High-Cell-Density Cultivation of Yeasts on Disaccharides in Oxygen-Limited Batch Cultures

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Many facultatively fermentative yeast species exhibit a "Kluyver effect": even under oxygen-limited growth conditions, certain disaccharides that support aerobic, respiratory growth are not fermented, even though the component monosaccharides are good fermentation substrates. This article investigates the applicability of this phenomenon for high-cell-density cultivation of yeasts. In glucose-grown batch cultures of *Candida utilis* CBS 621, the onset of oxygen limitation led to alcoholic fermentation and, consequently, a decrease of the biomass yield on sugar. In maltose-grown cultures, alcoholic fermentation did not occur and oxygen-limited growth resulted in high biomass concentrations (90 g dry weight L^{-1} from 200 g L^{-1} maltose monohydrate in a simple batch fermentation). It was subsequently investigated whether this principle could also be applied to *Kluyveromyces* species exhibiting a Kluyver effect for lactose. In oxygen-limited, glucose-grown chemostat cultures of *K. wickerhamii* CBS 2745, high ethanol concentrations and low biomass yields were observed. Conversely, ethanol was absent and biomass yields on sugar were high in oxygen-limited chemostat cultures grown on lactose. Batch cultures of *K. wickerhamii* grown on lactose exhibited the same growth characteristics as the maltose-grown *C. utilis* cultures: absence of ethanol formation and high biomass yields. Within the species *K. marxianus,* the occurrence of a Kluyver effect for lactose is known to be strain dependent. Thus, *K. marxianus* CBS 7894 could be grown to high biomass densities in lactose-grown batch cultures, whereas strain CBS 5795 produced ethanol after the onset of oxygen limitation and, consequently, yielded low amounts of biomass. Because the use of yeast strains exhibiting a Kluyver effect obviates the need for controlled substrate-feeding strategies to avoid oxygen limitation, such strains should be excellently suited for the production of biomass and growth-related products from low-cost disaccharide-containing feedstocks.

Key words: *Kluyveromyces* • *Candida utilis* • Kluyver effect • chemostat • biomass • whey

INTRODUCTION

During growth of facultatively fermentative yeasts on glucose, oxygen-limited growth conditions invariably result in the occurrence of alcoholic fermentation. Because the ATP yield from alcoholic fermentation is much lower than that from respiration, this leads to a reduction of the biomass yield on glucose. Furthermore, alcoholic fermentation negatively affects biomass yields due to the accumulation of toxic fermentation products.[21] Therefore, when facultatively fermentative yeasts are used to produce biomass, or products directly derived from biomass such as (heterologous) proteins, accurate control of the dissolved-oxygen concentration is a necessity when glucose is the carbon source.[1,7,19] In industrial processes, oxygen limitation is generally avoided by carefully controlled fed-batch strategies.[8,12,16]

In industrial feedstocks such as molasses, whey, and wort, disaccharides (sucrose, lactose, and maltose, respectively) are the predominant carbon sources. The common use of glucose as a model sugar for laboratory studies probably arises from the assumption that metabolism of disaccharides is similar to that of the component hexoses. Indeed, in *Saccharomyces cerevisiae,* sucrose is hydrolyzed extracellularly to glucose and fructose by invertase.[2,5] However, extracellular hydrolysis followed by uptake of the monosaccharides is not a common feature in yeasts. Not all yeasts hydrolyze sucrose extracellularly[2,9,25] and, moreover, in all cases thus far investigated, hydrolysis of the disaccharides maltose and lactose in yeasts is catalyzed by intracellular hydrolases.[2,23]

When disaccharides are used as a carbon source for the cultivation of facultatively fermentative yeasts, oxygen limitation does not always result in alcoholic fermentation. Already in 1940, Kluyver and Custers[11] noted that, in some yeasts, certain disaccharides do not support alcoholic fermentation, although the component hexoses are rapidly fermented. This phenomenon, which is widespread among facultatively fermentative yeasts and occurs for a wide range of disaccharides, is known as the Kluyver effect.[18,24]

The implications of the Kluyver effect on sugar metabolism can be clearly illustrated with the example of *Candida utilis.* In aerobic cultures, this yeast exhibits rapid growth on glucose and maltose. *C. utilis* is Crab-

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tree-negative (i.e., does not exhibit alcoholic fermentation under fully aerobic conditions). In oxygen-limited chemostat cultures, however, glucose is readily fermented to ethanol. In contrast, alcoholic fermentation does not occur in maltose-grown, oxygen-limited chemostat cultures. Instead, maltose metabolism in these cultures is fully respiratory and, as a result, the disaccharide is incompletely utilized.[10,24] The Kluyver effect does not result from an absence of the key enzymes of alcoholic fermentation: high activities of the fermentative key enzymes are present in maltose-grown cultures. Indeed, when oxygen-limited cultures of *C. utilis* grown on maltose were pulsed with glucose, alcoholic fermentation set in almost immediately.[24]

