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Heat Flux Measurements for the Fast Monitoring of Dynamic Responses to Glucose Additions by Yeasts That Were Subjected To Different Feeding Regimes in Continuous Culture

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Heat measurements have been used successfully as an analytical tool for the study of the dynamics of energy metabolism of *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous culture under fluctuating substrate supply. A low average dilution rate ($D = 0.05$ h^{-1}) was maintained either by adding the medium as continuously (dropwise) as possible or (blockwise) by adding the medium at high speed during a short period ($D = 0.5$ h^{-1} for 40 s) and not at all during the following period ($D = 0.00$ h^{-1} for 360 s). The resulting biological activity was monitored on-line with conventional (O_2 and CO_2) off-gas analyses, DOT measurements, and heat flux measurements. In *C. utilis* cultures, the biomass-specific maximum oxygen consumption rate ($q_{O_2,max}$), the biomass yield ($Y_{s,x}$), and the dynamic responses to a glucose pulse and to a change in feeding regime were not significantly affected by different preceding feeding regimes. In contrast, *S. cerevisiae* grown in continuous culture with blockwise feed showed a 50% increase in $q_{O_2,max}$ and a 25% drop in $Y_{s,x}$ compared to the culture grown with dropwise feed. The dynamic response to a glucose pulse (0.6 g L^{-1}) was slower for the continuous (dropwise) than for the blockwise grown *S. cerevisiae*. With a second testing method for the dynamic response of the yeasts, the feeding regime was changed. The blockwise fed *S. cerevisiae* proved to be better "trained" to cope with sudden changes in glucose supply and, therefore, was more "shockproof" toward a change in feeding regime. This clearly points to major differences in the intracellular metabolic flux control between the yeasts. These findings are of relevance for industrial baker's yeast production, where reactor mixing times of one to several minutes are not uncommon. The observed heat production, together with the dissolved oxygen concentration, appeared to give the fastest response to actual changes in the culture. It is suggested that heat measurements can be a very useful tool to monitor and control the growth of *S. cerevisiae* in laboratory and industrial fermenter operations.

Introduction

In industrial large-scale fermenters, microorganisms are exposed to a continuously changing environment. Changes in substrate and oxygen concentrations and in pH may occur in a time window of about 10–500 s (Sweere *et al.*, 1988b). Sweere *et al.* studied the influence of a periodically changing oxygen concentration on the growth of *Saccharomyces cerevisiae* (baker's yeast) in continuous culture. They found that relatively fast (several minutes period) fluctuations in dissolved oxygen tension can have a distinct influence on the growth yield and metabolite production by baker's yeast. Neijssel and Tempest (1976) found, with the bacterium *Klebsiella pneumoniae*, that a periodically changing feed rate of the carbon source leads to a higher oxygen uptake rate and lower yield values. Similar behavior was reported by Neubauer *et al.* (1995) for *Escherichia coli*.

The effect of glucose concentration on the metabolism of yeasts can be dramatic. An example is the glucose or Crabtree effect (De Deken, 1966). Crabtree-positive yeasts, including *S. cerevisiae*, will show alcoholic fermentation at high glucose concentrations even in the presence of high concentrations of oxygen in the broth. Since this oxidoreductive metabolism causes problems in baker's yeast production, glucose concentrations generally are minimized by the use of fed-batch techniques. However, due to mixing problems associated with the use of highly concentrated feeds, the microorganisms will still be transiently exposed to high glucose concentrations.

Sonnleitner and Käppeli (1986) proposed that a limited oxygen uptake capacity is the cause of aerobic fermentation in Crabtree-positive yeast strains. However, other explanations, such as a limited biosynthetic capacity, have been put forward (van Urk, 1989) since, during the uncoupling of growth by benzoate, very high oxygen uptake rates can be observed (Verduyn *et al.*, 1992). To our knowledge, only limited insight exists into the impact of feeding dynamics on the metabolic performance of yeasts. Recently, several findings were published on the reaction of yeasts to glucose pulses when the yeasts were oscillating spontaneously in continuous culture (Frandsen *et al.*, 1994) and on the effect on $q_{O_2,max}$ and biomass yield when the yeasts were subjected to glucose oscillations in fed-batch culture (George *et al.*, 1994).

