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Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode

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Summary. An alcohol electrode was constructed which consisted of an oxygen probe onto which alcohol oxidase was immobilized. This enzyme electrode was used, in combination with a reference oxygen electrode, to study the short-term kinetics of alcoholic fermentation by aerobic yeast suspensions after pulsing with glucose. The results demonstrate that this device is an excellent tool in obtaining quantitative data on the short-term expression of the Crabtree effect in yeasts.

Samples from aerobic glucose-limited chemostat cultures of *Saccharomyces cerevisiae* not producing ethanol, immediately (within 2 min) exhibited aerobic alcoholic fermentation after being pulsed with excess glucose. With chemostat-grown *Candida utilis,* however, ethanol production was not detectable even at high sugar concentrations. The Crabtree effect in *S. cerevisiae* was studied in more detail with commercial baker's yeast. Ethanol formation occurred only at initial glucose concentrations exceeding 150 mg · l^{-1}, and the rate of alcoholic fermentation increased with increasing glucose concentrations up to 1,000 mg · l^{-1} glucose.

Similar experiments with batch cultures of certain 'non-fermentative' yeasts revealed that these organisms are capable of alcoholic fermentation. Thus, even under fully aerobic conditions, *Hansenula nonfermentans* and *Candida buffonii* produced ethanol after being pulsed with glucose. In *C. buffonii* ethanol formation was already apparent at very low glucose concentrations (10 mg · l^{-1}) and alcoholic fermentation even proceeded at a higher rate than in *S. cerevisiae.* With *Rhodotorula rubra,* however, the rate of ethanol formation was below the detection limit, i.e., less than 0.1 mmol · g cells^{-1} · h^{-1}.

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Introduction

Of the environmental factors which influence the manifestation of alcoholic fermentation in yeast cultures, the concentrations of glucose and oxygen are probably the most important. It is well-known that the effects of these parameters are strongly dependent on the yeast strain. Thus, batch cultures of *Candida utilis* do not produce ethanol from glucose in the presence of oxygen; however, they exhibit alcoholic fermentation after the introduction of anaerobiosis (Bruinenberg et al. 1983a). In glucose-grown batch cultures of *Saccharomyces cerevisiae,* on the other hand, ethanol is produced under aerobic conditions and the rate of alcoholic fermentation is barely influenced by a change to anaerobiosis (Fiechter et al. 1981). In contrast, yeasts of the genus *Brettanomyces* show a transient inhibition of alcoholic fermentation upon transfer of aerobic cultures to anaerobic conditions (Scheffers et al. 1982). The manifestation of the Crabtree effect – defined here as a stimulation of alcoholic fermentation by excess glucose under aerobic conditions – has, so far, mainly been studied via discontinuous analysis of substrate consumption and metabolite production (Petrik et al. 1983).

In the course of our studies on the occurrence of the Crabtree effect in yeasts of various physiological categories, we have investigated the suitability of enzyme electrodes for continuous measurement of metabolic activity. In this paper it is shown that the recently developed alcohol electrode (Verduyn et al. 1983) offers promising possibilities in this respect. Its usefulness is illustrated by studies on the presence or absence of the Crabtree effect in classical cases (i.e., in *S. cerevisiae* and in *C. utilis*) and in addition by experiments with other yeasts (*C. buffonii* and *Hansenula nonfermentans*) which have so far not been subjected to studies on alcoholic fermentation since they were hitherto considered as fermentation-negative.

Materials and methods

Microorganisms and cultivation. *Saccharomyces cerevisiae* CBS 8066, *Rhodotorula rubra* CBS 17, *Hansenula nonfermentans* CBS 5764 and *Candida buffonii* CBS 2838 were grown in batch culture in 1-l erlenmeyer flasks containing 250 ml medium which consisted of 1% yeast extract and 2% glucose. Cultures were incubated at 28° C and shaken at 300 rpm on a rotatory shaker. Cells were harvested towards the end of the exponential growth phase and were used within 1 h after harvesting. Chemostat cultivation of *Candida utilis* CBS 621 and *S. cerevisiae* CBS 8066 was performed as described by Bruinenberg et al. (1983b). Compressed baker's yeast ('Koningsgist') was obtained from Gist-Brocades N.V., Delft, The Netherlands. Prior to being tested organisms were washed twice with mineral medium, pH 6.0, supplemented with vitamins, without carbon source (Bruinenberg et al. 1983b).

Instrumentation. The alcohol electrode was prepared according to Verduyn et al. (1983). The alcohol electrode and oxygen electrode (Yellow Springs Instr. Co.) were fitted into a 2-l laboratory fermenter (Harder et al. 1974) containing 800 ml mineral medium, pH 6.0, supplemented with vitamins (Bruinenberg et al. 1983b). Electrode signals were registered with a double-channel recorder (Kipp & Zonen, BD 40). For calibration, a standard ethanol solution was added to the fermenter at various rates with a peristaltic pump. Calibration was performed before each experiment under the conditions required (i.e., at the chosen temperature and at a dissolved oxygen tension corresponding to 95% air saturation in the medium). When not in use, the electrodes were kept in 100 mM potassium phosphate buffer, pH 7.5, at room temperature.

