1. Maltose (○) and lactose (●) uptake in non-energized CL-PMV. Sugar (2.5 mM) was added at t = 0. The fraction of the equilibrium value was calculated from the ratio of the internal sugar concentration per sample and the internal sugar concentration at the diffusion equilibrium.

Table 1. PMF and maltose (2.5 mM) transport activity of CL-PMV upon energization with ascorbate, cytochrome c and TMPD. CL-PMV were prepared by fusion of plasma membranes and cytochrome c oxidase-containing proteoliposomes at the different pH values indicated. The reconstitution efficiency of the maltose permease did not vary significantly at the different pH values. PMF was measured 2 min after ‘energization’. The maltose transport data were corrected for passive diffusion into vesicles not containing a maltose carrier, and are expressed in nmoles min⁻¹mg⁻¹ of phospholipid⁻¹. Z is a factor converting pH units into mV units, and is equal to 60 mV.

<table>
<thead>
<tr>
<th>pH</th>
<th>ΔΨ (mV)</th>
<th>ZpH</th>
<th>PMF (mV)</th>
<th>Initial rate of maltose uptake</th>
<th>Accumulation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>-74</td>
<td>85</td>
<td>-159</td>
<td>8.7</td>
<td>19.0</td>
</tr>
<tr>
<td>5.9</td>
<td>-98</td>
<td>62</td>
<td>-160</td>
<td>3.7</td>
<td>11.0</td>
</tr>
<tr>
<td>6.2</td>
<td>-106</td>
<td>63</td>
<td>-169</td>
<td>2.7</td>
<td>7.5</td>
</tr>
<tr>
<td>6.7</td>
<td>-108</td>
<td>37</td>
<td>-145</td>
<td>1.3</td>
<td>4.7</td>
</tr>
<tr>
<td>7.0</td>
<td>-110</td>
<td>31</td>
<td>-141</td>
<td>0.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Figure 2. Effect of ionophores on maltose uptake in energized CL-PMV at pH 5.5. Maltose (2.5 mM) was added at t = 0. Nigericin (37 nM, ■) or valinomycin (100 nM, □) was added at t = -2 min; ●, simultaneous addition of nigericin (37 nM) and valinomycin (100 nM) at t = -9 min; ○, no additions. Energization with ascorbate, TMPD and cytochrome c was performed at t = -1 min. All measurements were corrected for binding and diffusion. [S] is the internal maltose concentration in those vesicles that contained a maltose carrier.

by preparing vesicles at different lipid/protein ratios. This suggests that all vesicles participated in the generation of the PMF. This appears reasonable since cytochrome c oxidase was present in excess, resulting in a vesicle preparation devoid of oxidase-free liposomes as was shown by Driessen et al. (1986) and de Vrij et al. (1986). It can therefore be assumed that, in contrast with the maltose permease, apparently all vesicles contained cytochrome c oxidase, capable of generating a PMF with externally added cytochrome c. To determine PMF-driven transport, maltose (2.5 mM) was added to energized CL-PMV at pH 5.5. The intravesicular maltose concentration was calculated as the difference between maltose uptake and lactose diffusion, devided by the volume of the vesicles containing a maltose specific transport system (i.e. 30-35% of the internal volume of the total vesicle preparation). Uphill transport resulted in an accumulation ratio of 19 (figure 2, table 1), whereas glucose (2.5 mM) transport under the same conditions did not exceed the passive diffusion level (results not shown). Addition of the ionophores nigericin and valinomycin, which dissipate the ΔΨ and the ΔΨ⁺ respectively, lowered the level of accumulation. Moreover, addition of the uncoupler FCCP of nigericin and valinomycin simultaneously, resulted in efflux of accumulated maltose (figure 2). This maltose efflux proceeded by apparent biphasic kinetics, as has also been observed for galactose efflux from K. marxianus CL-PMV (van Leeuwen et al., 1991). The ratio-
Figure 3. Effect of pH on the maximal uptake rate of [14C]maltose in S. cerevisiae. The different symbols refer to cell suspensions from different steady states. For the calculation of these parameters, uptake experiments were performed with maltose concentrations ranging from 0.5 to 8.0 mM.

Table 2. Relationship between PMF and maltose uptake in CL-PMV at pH 5.5. All values were determined at the time steady-state sugar accumulation was reached. Maltose accumulation concerns only the vesicles containing a maltose carrier.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ΔΨ</th>
<th>ΔpH</th>
<th>PMF</th>
<th>Δmaltose/F</th>
<th>n_app.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-46</td>
<td>70</td>
<td>-116</td>
<td>91</td>
<td>0.8</td>
</tr>
<tr>
<td>Nigericin</td>
<td>-70</td>
<td>-70</td>
<td>-77</td>
<td>68</td>
<td>1.3</td>
</tr>
<tr>
<td>Valinomycin (37 nM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>68</td>
<td>0.9</td>
</tr>
<tr>
<td>Valinomycin (100 nM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>68</td>
<td>0.9</td>
</tr>
</tbody>
</table>

nane for the slow second efflux phase remains as yet unclear. At an external concentration of 15 μM maltose, accumulation ratios of 30-35 were reached. To determine the influence of the pH on influx velocity and the accumulation ratio of maltose, CL-PMV were prepared at pH values of respectively 7.0, 6.7, 6.2, 5.9 and 5.5. The total PMF, as measured with TPP+ distribution (ΔΨ) and the fluorescent probe pyranine (ΔpH), showed only a slight decrease with increasing pH (table 1). It was found that the electrical component of the PMF (ΔΨ) decreased with decreasing pH, whereas the ΔpH increased with decreasing pH, resulting in a virtually constant internal pH of 7.0-7.5. Although the PMF was high and should account for a high influx velocity and accumulation ratio over the total pH range, the values decreased dramatically with increasing external pH (table 1). Addition of nigericin inhibited maltose transport at low pH values (see also figure 2) whereas, at high pH, transport was not inhibited or even slightly stimulated. The stimulation is due to the fact that the high ΔΨ value at pH 6.7 and 7.0 increased upon addition of nigericin.

The kinetic constants of maltose transport both in CL-PMV and intact cells was measured as a function of the external pH. In CL-PMV, initial uptake rates of maltose and lactose at sugar concentrations ranging from 0.5 to 20.0 mM at pH 5.5 and 6.7 were determined. Analysis of these data by nonlinear regression, revealed that the affinity constant did not vary with the pH (Km = 9.9±2.4 mM at pH 5.5 and 10.6±1.2 mM at pH 6.7) whereas the Vmax at pH 6.7 was 6 times lower than at pH 5.5 (Vmax = 40.0±4.3 nmol.min⁻¹mg phospholipid⁻¹ at pH 5.5 and 6.3±0.3 at pH 6.7). In intact cells initial maltose uptake studies were performed at sugar concentrations ranging from 0.5 to 15.0 mM and at pH values from 3.0 to 7.0. Plots of these data also showed a small variation in maltose affinity (Km = 2.4 mM), whereas an optimum in Vmax was observed of 260 μmol.min⁻¹g dry weight⁻¹ at pH 4.0 to 4.5, decreasing to 50 μmol.min⁻¹g dry weight⁻¹ at pH 7.0 and to 100 μmol.min⁻¹g dry weight⁻¹ at pH 3.0 (figure 3).

The stoichiometry of the maltose/proton cotransport was determined in two ways. In intact cells the H⁺/maltose stoichiometry was determined by measuring proton uptake rates and [14C]maltose uptake rates at concentrations ranging from 0.5-15.0 mM at pH 5.0 and comparing Vmax values calculated assuming Michaelis Menten kinetics. The data obtained resulted in a H⁺/maltose stoichiometry of 0.8 (Vmax = 176±8 μmol.min⁻¹g dry weight⁻¹ for proton uptake and 218±30 for maltose influx). In energized vesicles the H⁺/maltose stoichiometry was determined from the steady-state maltose accumulation ratio at a tracer external maltose concentration (15 μM) and measurements of PMF at the time at which maximal maltose accumulation was observed, i.e. 15 to 20 min after sugar addition. Table 2 shows the values of the PMF and the chemical maltose gradient (Δμmaltose/F). Since the maltose uptake is both ΔΨ and ΔpH driven, data of experiments in the presence of nigericin or valinomycin are included. The stoichiometry, n_app., was calculated by the Mitchell equation (Driessen et al., 1987);

\[ n_{\text{app}} = \Delta \mu_{\text{maltose}} \times (F \times \text{PMF})^{-1} \]

The results are shown in table 2.

Discussion

S. cerevisiae was grown in chemostat culture under maltose limitation. The use of cells obtained via this cultivation technique offers several advantages for studies on active maltose transport as compared to batch cultures. Due to the low residual maltose concentration in the culture (approximately
0.1 mM), it can be expected that a possible low-affinity (passive) transport is absent, analogous to studies on glucose transport in chemostat-grown *Candida utilis* (Postma *et al.*, 1988). Furthermore, since cells are grown under essentially constant conditions, low batch variation in transport activity can be expected. Indeed, chemostat cultivation allowed for reproducible isolation of batches of plasma membrane vesicles with little variation in transport activity.

The results presented in this paper clearly establish that maltose uptake proceeds through a PMF-driven process. In vesicles this was concluded from the fact that PMF generation caused intravesicular accumulation of maltose. Accumulation ratios of 30-35 ($\Delta \mu_{\text{maltose}}/F = \pm 91 \text{ mV}$) were reached at low external concentrations of maltose (15 $\mu$m) (table 2). Since steady-state maltose accumulation ratios obtained are dependent on the maltose concentration used (figure 2 and table 2), this strongly suggests that passive transmembrane solute leakage becomes more important at high concentrations, as was concluded before (Driessen *et al.*, 1987; van Leeuwen *et al.*, 1991).

The influence of the extracellular or extravesicular pH on [14C]maltose uptake was very pronounced. Although the pH at which the vesicles were fused hardly affected the total PMF generated, it influenced the $\Delta V'$ and the $\Delta p$H in opposite ways (table 1). A similar relation between $\Delta V'$, $\Delta p$H and external pH was found for *Bacillus subtilis* membrane vesicles (de Vrij *et al.*, 1987). In contrast to the PMF, both accumulation ratio and initial influx velocity were negatively affected by increasing pH (table 1). This effect has also been observed for amino acid/proton symporter carriers in membrane vesicles of *Streptococcus cremoris* (Driessen *et al.*, 1987). The apparent influence of the pH on maltose accumulation is probably due to a counterbalancing of maltose uptake by passive maltose efflux. At high pH values, maltose influx is slow compared to outward diffusion, leading to a low accumulation ratio.

Kinetic studies showed that the $K_m$ in vesicles is higher than in intact cells. A similar difference has also been found for galactose transport in *K. marxianus* and for galactose uptake in *S. cerevisiae* (van Leeuwen *et al.*, 1991; Ramos *et al.*, 1989). This difference might indicate that the lipid environment of the carrier in vesicles is suboptimal compared with intact cells. Kinetic analysis in both vesicles and cells showed that, whereas the affinity of the carrier for maltose is not affected by pH, its maximal velocity decreases with decreasing external proton concentration. Studies on the effect of the external pH on the growth parameters of steady-state maltose-limited continuous culture of *S. cerevisiae* revealed similar conclusions (Olivero *et al.*, 1982). Plots of $V_{\text{max}}$ values of cells (figure 3) and the initial influx velocity of vesicles (in a pH range of 5.5-7.0) (table 1) against the external proton concentration, revealed an apparent pK of 5.7 and of 5.5 respectively. These values are slightly lower than those found for the sorbose/ and galactoside/proton symporters of *K. marxianus*, which were 6.2-6.4 (van den Broek & van Steveninck, 1980, 1982). For these carriers proton binding was suggested to proceed to the imidazole group of histidine, which has a pK of 6.0. Since the pK value for maltose transport is also close to 6, a histidine residue might be involved in the H⁺ binding in maltose/proton symport of *S. cerevisiae*. It should be noted that a pH sensitive $V_{\text{max}}$ and a pH insensitive $K_m$ has also been observed for other sugar transport systems in yeast (van den Broek & van Steveninck, 1980, 1982). This suggests that the symport mechanism in these yeasts might be similar and possibly has a random reaction sequence. Further studies are, however, needed to fully clarify the reaction mechanism.

The H⁺-maltose stoichiometry of the maltose symporter was estimated to be about 1, as follows from the ratio of H⁺ to maltose influx into intact cells (Eddy *et al.*, 1977; Serrano, 1977). This was confirmed in our cell-experiments as well as in CL-PMV studies. It is noteworthy that the latter value follows from the relation between the chemical gradient of solute ($\Delta \mu_{\text{sol}}/F$) and the PMF or its components (table 2). The data with CL-PMV in particular, clearly show that maltose transport is a PMF-driven process with a 1:1 H⁺/maltose stoichiometry. Moreover, the fact that maltose accumulation and the PMF reach a thermodynamic equilibrium in vesicles as well as the fact that maltose uptake kinetics in vesicles resemble those of intact cells, shows that our method of preparing plasma membrane vesicles from *S. cerevisiae* successfully reconstitutes maltose transport. Therefore it can be expected that studies with CL-PMV will greatly advance the knowledge of active transport mechanisms of *S. cerevisiae*.

**Abbreviations**

- PMF, proton-motive force; CL-PMV, hybrid cytochrome c oxidase liposome/plasma membrane vesicles, $\Delta V'$, transmembrane electrical potential; $\Delta p$H, transmembrane pH gradient; TPP⁺, tetraphenylphosphonium; TMPD, N,N,N,N-tetramethyl-p-phenylenediamine; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone.

**References**

- van den Broek PJA & van Steveninck J (1980) Kinetic analysis of simultaneously occurring proton-sorbose symport and passive sorbose transport in *Saccharomyces*
In vitro maltose transport in *S. cerevisiae* 45


Shinbo T, Kamo N, Kurihara K & Kobatake Y (1978) A PVC-based electrode sensitive to DDA+ as a device for

fragilis*. Biochim. Biophys. Acta 602: 419-432


monitoring the membrane potential in biological systems. Arch. Biochem. Biophys. 167: 414-422


Energetics and kinetics of maltose transport in *S. cerevisiae*

a continuous-culture study

by R. A. Weusthuis, H. Adams, W. A. Scheffers and J. P. van Dijken.

Abstract

In *Saccharomyces cerevisiae* maltose is transported by a proton symport mechanism, whereas glucose transport occurs via facilitated diffusion. The energy requirement for maltose transport was evaluated with a metabolic model based on an experimental value of $Y_{\text{ATP}}$ for growth on glucose and an ATP requirement for maltose transport of 1 mol-mol$^{-1}$. The predictions of the model were verified experimentally with anaerobic sugar-limited chemostat cultures growing on a range of maltose-glucose mixtures at a fixed dilution rate of 0.1 h$^{-1}$. The biomass yield (g cells/g sugar$^{-1}$) decreased linearly with increasing amounts of maltose in the mixture. During growth on maltose, the yield was 25% lower than on glucose, in agreement with the model predictions.

During sugar-limited growth the residual concentration of maltose and glucose in the culture increased in proportion to their relative concentration in the medium feed. From the residual maltose concentration, the *in situ* rates of maltose consumption by cultures and the $K_m$ of the maltose carrier for maltose, it was calculated that the amount of this carrier was proportional to the *in situ* maltose consumption rate. This was also found for the amount of intracellular maltase. These two maltose-specific enzymes therefore exert a high control over the maltose flux in *S. cerevisiae* in anaerobic sugar-limited steady state cultures.

Introduction

Energy required for transport processes is derived from the gradient of the solute over the membrane (passive transport) or is delivered by metabolic processes at the expense of metabolic energy (active transport). The energy costs of transport are especially important in cellular metabolism if the actively transported solute serves as the source of carbon and energy. This affects growth in two ways: ATP requirement for biomass formation is higher and energy yield of dissimilation is lower. The energy requirement for transport processes may take a large portion of the total energy budget of the cell if the energy yield of the substrate is low. An example is growth of *Pseudomonas oxalaticus* on oxalate. In this case, half the energy obtained in respiration of the growth substrate is required for transport of the dicarboxylic acid (Dijkhuizen et al., 1977a, b). However, also during growth of yeasts on sugars in mineral media, the energy requirement for sugar transport can be substantial. Verdun et al. (1991), calculated that the theoretical energy cost of sugar transport in the yeast *Candida utilis* growing on glucose is 8.2 mmol ATP/g biomass$^{-1}$ or 20% of the total ATP requirement.

