Antonie van Leeuwenhoek **50** (1984) 183–192

Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect)

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WIJSMAN, M. R., VAN DIJKEN, J. P., VAN KLEEFF, B. H. A. and SCHEFFERS, W. A. 1984. Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect). Antonie van Leeuwenhoek **50**: 183–192.

Aerobic growth of the yeast *Brettanomyces intermedius* CBS 1943 in batch culture on a medium containing glucose and yeast extract proceeded via a characteristic pattern. In the first phase of growth glucose was fermented to nearly equal amounts of ethanol and acetic acid. After glucose depletion, growth continued while the ethanol produced in the first phase was almost quantitatively converted to acetic acid. Finally, after a long lag phase, growth resumed with concomitant consumption of acetic acid.

When the culture was made anaerobic during the first phase, growth, glucose consumption and metabolite production stopped immediately. This Custers effect (inhibition of alcoholic fermentation as a result of anaerobic conditions) was transient. After 7–8 h the culture was adapted to anaerobiosis, and growth and ethanol production resumed. The lag phase could be shortened at will by the introduction of hydrogen acceptors, such as oxygen or acetoin, into the culture. Glycerol production was not observed during any phase of growth. These results support the hypothesis that the Custers effect in this yeast is due to a disturbance of the redox balance, resulting from the tendency of the organism to produce acetic acid, and its inability to restore the balance by production of glycerol.

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Introduction

Expression of fermentation capacity in facultatively fermentative yeasts is a function of environmental conditions. One important parameter is the availability of oxygen. For example, in batch cultures of *Candida utilis* growing with excess glucose, alcoholic fermentation is suppressed by the presence of oxygen but upon a shift to anaerobic conditions this yeast forms ethanol and glycerol almost instantaneously (Bruinenberg et al., 1983). In contrast to *C. utilis*, batch cultures of *Saccharomyces cerevisiae* express alcoholic fermentation despite the presence of oxygen (Fiechter et al., 1981); moreover, the rate of ethanol production is hardly affected by a change to anaerobiosis. In *S. cerevisiae* the Crabtree effect (repression of respiratory activity by excess glucose) overrules the Pasteur effect (inhibition of glycolysis by oxygen, manifested as an inhibition of ethanol formation). Thus, in growing cells of this yeast a Pasteur effect is virtually absent (Fiechter et al., 1981; Lagunas et al., 1982).

Although oxygen inhibits fermentation in *C. utilis* and is without effect in *S. cerevisiae*, it greatly enhances the rate of fermentation by washed cell suspensions of aerobically grown yeasts of the genera *Brettanomyces, Dekkera* and *Eeniella* (Wikén et al., 1961). This phenomenon has been first described by Custers and named negative Pasteur effect (Custers, 1940). Scheffers (1966) introduced the term Custers effect for the inhibition of alcoholic fermentation under anaerobic conditions. The phenomenon is wide-spread among glucose-fermenting yeasts (Scheffers and Wikén, 1969). Moreover, as recent studies have revealed, a Custers effect may also be observed during growth of yeasts on xylose (Bruinenberg et al., 1983).

This paper deals with the effect of oxygen on the kinetics of growth, substrate consumption and product formation by batch cultures of *B. intermedius*.

Materials and Methods

Microorganisms and growth conditions

Brettanomyces intermedius CBS 1943 was maintained on malt agar with 20 g $CaCO_3$ per litre and transferred monthly. The organism was grown at 30 °C in batch cultures in a laboratory fermenter (Chemap AG) with a working volume of 10 litres. The pH was controlled at 5.5 by automatic addition of 2 M KOH or 1 M H_2SO_4. The dissolved oxygen concentration was recorded with an oxygen electrode and during aerobic growth this parameter was kept between 40 and 70% of air saturation. The medium contained 15 g glucose per litre (83 mM) and 10 g yeast extract per litre (Difco); both components were heat-sterilized separately.

Gas analysis

Gas flows were regulated by a dual-channel thermal gas-flow controller

(Brooks) at 5 litres of air per min or 2 litres of ultra-pure nitrogen per min (oxygen content less than 4 μl/l). For anaerobic experiments, the fermenters were equipped with butyl rubber tubing. Carbon dioxide and oxygen concentrations in inlet and outlet gas were measured on-line with an infrared carbon dioxide analyser (Beckman model 864) and a paramagnetic oxygen analyser (Taylor Servomex 500). The analyses were standardized automatically every 6 min and data were collected every 2 min with the aid of a microcomputer and stored on magnetic medium (Van Kleeff, 1982).