Continuous ethanol measurement in yeast suspensions. In all experiments the dissolved oxygen tension was kept constant between 95 and 100% air saturation by employing a stirrer speed of 650 rpm and a constant air flow of 3.8 l · min^{-1}; pH control was not required since regular checks showed that the pH remained at 6.0. The temperature was maintained at the values indicated in the text. After equilibration of aeration and temperature, concentrated yeast suspensions were added to the fermenter to a final concentration of 1–3 g · l^{-1} wet-weight, and after further equilibration glucose was added to the concentration required. The maximal rate of ethanol formation was determined at 30° C at an initial glucose concentration of 5 g · l^{-1}. Higher glucose concentrations did not lead to increased ethanol production rates for any of the strains tested.

Analytical methods. For discontinuous measurements of ethanol and glucose concentrations, samples were withdrawn from the fermenter and centrifuged at high speed for 30 s in an Eppendorf centrifuge. Ethanol assays were performed by the colorimetric method described by Verduyn et al. (1984). This sensitive method was required since the range of ethanol concentrations which were measured with the alcohol electrode was below the detection limit of other methods such as gas chromatography.

Glucose was analyzed by the GOD-Perid method (Boehringer). For dry-weight determinations, 10-ml cell suspensions were transferred to vacuum-dried filters (Sartorius, pore size 0.45 µm), washed with two volumes of distilled water and dried at 70° C for 48 h.

Results

Since the alcohol electrode had to register ethanol production rates, calibration was performed by adding a standard ethanol solution to the fermenter with a peristaltic pump. The lag time of the electrode system was between 20 and 40 s for individual electrodes. The electrode gave a linear response to ethanol additions up to 7 mg · min^{-1} (Fig. 1). Therefore, in the experiments described below, such concentrations of yeast cells were chosen that the rate of ethanol production did not exceed this value.

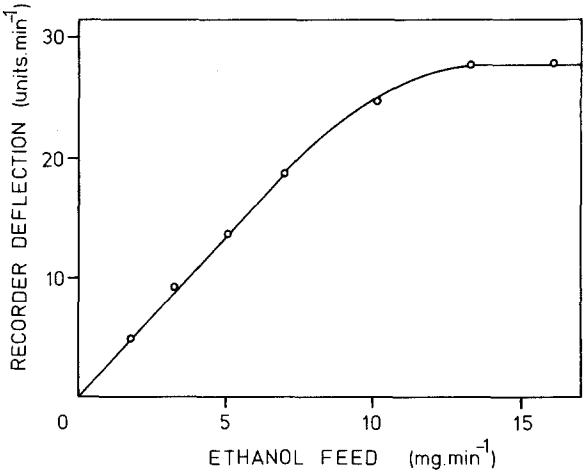

Fig. 1. Calibration of the alcohol electrode by continuous addition of a standard ethanol solution to the fermenter medium (at 30° C and pH 6.0) via a peristaltic pump

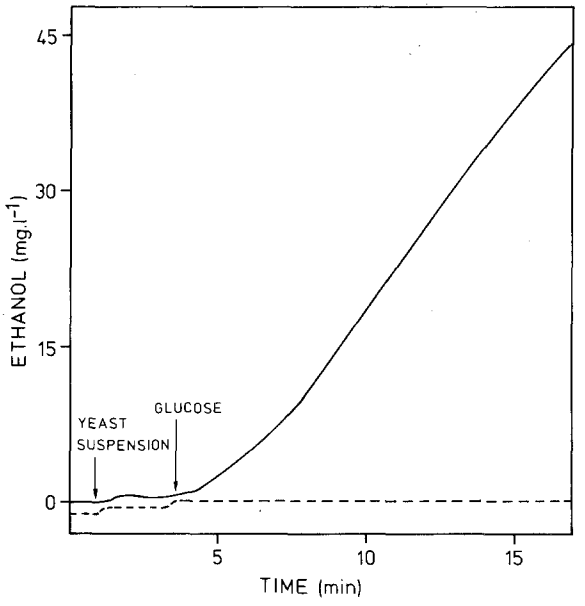

Fig. 2. Recorder tracings of the alcohol electrode (———) and the oxygen electrode (– – –) after the addition of glucose (initial concentration 5 g · l^{-1}) to commercial baker's yeast. The dissolved oxygen tension was between 95 and 100% air saturation

An important feature of this enzyme electrode is that it acts as an oxygen probe which measures the dissolved oxygen tension in the layer of alcohol oxidase immobilized onto the electrode (Verduyn et

al. 1983). A prerequisite for this type of electrode is that the oxygen tension is kept constant. Since the respiratory activity of the yeast might decrease the dissolved oxygen tension and thus false-positive results might be obtained, a reference oxygen electrode was also fitted into the fermenter. Measurements of the dissolved oxygen tension proved that the aeration was sufficient to maintain the dissolved oxygen tension between 95 and 100% air saturation.