Maltose is an important sugar in the production of beer and the leavening of certain doughs (Beudeker et al., 1960). So far, however, specific effects of maltose on yeast physiology, such as the energetics of growth, have received little attention. Most investigations were aimed at glucose metabolism. However, maltose is transported by proton symport in *S. cerevisiae* (Serrano, 1977; van Leeuwen et al., 1992), whereas glucose is taken up by facilitated diffusion. In this study an attempt is made to quantify the ATP-requirements of maltose transport in *S. cerevisiae*, via a comparison between growth on glucose and growth on maltose.

Material and methods

**Strain and growth conditions**

*S. cerevisiae* CBS 8066 was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slopes at 4 °C. Chemostat cultivation was performed in ADI 2-litre bioreactors (Applikon Dependable Instruments) at a dilution rate of 0.10 h$^{-1}$ and a working volume of 1.00 litre. Cultures were grown under carbon and energy limitation on a mineral medium described below. Removal of effluent by the standard procedure - continuous, upwards directed suction from the surface of the culture - gave rise to differences in cell density between culture and effluent of
up to 20%. Under these conditions the continuous-culture theory is not valid (See also Noorman et al., 1991). Removing effluent from the middle of the culture when the culture surface made contact with an electrical sensor did not give rise to such a difference and was used throughout this study.

The temperature was 30 °C and the stirrer speed was 750 rpm. The pH was kept constant at 5.0 by an ADI 1020 biocontroller, by the automatic addition of 2 M KOH. To assure anaerobic conditions the reactor and the reservoir vessel were flushed with nitrogen gas with a flow rate of 0.5 l/min. The flow rate was kept constant by a Brooks 5876 gas flow controller. The whole experimental set up (reactor, reservoir and waste vessel) was equipped with nonpre tubing (Cole-Palmer Corp.). The dissolved-oxygen tension of the culture was continuously monitored with an oxygen electrode (Ingold, 34 100 3002) and was below 0.1% air saturation.

The mineral medium contained per litre: 5.0 g (NH₄)₂SO₄, 3.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 15.0 mg EDTA, 4.5 mg ZnSO₄·7H₂O, 0.3 mg CoCl₂·6H₂O, 1.0 mg MnCl₂·4H₂O, 0.3 mg CuSO₄·5H₂O, 4.5 mg CaCl₂·2H₂O, 3.0 mg FeSO₄·7H₂O, 0.4 mg Na₂MoO₄·2H₂O, 1.0 mg H₂BO₃, 0.1 mg KI and 0.15 ml silicone antifoam (BDH). After heat sterilization at 120 °C and cooling, a filter-sterilized vitamin solution was added to final concentrations per litre of: 0.05 mg biotin, 1.0 mg calcium pantothenate, 1.0 mg nicotinic acid, 25.0 mg inositol, 1.0 mg thiamin·HCl and 0.2 mg p-aminobenzoic acid. Ergosterol and Tween-80 were dissolved in pure ethanol and steamed at 100 °C for 10 min before being added to the medium to final concentrations of 10 mg/l and 420 mg/l, respectively. Maltose monohydrate and glucose were sterilized separately (Postma & van den Broek, 1990) and added at the ratios indicated to a final sugar concentration of approximately 25 g/l.

**Determination of dry weight and elemental analysis**

The dry weight of the cultures was determined using a microwave oven and 0.45 μm filters according to Postma et al. (1989a). The carbon, hydrogen and nitrogen composition of the biomass was determined with an Elemental Analyzer 2408 (Perkin Elmer) (Verduyn et al., 1990a).

**Sugar analysis**

The sugar concentrations in the reservoir vessels were determined with a glucose analyzer (YSI 2000, Yellow Springs Instruments). Maltose was first hydrolysed to glucose by α-glucosidase (Boehringer 105 414) (Postma et al., 1990). Maltose was found to contain glucose and maltotriose as impurities (both approximately 3% (w/w) after heat sterilization). Maltotriose is also hydrolysed to glucose by α-glucosidase and is not metabolized by S. cerevisiae CBS 8066 (cf. Harris & Thompson, 1960; Yamamoto & Inoue, 1961). The maltose concentration in reservoir media and culture supernatants was therefore corrected for the amount of maltotriose and glucose present.

For the determination of residual substrate concentrations a culture sample was taken from the culture, frozen in liquid nitrogen within 2 seconds and stored at -40 °C. Prior to the determination of the concentrations, the sample was thawed and centrifuged at 0 °C. The residual concentrations of maltose and glucose were determined with Boehringer test kit 678543 before and after treatment of the supernatant with α-glucosidase. Although rapid sampling was performed throughout this study it can be calculated that this is not a prerequisite for obtaining accurate data on residual sugar concentrations. For example, the highest maltose consumption rate was 3.5 mmol·g⁻¹·h⁻¹ at a residual maltose concentration of 0.6 mm. Thus, with the amount of cells present (1.6 g/l), in two seconds maximally 1.6 × 3.5 / (60 × 30) = 0.003 mmol of maltose or 0.003 / 0.6 = 0.5 % disappeared during sampling.

**Metabolite analysis**

Ethanol, glycerol, maltotriose and organic acids (2-oxoglutaric acid, pyruvic acid, succinic acid and fumaric acid) were determined simultaneously by HPLC analysis using an HPLC-Biochromel Aminex exclusion column (300 × 7.8 mm, BioRad) at 30 °C. The column was eluted with 5 mm sulphuric acid at a flow rate of 0.6 ml/min⁻¹. Organic acids were detected by a Waters 441 UV-meter at 214 nm, coupled to a Waters 741 data module. Ethanol, glycerol and maltotriose were detected by an ERMA ERC-7515A refractive index detector coupled to a Hewlett Packard 3390A integrator. The amount of ethanol produced was corrected for the amount of ethanol in the reservoir (approximately 10 mm ethanol, originating from the addition of ergosterol). Acetic acid could not be determined with this HPLC method since it had the same retention time as one of the medium components. It was therefore determined using the Boehringer test kit 148261.

Metabolite fluxes were calculated as q = c × D / X in which q stands for flux (mmol·g biomass⁻¹·h⁻¹), c for the amount of substrate or product consumed or produced (mmol⁻¹), D for the dilution rate (h⁻¹) and X for the biomass concentration in the culture (g dry weight⁻¹).

**Gas analysis**

The gas flowing out of the reactor was cooled in a condenser (2 °C) and dried with a Perma Pure Dryer (PD-625-12P). CO₂ was determined with a Beckman model 864 infrared detector. The effluent gas flow rate was measured with a self-constructed device. It consisted of an inverted glass cylinder, filled with water. Under this cylinder, a water reser-
Enzyme analysis

Enzyme assays were performed with a Hitachi model 100-60 spectrophotometer at 30 °C. Reaction rates were linearly proportional to the amount of enzyme added. Preparation of cell-free extracts and assays of pyruvate decarboxylase (EC 4.1.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were done according to Postma et al. (1989b). Citrate synthase (EC 4.1.3.7) activity was determined according to Srere (1969) and hexokinase (EC 2.7.1.1) according Postma et al. (1988).

Maltose (EC 3.2.1.20) activity was measured with a discontinuous assay. The reaction mixture contained 100 mM acetate buffer pH 6.6 and 60 mM maltose. The reaction was carried out in 1.0 ml at 30 °C and was started by addition of cell free extract. The reaction was stopped at different time intervals by the addition of 10 μl 75 % (w/v) trichloric acid. Before the amount of glucose in the reaction mixture was determined with the glucose assay described above, the pH of the sample was neutralized by the addition of 4.5 μl 10 M NaOH. All enzyme activities are expressed as μmol substrate converted·min⁻¹·mg protein⁻¹.

Maltose transport assay

Maltose uptake rates were determined as described by van Leeuwen et al. (1992) by measuring alkalization of a weakly buffered cell suspensions after the addition of maltose.

Protein determination

The protein content of whole cells was determined by a modified biuret method (Verduyn et al., 1990a). The amount of protein in cell free extracts was determined by the Lowry method.

Graphical representation of data

All metabolic parameters are plotted as a function of the composition of the sugar mixture that is utilized in terms of hexose units on a molar basis according to 2 × (maltose in feed - residual maltose) / (2 × (maltose in feed - residual maltose) + glucose in feed - residual glucose).

Results

The disaccharide maltose is a good model substrate to evaluate the energy requirements of active sugar transport in S. cerevisiae. Since maltose hydrolysis does not require ATP, the only difference between the energetics of maltose and glucose metabolism resides in the transport step: transport of maltose is active, whereas transport of glucose is passive. It is therefore appropriate to use established data on the energetics of growth on glucose for predicting the growth efficiency on maltose.

Metabolic model

A metabolic model for the anaerobic growth of S. cerevisiae CBS 6066 with glucose as carbon and energy source (Verduyn et al., 1990a) is summarized in figure 1A. The overall equation (carbon compounds in mmol) for biomass formation is

\[
\text{5394 glucose} \rightarrow 100 \text{ g biomass} + 1102 \text{ glycerol} + 8240 \text{ ethanol} + 8825 \text{ CO}_2
\]

Maltose is regarded as two glucose units (in fact maltose consists of two glucose units minus one water molecule). It was therefore convenient to use maltose monohydrate as carbon source. Assuming that the ATP requirement for maltose transport is 1 ATP, caused by a proton/maltose stoichiometry of 1 of the maltose transporter (Serrano, 1977; van Leeuwen et al., 1992) and a proton/ATP stoichiometry of 1 of the plasma membrane ATPase (Malpartida & Serrano, 1981; Nelson & Taiz, 1989; Perlin et al., 1986), the net ATP production from 1 mol maltose is 3 mol ATP (figure 1B). Therefore, as compared to growth on glucose, a deficit of 5394 / 2 = 2697 mmol ATP will occur in the formation of 100 g biomass from an equivalent amount of maltose:

\[
\text{2697 maltose} \rightarrow 100 \text{ g biomass} + 1102 \text{ glycerol} + 8240 \text{ ethanol} + 8825 \text{ CO}_2 - 2697 \text{ ATP}
\]

This can be replenished by the additional dissimilation of 2697 / 3 = 899 mmol maltose:

\[
\text{899 maltose} \rightarrow 3596 \text{ ethanol} + 3596 \text{ CO}_2 + 2697 \text{ ATP}
\]

Therefore the overall equation for the formation of 100 g biomass from maltose under anaerobic conditions is

\[
\text{3596 maltose} \rightarrow 100 \text{ g biomass} + 1102 \text{ glycerol} + 11836 \text{ ethanol} + 12421 \text{ CO}_2
\]
This model can be used to predict the production of dry weight, ethanol, CO₂ and glycerol during growth of S. cerevisiae with glucose and maltose as carbon sources in chemostat cultures at a dilution rate of 0.1 h⁻¹. Thus, for example, whereas the biomass yield during growth on glucose is 100 / (5.394 × 180) = 0.103 g g⁻¹ (equation 1), the theoretical biomass yield on maltose is 100 / (3.596 × 360) = 0.077 (equation 4). The ethanol production during growth on glucose is 8240 / 100 = 82.4 mmol g biomass⁻¹ (equation 1). With a dilution rate of 0.1 h⁻¹ this is produced in 10 h or 8.24 mmol g biomass⁻¹ h⁻¹. Similarly, from equation 4 it follows that the theoretical specific production rate on maltose is 11936 / 100 × 0.1 = 11.84 mmol g bio-mass⁻¹ h⁻¹ (table 1).

Methodology of chemostat cultivation on sugar mixtures

For a comparison of anaerobic glucose and maltose metabolism it is important to keep the growth conditions the same, e.g. growth rate, temperature, pH, etc. The only suitable cultivation technique that can meet these requirements is the chemostat. Moreover, since carbon-limited chemostat cultivation gives rise to low residual substrate concentrations, it is possible to circumvent repression effects and to cultivate organisms on two carbon sources simultaneously (Harder & Dijkhuizen, 1976). This gives the opportunity to cultivate S. cerevisiae on various mixtures of glucose and maltose in the feed (Hågström & Cooney, 1984). The metabolic model can thus not only be tested for growth on glucose and maltose as sole carbon sources, but also for growth on various mixtures. The theoretical yields, production and consumption rates on mixtures of glucose and maltose can be calculated as the summation of the parameters for growth on the separate sugars. Thus, since a 25% decrease in cell yield is predicted according to the model (1), growth on a 1:1 mixture of maltose and glucose (in terms of hexose units) should result in a 12.5% decrease. The advantage of this approach is obvious: the calculation of the energetics of maltose transport is not dependent anymore on one measurement, but the model can be checked by many measurements.

During growth of S. cerevisiae CBS 8066 on mixtures of maltose and glucose a peculiar problem was encountered: cells tended to change their morphology upon changes in cultivation conditions. Cultures grown on mixtures of these sugars gave rise to the occurrence of pseudohyphae (figure 2). This was associated with a decrease in biomass densities in the culture after steady-state conditions should have been reached (i.e. after approximately 5 volume

---

**Table 1.** Predicted yields (Y, in g g sugar⁻¹) of biomass and ethanol and specific fluxes (q, in mmol g⁻¹ h⁻¹) of substrates and products in anaerobic carbon-limited chemostat cultures at D = 0.1 h⁻¹ of *Saccharomyces cerevisiae* CBS 8066 with maltose monohydrate as carbon sources. Established data for growth on glucose (Verduyn, 1990a) were used to predict growth parameters on maltose with the model outlined in figure 1. The values of these parameters for growth on maltose as percentages of the values on glucose are also given.

<table>
<thead>
<tr>
<th>carbon source</th>
<th>maltose/glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>biomass</td>
<td>0.103</td>
</tr>
<tr>
<td>ethanol</td>
<td>0.39</td>
</tr>
<tr>
<td>ethanol</td>
<td>8.24</td>
</tr>
<tr>
<td>CO₂</td>
<td>8.83</td>
</tr>
<tr>
<td>glycerol</td>
<td>1.10</td>
</tr>
<tr>
<td>glucose</td>
<td>5.29</td>
</tr>
<tr>
<td>maltose</td>
<td>0</td>
</tr>
<tr>
<td>glucose</td>
<td>5.29</td>
</tr>
</tbody>
</table>
changes). This phenomenon occurred despite the precautions with respect to the effluent removal system (see Material and Methods). Regular checks on the biomass densities in the culture and the culture effluent made clear that the surface level sensing for effluent removal was appropriate: The biomass density in the culture was always within 1% of that of the culture effluent, suggesting that occurrence and selection of pseudohyphae could not be ascribed to an inadequate effluent removal.

The formation of pseudohyphae only occurred during growth on glucose-maltose mixtures, not with glucose or maltose as the sole carbon source. It was not reproducible but seemed to be triggered by changes in the glucose-maltose composition of the medium. For example, when a culture was switched from a steady-state situation with maltose as sole carbon source to growth on a 75% glucose-25% maltose-feed mixture, no pseudohyphae were detected after one month. When this culture was subsequently grown on glucose as sole carbon source until steady state was established and then switched to a 25% glucose-75% maltose-feed mixture, pseudohyphae formation started after two days (figure 2). Plating of this culture on malt agar gave rise to the development of two colony types, one consisting of 'normal' cells and one of elongated cells. To exclude the possibility that the elongated cells were an infection, both colony types were tested by the Centraalbureau voor Schimmelcultures (CBS) using standard determination tests and were found to be phenotypically identical.

The volume/surface ratio of cells is known to influence the cellular energetics (Kooijman et al., 1991). In order to circumvent possible effects of cell morphology on the bioenergetics of growth, a standard procedure was adopted for steady-state cultivation on mixtures of maltose and glucose that avoided changes in morphology as follows: for each mixture, cultivation was started in a sterilized reactor by batch cultivation. The medium pump was switched on immediately after inoculation, cells were allowed to grow aerobically for two hours and then switched to anaerobic conditions. To check for a steady-state situation regular analysis of biomass and product concentrations was performed. Using this procedure steady states without pseudohyphae were obtained within six volume changes.

**Verification of the metabolic model**

*S. cerevisiae* CBS 8066 was grown anaerobically at D = 0.1 h⁻¹ on glucose-maltose mixtures ranging from 100% glucose to 100% maltose according to the procedure described above at a total reservoir sugar concentration of approximately 25 g l⁻¹. With increasing maltose concentrations the biomass decreased in a linear fashion from 2.42 g l⁻¹ on glucose to 1.62 g l⁻¹ on maltose. The glycerol concentration decreased in parallel to the dry weight. The ethanol concentration and the amount of CO₂ in the off-gas slightly increased (table 2). Not all products were identified by HPLC, but the unidentified products were found under all conditions and their concentrations did not change significantly. Furthermore, the carbon recovery of identified products in all steady states was between 98 and 104%.