The aim of this study was twofold. The use of heat flux measurement as an analytical tool in laboratory fermenters was studied to evaluate its precision and

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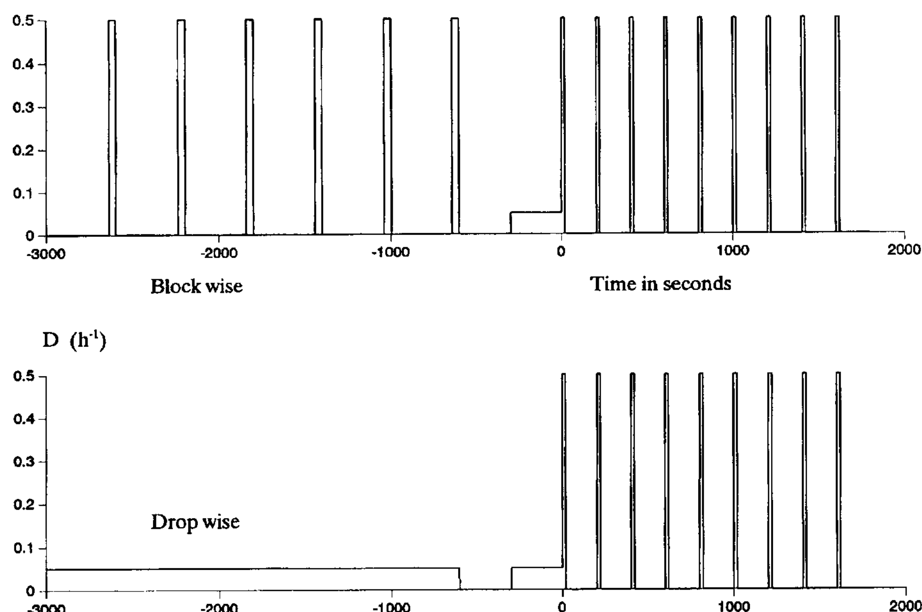


Figure 1. Setup of the regime change experiment. The upper drawing illustrates the setup for a change from blockwise 40 in 400 to the test regime 20 in 200. The lower drawing illustrates the setup for a change from dropwise to the test regime 20 in 200. Time is measured in seconds.

speed. Together with other methods this technique was used to study the influence of different feeding regimes on yeasts in continuous culture to show the resolution of the heat flux measurement in a biological system.

Yeasts were cultivated under two different feeding regimes in continuous culture. The dynamic properties of the yeast cells pregrown under these conditions were characterized with several experiments in which the glucose feed rate was suddenly changed. The fast and continuous method of heat flux measurement allowed a detailed analysis of the dynamics of the response of the yeast to the dynamics of glucose feeding. The Crabtree-positive yeast strain (*S. cerevisiae* CBS 8066) and the Crabtree-negative yeast strain (*Candida utilis* CBS 621) showed remarkable differences in their responses to dynamic growth and feeding regimes.

Materials and Methods

Yeast Strains. *S. cerevisiae* (CBS 8066) and *C. utilis* (CBS 621) were obtained from the Centraal Bureau voor Schimmelcultures (Yeast Division, Julianalaan 67, 2628 BC Delft, The Netherlands) and maintained by monthly subculturing on malt agar slopes.

Equipment. All continuous culture experiments were done in a calorimeter–fermenter that was described previously (van Kleeff *et al.*, 1993). This equipment allows the accurate and fast measurement of heat fluxes during the experiments, as well as the simultaneous monitoring of the more conventional fermentation parameters such as dissolved oxygen tension (measured with an Ingold polarographic electrode, response time = 45–90 s), CO_2 and O_2 concentrations in inlet and outlet gases, gas flow into and out of the fermenter, pH, acid and base additions, rpm, and power uptake of the stirrer motor.