Analytical methods

Glucose, acetic acid and glycerol were determined by the GOD-Perid method and the Test combinations for acetic acid and glycerol from Boehringer, respectively. Ethanol was determined by gas chromatography. Growth was followed by measuring optical density of cultures at 660 nm with a colorimeter (Vitatron). Dry weight of cultures was determined by weighing, after filtration of appropriate aliquots of culture on membrane filters (Millipore). Titres of KOH and H_2SO_4 were determined volumetrically with oxalic acid and boric acid, respectively.

Results

Growth of *Brettanomyces intermedius* in batch cultures on glucose under aerobic conditions proceeded with a characteristic pattern (Fig. 1). During the first phase (A) glucose was converted to biomass, ethanol, acetic acid and carbon dioxide. Shortly before the depletion of glucose the culture started to utilize the ethanol, without production of CO_2. During this second phase (B) ethanol was oxidized stoichiometrically to acetic acid and was thus serving as an energy source for growth on yeast extract. Finally, after a lag phase of 50 h (C) the acetic acid produced in the first and second phases was oxidized to CO_2 with the concomitant production of biomass (phase D).

When during the initial growth phase the culture was transferred to anaerobic conditions by gassing with pure nitrogen, production of biomass, ethanol, acetic acid and CO_2 ceased (Fig. 2). After a period of 7–8 h (phase C) growth resumed with concomitant production of ethanol (phase E). Hence the Custers effect (inhibition of alcoholic fermentation by anaerobiosis) was transient in *B. intermedius*.

An immediate release from the Custers effect was observed when during the anaerobic lag phase the culture was returned to aerobic conditions (Fig. 3). This resulted in the normal series of events: conversion of glucose to ethanol, acetic acid and CO_2 (phase A) followed by oxidation of ethanol to acetic acid (phase B) and a lag phase (C) before the final consumption of acetic acid (data not shown). Under all circumstances *B. intermedius* failed to produce glycerol.

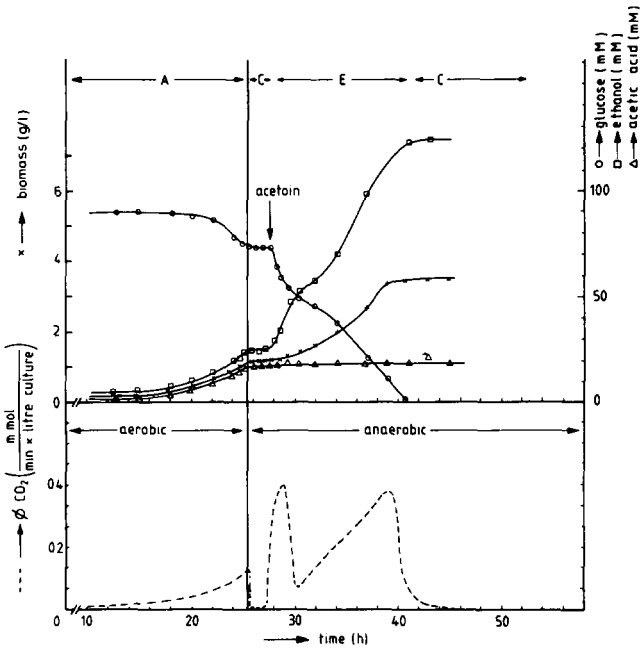

Fig. 1. Growth, glucose consumption and metabolite production in an aerobic batch culture of *Brettanomyces intermedius* CBS 1943 on a complex medium. The different phases of growth and metabolite production in Figs 1–4 have been indicated by capitals. They refer to the various dissimilatory events shown in Fig. 5.

Not only oxygen, also other electron acceptors such as acetoin can abolish the Custers effect (Scheffers, 1961). Addition of a small amount of acetoin, 3 h after the introduction of anaerobiosis, resulted in an immediate restoration of metabolic activity: consumption of glucose and production of ethanol and CO_2. These processes temporarily even proceeded at a much higher rate than before the anaerobic period (Fig. 4).