The experimental data of table 2 were used to calculate the actual biomass yields and specific fluxes of ethanol, glycerol, CO₂, glucose and maltose. These data, plotted in figure 3, fit well with the metabolic model (drawn lines). For example during growth on a mixture of 36.7 mm maltose and 52.2 mm glucose in the medium feed (table 2) it can be calculated that the biomass yield (defined as grams of biomass per gram of sugar) equals 1.92 / ((36.7 · 0.45) × 0.360 + (52.2 · 0.36) × 0.180) = 0.086 g sugar⁻¹ [g biomass / (maltose in feed - maltose in culture + glucose in feed - glucose in culture)]. The metabolic model (table 1) predicts a value of ((36.7 · 0.45) ×0.360 × 0.077 + (52.2 · 0.36) × 0.180 × 0.103) / ((36.7 · 0.45) × 0.360 + (52.2 · 0.36) × 0.180) = 0.088 g biomass/g sugar⁻¹ (((maltose in feed - maltose in culture) × biomass yield on maltose + (glucose in feed - glucose in culture) × biomass yield on glucose) / (maltose in feed - maltose in culture + glucose in feed - glucose in culture)) and is within the accuracy of the biomass weight assay. It should be noted that in this calculation the molecular weight of maltose is taken as 360 g mol⁻¹ rather than 342 g mol⁻¹ to make the biomass yield on maltose comparable to that on glucose when expressed on a gram/gram basis. For the calculation of the ethanol production it should be taken into account that the reservoir medium contained approximately 10 mm ethanol which was used to dissolve ergosterol and

![Figure 2. Phase-contrast micrograph of *Saccharomyces cerevisiae* CBS 8066 cultivated in carbon-limited chemostat culture at D = 0.1 h⁻¹ on a mixture of 75% maltose and 25% glucose. For explanation see text.](image-url)
Table 2. Reservoir and residual sugar concentrations, metabolite and biomass concentrations and standard deviations of single steady states in anaerobic chemostat cultures of *Saccharomyces cerevisiae* CBS 8066 growing at $D = 0.1 \text{ h}^{-1}$ on different glucose/maltose mixtures. The mixtures are defined as the percentage of maltose metabolized (in hexose units) per total amount sugar metabolized (in hexose units). The ethanol concentration in the culture was corrected for the amount of ethanol in the reservoir.

<table>
<thead>
<tr>
<th>% maltose</th>
<th>reservoir sugars</th>
<th>residual sugars</th>
<th>metabolites</th>
<th>carbon recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glucose (mM)</td>
<td>maltose (mM)</td>
<td>glucose (mM)</td>
<td>maltose (mM)</td>
</tr>
<tr>
<td>-0.1±3.7</td>
<td>132.1±1.2</td>
<td>0.0±2.3</td>
<td>0.48±0.01</td>
<td>0.06±0.06</td>
</tr>
<tr>
<td>18.5±2.9</td>
<td>107.5±0.0</td>
<td>12.3±2.2</td>
<td>0.54±0.01</td>
<td>0.23±0.06</td>
</tr>
<tr>
<td>37.3±1.6</td>
<td>75.1±1.4</td>
<td>22.4±1.0</td>
<td>0.43±0.02</td>
<td>0.34±0.08</td>
</tr>
<tr>
<td>58.5±1.1</td>
<td>52.2±0.6</td>
<td>36.7±1.2</td>
<td>0.36±0.01</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td>77.8±1.4</td>
<td>27.0±0.8</td>
<td>47.2±2.2</td>
<td>0.23±0.01</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td>97.2±0.1</td>
<td>3.3±0.0</td>
<td>57.5±2.2</td>
<td>0.08±0.01</td>
<td>0.59±0.06</td>
</tr>
</tbody>
</table>

Figure 3. Effect of the amount of maltose and glucose utilized on A) specific rates of glucose and maltose consumption and B) specific production rates of ethanol, CO$_2$ and glycerol and C) biomass yields of anaerobic carbon-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. The measured values are fitted with the metabolic model (solid curves) described in the results section. The model graphs for sugar consumption, ethanol and CO$_2$ production are curved due to the linear decrease in cell yield with increasing amount of maltose in the medium feed. The values on the x-axis are the percentages maltose of the total amount of sugar utilized.
Figure 4. Effect of the amount of maltose and glucose utilized on carbon (O), hydrogen (Q), nitrogen (■), sulphur (■) and protein (▲) content of the biomass in anaerobic carbon-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. The standard deviations for the nitrogen, carbon, hydrogen and sulphur contents are 0.08. The values on the x-axis are the percentages maltose of the total amount of sugar utilized.

Tween80. The ethanol yield is defined as (ethanol in culture - ethanol in feed) / (maltose in feed - maltose in culture + glucose in feed - glucose in culture). On glucose the ethanol yield equals $193 \times 0.046 / ((132.1 - 0.48) \times 0.180) = 0.37$ g ethanol/g biomass$^{-1}$. On the sugar mixture with the highest amount of maltose the ethanol yield is $197 \times 0.046 / ((57.5 - 0.59) \times 0.360 + (3.33 - 0.06) \times 0.180) = 0.43$ g ethanol/g biomass$^{-1}$. The higher ethanol production during growth on maltose is close to the predictions listed in Table 1.

The model assumes that the cell composition is constant. Differences in the cell composition may change the ATP requirements for biomass formation, thus leading to changes in the metabolite fluxes related to assimilation and dissimilation. Over the entire range of sugar mixtures the carbon, hydrogen and sulphur contents of the cells did not change significantly (figure 4). The nitrogen content, however, increased from 7.5% to 8.0%. The protein content of the cells increased from 45.3±0.5% on 100% glucose to 48.7±0.9% (g protein/g biomass$^{-1}$) on 100% maltose. In terms of the theoretical ATP requirement for biomass formation this increase in protein content would mean a 3% increase in energy expenditure. Therefore, this small change in biomass composition was neglected in the evaluation of the energetics of growth on maltose/glucose mixtures.

During anaerobic growth on glucose, *S. cerevisiae* produces organic acids that can be potent uncouplers. If the production rate of these acids would be proportional to the rate of maltose metabolism, this would have severe consequences for the validity of the model as this would cause a progressive decrease of the cell yield with increasing amounts of maltose in the reservoir feed. However, the amounts of acetic acid, pyruvic acid as well as 2-oxoglutaric acid and succinic acid were low and decreased with increasing maltose in the feed (table 2). The specific production rates of these acids were constant (data not shown). The decrease of fumaric acid was pronounced, but the absolute concentrations were very low. Uncoupling of metabolism by such low concentrations of acetic acid and pyruvic acid can be neglected (Verduyn et al., 1990b).

**Metabolic fluxes in relation to enzyme activities**

During growth of *S. cerevisiae* on glucose-maltose mixtures the specific rate of sugar consumption (expressed as mmol hexose-g cells$^{-1}$h$^{-1}$) slightly increased with increasing maltose concentrations in the medium feed (table 1). This is due to the lower biomass yield on maltose as compared to glucose. This small increase in flux had no significant effect on key enzymes of the glycolytic pathway such as hexokinase and pyruvate decarboxylase, which remain approximately constant over the whole range.
Figure 6. Relation between the rate of maltose utilization (mmol maltose g biomass⁻¹ h⁻¹) and the amount of A) maltase and B) maltose carrier during growth of *Saccharomyces cerevisiae* CBS 8066 on maltose/glucose mixtures in anaerobic carbon-limited chemostats. The \( V_{\text{max}} \) value for maltose uptake was calculated from the residual substrate concentrations, the rate of maltose utilization and a \( K_m \) of 4 mM.

of sugar concentrations. The same was true for glucose-6-P-dehydrogenase and citrate synthase (which under anaerobic conditions only fulfil an assimilatory function (figure 5). As expected, a different pattern was encountered for the maltose-specific enzymes: maltase and the maltose carrier. Maltase was present in glucose-limited cultures at an activity of approximately 1 unit mg protein⁻¹. Its amount increased nearly sixfold with increasing maltose in the feed (figure 6a). Also the amount of maltose carrier increased with increasing maltose in the feed. This may be envisaged as follows: the amount of maltose carrier can be calculated according to the equation \( V = V_{\text{max}} \times s / (K_m + s) \) in which \( V \) equals the in situ maltose consumption rate in the culture; \( V_{\text{max}} \) is equivalent to the amount of the carrier; \( s \), the residual maltose concentration in the culture (figure 7) and \( K_m \), the Michaelis Menten constant of the carrier for maltose. Using a value of 4 mM for the latter parameter (van Leeuwen *et al.*, 1992) it can be calculated that also the amount of maltose carrier (ex-

![Graph](image)

Figure 7. Effect of the amount of maltose and glucose utilized by *Saccharomyces cerevisiae* CBS 8066, cultivated in anaerobic carbon-limited chemostats, on the residual glucose (□) and maltose (○) concentration. The values on the x-axis are the percentages maltose of the total amount of sugar utilized.

pressed as \( V_{\text{max}} \) in figure 6b) increased with increasing amounts of maltose in the medium feed.

**Discussion**

A quantitative analysis of anaerobic chemostat cultures of *S. cerevisiae* CBS 8066 growing on mixtures of maltose and glucose showed that growth and product formation fitted in a model based on an ATP requirement for maltose transport of 1 mol ATP/mol maltose (figure 1). The ATP requirement for maltose transport is due to the symport with protons which subsequently must be expelled by the plasma membrane-ATPase at the expense of ATP. The model as presented in figure 1 predicts the various parameters for anaerobic growth as listed in table 1. The experimental data (figure 3) show an excellent fit with these predictions. For example the cell yield on maltose was 25% lower than on glucose. As expected this decrease in cell yield is proportional to the amount of maltose utilized.

**Prerequisites for validation of the model**

Relating cell yields to medium composition is often very difficult since many factors, such as biomass composition and formation of byproducts, may affect cell yields. For this reason it was decided to study not only cell yields on the individual sugars but also on mixtures. In this way, the model could not only be tested for growth on maltose as sole carbon source, but also verified under additional conditions. A slight increase in protein content was observed with increasing maltose concentrations in the medium feed. This, however, cannot explain the strong decrease in biomass yield. Also a maltose-associated increase in maintenance energy re-
qurement due to uncoupling could be excluded (table 2).

Since the model is based on the assumption that the only difference between growth on glucose and on maltose is the transport step, other factors such as morphological changes due to a different medium composition should be avoided, especially since the surface/volume ratio can affect the cellular energetics (Kooijman et al., 1991). The irregular formation of pseudohyphae by *S. cerevisiae* growing under sugar limitation on mixtures of glucose and maltose interfered with attempts to quantify the energetics of maltose transport (figure 2). Usually, pseudohyphae formation by *S. cerevisiae* is associated with nutrient limitation (Brown & Hough, 1965). Since the cell elongation did not occur in sugar-limited cultures growing on glucose and maltose alone, nutrient limitation cannot be the only cause for the formation of pseudohyphae in this case. Fortunately, pseudohyphae formation could be avoided by adopting a standard procedure that allowed steady-state growth with uniform yeast-like morphology.

**Kinetics of mixed substrate utilization**

Our results confirm and extend the original observations by Egli et al. (1993), obtained for *Escherichia coli*, that during growth on mixed substrates the residual concentration of the individual sugars is lower than during growth on the single substrate (figure 7). The residual sugar concentration depended on the composition of the medium feed. The residual sugar concentrations in carbon-limited chemostat cultures of *S. cerevisiae* were two orders of magnitude higher than in carbon-limited *E. coli* cultures. This is a reflection of the much lower affinity of the sugar uptake systems in *S. cerevisiae* as compared to *E. coli*.

Using the Michaelis Menten equation, the residual substrate concentrations and the $K_m$ of the maltose carrier for its substrate, it was calculated that the amount of maltose carrier increased with increasing maltose consumption rates. In a separate experiment it was established that a small but significant amount of maltose carrier was still present in glucose-limited cultures. The same was true for the amount of maltase. The most drastic adaptation in enzyme levels occurred at low maltose concentrations in the medium feed (i.e. at low maltose consumption rates) Above a $q_{maltose}$ of 0.5 mmol·g$^{-1}$·h$^{-1}$ the amount of these enzymes was linearly proportional to the consumption rate (figure 6). The clear correlation of the amount of both enzymes with the *in situ* rate of maltose consumption by the cultures confirms that these enzymes exert a strong control over the maltose flux in the cells.

**Anaerobic cultivation for the evaluation of the bioenergetics of sugar transport**

For the study of the ATP requirement of maltose/proton symport anaerobic growth conditions were chosen since particularly during anaerobic growth the energy requirements of sugar transport become apparent. This is due to the low ATP yield of catabolism as compared to that under aerobic growth conditions. Under anaerobic conditions a 25% difference can be expected between cell yield on glucose and maltose (table 1, figure 3). During aerobic growth the relative effect of energy requirement for sugar transport is much smaller. For example, with a P/O ratio of 1, aerobic dissimilation of 1 mol maltose yields 32 ATP. If maltose transport requires 1 ATP, this would be only 1/32 of the ATP produced in catabolism. As a result, the cell yield (g cells/g maltose$^{-1}$) would be only 3% lower on maltose than on glucose, which is almost within the accuracy of yield determination.

**Practical implications of active maltose transport**

The results described above may have practical implications for the ethanol production with yeasts. When starch (cereals) are used as a feedstock, it may be profitable to use hydrolysates of high maltose content: in this way a higher yield of ethanol (g ethanol/g sugar$^{-1}$) can be expected (table 1). With respect to the effects of sugar transport on cellular energetics it would also be of interest to study various *Saccharomyces* strains that differ with respect to their mode of hexose uptake. It has been reported, for example, that certain strains of brewer's yeast have the ability to take up fructose with a sugar/proton mechanism (Cason et al., 1966). In such a case the energetics of growth on sucrose and maltose would be comparable. In contrast to maltose, sucrose is not taken up by yeasts but hydrolysed extracellularly by invertase or inulinase to glucose and fructose. If fructose transport would require 1 ATP, the ATP yield of anaerobic catabolism of sucrose would be 3, identical to that of maltose.

**Acknowledgements**

We are indebted to Dr. C. Verduyn (BIRD Engineering, Schiedam, The Netherlands) for valuable discussions and to Drs. M. H. Smith of the Centraalbureau voor Schimmelcultures (CBS) for the taxonomic analysis of the pseudohyphae-forming variants.
References


Kooljmen SALM, Muller EB & Stouthamer AH (1991) Microbial growth dynamics on the basis of individual budgets. Antonie van Leeuwenhoek 60: 159-174


Effect of oxygen limitation on sugar metabolism in yeasts

a continuous-culture study of the Kluyver effect

by R. A. Weusthuis, W. Visser, J. T. Pronk, W. A. Scheffers and J. P. van Dijken.

Abstract

Growth and metabolite formation was studied in oxygen-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 growing on glucose or maltose at a dilution rate of 0.1 h \(^{-1}\). With either glucose or maltose *S. cerevisiae* could be grown under dual limitation of oxygen and sugar. Respiration and alcoholic fermentation occurred simultaneously and the catabolite fluxes through these processes were dependent on the magnitude of the oxygen feed.

Also *C. utilis* could be grown under dual limitation of glucose and oxygen. However, at very low oxygen feed rates (i.e. below 4 mmol l \(^{-1}\)h \(^{-1}\)) growth was limited by oxygen only as indicated by the high residual glucose concentration in the culture. In contrast to *S. cerevisiae*, *C. utilis* could not be grown anaerobically at a dilution rate of 0.1 h \(^{-1}\). With *C. utilis* absence of oxygen resulted in wash-out, despite the presence of ergosterol and Tween-80 in the medium.

The behaviour of *C. utilis* with respect to maltose utilization in oxygen-limited cultures was exceptional: alcoholic fermentation did not occur in such cultures and the amount of maltose metabolized was dependent on the oxygen supply. Oxygen-limited cultures of *C. utilis* growing on maltose always contained high residual sugar concentrations.

These observations throw new light on the so-called Kluyver effect. Apparently, maltose is a non-fermentable sugar for *C. utilis* despite the fact that it can serve as a substrate for growth of this facultatively fermentative yeast. This is not due to the absence of key enzymes of alcoholic fermentation. Pyruvate decarboxylase and alcohol dehydrogenase were present at high levels in maltose-utilizing cells of *C. utilis* grown under oxygen limitation.

It is concluded that the Kluyver effect, in *C. utilis* on maltose, results from a regulatory mechanism that prevents the sugar from being fermented. Oxygen is not a key factor in this phenomenon since under oxygen limitation alcoholic fermentation of maltose was not triggered.

Introduction

Oxygen is a key factor in the regulation of sugar metabolism in yeasts. In the presence of oxygen, virtually all yeasts can respire sugars to carbon dioxide and water. The majority of the yeast species described so far are also capable of fermenting sugars to ethanol and carbon dioxide (van Dijken *et al.*, 1986). The capacity to ferment sugars to ethanol does not imply the ability to grow under anaerobic conditions. In fact, most facultatively fermentative yeasts do not grow well in the complete absence of oxygen, not even in complex media (Visser *et al.*, 1990).