Figure 2 gives the recorder tracings of the alcohol and oxygen electrodes after a pulse of glucose to a suspension of commercial baker's yeast. Within 2 min ethanol was detected. Approximately 5 min after glucose addition the maximal rate of ethanol formation was attained. Discontinuous ethanol assays by the colorimetric method corroborated these results. It must be noted here that ethanol formation can only be followed for a short period of time due to the high sensitivity of the electrode. The response of the alcohol electrode towards ethanol at pH 6.0 and 30° C is only linear up to 35 mg · l^{-1}, hence an ethanol production rate of for example 3 mg · l^{-1} · min^{-1} can only be recorded for 10 min.

When a lower concentration of glucose was added to the baker's yeast suspension a different pattern emerged. After addition of 400 mg · l^{-1} of glucose ethanol was formed but soon disappeared again (Fig. 3). Control experiments with mineral medium containing similar amounts of ethanol (10 mg · l^{-1}) proved that the disappearance of ethanol was not due to evaporation which was only 0.1 mg · l^{-1} · h^{-1}, and hence must be ascribed to metabolic activity. Discontinuous glucose assays showed that the rate of ethanol consumption exceeded its rate of production at a glucose concentration of approximately 150 mg · l^{-1} (Fig. 3). Furthermore, from a comparison with Fig. 2 it is evident that ethanol formation at this lower glucose concentration was much slower. The data presented in Fig. 3 also demonstrate that under the conditions employed only a minor part of the glucose added was fermented to ethanol. Probably respiration and synthesis of glycogen account for the major part (95%) of the glucose consumption.

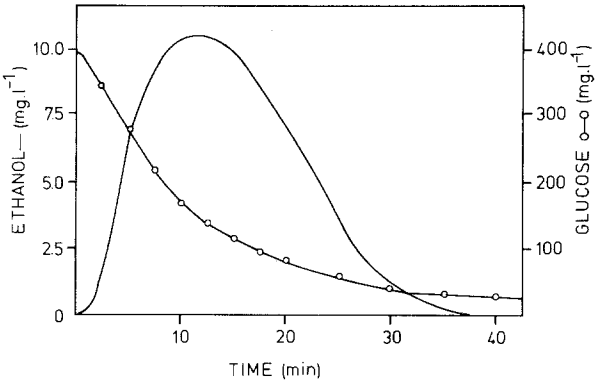

Fig. 3. Recorder tracing of ethanol formation and consumption after addition of glucose (400 mg · l^{-1}) to commercial baker's yeast and time course of the glucose concentration of the suspension (O–O)

The response of baker's yeast towards different initial glucose concentrations is illustrated in Fig. 4. Glucose concentrations below 150 mg · l^{-1} did not result in ethanol production. Above this value ethanol was formed with rates increasing up to a glucose concentration of 1,000 mg · l^{-1}. Thus, above 150 mg · l^{-1} glucose the organism exhibited a Crabtree effect. It is noteworthy that this glucose concentration corresponds to the glucose concentration at which net ethanol consumption started in experiments in which glucose was added at low initial levels (Fig. 3). Although the rate of ethanol formation at saturating glucose concentrations was a function of temperature (Table 1), the critical glucose concentration for ethanol formation was 150 mg · l^{-1} at all temperatures.

The rapid response of the electrode and its high sensitivity enable a rapid estimation of the fermentative capacity of yeast cultures under aerobic

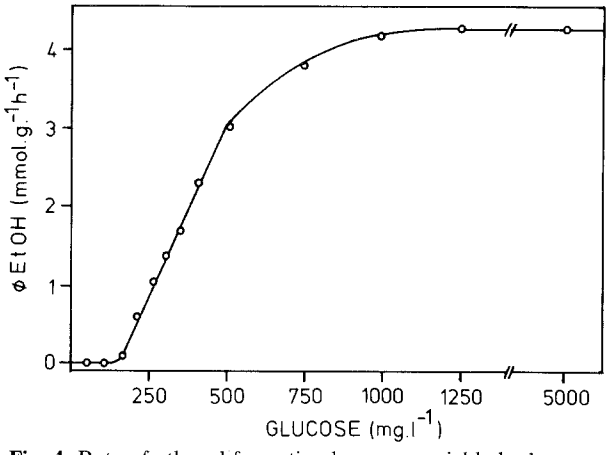

Fig. 4. Rate of ethanol formation by commercial baker's yeast at various initial glucose concentrations