The aim of this study was to investigate whether the widespread occurrence of the Kluyver effect in facultatively fermentative yeasts can be applied for the straightforward high-cell-density cultivation of yeasts on disaccharides. This was approached experimentally by first investigating growth of the model organism *C. utilis* on maltose. Because maltose is not a commercially interesting substrate for biomass production, we subsequently also investigated the possibility of exploiting the Kluyver effect for lactose in *Kluyveromyces* yeasts. High-cell-density cultivation of these organisms on the lactose-containing feedstock whey may be of commercial interest for the production of single-cell protein, β-galactosidase, and heterologous proteins.[4,13,14]

MATERIALS AND METHODS

Organisms and Maintenance

Kluyveromyces wickerhamii CBS 2745, *Kluyveromyces marxianus* CBS 5795 and 7894, and *Candida 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-L fermentors (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-1020 biocontroller. Aerobic, sugar-limited cultivation was performed by maintaining an air flow through the culture of 0.55 L min^{-1}. This was administered by two routes: 0.5 L min^{-1} via a Brooks 5876 mass-flow controller (Brooks, Veenendaal, The Netherlands) and 50 mL min^{-1} via a Masterflex peristaltic pump. The dissolved oxygen concentration in aerobic, sugar-limited chemostat cultures, as measured with an Ingold oxygen electrode, was above 50% of saturation. The working volume of the culture was kept at 1.0 L by removal of effluent from below the surface of the culture, using an electrical level controller. This set-up ensured that biomass concentrations in the effluent differed by less than 1% from those in samples taken directly from the culture.[14] To obtain reproducible gas transfer properties, positions of baffles, pipes, impellers, and sensors were identical in the fermentors used in this study. To avoid loss of volatile metabolites the off-gas condenser was cooled to 2°C. The mineral medium was prepared according to van Leeuwen et al.[20] Lactose or glucose and vitamins were added to the cultures after separate sterilization to result in a final sugar concentration of 10 g L^{-1} in the input vessel.[20] The purity of the chemostat cultures was routinely checked by phase-contrast microscopy at 1000× magnification.

Transient-State Experiments

The effect of a transition from aerobic conditions to oxygen limitation was investigated by applying a sudden decrease in the oxygen feed. This was achieved by switching the air flow (0.55 L min^{-1}) to a mixture of nitrogen gas (0.5 L min^{-1}) and air (50 mL min^{-1}). As a consequence, the dissolved-oxygen concentration in the culture decreased from above 60% air saturation to below 0.1% of air saturation within 2 min. To minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up (including medium reservoir and effluent vessel) was equipped with Norprene tubing (Cole Parmer Inc.). At appropriate intervals, samples from the effluent line were collected on ice. The residence time in the effluent line was approximately 1 min. Samples were analyzed for culture dry weight and, after centrifugation at 10,000*g*, for metabolites. Cell pellets for preparation of cell-free extracts were resuspended in 10 m*M* potassium phosphate buffer (pH 7.5) containing 2 m*M* EDTA, frozen and stored at −20°C.[17]

High-Cell-Density Batch Cultivation

Yeasts were pregrown in 250-mL shake flasks on 100 mL of the mineral medium described above with an initial pH of 6.0 containing 5.0 g L^{-1} of sugar. Batch cultivation took place in the fermentors previously described with an initial working volume of 1.5 L. The airflow rate into the culture was 0.75 L min^{-1}. In the case of *C. utilis* studies, stirring speeds of 750 or 1000 rpm and a temperature of 37°C were used. Maltose or glucose was added to give an initial concentration of 200 g L^{-1}. Cultivation of *Kluyveromyces* strains was performed at 750 rpm and 30°C. Lactose or and glucose was added to an initial concentration of 100 g L^{-1}. The mineral medium consisted of (per liter): $(NH_4)_2SO_4$, 5.0 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 5.0 g; EDTA (disodium salt), 0.3 g; $ZnSO_4 \cdot 7H_2O$, 90 mg; $MnCl_2 \cdot 2H_2O$, 20 mg; $CoCl_2 \cdot 6H_2O$, 6 mg; $CuSO_4 \cdot 5H_2O$, 6 mg; $Na_2MoO_4 \cdot 2H_2O$, 80 mg; $CaCl_2 \cdot 2H_2O$, 90 mg; $FeSO_4 \cdot$

$7H_2O$, 60 mg; KI, 2 mg; and antifoaming agent Struktol J673 (Struktol Co.), 0.5 mL. After heat sterilization at 120°C and cooling, a filter sterilized vitamin solution was added, giving final concentrations (per liter) of: D-biotine, 1.0 mg; calcium pantothenate, 20 mg; nicotinic acid, 20 mg: *myo*-inositol, 0.5 g; thiamine hydrochloride, 20 mg: pyridoxin hydrochloride, 20 mg; and *p*-aminobenzoic acid, 4 mg. Sugars were heat-sterilized separately at 110°C. The pH was controlled between 4.9 and 5.1 by automatic addition of 10 *M* NH_4OH or 2 *M* H_2SO_4. At appropriate intervals, culture samples were collected for analysis of dry weight and metabolites.