Oxygen-related physiological phenomena in yeasts have been categorized as four ‘effects’, the occurrence of which depends both on the yeast species and on the sugar substrate. The Pasteur effect has been defined as an inhibition of fermentative sugar metabolism by oxygen (Lagunas, 1986; Lagunas *et al.*, 1982; Busturia & Lagunas, 1986; Lloyd & James, 1987). In contrast, the Custers effect describes the phenomenon that, in certain yeasts, fermentation is inhibited by the absence of oxygen (Custers, 1940; Scheffers, 1966; Carrascosa *et al.*, 1981; Wijsman *et al.*, 1984; Gaunt *et al.*, 1988). The Crabtree effect, defined as the occurrence of fermentative metabolism in the presence of oxygen, occurs in some yeasts when these are exposed to excess sugar (Fiechter *et al.*, 1981; Petrik *et al.*, 1983; Käppeli, 1986; Postma *et al.*, 1988; 1999a, b; Käppeli *et al.*, 1985a, b; Käppeli & Sonnleitner, 1986; van Urk *et al.*, 1988; 1989).

A fourth phenomenon, the Kluyver effect (Sims & Barnett, 1976), is probably the least understood of these oxygen-related metabolic responses. It has been defined as follows: ‘... certain yeasts can utilize particular disaccharides aerobically but not anaerobically, although these yeasts can use one or more of the component hexoses anaerobically.'
The data presented in the original screening of yeast species by Sims & Barnett (1978) originated from two types of taxonomic tests. ‘Aerobic’ growth tests were performed in slowly shaking test tubes; ‘anaerobic’ growth and fermentation tests were performed in static incubation of Durham tubes containing an inverted vial for monitoring gas production. It is clear that in the screening by Sims & Barnett (1978) the ‘aerobic’ cultures are likely to have been oxygen-limited. On the other hand, some oxygen must also have entered the static ‘anaerobic’ cultures.

Since none of the facultatively fermentative yeasts that exhibit the Kluyver effect are capable of rapid anaerobic growth (Visser et al., 1990), occurrence of the Kluyver effect must be confined to oxygen-limited growth conditions. Although oxygen has often been implicated as a key factor in its occurrence, the Kluyver effect has not yet been studied under controlled oxygen feed regimes.

The aim of the present work was to investigate the function of oxygen in the Kluyver effect. For this purpose, the effect of the oxygen feed rate on sugar metabolism was studied in sugar-limited chemostat cultures. C. utilis CBS 621 was chosen as a model organism that exhibits the Kluyver effect for maltose (Sims & Barnett, 1978), whereas S. cerevisiae CBS 8066 is Kluyver-negative for this disaccharide (Weusthuis et al., 1993). Since both strains are capable of respiratory and fermentative glucose metabolism, cultures grown on glucose were included as references. The experimental results were compared to those predicted by a simple metabolic model.

Material and Methods

Organisms and maintenance

S. cerevisiae CBS 8066 and C. utilis CBS 621 were obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slants at 4°C.

Chemostat cultivation

Chemostat cultivation was performed in 2-litre fermenters (Applikon, Schiedam, The Netherlands) at a dilution rate of 0.10 h⁻¹, a temperature of 30°C and a stirrer speed of 750 rpm. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH, via an Applikon ADI-1030 biocinstaller. The working volume of the culture was kept at 1.1 litre by removal of effluent from the middle of the culture, via an Applikon electrical level controller. This set-up ensured that biomass concentrations in the effluent line differed by less than 1% from those in samples taken directly from the culture. To achieve an identical fermenter geometry, positions of baffles, pipes,
impellers and sensors were kept the same in all experiments. To avoid loss of volatile metabolites, the condenser was cooled to 2 °C, using a cryostat. The mineral medium, supplemented with vitamins, trace elements and the anaerobic growth factors ergosterol (5, 7, 22-ergostatrien-3β-ol, Sigma E-6510) and Tween-80 (polyoxyethylene-sorbitanmono-
oleate, Merck 822187) (see Andreassen & Stier, 1953; 1954) was prepared as described by Weusthuis et al. (1993). Glucose or maltose monohydrate, the growth factors and vitamins were added to the media after separate sterilization (Weusthuis et al., 1993). Both the oxygen transfer properties of the cultures and the optimum Tween-80 concentration (Verduyn et al., 1990) are functions of the biomass concentration in the reactor. Therefore, it was attempted to keep the biomass concentration in the cultures constant by increasing the reservoir concentration of the sugars with decreasing oxygen feed. In practice, biomass concentrations in the cultures typically varied between 2 and 3 g dry weight l⁻¹, with sugar concentrations in the reservoir medium ranging from 5 to 50 g l⁻¹. The purity of the chemostat cultures was routinely checked by phase contrast microscopy at 1000× magnification.

Oxygenation of the chemostat cultures

Oxygen was added to the cultures as air, using a peristaltic pump. The air flow rates ranged from 0 to 100 ml min⁻¹. The temperature of the ingoing air was kept constant at 20 °C. The overall gas flow into the cultures was maintained at 0.5 l min⁻¹ by the supplementary addition of nitrogen gas, using a Brooks 5876 mass flow controller (Brooks, The Netherlands). Addition of nitrogen gas assured good mixing of the air with the culture fluid and promoted anaerobiosis when air was not added. To minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up (including medium reservoir and effluent vessel) was equipped with Norpren tubing (Cole Parmer Inc., U.S.A.), and the reservoir vessel was flushed with nitrogen gas. The dissolved-oxygen concentration in the cultures was monitored with a polarographic oxygen electrode (Ingold, Switzerland).

Gas analysis.

Gas flows were measured with a self-constructed device, consisting of an inverted glass cylinder, filled with water. The cylinder was placed in a water-filled reservoir to prevent outflow of water, without touching the reservoir walls. A gas flow directed into the cylinder causes water to flow into the reservoir, which rested on an electronic balance. The weight of the water displaced per unit of time could, after the necessary corrections (e.g. ambient air pressure, temperature, pressure falls), be used to calculate the gas flow. Using this device, measurements were reproducible within 0.5%. Since the gas flows into the cultures (N₂ and air) had to be interrupted before measurement, they were measured both before and immediately after steady-state analysis. Due to a slight loss of resilience of new tubing, usually a small difference between these measurements were found (on average 2%). The steady state value was used in the calculations. The exhaust gas flow was determined on-line during the steady states. The oxygen content of the exhaust gas was determined with a Servomex oxygen analyser, the carbon dioxide content with a Beckman infrared CO₂ analyser. The exhaust gas entering both analysers was dried with a Perma Pure Dryer (PD-625-12P). Specific rates of carbon dioxide production and oxygen consumption were calculated according to van Urk et al. (1988). The amount of CO₂ leaving the culture with the effluent was negligible.

Metabolite analysis

Glucose and maltose concentrations were determined as described by Weusthuis et al. (1993). Ethanol, glycerol and organic acids as pyruvate, succinate, fumarate and 2-oxoglutarate were determined by HPLC (Weusthuis et al., 1993). Ethanol concentrations were also determined with an enzymatic assay (based on alcohol oxidase, EK 001 Leeds Biochemicals). Both methods gave identical results.

Culture dry weights

Dry weights of culture samples were determined using a microwave oven and 0.45-μm membrane filters as described by Postma et al. (1989b). Parallel samples varied by less than 1 %.

Enzyme assays

Preparation of cell-free extracts and assays of pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) activity were performed as described by Postma et al. (1989b).

Presentation of data

Several experimental approaches can be used to study the effects of oxygen on yeast metabolism. One possibility is to study the effect of dissolved oxygen concentration (Brown & Rose, 1969; Laplacet et al., 1991; Furukawa et al., 1983; Nishizawa et al., 1980; Cysewski & Wilke, 1976; Moss et al., 1969). However, at limiting oxygen supply rates, the dissolved-oxygen concentration falls below 1 % air saturation and becomes difficult to measure accurately. Moreover, the anaerobic growth factors Tween-80 and ergosterol tend to foul oxygen electrode membranes, thereby further reducing the reliability of the measurements.

An alternative approach is to study the effect of oxygen feed rate (Grosz & Stephanopouloes, 1990;
Kuriyama & Kobayashi, 1993; Oura, 1972). The effect of oxygen feed rate on growth and metabolism is strongly influenced by the gas transfer characteristics of the culture, which are affected by biomass density and fermenter geometry. However, when oxygen feed rates are varied in identical fermentation set-ups with approximately equal biomass concentrations, this should allow a comparative study involving different carbon sources and yeast species. Nevertheless, data from this type of comparative studies cannot easily be extrapolated to alternative experimental set-ups.

In well-mixed systems, effects of gas transfer characteristics can be eliminated by using the specific oxygen uptake rate \( (q_{O_2}) \) as the experimental variable. This should allow extrapolation to other well-mixed fermenter set-ups, even if these exhibit different gas transfer properties. Unfortunately, since \( q_{O_2} \) is a derived parameter, its use will inevitably result in more scatter of the experimental data. In the present study, we have tried to relate experimental data to specific oxygen uptake rates whenever possible. However, at very low oxygen feeds, the off-gas oxygen analysis was not sufficiently sensitive to accurately calculate \( q_{O_2} \). In some cases, this even resulted in negative apparent oxygen uptake rates. To enable comparison, even at low oxygen feed rates, of the four sets of experiments (two yeast species and two substrates), it was tried to keep fermenter geometry and biomass concentrations in all experiments constant (see above).

**Results**

**Relation between oxygen consumption rate and metabolic fluxes in S. cerevisiae: a simplified model**

When the oxygen feed to sugar-limited cultures of the facultatively anaerobic yeast *S. cerevisiae* is varied, the biomass yield on sugar can be expected to vary between the growth yield observed under anaerobic conditions and the aerobic, 'respiratory' biomass yield. Because of the higher energetic efficiency of respiratory sugar metabolism, the biomass yield during respiratory growth is ca. 5-fold higher than that under strictly anaerobic conditions (Verduyn et al., 1990; 1991). Over this range of oxygen feeds, the specific oxygen uptake rate can be expected to vary between zero (under anaerobic conditions) and the rate corresponding to sugar-limited, fully respiratory growth.

At submaximal oxygen consumption rates, both respiration and fermentation can contribute to glucose metabolism. An increase of the oxygen consumption rate implies that more sugar is respired, thereby increasing the biomass yield. Alternatively, an increase of the biomass yield can be achieved by increasing the amount of sugar in the feed, which allows more sugar to be fermented. Growth under these conditions can therefore be described as dually limited by oxygen and glucose.

A simplified model describing biomass yield and metabolic fluxes in *S. cerevisiae* cultures growing under this dual limitation can be constructed by assuming that the energetic efficiency of fermentative and respiratory sugar metabolism is not affected by the simultaneous occurrence of both processes. If this assumption holds, and products other than ethanol, carbon dioxide, biomass and water are neglected, the biomass yield in the oxygen- and sugar-limited cultures will be a simple function of the fraction of glucose that is metabolised by respiration. This fraction is equal to the ratio of the actual specific oxygen uptake rate \( (q_{O_2}) \) and the specific oxygen uptake rate during fully respiratory growth \( (q_{O_2,R}) \). The actual biomass yield on glucose is given by equation 1, in which \( Y \) is the actual biomass yield, \( Y_F \) is the anaerobic, fermentative biomass yield and \( Y_R \) is the aerobic, respiratory biomass yield on sugar.

\[
\frac{1}{Y} = \frac{1}{Y_F} \left( 1 - \frac{q_{O_2}}{q_{O_2,R}} \right) + \frac{1}{Y_R} \frac{q_{O_2}}{q_{O_2,R}}
\]

(1)

Equation 1 is based on the assumption that the fraction of sugar that is fermented \( (1/Y_F) \) decreases linearly with increasing \( q_{O_2} \). Correspondingly, with increasing \( q_{O_2} \), the specific ethanol production rate \( (q_{ethanol}) \) will decrease linearly from the rate that is observed during anaerobic growth \( (q_{ethanol,F}) \) to zero, according to equation 2.

\[
q_{ethanol} = q_{ethanol,F} \left( 1 - \frac{q_{O_2}}{q_{O_2,R}} \right)
\]

(2)

**Dissimilatory production of CO₂ occurs both during respiration and fermentation.** During complete respiratory dissimilation of glucose, the rate of carbon dioxide production is equal to the oxygen consumption rate (the respiratory quotient, RO, is being one). Fermentative CO₂ production during alcoholic fermentation should be equal to the specific ethanol production rate given by equation 2. Because yeast biomass is more oxidized than the substrate sugars, production of CO₂ also occurs as a result of assimilatory processes (see e.g. Bruinenberg et al., 1984; Gommers et al., 1988). If it is assumed that the biomass composition does not vary substantially, the specific rate of assimilatory CO₂ production should not be influenced by the oxygen feed rate. For *S. cerevisiae* cultures growing at a dilution rate of 0.10 h⁻¹, an assimilatory \( q_{CO_2,A} \) of 0.58 mmol G⁻¹ h⁻¹ has been reported by Verduyn et al. (1990). When the three sources of carbon dioxide production are
Table 1. Effect of oxygen feed rate on oxygen and substrate utilization and production of ethanol and biomass by Saccharomyces cerevisiae CBS 8066 grown in chemostat cultures (\(D = 0.10 \ h^{-1}\)) with glucose as a carbon and energy source.

<table>
<thead>
<tr>
<th>oxygen (mmol l(^{-1}) h(^{-1}))</th>
<th>glucose (g l(^{-1}))</th>
<th>dry weight</th>
<th>ethanol (g l(^{-1}))</th>
<th>(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in(^a) out(^b)</td>
<td>in(^c) out(^d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32 24.6 &lt;0.1 2.52 242</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.70 22.8 &lt;0.1 2.64 201</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.93 23.7 &lt;0.1 2.70 240</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7 15.5 &lt;0.1 2.56 172</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.3 11.4 &lt;0.1 1.99 99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.5 8.9 &lt;0.1 2.07 58</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.5 13.7 &lt;0.1 2.15 41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.2 18.2 &lt;0.1 2.67 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33.1 25.6 &lt;0.1 3.18 &lt;1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46.6 39.3 &lt;0.1 2.79 &lt;1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) oxygen feed rate; \(^b\) oxygen leaving the culture; \(^c\) reservoir sugar concentration; \(^d\) residual sugar concentration.

Table 2. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass and ethanol by Saccharomyces cerevisiae CBS 8066 grown in chemostat cultures (\(D = 0.10 \ h^{-1}\)) with maltose as a carbon and energy source.

<table>
<thead>
<tr>
<th>oxygen (mmol l(^{-1}) h(^{-1}))</th>
<th>glucose (g l(^{-1}))</th>
<th>dry weight</th>
<th>ethanol (g l(^{-1}))</th>
<th>(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in(^a) out(^b)</td>
<td>in(^c) out(^d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 30.0 &lt;0.1 2.16 259</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 30.3 0.1 2.13 259</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.4 24.0 &lt;0.1 2.40 211</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8 20.5 &lt;0.1 2.55 121</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.7 14.1 &lt;0.1 2.36 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.3 17.3 &lt;0.1 2.51 41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.8 19.1 &lt;0.1 2.68 26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.8 24.8 &lt;0.1 2.41 35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35.3 29.6 &lt;0.1 2.52 &lt;1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40.2 34.0 &lt;0.1 2.51 &lt;1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45.8 39.8 &lt;0.1 2.71 &lt;1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of oxygen feed on growth of S. cerevisiae on glucose and maltose.

The effects of oxygen on the physiology of S. cerevisiae were studied by varying the oxygen feed rate to glucose- and maltose-limited chemostat cultures grown at a dilution rate of 0.10 h\(^{-1}\). At oxygen feed rates above 30 mmol l\(^{-1}\) h\(^{-1}\) and sugar concentrations below 5 g l\(^{-1}\), growth of S. cerevisiae was not oxygen-limited; a further increase in air supply did not result in higher biomass yields (tables 1 & 2). Growth was fully respiratory, as was evident from the absence of ethanol in the culture supernatants (tables 1 & 2) and an RQ of approximately 1 (figure 3). The q\(_{O_2}\) of these oxygen-sufficient, glucose-limited chemostat cultures was ca. 2.5 mmol g\(^{-1}\) h\(^{-1}\) (figure 2). When q\(_{O_2}\) was reduced by decreasing the oxygen feed rate, alcoholic fermentation set in (tables 1 & 2; figure 3). This coincided with a decrease of the biomass yield (figure 2).