Table 1. Effect of temperature on the maximal rate of ethanol formation, ϕ_{EtOH}^{max}, by the commercial baker's yeast[a]

Temperature (°C)	ϕ_{EtOH}^{max} (mmol · g cells^{-1} · h^{-1})
20	1.9
25	2.8
30	4.3
35	5.4

[a] Since the sensitivity of the alcohol electrode is a function of temperature, calibration was performed at each temperature

Table 2. Maximal rate of ethanol formation, ϕ_{EtOH}^{max}, of fermentative and 'non-fermentative' yeasts under standard assay conditions

Yeast strain	ϕ_{EtOH}^{max} (mmol · g cells^{-1} · h^{-1})
Saccharomyces cerevisiae[a]	4.3
Saccharomyces cerevisiae CBS 8066[b]	5.8
Saccharomyces cerevisiae CBS 8066[c]	4.0
Candida utilis CBS 621[c]	0[d]
Rhodotorula rubra CBS 17[b]	0[d]
Hansenula nonfermentans CBS 5764[b]	2.8
Candida buffonii CBS 2838[b]	12.7

[a] Baker's yeast
[b] Sample from shake-flask culture grown on yeast extract + glucose
[c] Sample from aerobic glucose-limited chemostat culture grown at $D = 0.1\ h^{-1}$
[d] Below detection limit (i.e., less than 0.1 mmol · g cells^{-1} · h^{-1})

conditions. For example, cells from aerobic glucose-limited chemostat cultures of *S. cerevisiae* growing at a low dilution rate where no ethanol formation occurred, started ethanol production within 2 min after addition of excess glucose. The rate of aerobic ethanol formation was similar to that of commercial baker's yeast (Table 2). Cells from cultures of *C. utilis* growing at the same conditions, however, did not show ethanol production after pulsing with excess glucose.

In the course of our studies on the regulation of glucose metabolism in yeasts it was noted that batch cultures of several non-fermentative yeasts produced ethanol. In order to study whether ethanol formation by these organisms was due to insufficient aeration or whether these yeasts display a Crabtree effect, cells from shake-flask cultures of *Hansenula nonfermentans* and *Candida buffonii* were analyzed for ethanol production with the electrode system. It was established that ethanol production in these organisms may occur under aerobic conditions. The rate of alcoholic fermentation in *C. buffonii* was even 2–3 times higher than that of *S. cerevisiae* (Table 2). Furthermore, *C. buffonii* proved to be extremely glucose-sensitive. In contrast to *S. cerevisiae* this yeast exhibited aerobic alcoholic fermentation at all glucose concentrations tested (down to 10 mg · l^{-1}). Another non-fermentative yeast, *Rhodotorula rubra,* however, did not produce detectable quantities of ethanol under the test conditions.

Discussion

The data presented above clearly illustrate that the enzyme electrode is a useful tool for the study of ethanol production by yeasts in short-term experiments. Its preparation is simple and the sensor is cheap, very sensitive and accurate (Verduyn et al. 1983). Major drawbacks of the use of the enzyme electrode as compared to on-line measurement of ethanol in exhaust gas of fermenters (Bach et al. 1978) are: 1) the narrow range of ethanol concentrations for which a linear response is obtained, and 2) its dependence on oxygen. Furthermore, the enzyme has only a short lifetime in yeast cultures. Although in discontinuous assays of ethanol in phosphate buffers of neutral pH the electrode gives a stable response for several weeks (Verduyn et al. 1983), in the experiments described above the electrode was stable for only 3–5 days.

The results obtained with *S. cerevisiae* (Figs. 3, 4) confirm the well-known behaviour of this yeast with respect to the glucose concentration in the culture. The critical glucose concentration at which aerobic fermentation started is close to that reported by Woehrer and Roehr (1981) who obtained values between 90 and 100 mg · l^{-1} glucose for glucose-limited *S. cerevisiae.* These authors demonstrated that the conditions at which the cells are precultivated are of crucial importance for the occurrence of alcoholic fermentation. Therefore the absence of alcoholic fermentation under the conditions employed (i.e., air saturation, excess glucose) with *C. utilis* and *Rhodotorula rubra* cannot be taken as an absolute measure for their behaviour under these conditions.

The results obtained with *H. nonfermentans* and *C. buffonii,* organisms which are classified as non-fermentative yeasts (Lodder 1970; Barnett et al. 1979) further illustrate that the taxonomic tests for the fermentative abilities of yeasts (i.e., gas formation in Durham tubes in complex media under static incubation) are inadequate for biochemical characterization. Further work is required to establish the factors which govern the physiological behaviour of these representatives of so-called non-fermentative yeasts. The alcohol electrode is a useful tool to quantify their capacity to perform aerobic alcoholic fermentation when grown under various conditions.

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