Metabolite Analysis

Enzymatic analysis of glucose and disaccharide concentrations and HPLC analysis of ethanol, glycerol, lactate, and other low-molecular-weight metabolites was performed as described elsewhere.[22] Lactose was first hydrolyzed by β-galactosidase (Sigma G 6008, Sigma Co., St. Louis, MO) and the obtained glucose was determined enzymatically as previously described. Ethanol was also determined with an enzymic assay (based on alcohol oxidase, EK001, Leeds, UK), which is more sensitive than the HPLC method.

Culture Dry Weights

Dry weights of culture samples were determined using 0.45-μm membrane filters and a microwave oven as described elsewhere.[17] 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) activities were performed as described elsewhere.[17] In all enzyme assays, reaction rates were linearly proportional to the amount of enzyme added.

RESULTS

Cultivation of *Candida utilis* CBS 621 in Oxygen-Limited Batch Cultures

The facultatively fermentative yeast, *Candida utilis* ("fodder yeast"), exhibits a Kluyver effect for maltose,[18] and effects of oxygen limitation on its glucose and maltose metabolism have been studied in detail in chemostat cultures.[10,23,24] This yeast was, therefore, chosen as a model organism to investigate the applicability of the Kluyver effect for high-cell-density cultivation of yeasts.

As a reference experiment, *C. utilis* CBS 621 was grown in a stirred and aerated laboratory fermentor on 200 g L^{-1} glucose. Under these conditions, its behavior was typical of facultatively fermentative yeasts which are Crabtree negative (i.e., yeasts which do not exhibit alcoholic fermentation under aerobic conditions). During the first hours of the experiment, growth proceeded exponentially without significant accumulation of metabolites. After approximately 12 h, the dissolved-oxygen concentration in the culture fell below 0.1% air saturation, due to the limited oxygen-transfer capacity of the fermentor. This resulted in the immediate onset of alcoholic fermentation (Fig. 1A). Probably as a result of the accumulation of toxic fermentation products, growth ceased before glucose was exhausted, with the biomass concentration not exceeding 20 g dry weight L^{-1} (Fig. 1A).

A similar growth experiment was performed with 200 g L^{-1} maltose as the carbon source (Fig. 1B). As in the glucose experiment, exponential growth occurred during the first hours and was accompanied by a decrease of the dissolved-oxygen concentration. However, during growth on maltose, the onset of oxygen limitation did not trigger alcoholic fermentation (Fig. 1B). Instead, growth proceeded linearly until maltose was exhausted. At this stage, the biomass concentration in the culture was 89 g dry mass L^{-1}. The overall biomass yield (89/200 = 0.45 g biomass · g maltose^{-1}) was close to the biomass yield of 0.50 g · g maltose^{-1} observed in aerobic, maltose-limited chemostat cultures of *C. utilis*.[10]

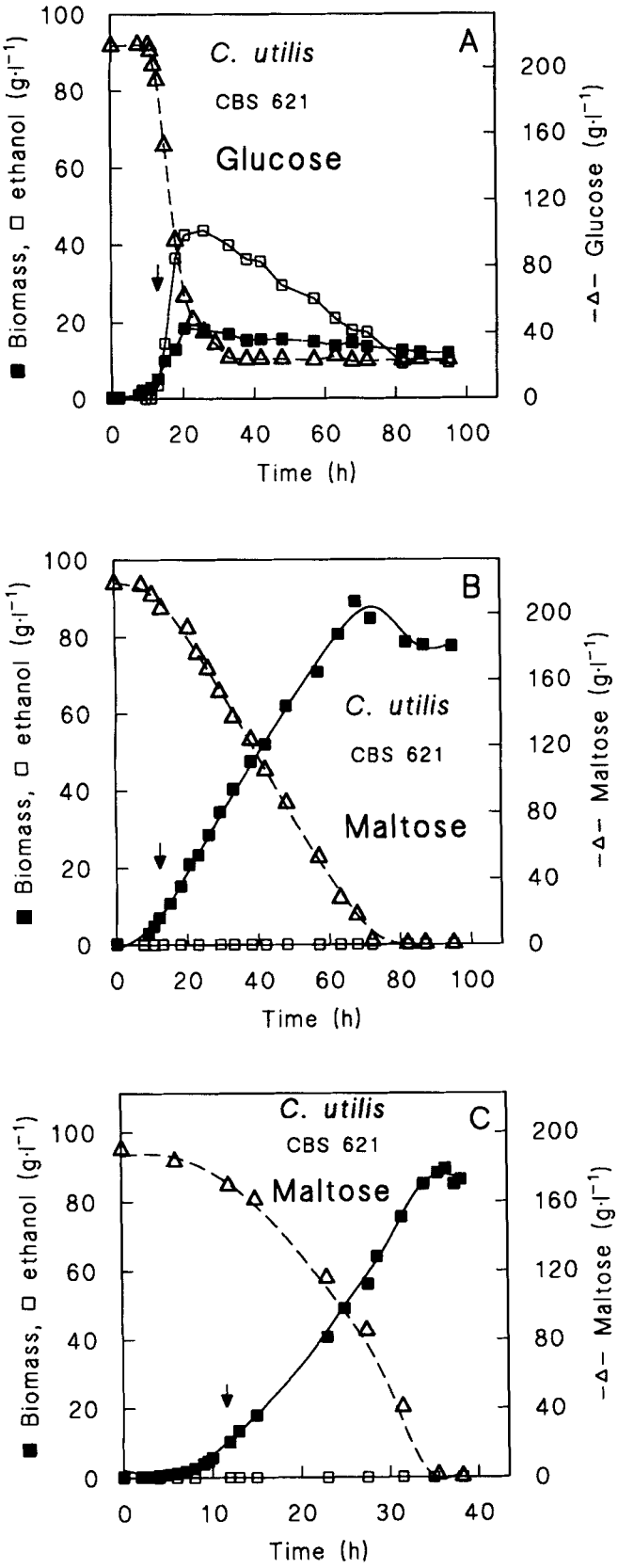
The linear increase of the biomass concentration in the maltose-grown cultures was limited by the oxygen-transfer rate. Indeed, when the oxygen-transfer rate was improved by increasing the stirring speed from 750 to 1000 rpm, the total fermentation time was reduced from 70 to 35 h (compare Fig. 1B and C). The increased stirring speed did not substantially influence the final biomass yield on maltose (Fig. 1B and C) or the growth pattern of glucose-grown cultures (data not shown).