Over a range of oxygen consumption rates, respiratory and fermentative glucose metabolism occurred simultaneously. In these cultures, the biomass concentration could be increased either by increasing the oxygen feed rate (figure 2), or by increasing the sugar concentration in the reservoir medium (which led to increased alcoholic fermentation; data not shown). Therefore, such cultures grew under a dual limitation of sugar and oxygen.

Qualitatively, equation 1 gave a good description of the observed biomass yields at non-saturating oxygen feeds (figure 2). However, the apparent combined, this results in equation 3:

\[
q_{O_2} = q_{O_2,A} + q_{O_2} + q_{ethanol}
\]  

Substitution of equation 2 in equation 3 gives equation 4, which indicates that also the specific carbon dioxide production rate by the cultures is a linear function of q\(_{O_2}\).

\[
q_{CO_2} = q_{CO_2,A} + q_{CO_2} + q_{ethanol}F \left(1 - \frac{q_{O_2}}{q_{O_2,R}}\right)
\]

Figure 2. Relation between the specific oxygen uptake rate (q\(_{O_2}\)) and biomass yield of Saccharomyces cerevisiae CBS 8066, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h\(^{-1}\)), with glucose (●) or maltose (○) as a carbon and energy source. The data are fitted with equation 1 (dashed lines). Note that in this and the following figures also some negative q\(_{O_2}\) values are presented. This is due to the inaccuracy of the gas analysis at very low oxygen feed rates (see materials and methods).
Figure 3. Relation between the specific oxygen uptake rate ($q_{O_2}$) and the specific production rates of ethanol ($q_{\text{ethanol}}$) and carbon dioxide ($q_{\text{CO}_2}$) of Saccharomyces cerevisiae CBS 8066 grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with glucose (A) or maltose (B) as a carbon and energy source. The ethanol data are fitted with equation 2, the carbon dioxide data with equation 4 (dashed lines).

Table 3. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass, ethanol and pyruvate by Candida utilis CBS 621 grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose as a carbon and energy source. eth. = ethanol; pyr. = pyruvate.

<table>
<thead>
<tr>
<th>oxygen</th>
<th>glucose</th>
<th>dry weight</th>
<th>eth.</th>
<th>pyr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>in ($\text{mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)</td>
<td>out ($\text{g} \cdot \text{t}^{-1}$)</td>
<td>in ($\text{mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)</td>
<td>out ($\text{g} \cdot \text{t}^{-1}$)</td>
<td>(mm)</td>
</tr>
<tr>
<td>0.17</td>
<td>0.51</td>
<td>45.5</td>
<td>28.3</td>
<td>1.50</td>
</tr>
<tr>
<td>0.33</td>
<td>0.61</td>
<td>45.5</td>
<td>17.3</td>
<td>1.99</td>
</tr>
<tr>
<td>0.77</td>
<td>0.69</td>
<td>45.5</td>
<td>17.8</td>
<td>2.00</td>
</tr>
<tr>
<td>1.8</td>
<td>1.3</td>
<td>45.5</td>
<td>0.9</td>
<td>2.22</td>
</tr>
<tr>
<td>2.9</td>
<td>2.3</td>
<td>45.5</td>
<td>16.2</td>
<td>2.50</td>
</tr>
<tr>
<td>4.3</td>
<td>3.0</td>
<td>28.4</td>
<td>&lt;0.1</td>
<td>3.20</td>
</tr>
<tr>
<td>9.3</td>
<td>5.8</td>
<td>14.2</td>
<td>&lt;0.1</td>
<td>2.70</td>
</tr>
<tr>
<td>11.4</td>
<td>8.9</td>
<td>10.5</td>
<td>&lt;0.1</td>
<td>2.21</td>
</tr>
<tr>
<td>16.7</td>
<td>13.1</td>
<td>7.7</td>
<td>&lt;0.1</td>
<td>2.38</td>
</tr>
<tr>
<td>18.6</td>
<td>14.9</td>
<td>7.7</td>
<td>&lt;0.1</td>
<td>2.57</td>
</tr>
<tr>
<td>24.4</td>
<td>18.8</td>
<td>6.4</td>
<td>&lt;0.1</td>
<td>2.99</td>
</tr>
<tr>
<td>22.3</td>
<td>15.9</td>
<td>5.9</td>
<td>&lt;0.1</td>
<td>3.14</td>
</tr>
<tr>
<td>32.6</td>
<td>25.3</td>
<td>4.6</td>
<td>&lt;0.1</td>
<td>3.03</td>
</tr>
<tr>
<td>47.8</td>
<td>40.1</td>
<td>4.5</td>
<td>&lt;0.1</td>
<td>2.96</td>
</tr>
</tbody>
</table>

$q_{\text{CO}_2}$ increased with decreasing $q_{O_2}$ to reach a maximum in the anaerobic cultures (figure 3). Equations 2 and 4 gave a good fit of the experimental data.

The main difference between growth of S. cerevisiae on glucose and maltose was the consistently lower biomass yield (figure 2) and higher $q_{\text{ethanol}}$ (figure 3) during oxygen-limited growth on maltose. The lower growth efficiency with maltose as the energy source can be fully explained by the fact that, unlike glucose, maltose is transported via a proton symport mechanism in S. cerevisiae. The net energy requirement for maltose transport is 1 mol ATP per mol maltose. Therefore, when maltose metabolism is fully fermentative, 1 out of the 4 ATP produced in glycolysis is used for maltose uptake, leading to a 25% lower biomass yield and a 43% higher $q_{\text{ethanol}}$ (Weusthuis et al., 1993). This tendency was indeed observed during anaerobic growth on maltose and glucose (figures 2 & 3). When sugar metabolism is respiratory, the ATP yield on maltose is much higher, and it is no longer possible to detect significant differences between the biomass yields on glucose and maltose (figure 2).

Effects of oxygen feed on growth of C. utilis on glucose

As in S. cerevisiae, glucose metabolism of C. utilis was fully respiratory at oxygen feed rates above 30 mmol·h⁻¹ (table 3). When the oxygen feed rate was reduced below this value, corresponding to an oxygen consumption rate of 2.5 mmol·h⁻¹, the biomass yield decreased (figure 4) and alcoholic fermentation set in (table 3, figure 5).
The role of oxygen in the Kluyver effect

Figure 4. Relation between the specific oxygen uptake rate \( (q_{O_2}) \) and the biomass yield of \textit{Candida utilis} CBS 621, grown at different oxygen feed rates in chemostat cultures \( (D = 0.10 \text{ h}^{-1}) \), with glucose as a carbon and energy source.

Figure 5. Relation between the specific oxygen uptake rate \( (q_{O_2}) \) and the specific production rates of ethanol \( (q_{\text{ethanol}}) \) and carbon dioxide \( (q_{\text{CO}_2}) \) of \textit{Candida utilis} CBS 621, grown at different oxygen feed rates in chemostat cultures \( (D = 0.10 \text{ h}^{-1}) \), with glucose as a carbon and energy source.

At oxygen feed rates between 4.3 and 30 mmol\( \cdot \)h\(^{-1}\) \cdot h\(^{-1}\), virtually all glucose was consumed (table 3), with respiration and fermentation occurring simultaneously (figure 5). Qualitatively, the behaviour of the \textit{C. utilis} cultures over this range of oxygen feeds was very similar to that of \textit{S. cerevisiae} and growth could be considered as dually limited by glucose and oxygen (table 3, figures 4 & 5). However, when the oxygen feed rate was decreased below 4.3 mmol\( \cdot \)h\(^{-1}\), the residual glucose concentration in the cultures increased from below 0.1 g\( \cdot \)l\(^{-1}\) to 28.3 g\( \cdot \)l\(^{-1}\), resulting in a decrease of the culture dry weights (table 3). At these low oxygen feed rates, the biomass concentration in the cultures could no longer be increased by adding more glucose to the reservoir media. Apparently, at these low oxygen feed rates, growth of \textit{C. utilis} was only limited by oxygen.

Attempts to grow \textit{C. utilis} on glucose at oxygen consumption rates below 0.17 mmol\( \cdot \)g\(^{-1}\)\cdot h\(^{-1}\) resulted in wash-out. Apparently, the maximum anaerobic growth rate of \textit{C. utilis} is lower than the dilution rate (0.10 h\(^{-1}\)). This is in agreement with the reported maximum growth rate of \textit{C. utilis} in anaerobic batch cultures (0.01 h\(^{-1}\); Visser \textit{et al.}, 1990).

As mentioned above for \textit{S. cerevisiae}, the biomass yield of \textit{C. utilis} under fully aerobic, respiratory conditions was higher than the value of 0.51 g\( \cdot \)g\(^{-1}\) reported by Verduyn \textit{et al.} (1991), probably due to the presence of ethanol and oleic acid in the media. In view of the inability of \textit{C. utilis} to grow anaerobically, the experimental data could not be fitted with the equations 1-4. However, it is clear from figure 5 that during dual substrate-limited growth, \( q_{\text{ethanol}} \) and \( q_{\text{CO}_2} \) did not increase linearly with decreasing \( q_{O_2} \), as predicted by equations 2 and 4. The observed non-linearity suggests that either fermentative metabolism or respiratory metabolism changes under dual substrate limitation. Factors that may be involved are changes in biomass composition or composition of the respiratory chain.

Compared to \textit{S. cerevisiae}, the maximum \( q_{\text{ethanol}} \) of \textit{C. utilis} at very low oxygen feeds was substantially higher and the biomass yield correspondingly lower (figures 4 & 5). This lower efficiency of fermentative growth may be due to the involvement of a proton symport carrier in glucose transport by \textit{C. utilis}. Also increased maintenance requirements, for example caused by the production of uncoupling weak acids, may contribute to the high \( q_{\text{ethanol}} \) at low oxygen feeds. Indeed, organic acid concentrations increased with decreasing oxygen feed (data not shown), with pyruvate concentrations up to 8 mM occurring in oxygen-limited \textit{C. utilis} cultures (table 3).

Effects of oxygen feed on growth of \textit{C. utilis} on maltose

The effect of the oxygen feed rate on growth of \textit{C. utilis} on maltose was markedly different from the three situations described above (figure 6). In all cultures, the ethanol concentration was lower than in the reservoir medium (table 4). Apparently, alcoholic fermentation did not occur at any of the oxygen feed rates tested. Instead, maltose metabolism was respiratory over the whole range of oxygen feed rates tested, as indicated by the constant \( q_{\text{CO}_2} \) and \( q_{O_2} \) (figure 6). Since the \( q_{O_2} \) hardly changed, culture parameters were plotted against the oxygen feed rate. In contrast to the results on glucose, organic acids could not be detected in any of the cultures (only shown for pyruvate, table 4).
Figure 6. Relation between oxygen feed rate and the dry weight (■), specific production rate of carbon dioxide (q_{CO_2} ●) and specific consumption rate of oxygen (q_{O_2}) of Candida utilis CBS 621, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with maltose as a carbon and energy source. Note that on the x-axis the oxygen feed rate is plotted and not, as in the other graphs, q_{O_2}.

Table 4. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass, ethanol and pyruvate by Candida utilis CBS 621 grown in chemostat (D = 0.10 h^{-1}) with maltose as a carbon and energy source. The presence of small amounts of ethanol in cultures growing at low oxygen feed, results from the presence of this compound in the reservoir medium. nd = not determined; eth. = ethanol; pyr. = pyruvate.

<table>
<thead>
<tr>
<th>oxygen (mmol l^{-1} h^{-1})</th>
<th>maltose (g l^{-1})</th>
<th>dry weight (g l^{-1})</th>
<th>eth. (mm)</th>
<th>pyr. (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in</td>
<td>out</td>
<td>in</td>
<td>out</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(g l^{-1})</td>
<td>(g l^{-1})</td>
<td>(mm)</td>
</tr>
<tr>
<td>2.5</td>
<td>2.0</td>
<td>4.6</td>
<td>nd</td>
<td>0.28</td>
</tr>
<tr>
<td>4.7</td>
<td>4.0</td>
<td>45.5</td>
<td>48.4</td>
<td>0.43</td>
</tr>
<tr>
<td>4.7</td>
<td>3.8</td>
<td>4.6</td>
<td>4.6</td>
<td>0.38</td>
</tr>
<tr>
<td>10.9</td>
<td>7.0</td>
<td>12.3</td>
<td>11.0</td>
<td>1.88</td>
</tr>
<tr>
<td>14.5</td>
<td>11.1</td>
<td>10.5</td>
<td>9.2</td>
<td>1.88</td>
</tr>
<tr>
<td>14.8</td>
<td>11.2</td>
<td>4.6</td>
<td>2.6</td>
<td>1.76</td>
</tr>
<tr>
<td>15.6</td>
<td>11.3</td>
<td>4.6</td>
<td>nd</td>
<td>1.97</td>
</tr>
<tr>
<td>18.0</td>
<td>12.7</td>
<td>6.8</td>
<td>1.8</td>
<td>3.46</td>
</tr>
<tr>
<td>18.1</td>
<td>12.8</td>
<td>4.6</td>
<td>nd</td>
<td>3.07</td>
</tr>
<tr>
<td>21.1</td>
<td>17.2</td>
<td>6.8</td>
<td>0.8</td>
<td>3.21</td>
</tr>
<tr>
<td>22.8</td>
<td>19.2</td>
<td>5.5</td>
<td>4.0</td>
<td>2.45</td>
</tr>
<tr>
<td>32.5</td>
<td>26.2</td>
<td>4.6</td>
<td>0.7</td>
<td>2.87</td>
</tr>
<tr>
<td>47.6</td>
<td>40.4</td>
<td>4.6</td>
<td>nd</td>
<td>2.90</td>
</tr>
</tbody>
</table>

At oxygen feed rates below 30 mmol l^{-1} h^{-1}, residual maltose concentrations in the cultures increased and the amount of biomass decreased (table 4, figure 6). At these suboptimal oxygen feed rates, the culture dry weight could not be increased by adding maltose to the reservoir media (table 4). A dual limitation of sugar and oxygen, as observed during growth of C. utilis on glucose, could not be attained with maltose, and growth was only limited by oxygen. The high residual maltose concentrations resulted in small differences between culture and reservoir sugar concentrations. As a consequence, large variation occurred in the calculated biomass yields and maltose fluxes (results not shown).

Due to the low culture dry weights at low oxygen feed rates, it was not possible to test at which oxygen feed rate maltose-grown cultures of C. utilis washed out. The ethanol added to the medium reservoir accumulated in the culture at oxygen feed rates below 10.9 mmol l^{-1} h^{-1}. With a biomass yield of C. utilis on ethanol of 0.69 g g^{-1} (Verduyn et al., 1991), ethanol present in the media (12 mM) could only account for 0.38 g l^{-1} biomass. The observed biomass concentrations and ethanol concentrations in the culture (table 4) hence indicate that respiratory growth of C. utilis on maltose occurred at all oxygen feed rates.

C. utilis exhibited a clear Kluyver effect for maltose: alcoholic fermentation of the disaccharide was not observed, while both respiratory metabolism of maltose and fermentation of its hydrolysis product glucose were possible (Kluyver & Custers, 1940; Sima & Barnett, 1978). An explanation for this apparent intrinsic inability to ferment maltose might be the absence of the fermentative key enzymes pyruvate decarboxylase and/or alcohol dehydrogenase during growth on the disaccharide. Therefore, the activities of these enzymes were assayed in cell-free extracts of maltose-grown C. utilis.

Effect of oxygen on pyruvate decarboxylase and alcohol dehydrogenase activities of C. utilis

Pyruvate decarboxylase (PDC) activities in glucose-grown C. utilis increased from 0.3 to 2.2 U mg protein^{-1} with decreasing oxygen feed rates, parallel
The role of oxygen in the Kluyver effect

Figure 8. Relation between oxygen feed rate and ethanol (O)- and pentanol (●)-dependent alcohol dehydrogenase activity of Candida utilis CBS 621, grown in chemostat cultures (D = 0.10 h⁻¹) with glucose (A) or maltose (B) as a carbon and energy source.

to the q ethanol. An increase of PDC activities was also observed in the maltose-grown cultures: with decreasing oxygen feed, PDC activities increased from 0.2 to 0.6 U·mg protein⁻¹ (figure 7). The maximum fermentation rates that can be sustained by the PDC activities measured in cell-free extracts can be calculated by assuming a soluble protein content of yeast biomass of 30%. Thus, the maximum observed PDC activity of 0.6 U·mg protein⁻¹ would be sufficient to account for a maximum rate of ethanol formation of as high as 11 mmol·g dry weight⁻¹·h⁻¹.