Discussion

During aerobic growth of *Brettanomyces intermedius* in a complex medium glucose was fermented to ethanol and acetic acid (Fig. 1). The relative amounts of these metabolites, formed during the first phase of growth (A) varied between individual experiments. Alkali consumption in this phase (A) and the following (B) matched acetic acid production, indicating that acetic acid was the only acid formed (Custers, 1940). The analysis of carbon dioxide production made clear

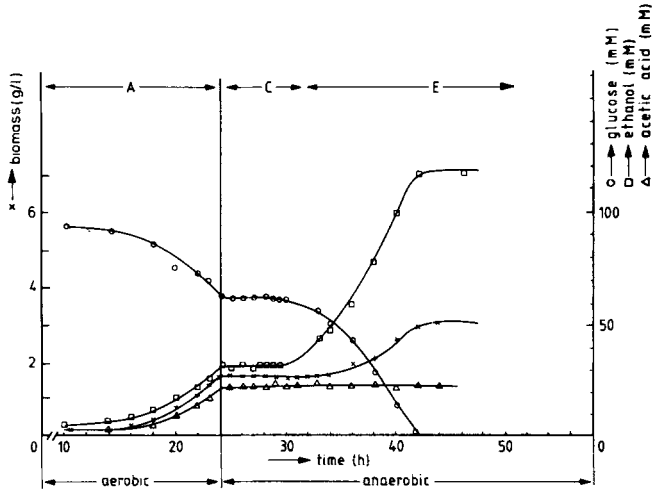

Fig. 2. Effect of a shift from aerobic to anaerobic culture conditions on the growth kinetics of *Brettanomyces intermedius*. From t = 24 h onwards, the culture was kept anaerobic by gassing with nitrogen.

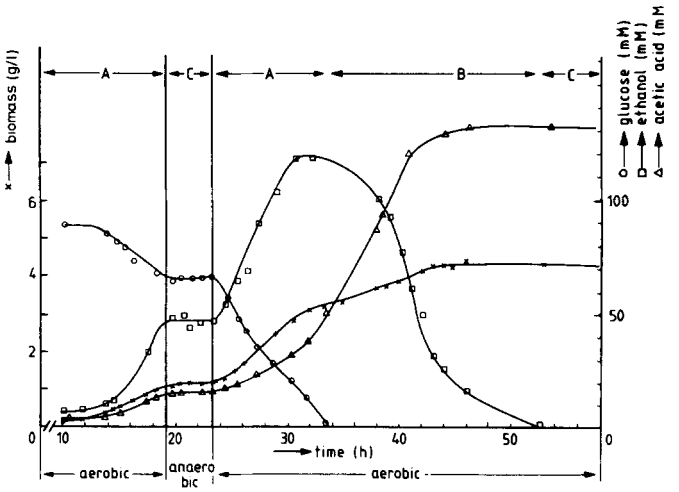

Fig. 3. Elimination of the Custers effect in *Brettanomyces intermedius* as a result of a shift to aerobic conditions during the anaerobic lag phase.

that at most 10% of the glucose consumed in the first phase of growth could not be accounted for by the formation of ethanol and acetic acid. Although the use of a complex medium prevented the exact calculation of mass balances, it can be deduced that the contribution of the TCA-cycle to the dissimilation

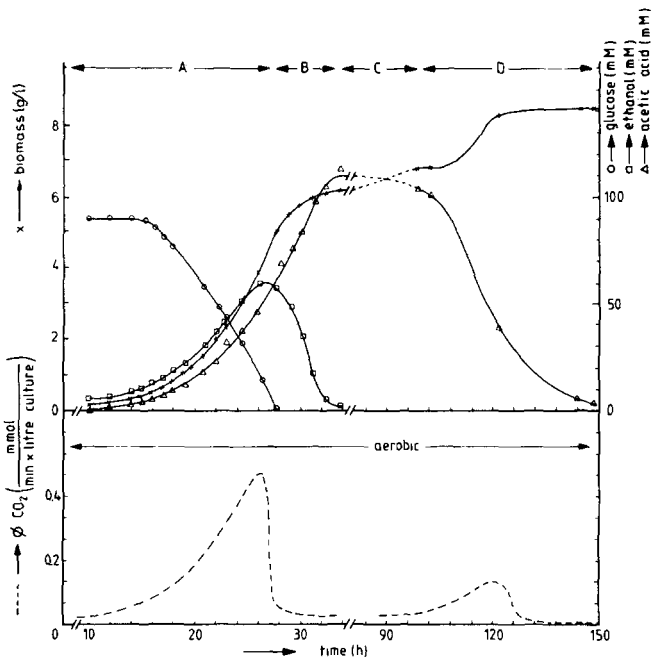

Fig. 4. Elimination of the Custers effect in *Brettanomyces intermedius* by the addition of acetoin (4 mM).