For the sake of clarity, only the main fermentation product ethanol is shown in Figure 1. However, the onset of oxygen limitation in the glucose-grown cultures resulted in the accumulation of a variety of fermentation products, including acetate, succinate, acetoin, and 2,3-butanediol, as well as a variety of other low-molecular-weight organic acids. Concentrations of these metabolites in maltose-grown cultures were negligible (Fig. 2).

Lactose Metabolism in Chemostat Cultures of *Kluyveromyces* Yeasts

The above results clearly demonstrate the potential of yeasts that exhibit a Kluyver effect for straightforward high-cell-density cultivation. Several *Kluyveromyces* strains have been reported to exhibit a Kluyver effect for lactose,[3,18] but their applicability for biomass production from lactose-containing feedstocks has not been investigated.

Previous reports on the occurrence of a Kluyver effect in *Kluyveromyces* yeasts were mainly based on the pres-



ence or absence of gas formation in taxonomic tests. As recently reported,[9] this does not always give a reliable indication about the absence of alcoholic fermentation. Therefore, the response of two *Kluyveromyces* strains to oxygen limitation was investigated in glucose- and lactose-grown chemostat cultures. *K. marxianus* CBS 5795 is a lactose-fermenting strain, whereas *K. wickerhamii* CBS 2745 has been reported to exhibit a Kluyver effect for lactose.[18]

When aerobic, glucose-limited chemostat cultures ($D = 0.10\ h^{-1}$) were subjected to oxygen limitation, a similar response was observed in the two yeasts (Fig. 3A and C). Alcoholic fermentation started immediately after the switch to oxygen limitation and the ethanol concentration in the cultures increased until a new steady state was reached, in which respiration and fermentation occurred simultaneously and the culture was dually limited by oxygen and glucose.

Lactose-grown cultures of the two yeasts exhibited a strikingly different response to oxygen limitation. The response of aerobic, lactose-limited chemostat cultures of *K. marxianus* CBS 5795 was virtually the same as that of glucose-limited cultures (Fig. 3B). In contrast, alcoholic fermentation by lactose-grown cultures of *K. wickerhamii* CBS 2745 was a transient phenomenon, with ethanol production only occurring immediately after the switch to oxygen limitation (Fig. 3D). Within hours after the switch, lactose started to accumulate in the cultures and growth became limited by oxygen only. In the oxygen-limited steady state that was eventually reached, metabolism was exclusively respiratory. The disappearance of alcoholic fermentation in the oxygen-limited cultures of *K. wickerhamii* was not due to repression of the synthesis of the key enzymes pyruvate decarboxylase and alcohol dehydrogenase. Activities of these enzymes in cell extracts were similar to those observed in extracts of oxygen-limited, glucose-grown cultures (data not shown).

The response of lactose-grown chemostat cultures of *K. wickerhamii* to oxygen limitation was comparable to that observed with maltose-grown chemostat cultures of *C. utilis*.[10] To investigate whether this similarity could be extended to high-cell-density batch cultivation, growth of *K. wickerhamii* and *K. marxianus* was studied in lactose-grown batch cultures.

Figure 1. Concentrations of sugar, ethanol, and biomass in pH-controlled batch cultures of *Candida utilis* CBS 621 grown on glucose or maltose. Fermentations were performed in a 1.5-L working volume laboratory fermentor. Growth conditions: $T = 30°C$, pH 5; air supply: 0.75 L min^{-1}. The arrows indicate the moment at which the dissolved-oxygen concentration fell below 0.1% of air saturation. (A) Carbon source: 200 g L^{-1} glucose, stirring speed 750 rpm. (B) Carbon source 200 g L^{-1} maltose, stirring speed 750 rpm. (C) Carbon source: 200 g L^{-1} maltose monohydrate, stirring speed 1000 rpm.