Various forms of the second key enzyme in alcoholic fermentation, alcohol dehydrogenase (ADH), occur in yeasts (Verduyn et al., 1988). S. cerevisiae, for example, contains three isoenzymes: a constitutive cytoplasmic ADH functioning in alcoholic fermentation, an inducible cytoplasmic ADH active during growth on ethanol, and a mitochondrial ADH of which the function is unknown. The ‘fermentative’ ADH activity can be discerned from the other two ADH activities by its inability to use pentanol as a substrate. A similar situation probably exists in C. utilis, although the role of the several isoenzymes has been less well studied. During growth of C. utilis on glucose, the pentanol-dependent ADH activity was approximately 4 U·mg protein⁻¹ at high oxygen feed rates and decreased sharply to a basal level of 0.2 U·mg protein⁻¹ when the oxygen feed rate was reduced. The high pentanol-dependent ADH activities coincided with the consumption of ethanol present in the reservoir media. Ethanol-dependent ADH activity showed a similar pattern, but was approximately 2 U·mg protein⁻¹ higher in all cultures, suggesting a constitutive expression of the ‘fermentative’ ADH. During growth on maltose, pentanol-dependent ADH activity remained high over a broader oxygen feed range than during growth on glucose. Also in this case a good correlation existed with the occurrence of ethanol co-metabolism. Under oxygen-limited conditions, the ADH activities of maltose-grown cells were equal to or higher than those of glucose-grown cells (figure 8).

Production of glycerol

In the equations 1 to 4, fermentative sugar metabolism in yeasts has been simplified by assuming that ethanol is the sole fermentation product. However, it is well-known that in additional to ethanol, other metabolites may be excreted by yeasts. Quantitatively, glycerol is one of the major byproducts of alcoholic fermentation under anaerobic conditions (Verduyn et al., 1990).

Glycerol formation by yeasts may have various functions, some involved in osmoregulation. Under anaerobic conditions, however, the main physiologi-
cal role of glycerol formation is related to redox metabolism. Since, as mentioned above, yeast biomass is more oxidized than the carbohydrate substrate, NADH is produced during assimilation of sugars. In the presence of oxygen, reduced cofactors can be reoxidized by respiration. When respiration is not possible, yeasts can reduce dihydroxyacetone phosphate to glycerol to close the redox balance (Holzer et al., 1963; Gancedo et al., 1968; Oura, 1977).

Glycerol formation branches off from the glycolytic pathway before the reactions that involve substrate-level phosphorylation. Therefore, formation of one mole of glycerol from glucose requires the net hydrolysis of one mole of ATP. In contrast, respiratory re-oxidation of reduced cofactors yields metabolic energy by oxidative phosphorylation. To investigate the regulation of redox metabolism in yeasts, we studied the formation of glycerol at limiting oxygen feed rates.

Verduyn et al. (1990) reported that formation of 1 g S. cerevisiae biomass results in the formation of 11 mmol NADH. Under respiratory conditions, this NADH can be oxidized with 5.5 mmol oxygen. If redox metabolism in yeasts is regulated to minimize ATP expenditure for glycerol formation, glycerol should not be produced at specific oxygen consumption rates above 5.5 × 0.1 = 0.55 mmol·g⁻¹·h⁻¹ (product of oxygen requirement per unit yeast biomass and dilution rate). This could indeed be confirmed experimentally for growth of S. cerevisiae on either maltose or glucose, and for C. utilis grown on glucose (figure 9). Cultures in which qO₂ was decreased below this threshold invariably produced glycerol (figure 9). The glycerol production rates in anaerobic cultures of S. cerevisiae were in good agreement with the data of Verduyn et al. (1990). No glycerol formation was observed in the cultures of C. utilis grown on maltose (data not shown), consistent with their respiratory mode of metabolism.

The physiological necessity of glycerol production at very low oxygen feeds implies that the energetic efficiency of sugar metabolism is dependent on the oxygen feed. When the reducing equivalents produced during anaerobic processes are oxidized by respiration, ATP is produced. However, when reoxidation occurs via glycerol formation, ATP is consumed. Consequently, equations 1 to 4 that were used to model growth and alcoholic fermentation in oxygen-limited cultures are an oversimplification.

Discussion

Regulation of alcoholic fermentation in yeasts

Crabtree-positive yeasts, including S. cerevisiae, have a very strong tendency towards alcoholic fermentation. In practice, ethanol formation by these yeasts can only be avoided by growth under fully aerobic conditions with a limited supply of sugar. In contrast, fermentative metabolism in Crabtree-negative yeasts, including C. utilis, can not be induced in the presence of excess oxygen. However, under oxygen-limited growth conditions, fermentation rates in Crabtree-positive and Crabtree-negative yeasts are comparable.

In the Crabtree-positive yeast S. cerevisiae, high levels of the fermentative key enzyme pyruvate decarboxylase are present under aerobic, glucose-limited growth conditions. These activities increase only approximately twofold upon a switch to respiration-fermentative growth. In contrast, only low pyruvate decarboxylase activities could be detected in aerobic, glucose-limited cultures of the Crabtree-negative yeast C. utilis. These activities increased sharply when C. utilis was grown under oxygen limitation (figure 7), suggesting that oxygen may be a key factor in the regulation of pyruvate decarboxylase activity in this yeast. In fact, also in cultures in which growth was only limited by glucose (i.e., at dissolved-oxygen concentrations above ca. 1 % air saturation) the actual dissolved-oxygen concentration has a significant effect on pyruvate decarboxylase activities (data not shown). These data suggest that regulation of sugar metabolism at the level of pyruvate may well be responsible for the different behaviour of Crabtree-positive and Crabtree-negative yeasts.

Oxygen requirements for growth

Oxygen plays a dual role in yeast physiology: it is used as the terminal electron acceptor for mitochondrial respiration and for assimilatory oxygenation reactions. Respiration has two major functions: oxidation of reduced cofactors and generation of metabolic energy in the form of ATP. Under oxygen-limited conditions, energy transduction can be taken over by alcoholic fermentation. However, the conversion of glucose into ethanol is redox-neutral, and can therefore not be used to reoxidize the 'excess' reducing equivalents generated in assimilation. Glycerol production can serve as an alternative redox sink but requires a net input of ATP (van Dijken & Scheffers, 1985). Theoretically, therefore, yeasts can optimize their sugar metabolism under oxygen-limited growth conditions by preferentially using oxygen for the regeneration of 'excess' NADH. The data presented in figure 9 indicate that in both S. cerevisiae and C. utilis, sugar metabolism is regulated in this way. At present, it is unclear at which level the preferential use of oxygen as an electron acceptor for NADH regeneration is regulated. At the kinetic level, a higher affinity of the mitochondrial external NADH dehydrogenase complex for NADH as compared to the cytosolic dihydroxyacetone phosphate reductase could be significant.
In addition to its role in respiration, oxygen can also fulfill an essential role in assimilatory oxygenation reactions. Cellular constituents, the synthesis of which requires molecular oxygen must be added to growth media to allow anaerobic growth. In the case of *S. cerevisiae*, these ‘anaerobic growth factors’ are well-defined, and the organism grows well anaerobically in defined media supplemented with ergosterol, unsaturated fatty acids and nicotinic acid (Andreasen & Stier, 1953; 1954)(table 1, figures 2 & 3). In the same medium, however, *C. utilis* was not able to grow at a rate of 0.10 h⁻¹ at zero oxygen feed (table 4, figure 6). This confirmed earlier reports that *C. utilis* is unable to grow anaerobically at rates above 0.01 h⁻¹ (Visser et al., 1990). *C. utilis* is not an exception in this respect: apart from *S. cerevisiae*, none of the type species of the 75 yeast genera studied were able to grow under strictly anaerobic conditions with specific growth rates higher than 0.10 h⁻¹ (Visser et al., 1990). It is as yet unclear if unidentified assimilatory oxygen requirements are involved, or that these yeasts require oxygen for other cellular processes.

This hitherto unexplained inability of non-*S. cerevisiae* yeasts to grow anaerobically is of great importance for some industrial applications of these organisms. The range of sugar substrates is much larger for these yeasts than for *S. cerevisiae*. Numerous processes have been suggested in which non-*Saccharomyces* yeasts are used for the production of ethanol from waste streams and complex raw materials. A fact, overlooked in many of these publications, is that oxygen is required by these yeasts, even during fermentative growth. At very low oxygen feeds, growth (and eventually fermentation) becomes inhibited due to the intrinsic inability of these yeasts to grow anaerobically. At higher oxygen feeds, the glycolytic flux is preferentially directed towards respiration, thereby lowering the ethanol yield. This makes it rather difficult to optimize ethanol yields in large-scale industrial fermentations using non-*Saccharomyces* yeasts. However, in small-scale laboratory experiments, the oxygen requirements of these yeasts may easily go unnoticed. For example, if in the present study oxygen-permeable silicone tubing (instead of Norprene) was used on the fermenters, *C. utilis* could be grown at a dilution rate of 0.10 h⁻¹ in cultures flushed with pure nitrogen gas.

The Kluvyer effect

The observation of Sims & Barnett (1978) that *C. utilis* exhibits a Kluvyer effect during growth on maltose is clearly supported by the results presented in this study. Over a range of oxygen feed rates, *C. utilis* grew strictly respiratory on maltose, without the occurrence of alcoholic fermentation (figure 6, table 4). Our results imply, that even in a situation where the enzymes for maltose uptake and hydrolysis were present, *C. utilis* did not ferment this disaccharide. In contrast, during oxygen-limited growth of *C. utilis* on glucose, respiration and fermentation occurred simultaneously (figure 5). The fermentation rates in these cultures (figure 5) were equal to or even exceeding those of the Kluvyer-negative yeast *S. cerevisiae*, which exhibited a respiro-fermentative metabolism during oxygen-limited growth on both glucose and maltose (figure 3).

The intrinsic inability of *C. utilis* to ferment maltose is important for the interpretation of the results of Sims & Barnett (1978). From experiments with a CO₂ electrode, these authors concluded that the fermentative activity of this yeast with maltose responded rapidly and reversibly to changes in the oxygen concentration. The results presented in this paper indicate that the CO₂ production measured in the experiments of Sims & Barnett (1978) was due to respiratory rather than fermentative maltose metabolism.

In earlier reports, low pyruvate decarboxylase activities have been reported for Kluvyer-positive yeasts grown on disaccharides (Sims & Barnett, 1991; Sims et al., 1991). In the present study, the pyruvate decarboxylase and alcohol dehydrogenase activities in *C. utilis* grown on maltose in oxygen-limited chemostat cultures (figures 7 & 6) were theoretically sufficient to sustain a *α*₀ of 11 mmol/g⁻¹·h⁻¹. This theoretical flux is even higher than that observed in *S. cerevisiae* grown under the same conditions. Apparently, in *C. utilis* the Kluvyer effect for maltose is not caused by an insufficient capacity of pyruvate decarboxylase and alcohol dehydrogenase. Also the absence of significant concentrations of organic acids in the maltose-grown *C. utilis* cultures (table 4) indicates that a limited capacity of these fermentative enzymes is unlikely to cause the Kluvyer effect. Instead, maltose metabolism appears to be regulated before the level of pyruvate, i.e. at the level of disaccharide uptake, hydrolysis or glycolysis.

An inhibition of disaccharide transport activity by the absence of oxygen has been proposed as one of the possible causes of the Kluvyer effect (Sims & Barnett, 1978; Schulz & Höfer, 1986). However, low, but significant disaccharide uptake rates have been reported for Kluvyer-positive yeasts (Sims & Barnett, 1978; Schulz & Höfer, 1986). Barnett & Sims (1982) therefore concluded that the Kluvyer effect cannot be solely caused by absence of transport activity. This conclusion is supported by the present study: under all oxygen feed regimes studied, *C. utilis* continued to take up and respire maltose. The total absence of alcoholic fermentation in maltose-grown, oxygen-limited cultures of *C. utilis* indicates that uptake and hydrolysis of maltose was stoichiometrically balanced with the amount of this disaccharide that could be respired. A mechanism which tunes
disaccharide uptake and hydrolysis in response to oxygen concentration or redox potential may indeed be responsible for this phenomenon.

The original description of the Kluyver effect (Sims & Barnett, 1978) discriminated between aerobic and anaerobic utilization of sugars. However, C. utilis and other yeasts exhibiting the Kluyver effect are not capable of anaerobic growth (Visser et al., 1990). Furthermore, the data presented here indicate that occurrence of the Kluyver effect does not depend on the oxygen concentration, but reflects an intrinsic inability to perform fermentative metabolism with the disaccharide. We therefore propose a new definition of the Kluyver effect: 'The inability to ferment certain disaccharides to ethanol and carbon dioxide, even though respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexose(s) are possible'.

Acknowledgements

We are indebted to Dr. C. Verduyn (BIRO Engineering, Schiedam, The Netherlands) for valuable discussions and Kjeld Bangma and Yvonne van Kiel for their skilful technical assistance.

References


Kluyver AJ & Custers MTJ (1940) The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. Antonie van Leeuwenhoek J. Microbiol. Serol. 6: 121-162


Lagunas R (1986) Misconceptions about the energy metabolism of Saccharomyces cerevisiae. Yeast 2: 221-228


Postma E, Scheffers WA & van Dijken JP (1989a) Kinetics of growth and glucose transport in glucose-limited chemostat cultures of Saccharomyces cerevisiae CBS 8066. Yeast 5: 159-165


Is the Kluvyer effect caused by product inhibition?


Abstract

*Candida utilis* CBS 621 exhibits the Kluvyer effect for maltose, i.e. this yeast can respire maltose and is able to ferment glucose, but is unable to ferment maltose. When glucose was pulsed to a maltose-grown, oxygen-limited chemostat culture of *C. utilis*, ethanol formation from glucose started almost instantaneously, indicating that the enzymes needed for alcoholic fermentation are expressed in maltose-grown cells. However, the addition of glucose inhibited maltose metabolism. To eliminate a possible catabolite inhibition and/or repression of enzyme activities involved in maltose metabolism, the effect of simultaneously feeding glucose and maltose to an oxygen-limited, maltose-grown chemostat culture was studied. In this case, the glucose concentration in the culture remained below 0.1 mM, which makes glucose catabolite repression unlikely. Nevertheless, maltose metabolism appeared to cease when the culture was switched to the mixed feed. Based on the outcome of the mixed-substrate studies, it was postulated that the Kluvyer effect may be caused by feedback inhibition of maltose utilization by ethanol, the product of its fermentative metabolism. If ethanol suppresses the utilization of non-fermentable disaccharides, this would provide a phenomenological explanation for the occurrence of the Kluvyer effect: accumulation would then not occur and the rate of maltose metabolism would be tuned to the culture's respiratory capacity. This hypothesis was tested by studying growth of *C. utilis* CBS 621 and *Debaryomyces castellii* CBS 2932 in aerobic batch cultures on mixtures of sugars and ethanol. Indeed, with both yeasts, diauxic growth was observed on mixtures of ethanol and a disaccharide that gives rise to the Kluvyer effect, with ethanol being the preferred substrate. In contrast, sugars which could be fermented were either utilized simultaneously with ethanol or preferred over this substrate.

Introduction

Many facultatively fermentative yeasts species show a peculiar behaviour with respect to the utilization of certain disaccharides. Depending on the yeast species, some disaccharides cannot be fermented, although respiration of the disaccharides and fermentation of the component hexose(s) are both possible (Weusthuis *et al.*, 1994). This physiological phenomenon is called the Kluvyer effect (Kluvyer & Custers, 1940; Sims & Barnett, 1978). Of the 215 glucose fermenting yeast species, 96 exhibit the Kluvyer effect for at least one disaccharide. Table 1 shows the widespread occurrence of the Kluvyer effect among the facultatively fermentative yeasts that are presently known.

Although the mechanism responsible for the Kluvyer effect has not yet been elucidated, it must somehow be related to differences in the metabolism of disaccharides and monosaccharides. Target reactions at which control of disaccharide metabolism may be exerted are sugar uptake and/or disaccharide hydrolysis. Also the possibility that specific kinases are involved in transport-associated phosphorylation of hexoses and phosphorylation of cytosolic hexose molecules generated from disaccharide hydrolysis (Clifton *et al.*, 1993) cannot be neglected as a possibility.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Number of yeast species that show Fermentation</th>
<th>Kluvyer effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>15</td>
<td>41</td>
</tr>
<tr>
<td>Methyl-α-D-glucopyranoside</td>
<td>7</td>
<td>45</td>
</tr>
<tr>
<td>Sucrose</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Trehalose</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>Melibiose</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Lactose</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Melezitose</td>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>Raffinose</td>
<td>19</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1. Screening of facultatively fermentative yeast species for their ability to ferment a number of disaccharides and for the occurrence of the Kluvyer effect. Yeasts included in the screening could grow on the disaccharide listed and were able to ferment glucose. Data were obtained from Barnett *et al.* (1990). Only yeasts species of which all strains showed positive results were used.
ble cause of the Kluvyer effect.