of glucose was low. This may be due to a low activity, or to the absence of one or more key enzymes of this pathway. This is also indicated by the fact that during the second phase (B) the ethanol produced in the first phase was converted stoichiometrically to acetic acid. Only after a long lag phase the culture adapted to oxidation of acetic acid via the TCA-cycle (Figs 1 and 5), resulting in additional growth. A similar series of events has been reported for *B. lambicus* in semi-synthetic medium by Wöhrer et al. (1981).

The results presented in Fig. 1 demonstrate that the classical definition of the Crabtree effect – repression of respiration by excess glucose (Fiechter et al., 1981) – only partially applies to growth of *B. intermedius* in batch cultures on excess glucose. Although during aerobic fermentative metabolism of glucose the activity of the TCA-cycle may be very low, cultures nevertheless had respiratory activity, due to the oxidation of reducing equivalents produced in the formation of acetic acid (Fig. 5A). Not only in the case of yeasts such as *B. intermedius* the classical definition of the Crabtree effect seems inappropriate. In chemostat cultures of *Saccharomyces uvarum* oxygen consumption is not repressed during fermentative growth (Petrik et al., 1983). Since, furthermore, alcoholic fermentation in yeasts may be provoked without repression or inhibition of respiration,

for example after giving a glucose pulse to glucose-limited cultures (Petrik et al., 1983; Van Dijken et al., 1984; Verduyn et al., 1984) it seems more appropriate to describe the glucose effect in yeasts in phenomenologic rather than in mechanistic terms, namely as a stimulation of alcoholic fermentation by glucose. Depending on the yeast species and the environmental conditions, this phenomenon may or may not be associated with a lower respiratory activity.

When shifted to anaerobiosis, batch cultures of *B. intermedius* exhibited a transient inhibition of alcoholic fermentation (Custers effect). Thus, phenomenologically the Custers effect is opposite to the Pasteur effect, which may become apparent as a stimulation of the fermentation under conditions of limited oxygen supply. It must be stressed, however, that the term Pasteur effect is meant to indicate a higher rate of glycolysis as a result of anaerobiosis (Fiechter et al., 1981). Whether an increased rate of ethanol production reflects a higher rate of glycolysis can only be established via a detailed comparison of the carbon flows over intermediary pathways (Fiechter et al., 1981). To the authors' knowledge, so far no examples are available to show that in growing yeasts absence of oxygen accelerates the rate of glycolysis. Even in the case of *C. utilis,* in which alcoholic fermentation of glucose can only be triggered by anaerobiosis, ethanol production is not accompanied by a significant increase in the glycolytic flux (Bruinenberg et al., 1983). In contrast to the Pasteur effect, the Custers effect is much easier to interpret in terms of glycolytic activity, since in this case anaerobiosis triggers an on-off mechanism. Upon a shift of an aerobic culture of *B. intermedius* to anaerobic conditions glycolysis is temporarily stagnating, as is evident from the absence of glucose consumption and CO_2 production (Fig. 2).

The inhibition of growth and alcoholic fermentation after shifting to anaerobiosis (Figs 2–4) cannot be explained by a lack of essential nutrients, since the medium used contained all the required ingredients for anaerobic growth, including fatty acids and sterols (Andreasen and Stier, 1954). This is also evident from the fact that inhibition of growth and ethanol production was a transient phenomenon (Fig. 2). Neither can catabolite inactivation (Holzer, 1976) of essential enzymes in the fermentation pathway explain this inhibition which was readily reversible (Figs 3 and 4). One explanation could be that oxygen is required for glucose transport which might be blocked by a low redox potential. The lag phase observed after transition to anaerobiosis then could be required for the synthesis of an oxygen-independent transport system, or for a slow readjustment of the redox potential, and the effect of acetoin (Fig. 4) might result from an enhanced intracellular redox potential via NADH-dependent reduction of acetoin.