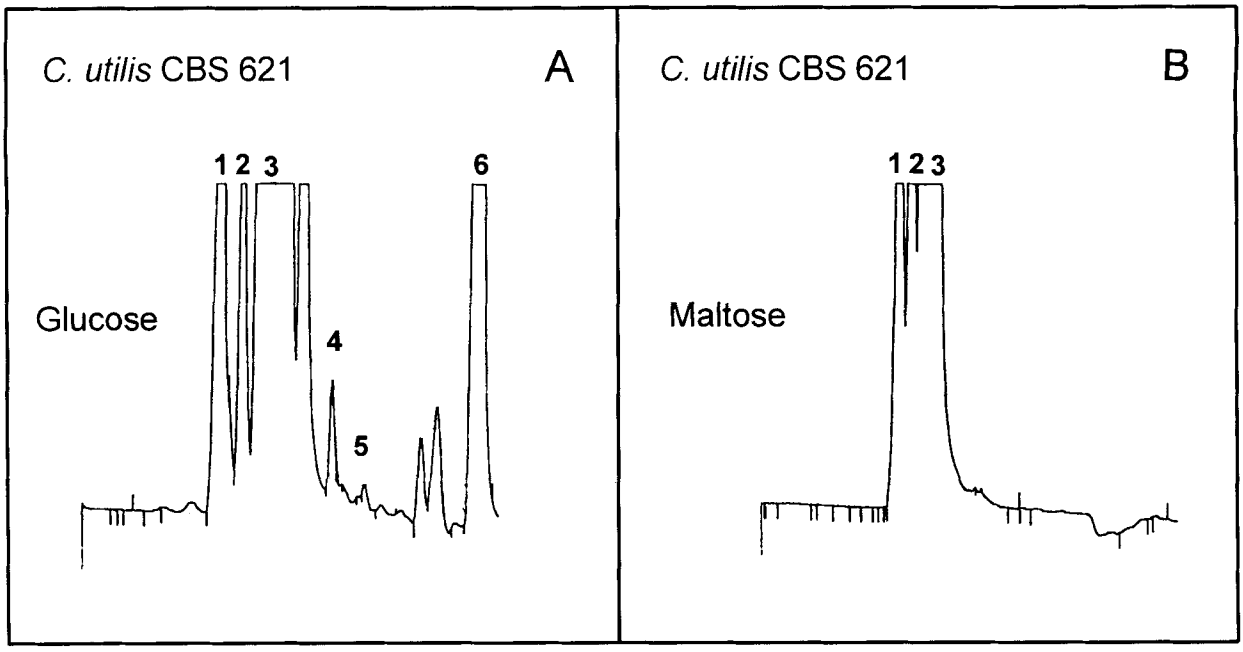


Figure 2. HPLC chromatograms (refractive-index detection) of batch cultures of *Candida utilis* CBS 621 on glucose (A) and maltose (B). Stirring speed: 1000 rpm, other growth conditions as in Figure 1. Samples were taken after 13 h (i.e., within 1 h after the onset of oxygen limitation). The biomass concentration in both cultures was 14 g L^{-1}. Identified peaks: 1 and 2, mineral medium; 3, sugar (glucose or maltose); 4, succinate; 5, acetate; 6, ethanol.

Cultivation of *Kluyveromyces* Yeasts in Oxygen-Limited Batch Cultures

In view of the limited solubility of lactose, batch cultivation of *Kluyveromyces* strains on glucose or lactose was performed at an initial sugar concentration of 100 g L^{-1}. The growth pattern of CBS 5795, a strain which does not exhibit a Kluyver effect for lactose, confirmed its lactose-fermenting capacity (Fig. 4A). After a brief period of exponential growth, the culture became oxygen-limited, which triggered alcoholic fermentation. After the sugar was exhausted, the ethanol was reconsumed. Similar results were obtained with glucose-grown batch cultures of this strain (data not shown). The final biomass concentration in glucose- and lactose-grown cultures was ca. 18 g dry weight L^{-1}.

The growth of *K. wickerhamii* CBS 2745 on lactose was somewhat slower than that of *K. marxianus* CBS 5795. As a result, oxygen limitation occurred at a later stage (Fig. 4B). After the onset of oxygen limitation, however, alcoholic fermentation did not occur in the lactose-grown culture, which reached a final biomass concentration of 45 g dry weight L^{-1}, corresponding to a biomass yield of 0.45 g · (g lactose)$^{-1}$. This biomass yield is comparable to the biomass yield in aerobic, lactose-limited chemostat cultures (Fig. 3D).

The fermentative behavior of *K. marxianus* CBS 5795 in lactose-grown cultures is not a characteristic of all strains of this species: some strains exhibit a Kluyver effect for lactose.[2] In view of the significance of *K. marxianus* as an industrial yeast, growth of one of these strains, *K. marxianus* CBS 7894, was studied in batch cultures grown on 100 g L^{-1} glucose or lactose (Fig. 4C and D). In glucose-grown cultures, respirofermentative growth was observed at the onset of oxygen limitation (Fig. 4C) and final biomass concentrations did not exceed 20 g dry weight L^{-1}. Conversely, lactose-grown batch cultures of this "Kluyver-positive" *K. marxianus* strain exhibited a fully respiratory metabolism and a final biomass concentration of 45 g dry weight L^{-1} (Fig. 4D). In fact, the final biomass concentration was limited by the solubility of lactose: when additional lactose was supplied to a stationary-phase culture, growth continued (results not shown).