In oxygen-limited chemostat cultures of *C. utilis* grown on maltose, the amount of maltose that can be metabolized is limited by the amount of oxygen available for respiration. When the oxygen feed to these cultures is decreased, maltose is only partially consumed and alcoholic fermentation is not observed (Weusthuis et al., 1994). In contrast, glucose-grown cultures of *C. utilis* simultaneously respire and ferment glucose. These experiments indicate that in *C. utilis*, oxygen availability is not a key factor in the Kluvyer effect for maltose. Instead, the Kluvyer effect appears to reflect an intrinsic inability to ferment particular disaccharides (Weusthuis et al., 1994).

Regardless of the molecular mechanism, the observed inability of Kluvyer-positive yeasts to form ethanol from disaccharides may be caused in two distinct ways: either the presence of disaccharides directly or indirectly inhibits the occurrence of alcoholic fermentation or, alternatively, extracellular free monosaccharides are required to induce alcoholic fermentation. The primary aim of the present work was to discriminate between the above two possibilities by studying the metabolism of mixtures of glucose and maltose by *C. utilis*. To avoid repression of maltose-metabolizing enzymes, glucose-limited chemostat cultures were used, in which the low residual glucose concentrations prevent such phenomena.

Based on the experimental results, a hypothesis was formulated which gives a phenomenological explanation of the Kluvyer effect. This hypothesis was verified by studying the growth of the Kluvyer-positive yeasts *C. utilis* and *D. castellii* on mixtures of various sugars and ethanol.

### Material and Methods

**Organisms and maintenance**

*C. utilis* CBS 621 and *D. castellii* CBS 2923 were obtained from the Centraalbureau voor Schimmel-cultures (Delft, The Netherlands) and maintained on malt agar slants at 4 °C.

**Chemostat cultivation**

Chemostat cultivation was performed in 2-litre fermenters (Applikon, Schiedam, The Netherlands) at a dilution rate of 0.10 h⁻¹, a temperature of 30 °C and a stirrer speed of 760 rpm. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH, controlled by an Applikon ADI-1020 biocontroller. To avoid loss of volatile metabolites, the condenser was cooled to 2 °C, using a cryostat. The working volume of the culture was kept at 1.0 litre by removal of effluent from below the surface of the culture, controlled by an Applikon electrical level controller. This set-up ensured that biomass concentrations in the effluent line differed by less than 1% from those in samples taken directly from the culture. Samples for biomass, substrate and product analysis were taken from the effluent line. The mineral medium, supplemented with vitamins and trace elements was prepared as described by van Leeuwen et al. (1992). Glucose or maltose were added to the media after separate sterilization (Weusthuis et al., 1993). The purity of the chemostat cultures was routinely checked by phase-contrast microscopy at 1000x magnification.

**Oxygenation of the chemostat cultures**

Oxygen was added to the cultures as air (60 ml·min⁻¹), with a constant temperature of 20 °C, using a Masterflex peristaltic pump. In the oxygen-limited cultures, the overall gas-flow rate into the cultures was maintained at 560 ml·min⁻¹ by supplementary addition of nitrogen gas via a mass-flow controller (Brooks, Veenendaal, The Netherlands). Addition of nitrogen gas assured good mixing of the air with the culture fluid. To minimize diffusion of atmospheric oxygen into the oxygen-limited cultures, the entire fermentation set-up (including medium reservoir and effluent vessel) was equipped with Norprene tubing (Cole Parmer Inc.). The dissolved-oxygen concentration in the cultures was monitored with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland).

**Batch cultivation**

Yeasts were pregrown in 100 ml shake flasks on 20 ml of the mineral medium described above, with an initial pH of 6.0 and 5.0 g l⁻¹ glucose. Batch cultivation took place in the fermenters described above with an initial working volume of 1.5 l. The pH was controlled between 4.9 and 5.1 by automatic addition of 2 M KOH or 1 M H₂SO₄. The stirrer speed was kept between 750 and 1250 rpm, the air-flow rate into the culture was 1.0 l·min⁻¹. The dissolved oxygen tension in the culture was maintained above 50% air saturation by manual adjustment of the stirrer speed. The temperature was 30 °C for cultures of *C. utilis* or 25 °C for cultures of *D. castellii*.

**Metabolite analysis**

Glucose and maltose concentrations were determined as described by Weusthuis et al. (1993). The lactose concentration was measured with the same assay as maltose, using β-galactosidase instead of α-glucosidase. Acetate was determined by HPLC (Weusthuis et al., 1993). Ethanol concentrations were determined with an colorimetric assay kit (EK 001 Leeds Biochemicals, UK). The maximal standard deviation for glucose and lactose was 0.2 g l⁻¹, for ethanol 0.1 g l⁻¹, for maltose 0.2 g l⁻¹ in absence of glucose and 0.8 g l⁻¹ in presence of glucose.

**Biomass determinations**

Dry weights of culture samples were determined
using a microwave oven and 0.45-μm membrane filters as described by Postma et al. (1989). Parallel samples varied by less than 1%.

Optical densities at 660 nm were measured with a Vita Lab 20 (Vita Scientific, Dieren, The Netherlands). When the optical density was above 0.3 the samples were diluted with demineralized water. Control experiments showed that this procedure assured a linear relationship between optical density and biomass dry weight. The standard deviation of the measurements was less than 2% of the measured value.

Results

Fermentative capacity of maltose-grown cells

One of the proposed causes of the Kluyver effect is the absence, during growth on disaccharides, of one of the enzyme activities responsible for the conversion of glucose into ethanol. For example, Sims et al. (1991) and Sims & Barnett (1991) proposed that the Kluyver effect could be caused by the absence of the enzymes pyruvate decarboxylase and/or alcohol dehydrogenase. However, in our studies it has been observed that during oxygen-limited growth of the Kluyver-positive yeast C. utilis on maltose, these enzymes were present at high levels (Weusthuis et al., 1984). This indicates that in C. utilis the apparent inability to perform alcoholic fermentation with maltose is not due to the absence of these fermentative key enzymes. Nevertheless, the detection of these enzyme activities in cell-free extracts does not necessarily imply that they are also functional in vivo. To study whether maltose-grown C. utilis is capable of fermenting glucose, addition of glucose to oxygen-limited, maltose-grown cultures of this yeast was studied.

In a steady-state oxygen-limited culture of C. utilis grown on 10 g l⁻¹ maltose, alcoholic fermentation did not occur. Instead, the amount of maltose that could not be respired was not consumed, resulting in a residual maltose concentration of 4 g l⁻¹. When glucose (10 g l⁻¹) was added to this culture (figure 1A), ethanol formation could be detected in culture supernatants approximately 2 minutes after the pulse (figure 1B). The rate of ethanol formation was linear during the first 15 minutes, confirming that all enzymes needed for the conversion of glucose into ethanol were present in the maltose-grown culture. The apparent 2-minute delay before the onset of ethanol formation may be caused by the experimental procedures (sampling time, sensitivity of the alcohol assay). Also, the possibility of short-term activation/inactivation processes involving key enzymes of fermentative glucose metabolism cannot be excluded.

During the glucose pulse experiment, both the addition of maltose-containing medium to the fermenter and the removal of culture effluent continued. Upon the addition of glucose, an increase of the maltose concentration in the culture was observed (figure 1), suggesting that the presence of glucose interfered with maltose utilization. One of the possible causes for this interference is catabolite repression of maltose utilization by glucose, a phenomenon well documented in e.g. Saccharomyces cerevisiae (Göts, 1969; Peinado & Loureiro-Dias, 1986). In theory, glucose repression can be circumvented if a steady-state culture growing on maltose is switched to a medium feed containing both maltose and glucose. In this way, the glucose enters the culture slowly and can in principle be used immediately, thus avoiding accumulation of repressing glucose concentrations in the culture. Previous experiments with S. cerevisiae had shown that switching maltose-grown cultures to mixtures of maltose and glucose indeed did not result
in glucose accumulation, but allowed simultaneous utilization of glucose and maltose. The residual glucose concentrations in these cultures were sufficiently low to prevent glucose catabolism repression of maltose-utilizing enzyme systems (Weusthuis et al., 1990).

Chemostat cultivation of C. utilis on mixtures of glucose and maltose

Addition of glucose (10 g·l⁻¹) to the reservoir medium of an oxygen-limited, steady-state chemostat culture of C. utilis growing on 10 g·l⁻¹ maltose, caused an increase of the residual maltose concentration in the culture. This increase followed wash-in kinetics until, eventually, the maltose concentration in the culture became equal to the concentration in the reservoir medium (figure 2). Apparently, simultaneous feeding of glucose and maltose caused a complete inhibition or suppression of maltose utilization.

During the transient-state experiment shown in figure 2, the glucose concentration in the culture remained below the detection limit of the glucose assay (approximately 0.1 mM). Therefore, suppression of maltose utilization by glucose catabolism repression or inactivation seemed unlikely, although we could not exclude the possibility that even extremely low glucose concentrations inhibit maltose utilization in C. utilis. However, the rapid accumulation of ethanol after addition of glucose to oxygen-limited, maltose-grown cultures (figures 1 & 2) indicates another explanation, namely an effect of ethanol or related product of fermentative glucose metabolism on maltose metabolism. The product of fermentative metabolism of maltose and glucose are expected to be identical. Therefore, if fermentation products suppress or inhibit maltose metabolism, this should result in a feed-back inhibition of maltose utilization once ethanol is formed from this substrate. This mechanism would offer a phenomenological explanation of the Kluiver effect that has so far not been mentioned in the literature: it would limit the rate of maltose utilization to the rate that can be accomplished without the occurrence of alcoholic fermentation (figure 3). The most likely target for this type of regulation would appear to be the disaccharide permease, since regulation of subsequent metabolic reactions could result in intracellular accumulation of disaccharides or other metabolites.

An indication that product inhibition may indeed be involved in the Kluiver effect is provided by the data shown in figure 1A. After the complete consumption of the glucose that was pulsed into the culture, the concentration of ethanol decreased faster than wash-out kinetics predicts, indicating ethanol consumption by the culture. Maltose, however, was still not utilized. As long as ethanol was present in the culture, the maltose concentration continued to increase according to wash-in kinetics, as a result of the feed of medium to the culture. If ethanol, or metabolites directly derived from ethanol, cause the Kluiver effect by inhibition of disaccharide utilization, this should imply that yeasts exhibiting the Kluiver for particular
short lag period in which no growth occurred (figure 4B).

In a control experiment, *C. utilis* was grown under identical conditions on a mixture of glucose and ethanol (5.0 and 4.0 g l\(^{-1}\) respectively). In this case, diauxic growth was not observed (figure 4A). Glucose and ethanol were utilized simultaneously, until glucose was completely consumed. Growth on ethanol as sole carbon source occurred after a small lag phase, a period possibly needed to induce the enzymes of gluconeogenesis and/or the glyoxylate cycle (figure 4A).

To investigate whether preferential utilization of ethanol over disaccharides also occurs in other yeasts exhibiting the Kluyver effect, growth of *D. castellii* CBS 2923 on mixtures of sugars and ethanol was studied. *D. castellii* is a facultatively fermentative yeast which exhibits the Kluyver effect for lactose, but not for maltose (Sims & Barnett, 1991).

During growth of *D. castellii* on a mixture of either glucose or maltose and ethanol, diauxic growth was observed (figures 5A & 5B, respectively). These two sugars were preferred over ethanol. After the sugars were completely exhausted, utilization of ethanol proceeded and was accompanied by a transient accumulation of acetate. After this period growth resumed with acetate as carbon source. A diauxic growth pattern was also observed during growth of *D. castellii* on mixtures of lactose and ethanol. However, in this case ethanol was preferred over the sugar (figure 5C).

**Discussion**

The experiments shown in figures 1 and 2 confirm the conclusion from earlier work that *C. utilis* exhibits the Kluyver effect: this yeast is unable to produce ethanol from maltose, although respiratory metabolism of maltose and fermentation of glucose are both possible. Furthermore, the almost instantaneous occurrence of ethanol formation after the pulse-wise addition of glucose to an oxygen-limited, maltose-grown culture (figure 1) confirms our earlier conclusion that *C. utilis* expresses all enzymes required for alcoholic fermentation during oxygen-limited growth on maltose (Weusthuis et al., 1994). The original aim of this work was to see if the apparent inability of *C. utilis* to produce ethanol from maltose is due to a requirement for extracellular glucose to induce maltose fermentation or that, alternatively, the presence of maltose inhibits glucose fermentation. The results indicate that addition of extracellular glucose does not induce maltose fermentation but, instead, inhibits respiratory maltose metabolism. The observation that this negative effect of glucose addition even occurs under conditions where glucose catabolite repression is not expected (figure 2), suggests an alternative explanation for the
batch-growth experiments involving two yeasts, between the occurrence of the Kluvyer effect for a disaccharide and the preferential utilization of ethanol during growth on sugar-ethanol mixtures (figures 4 & 5) strongly supports the above hypothesis. In fact, the Kluvyer effect is an inevitable consequence of the observed preferential use of ethanol over the disaccharides: if, during batch growth, a product is preferred as a growth substrate over the compound from which it is formed, net accumulation of the metabolite can by definition not occur.

At present, it is not possible to assess whether regulation is exerted at the level of enzyme synthesis or at the level of enzyme activity or indeed which step in disaccharide metabolism is the target for regulation. Therefore, although the experimental data presented in this paper provide a phenomenological explanation for the occurrence of the Kluvyer effect, further work is needed to elucidate the molecular mechanism that causes the apparent down-regulation of disaccharide metabolism in the presence of ethanol. In this respect, it may be necessary to extend studies on the Kluvyer effect to yeast species that are well accessible for molecular genetic techniques.

References


Kluvyer AJ & Custers MTJ (1940) The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. Antonie van Leeuwenhoek J. Microbiol. Serol. 6: 121-162


Figure 5. Growth and substrate consumption of D. castellii CBS 2923 in aerobic, pH-controlled batch cultures on mixtures of A) glucose and ethanol, B) maltose monohydrate and ethanol and C) lactose monohydrate and ethanol. The medium contained 5.0 g l⁻¹ sugar and 4.0 g l⁻¹ ethanol.

Kluvyer effect, namely that alcoholic fermentation, which occurs after the addition of glucose, inhibits or suppresses maltose metabolism via a feed-back mechanism involving ethanol or a related metabolite (figure 3).

The striking correlation, observed in aerobic


Future prospects

Chapters 5 and 6 of this thesis deal with an investigation of the Kluyver effect: the inability to ferment certain disaccharides under anaerobic conditions, whereas aerobic growth on the disaccharide and the anaerobic fermentation of the component hexose(s) is possible (Sims & Barnett, 1978). In chapter 5 the influence of oxygen on the occurrence of the Kluyver effect was studied and it was concluded that oxygen is not directly involved in this effect. The Kluyver effect seems to be caused by an inherent inability to ferment certain disaccharides. From the experiments described in chapter 6 it appears that the occurrence of the Kluyver effect coincides with the preferential consumption of ethanol over the disaccharides in batch cultures. Glucose and disaccharides that do not give rise to the Kluyver effect were used before ethanol. This led to the hypothesis that the Kluyver effect is caused by a feedback inhibition of disaccharide transport and/or hydrolysis by ethanol or related compounds. These experiments have been performed with two disaccharides and two yeasts. Clearly, to show that the coincidence of the Kluyver effect and preferential ethanol utilization in batch cultures is a general phenomenon, additional yeasts and sugars have to be investigated.

The feedback inhibition of disaccharide utilization by ethanol or related compounds is a phenomenological description of the Kluyver effect. The mechanism on molecular level is still unknown. Initially, further research concerning the mechanism has to focus on three topics:

1. The identity of the effector: although ethanol was used in the experiments of chapter 6, it could also be a metabolic derivative of ethanol like acetate or acetaldehyde.
2. The target enzyme: is it the disaccharide transport protein, the disaccharide hydrolase or an as yet unknown protein? From previous studies (See chapter 1) the transport step appears to be the most likely candidate.
3. The regulation of the Kluyver effect: is it regulated on the level of enzyme activity or synthesis?

As pointed out in the general introduction the Kluyver effect is a general phenomenon occurring in many yeasts with a variety of disaccharides. Surprisingly, also sucrose gives rise to the Kluyver effect, for example, in the yeast Hansenula polymorpha (presently known as Pichia angusta). It seems likely therefore that metabolism of sucrose in this yeast involves intracellular rather than extra-cellular hydrolysis of the sugar. If sucrose hydrolysis would occur extracellularly, as in S. cerevisiae, glucose and fructose would be formed outside the cell. Since these monosaccharides are readily fermented by H. polymorpha, an extracellular localization of invertase is at variance with its inability to ferment sucrose. Further research on the localization of invertase in this and other yeasts that exhibit the Kluyver effect for sucrose is required to show that the inability to ferment sucrose coincides with an intracellular localization of invertase.