Although redox limitations at the level of sugar transport may contribute to the occurrence of the Custers effect, they are not the cause of the disturbed redox balance. The origin of the Custers effect in *B. intermedius* must be sought in the capacity of this organism, and other representatives of the genera *Brettanomyces, Dekkera* and *Eeniella,* to form acetic acid (Custers, 1940; Scheffers and

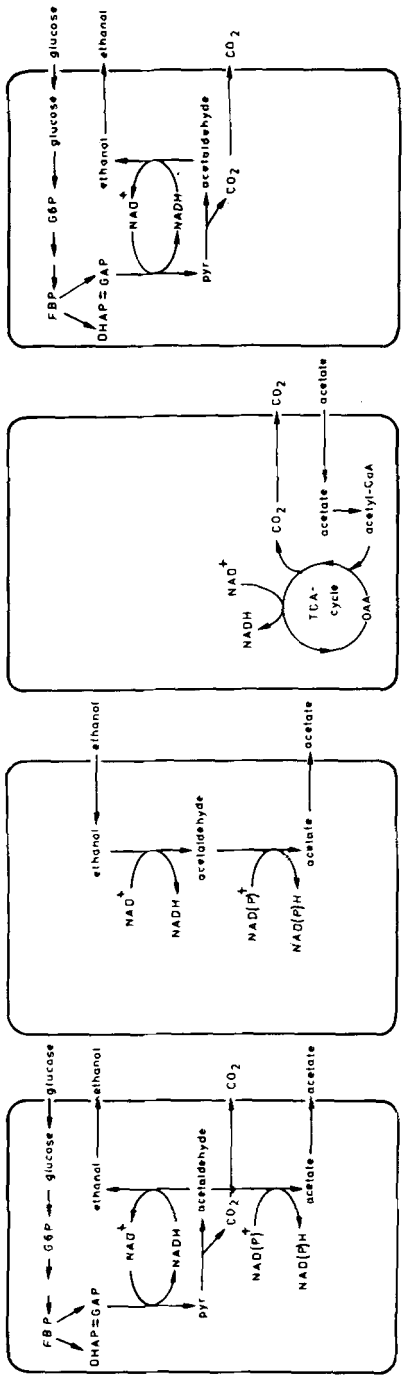

Fig. 5. Dissimilatory pathways during the various phases of growth of *Brettanomyces intermedius* in batch cultures. The capitals refer to the phases indicated in Figs 1–4.

Misset, 1974; Smith et al., 1981). Formation of acetic acid from glucose results in a net production of two reduced pyridine nucleotides per mol of acetic acid via glyceraldehyde 3-phosphate dehydrogenase and the acetaldehyde dehydrogenases (Fig. 5A; Carrascosa et al., 1981). After a shift of *B. intermedius* to anaerobic conditions, continued drainage of NAD^+ by way of the irreversible conversion of acetaldehyde to acetic acid, even to a small extent, would result in a standstill of glycolysis due to a lack of NAD^+. Addition of acetoin can counteract this via an NADH-dependent reduction of acetoin to 2,3-butanediol which regenerates NAD^+ (Scheffers, 1967). This reaction was shown to be catalysed by cell-free extracts of *B. intermedius* (unpublished results). The observation that even small amounts of acetoin were sufficient to restore glycolytic activity implies that under anaerobic conditions the rate of acetic acid production is very low. So far it remains unclear why acetoin even stimulates the rates of glucose consumption, and ethanol and CO_2 production as compared to those before entrance of the culture in the anaerobic state (Fig. 4).

Only the addition of external hydrogen acceptors can shorten the transient lag phase after the shift of *B. intermedius* to anaerobic conditions. Apparently, intracellular reoxidation of NADH via other routes is too slow. In this context it is relevant that formation of glycerol from glucose, which is an important redox valve for other yeasts under anaerobic conditions (Oura, 1977), was not observed in our experiments with *B. intermedius*. This may be explained by the absence of glycerol 3-phosphate phosphatase activity in *B. intermedius* (unpublished results). Thus, the Custers effect in this yeast is due to the tendency of the organism to form acetic acid, in combination with its inability to restore the redox balance via production of reduced metabolites such as glycerol.

Received 3 December 1983

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