DISCUSSION

Effects of Oxygen Limitation on Sugar Metabolism in Chemostat Cultures

In glucose-grown cultures, the four facultatively fermentative yeasts used in this study reacted uniformly to the onset of oxygen limitation by triggering alcoholic fermentation. The biomass yield of yeasts during sugar-limited fermentative growth is characteristically ca. one fifth that during respiratory growth.[21] This effect of alcoholic fermentation on biomass yields was evident in the oxygen-limited glucose-grown chemostat cultures and in the lactose-grown chemostat cultures of the lactose-fermenting strain *K. marxianus* CBS 5795 (Fig. 3). These cultures showed a biomass yield on sugar which was only about half that of the corresponding aerobic cul-

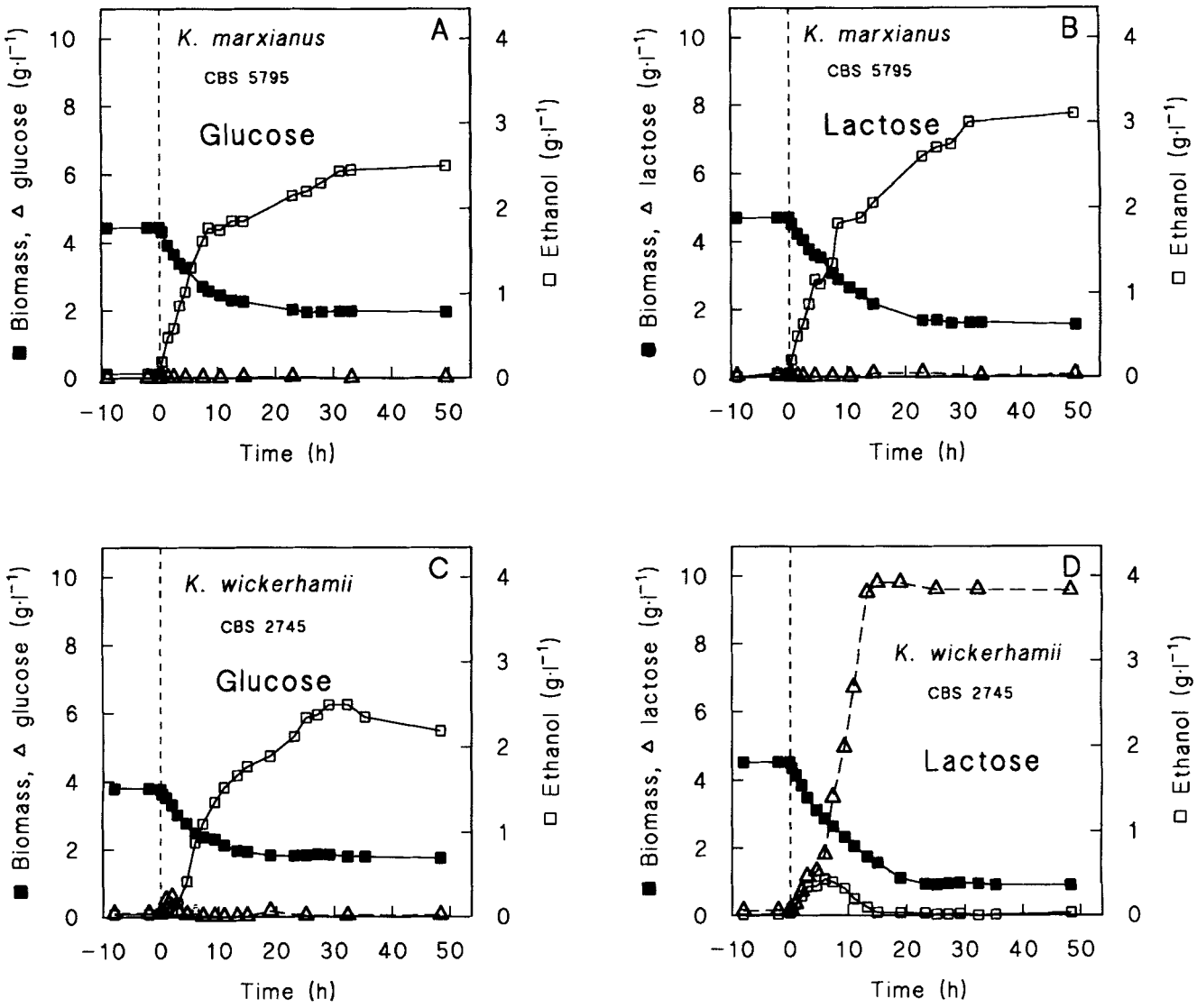


Figure 3. Effects of a sudden shift from aerobic to oxygen-limited conditions on concentrations of biomass, ethanol, and sugar in glucose- and lactose-limited chemostat cultures (D = 0.10 h^{-1}) of *Kluyveromyces* yeasts. The dashed lines indicate the time at which the oxygen feed to the cultures was decreased. (A) *K. marxianus* CBS 5795 grown on glucose. (B) *K. marxianus* CBS 5795 grown on lactose. (C) *K. wickerhamii* CBS 2745 grown on glucose. (D) *K. wickerhamii* CBS 2745 grown on lactose.

tures. This intermediate growth yield is due to the occurrence of a mixed respirofermentative metabolism.