The inability of H. polymorpha to ferment sucrose might be advantageous in high cell density cultivation of this yeast for the production of heterologous proteins (Giuseppin et al., 1993). Sucrose is the main sugar in molasses, which is a popular feed stock in industrial fermentations. Therefore, growth of H. polymorpha on molasses to high cell densities will not lead to unwanted ethanol formation as a result of oxygen shortage since the sugar cannot be fermented. Stringent control of the dissolved oxygen concentration in such fermentations is therefore not as crucial as with S. cerevisiae. In this yeast shortage of oxygen rapidly triggers alcoholic fermentation.

References


Summary

The utilization of disaccharides may differ from monosaccharide metabolism in only two steps: transport of the sugar over the plasma membrane and hydrolysis. Disaccharide metabolism therefore seems to be similar to monosaccharide metabolism. Nevertheless, it is known that some yeasts are not able to ferment certain disaccharides, although respiration of the disaccharide and fermentation of both monosaccharides is possible. This phenomenon, known as the Kluyver effect, indicates that disaccharide metabolism is not just the summation of the metabolism of the component hexoses. This thesis deals with growth of yeasts on disaccharides, concentrating on the differences with growth on monosaccharides. Special attention is paid to the role of sugar transport and to the Kluyver effect.

Chapter 2 is about sugar transport systems in yeasts and how they are studied. The kinetics and energetics of sugar transport in yeasts strongly depend on environmental conditions. The specific rate of sugar consumption ($q_s$) in growing cultures is given by specific growth rate $\times$ biomass yield$^{-1}$. So, $q_s$ can be manipulated either by variation of the growth rate or by variation of the growth yield on the sugar. In chemostats, the former can be accomplished by varying the dilution rate, the latter by increasing the maintenance-energy requirement of yeast cultures by the inclusion of non-metabolizable weak acids in the growth medium, by manipulation of the oxygen supply to cultures, or by growth on mixtures of sugars. This type of controlled variation of metabolic fluxes cannot be achieved in batch cultures, where various parameters that are decisive for the kinetics of sugar transport cannot be fixed.

A key factor with respect to transport kinetics is the sugar concentration in the culture. In chemostat cultures, yeasts can be grown continuously under sugar limitation. As a result, cells adapt their uptake systems to cope with low sugar concentrations, often in the micromolar range. At low environmental sugar concentrations, yeasts that possess high-affinity proton-symport sugar-uptake systems have a competitive advantage over yeasts that transport sugars via facilitated diffusion.

Manipulation of growth conditions in chemostat cultures can be used to vary the contribution of various transport mechanisms to the overall sugar-uptake capacity in a controlled and reproducible manner.

Chapter 3 describes the study of maltose/proton co-transport in intact cells and in plasma-membrane vesicles of the yeast *Saccharomyces cerevisiae*. In order to determine uphill transport in vesicles, plasma membranes were fused with proteoliposomes containing cytochrome c oxidase as a proton-motive force-generating system. Maltose accumulation, dependent on the electrical and pH-gradients, was observed. The initial uptake velocity and accumulation ratio in vesicles proved to be dependent on the external pH. Moreover, kinetic analysis of maltose transport showed that $V_{\text{max}}$ greatly decreased with increasing pH, whereas the $K_m$ remained virtually constant. These observations were in good agreement with results obtained with intact cells, and suggest that proton binding to the carrier proceeds with an apparent pK of 5.7. The observation with intact cells that maltose is co-transported with protons in a one-to-one stoichiometry was ascertained in the vesicle system by measuring the balance between proton-motive force and the chemical maltose gradient. These results show that maltose transport in vesicles prepared by fusion of plasma membranes with cytochrome c oxidase proteoliposomes behaves in a similar way as in intact cells. It is therefore concluded that this vesicle model system offers a wide range of new possibilities for the study of maltose/proton co-transport in more detail.

Chapter 4 deals with the effect of energy requirements of transport systems on growth and product formation. This study was performed with *S. cerevisiae* in which maltose is transported by a proton symport mechanism, whereas glucose transport occurs via facilitated diffusion. The energy requirement for maltose transport was evaluated with a metabolic model based on an experimental value of $Y_{\text{ATP}}$ for growth on glucose and an ATP requirement for maltose transport of 1 mol mol$^{-1}$. 

81
The predictions of the model were verified experimentally with anaerobic sugar-limited chemostat cultures growing on a range of maltose-glucose mixtures at a fixed dilution rate of 0.1 h\(^{-1}\). The biomass yield (g cells/g sugar\(^{-1}\)) decreased linearly with increasing amounts of maltose in the mixture. During growth on maltose, the yield was 25% lower than on glucose, in agreement with the model predictions. Due to the enhanced energy requirement for growth on maltose, 8% more ethanol was produced compared with growth on glucose.

Chapter 5 and 6 deal with the Kluyver effect. The definition of the Kluyver effect in the literature suggests an important role for oxygen. The effect has however been studied in batch cultures, which is an unsuitable cultivation method for a quantitative investigation of the role of oxygen. Chapter 5 describes a study of growth and metabolite formation in oxygen-limited chemostat cultures of \(S.\) \textit{cerevisiae} and \(C.\) \textit{utilis} growing on glucose or maltose at a dilution rate of 0.1 h\(^{-1}\). \(C.\) \textit{utilis} is known to show the Kluyver effect for maltose, whereas \(S.\) \textit{cerevisiae} does not. With either glucose or maltose \(S.\) \textit{cerevisiae} could be grown under dual limitation of oxygen and sugar. Respiration and alcholic fermentation occurred simultaneously and the catabolite fluxes through these processes were dependent on the magnitude of the oxygen feed.

Also \(C.\) \textit{utilis} could be grown under dual limitation of glucose and oxygen. However, at very low oxygen feed rates (i.e. below 4 mmol\(\) l\(^{-1}\) h\(^{-1}\)) growth was limited by oxygen only, as indicated by the high residual glucose concentration in the culture. In contrast to \(S.\) \textit{cerevisiae}, \(C.\) \textit{utilis} could not be grown anaerobically at a dilution rate of 0.1 h\(^{-1}\). With \(C.\) \textit{utilis}, absence of oxygen resulted in wash-out, despite the presence of ergosterol and Tween-80 in the growth medium.

The behaviour of \(C.\) \textit{utilis} with respect to maltose utilization in oxygen-limited cultures was exceptional: alcoholic fermentation did not occur in such cultures and the amount of maltose metabolized was dependent on the oxygen supply. Oxygen-limited cultures of \(C.\) \textit{utilis} growing on maltose always contained high residual sugar concentrations.

These observations throw new light on the so-called Kluyver effect. Apparently, maltose is a non-fermentable sugar for \(C.\) \textit{utilis} despite the fact that it can serve as a substrate for growth of this facultatively-fermentative yeast. This is not due to the absence of key enzymes of alcoholic fermentation. Pyruvate decarboxylase and alcohol dehydrogenase were present at high levels in maltose-utilizing cells of \(C.\) \textit{utilis} grown under oxygen limitation.

In this chapter it is concluded that the Kluyver effect, in \(C.\) \textit{utilis} on maltose, results from a regulatory mechanism that prevents the sugar from being fermented. Oxygen is not a key factor in this phenomenon since under oxygen limitation alcoholic fermentation of maltose was not triggered.

In chapter 6 the Kluyver effect was studied more closely. This was done by supplying \(C.\) \textit{utilis} with both maltose and glucose at the same time, as described for \(S.\) \textit{cerevisiae} in chapter 4. When glucose was pulsed to a maltose-grown, oxygen-limited chemostat culture of \(C.\) \textit{utilis}, ethanol formation from glucose started almost instantaneously, indicating that the enzymes needed for alcoholic fermentation are expressed in maltose-grown cells. However, the addition of glucose inhibited maltose metabolism. To eliminate a possible catabolite inhibition and/or repression of enzyme activities involved in maltose metabolism, the effect of simultaneously feeding glucose and maltose to an oxygen-limited, maltose-grown chemostat culture was studied. In this case, the glucose concentration in the culture remained below 0.1 mm, which makes glucose catabolite repression unlikely. Nevertheless, maltose metabolism appeared to cease when the culture was switched to the mixed feed. Based on the outcome of the mixed-substrate studies, it was postulated that the Kluyver effect may be caused by feedback inhibition of maltose utilization by ethanol, the product of its fermentative metabolism. If ethanol suppresses the utilization of non-fermentable disaccharides, this would provide a phenomenological explanation for the occurrence of the Kluyver effect: accumulation would then not occur and the rate of maltose metabolism would be tuned to the culture's respiratory capacity. This hypothesis was tested by studying growth of \(C.\) \textit{utilis} and \(D.\) \textit{bayronomyces castellii} in aerobic batch cultures on mixtures of sugars and ethanol. Indeed, with both yeasts, diauxic growth was observed on mixtures of ethanol and a disaccharide that gives rise to the Kluyver effect, with ethanol being the preferred substrate. In contrast, sugars which could be fermented were either utilized simultaneously with ethanol or preferred over this substrate.
Samenvatting

Het metabolisme van disacchariden door gisten kan in slechts twee stappen verschillen van het monosaccharide-metabolisme: het transport van de disacchariden over de plasmamembraan en de hydrolyse. Disaccharide-metabolisme lijkt daarom groterdeels gelijk te zijn aan monosaccharide-metabolisme. Toch is bekend dat sommige gisten niet in staat zijn bepaalde disacchariden te vergisten, terwijl deze wel kunnen worden verademd en de fermentatie van de samenstellende monosacchariden wel mogelijk is. Uit dit fenomeen, bekend als het Kluyver-effect, blijkt dat disaccharide-metabolisme niet simpelweg hetzelfde is als de optelling van het metabolisme van de hexosen waaruit het disaccharide bestaat. Dit proefschrift gaat over de groei van gisten op disacchariden en met name over de verschillen met groei op monosacchariden. Speciale aandacht wordt besteed aan de rol van het suikertransport en aan het Kluyver-effect.

Hoofdstuk 2 beschrijft suikertransportsystemen in gisten en de manier waarop deze worden beschreven. De kinetiek en energetica van suikertransport in gisten is sterk afhankelijk van omgevingsfactoren. De specifieke consumptiesnelheid (qₘ) in groeiende culturen wordt gegeven door specifieke groeiensnelheid x biomassaoendering.ц 1. Dus kan qₘ gemanipuleerd worden door de groeiensnelheid of de biomassaoendering te variëren. In chemostaten kan het eerste worden bereikt door de verdunningsniveau te variëren; het laatste door de maintenance-energie te verhogen via toevoeging van niet-metaboliseerbare zwakke zuren aan het medium, door verandering van de zuurstoftoevoer naar de cultuur, of door kweken op mengsels van suikers. Dit soort gecontroleerde variatie van metabole fluxen kan niet worden bereikt met batchculturen, waarin verscheidende parameters die belangrijk zijn voor de kinetiek van suikertransport niet constant kunnen worden gehouden.

Een sleutelfactor inzake transportkinetiek is de suikerconcentratie in de cultuur. Gisten kunnen in chemostataculturen worden gekweekt onder suikerlimitatie. Om deze lage suikerconcentraties (in het micromolair gebied) te kunnen gebruiken passen cellen hun opnamesysteem aan. Gisten die een protonsuikerscotransportsysteem hebben met een hoge af
De energiekosten van maltosetransport worden geëvalueerd met een metabool model dat gebaseerd is op een experimentele $Y_{ATP}$-waarde voor groei op glucose en een ATP-behoefte voor maltosetransport van 1 mol-mol$^{-1}$. De modellmatige voorspellingen werden experimenteel geverifieerd met anaërobe suiker-gelimeerde chemostaatcultures op verschillende mengsels van glucose en maltose bij een vaste verdunningsnelheid van 0.1 h$^{-1}$. De biomassa-opbrengst (g cellen-g suiker$^{-1}$) nam lineair af met toenemende hoeveelheden maltose in het mengsel. Bij groei op maltose was de biomassa-opbrengst 25% lager dan op glucose, in overeenstemming met de waarde die door het model werd voorspeld. Dankzij de toegenomen energiebehoefte veroorzaakt door groei op maltose werd er 8% meer ethanol geproduceerd, vergeleken met groei op glucose.

Hoofdstuk 5 en 6 gaan over het Kluyver-effect. De definitie van het Kluyver-effect zoals die in de literatuur is gegeven suggereert een grote rol van zuurstof in het veroorzaken van dit effect. Het effect is echter alleen onderzocht in batchcultures, een kweektechniek die ongeschikt is voor een kwantitatief onderzoek naar de rol van zuurstof. Hoofdstuk 5 beschrijft onderzoek naar groei en metabolievorming in zuurstof-gelimeerde chemostaatcultures van S. cerevisiae en Candida utilis op glucose en maltose bij een verdunningsnelheid van 0.1 h$^{-1}$. Bekend is dat C. utilis wel het Kluyver-effect vertoont voor maltose en S. cerevisiae niet.

S. cerevisiae kon zowel op glucose als op maltose worden gekweekt onder een dubbele limiatie van suiker en zuurstof. Ademhaling en fermentatie vonden gelijktijdig plaats en de katabolietfluxen uit deze processen waren afhankelijk van de zuurstoftoevoer.

Ook C. utilis kon worden gekweekt onder een dubbele limiatie van glucose en zuurstof. Echter, bij zeer lage zuurstofvoersnelheden (onder 4 mmol$^{-1}$ h$^{-1}$) werd groei alleen gelimeerd door zuurstof, hetgeen weergegeven door de hoge residuële glucoseconcentratie in de cultuur. In tegenstelling tot S. cerevisiae kon C. utilis niet worden gekweekt onder strikt anaërobe condities bij een verdunningsnelheid van 0.1 h$^{-1}$. De afwezigheid van zuurstof leidde tot de uitspoeling van C. utilis, ondanks de aanwezigheid van ergosterol en Tween-80 in het medium.

Het gedrag van C. utilis met betrekking tot maltosegebruik in zuurstof-gelimeerde culturen was uitzonderlijk: alcoholische gisting vond niet plaats in deze culturen en het maltoseverbruik was afhankelijk van de zuurstoftoevoer. Zuurstof-gelimeerde culturen van C. utilis bevatten altijd hoge residuële suiker-concentraties.


In hoofdstuk 6 wordt het Kluyver-effect nader bekeken. Dit werd gedaan door C. utilis gelijktijdig maltose als glucose aan te bieden, zoals ook is beschreven voor S. cerevisiae in hoofdstuk 4. Pulsgewijze toevoeging van glucose aan een zuurstof-gelimeerde chemostaatcultuur van C. utilis op maltose leidde tot een onmiddellijke omdoening van glucose in ethanol, hetgeen impliceert dat de enzymen die nodig zijn voor alcoholische gisting aanwezig zijn in op maltose gekweekte cellen. Echter, de toevoeging van glucose leidde tot remming van het maltosemetabolisme. Om een mogelijke kataboliet-inhibiti en of -repressie van enzymen, betrokken bij het maltosemetabolisme te elimineren, werd het effect van het gelijktijdig toevoegen van glucose en maltose aan een zuurstof-gelimeerde chemostaatcultuur bestudeerd. In dit geval bleef de glucoseconcentratie onder de 0.1 mm, hetgeen katabolietrepressie door glucose onwaarschijnlijk maakt. De repressiemekanisme van de maltosemetabolisme te stoppen op het moment dat de cultuur werd overgeschakeld op de gemengde voeding. Gebaseerd op de uitkomsten van dit onderzoek met gemengde substraten werd verondersteld dat het Kluyver-effect veroorzaakt kan worden door feedback-inhibiet van maltosegebruik door het produkt van zijn fermentatieve stofwisseling: ethanol. Indien ethanol het verbruik van niet-fermenteerbare dissacchariden zou onderdrukken, dan zou dit een fenomenologische verklaring geven voor het optreden van het Kluyver-effect: accumulatie van ethanol zou dan niet plaatsvinden en de snelheid van maltoseverbruik zou afhankelijk zijn van de ademhalingscapaciteit van de cultuur. Deze hypothese werd getest door de groei van C. utilis en Debaryomyces castellii te onderzoeken in anaërobe batchcultures op mengsels van suiker en ethanol. Inderdaad bleek bij beide gisten duidelijk waargenomen op mengsels van ethanol en een di-saccharide dat aanleiding geeft tot het Kluyver-effect, met ethanol als het geprefereerde substraat. Daarentegen werden suikers die gefermenteerd kunnen worden gelijktijdig of eerder verbruikt dan ethanol.
Publications presented in this thesis

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Other publications in refereed journals