No effect of oxygen limitation on the biomass yield was observed in situations in which a Kluyver effect occurred. For example, the biomass yield of *K. wickerhamii* CBS 2745 on lactose was virtually identical in aerobic, lactose-limited chemostat cultures and in oxygen-limited cultures grown on lactose (Fig. 3D). The specific rate of sugar consumption is inversely related to the specific rate of sugar consumption (q_s) according to:

$$q_s = \mu / Y_{sx} \quad (1)$$

In Eq. (1), μ is the specific growth rate (equal to the dilution rate in steady-state chemostat cultures) and Y_{sx} is the biomass yield on the growth-limiting substrate. Because the experiment shown in Figure 3D was performed at a fixed dilution rate of 0.10 h^{-1}, it follows from Eq. (1) that q_s was also identical in these two steady-state situations. Nevertheless, important changes in the kinetics of lactose metabolism have to occur during the switch from aerobic to oxygen-limited conditions. These changes will be briefly discussed.

In the aerobic, lactose-limited chemostat cultures of *K. wickerhamii,* the residual lactose concentration was below 0.3 m*M*. This is much lower than the apparent substrate-saturation constant of the lactose carrier (K_s ca. 3 m*M*[6]). As a result, the capacity of the lactose carrier in the cell is not completely used under these conditions. Conversely, in the oxygen-limited steady-state culture, the residual lactose concentration was ca. 30 m*M* (Fig. 3). This concentration is an order of magnitude higher than the saturation constant of the lactose carrier, which must therefore be operating at a rate close to its maximum capacity. This implies that a decrease of the amount of lactose carrier in the cells must occur during the transition from aerobic, lactose-limited growth to

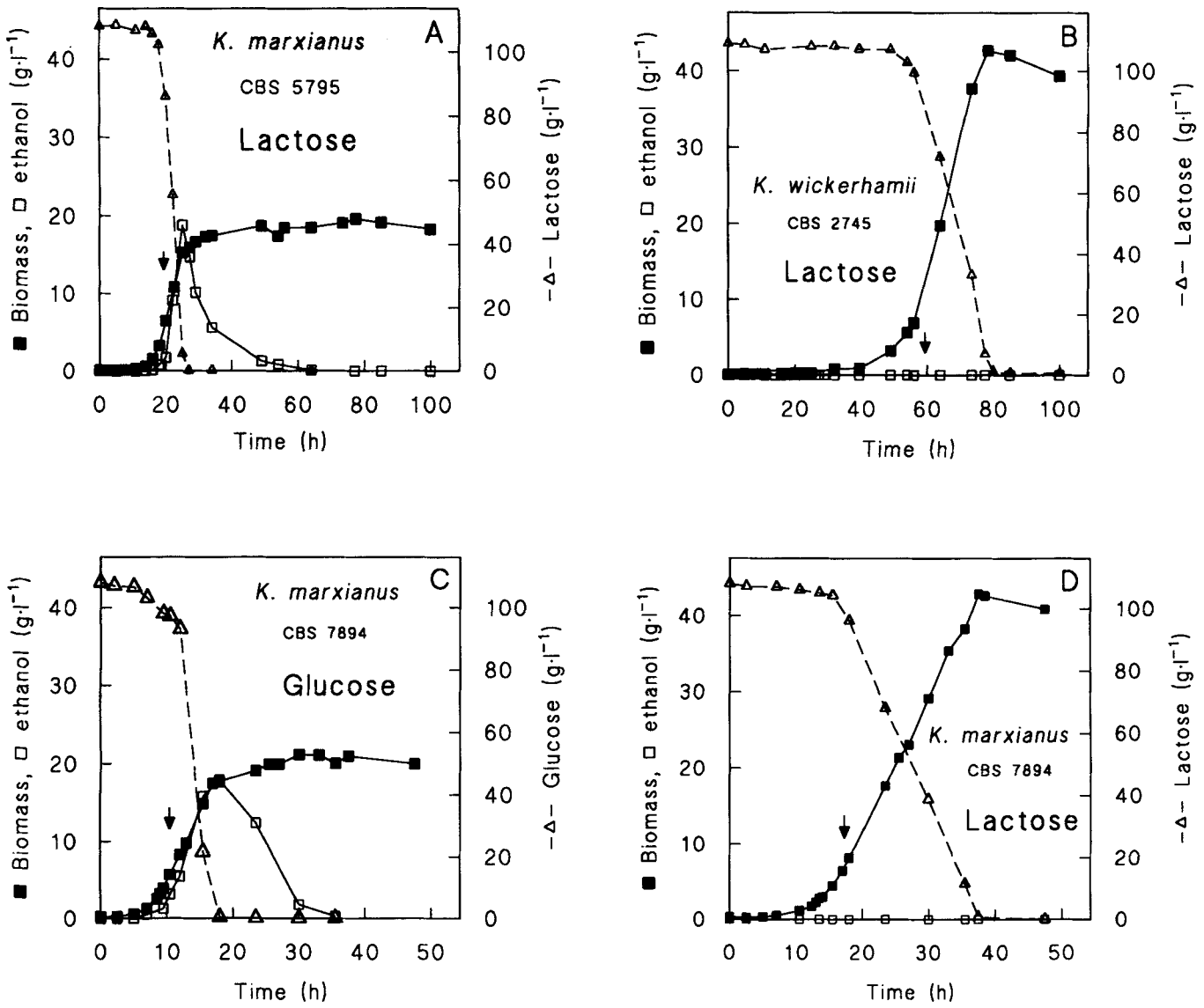


Figure 4. Concentrations of sugar, ethanol, and biomass in pH-controlled batch cultures of *Kluyveromyces* yeasts grown on glucose or lactose. Fermentations were performed in a 1.5-L working volume laboratory fermentor. Growth conditions: T = 30°C, pH 5; air supply 0.75 L min^{-1}; stirring speed 750 rpm. Arrows indicate the moment at which the dissolved-oxygen concentration in the cultures fell below 0.1% of air saturation. (A) *K. marxianus* CBS 5795; carbon source: 100 g L^{-1} lactose. (B) *K. wickerhamii* CBS 2745, carbon source: 100 g L^{-1} lactose. (C) *K. marxianus* CBS 7894; carbon source: 100 g L^{-1} glucose. (D) *K. marxianus* CBS 7894; carbon source 100 g L^{-1} lactose.

oxygen limitation. Over a period of ca. 3 h, after the switch to oxygen limitation, the glycolytic flux increased and alcoholic fermentation of lactose occurred (Fig. 3D). A similar transient response to oxygen limitation has been observed in maltose-limited chemostat cultures of *Candida utilis*.[10] This transient behavior suggests that the decrease of the carrier content occurred by a downregulation of carrier synthesis, rather than by rapid inactivation of existing carrier molecules.

Effects of Oxygen Limitation on Batch Cultures

Aerobic batch cultures of yeasts invariably become oxygen-limited when a high initial concentration of the carbon substrate is present. As shown in Figures 1 to 4, this does not only hold for shake-flask cultures, but also for well-stirred, aerated laboratory fermentors. The occurrence of alcoholic fermentation during oxygen-limited growth goes at the expense of biomass formation and may lead to the accumulation of toxic metabolites in the culture medium. This latter aspect was illustrated during growth of *C. utilis* on glucose, where growth ceased before glucose was exhausted (Fig. 1A).

Neither alcoholic fermentation nor accumulation of other metabolites occurred during growth on disaccharides of yeasts which exhibited a Kluyver effect for the carbon source. Instead, after the onset of oxygen limitation, the increase of the biomass concentration in such cultures was solely dependent on the constant rate of oxygen transfer into the cultures, resulting in linear growth kinetics (Figs. 1 and 4). The transient accumulation of ethanol that was observed during switch experi-

ments with maltose-limited chemostat cultures of *C. utilis*[10] and lactose-limited chemostat cultures of *K. wickerhamii* (Fig. 3D) was not observed in the corresponding batch cultures (Figs. 1B and C and 4B). This can be explained from the high disaccharide concentrations present at the moment of transition to oxygen limitation, as a result of which disaccharide carriers present in the cultures were operating at or near their maximum capacity. Consequently, the yeast cells in these batch cultures cannot instantaneously increase their specific rate of disaccharide uptake in response to a decreased oxygen availability: at saturating sugar concentrations, an increase of the specific rate of sugar uptake requires de novo synthesis of sugar carriers.

The Kluyver effect for disaccharides (including maltose, lactose, and sucrose) is widespread among yeast species.[18] As demonstrated in this article, use of yeast strains that exhibit a Kluyver effect obviates the necessity of elaborate and expensive systems for monitoring and control of the dissolved-oxygen concentration to avoid occurrence of alcoholic fermentation. Therefore, it seems worthwhile to include this feature in screening programs for yeast strains that are to be used for the production of biomass or growth-related products from disaccharide-containing feedstocks.

Recent work suggests that regulation of disaccharide transport across the yeast plasma membrane is a key factor in the coordination of disaccharide metabolism and respiratory activity in yeasts exhibiting a Kluyver effect.[3,9,10] Still, over half a century after Kluyver and Custers[11] first mentioned the "nonfermentability" of certain disaccharides by some yeasts, the exact molecular mechanism responsible for the Kluyver effect remains unknown. Possibly, the potential industrial significance of the Kluyver effect will give new impetus to studies into the molecular biology of this intriguing phenomenon.

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