Continuous Culture Conditions. The growth medium with glucose (10 g L^{-1}) as the limiting carbon and energy source was described previously (van Urk *et al.*, 1988). A 2 L volume fermenter (Applikon) was used with a 1.5 L culture volume. The culture conditions were as follows: pH, 5.0 ± 0.1; stirring speed, 1000 rpm; temperature, 30 °C. Dissolved oxygen tension, also during all transient experiments, was above 40% of air saturation.

Feeding Regimes. Both the Crabtree-positive yeast strain *S. cerevisiae* and the Crabtree-negative yeast strain *C. utilis* were grown at a low average dilution rate (0.05 h^{-1}) under two different regimes of substrate feeding. Both regimes are illustrated in the left part of Figure 1.

The first feeding regime was dropwise. In these experiments, the yeasts were grown under normal continuous culture conditions. The medium was added to the fermenter as continuously as possible. However, this still means that there is not a real continuous flow of medium since the small flow of medium breaks up into separate droplets.

The second glucose addition regime was blockwise. In these experiments, the total medium normally fed over a 400 s interval was added over 40 s with a high pump speed (D = 0.5 h^{-1}). After this feeding block the pump stopped (D = 0.00 h^{-1}) for a period of 360 s. The effluent pump was operated in parallel. This block feeding cycle was repeated for at least five reactor volume changes until a dynamic steady state had been reached.

Analytical Procedures. For all experiments, O_2 uptake, CO_2 production, DOT, and heat production were measured on-line. The fermenter off-gas was analyzed with an infrared CO_2 analyzer (Beckman 870) and a paramagnetic O_2 analyzer (Servomex 1100). No efforts were made to optimize the gas analysis; rather, the fermenter was used with the usual broth volume (1.5 L) and head space (0.5 L) to avoid foam problems. When samples were needed from the broth, their volumes were kept small (5 mL), and the total sample volume in any hour was kept below the medium flow per hour so as not to disturb the dilution rate. During the dynamic steady states, samples were analyzed for ethanol, acetate, and glycerol with HPLC techniques as described by Kaliterna *et al.* (1995). Glucose was determined by the Boehringer Mannheim GOD–Perid method. TOC and TC were determined in a Dohrman DC190 analyzer. Yeast dry

Table 1. Steady State Values with Standard Deviations for Continuous Cultures of *S. cerevisiae* and *C. utilis* Grown under Different Feeding Regimes

	S. cerevisiae, dropwise, $D = 0.054\ h^{-1}$ $CH_{1.76}O_{0.58}N_{0.144}$	*C. utilis*, dropwise, $D = 0.056\ h^{-1}$ $CH_{1.69}O_{0.49}N_{0.179}$	unit
glucose flow into fermenter	7.5 ± 0.1	7.8 ± 0.1	μCmol s^{-1}
yield (biomass$_{ash\ free}$/glucose)	0.47 ± 0.03	0.47 ± 0.03	g g^{-1}
	0.54 ± 0.04	0.59 ± 0.04	Cmol $Cmol^{-1}$
biomass flow out of fermenter	4.0 ± 0.1	4.5 ± 0.1	μCmol s^{-1}
CO_2 production	3.4 ± 0.1	3.6 ± 0.1	μmol s^{-1}
O_2 consumption	3.2 ± 0.1	3.4 ± 0.1	μmol s^{-1}
heat production	1.42 ± 0.05	1.47 ± 0.05	W
carbon recovery	99 ± 4	104 ± 4	%
energy recovery	101 ± 6	104 ± 6	%
degree of reduction balance	99 ± 6	104 ± 6	%
heat production/O_2 consumption	445 ± 35	432 ± 35	kJ mol^{-1}
	blockwise, $D = 0.053\ h^{-1}$	blockwise, $D = 0.057\ h^{-1}$	unit
glucose flow into fermenter	7.4 ± 0.1	7.8 ± 0.1	μCmol s^{-1}
yield (biomass$_{ash\ free}$/glucose)	0.36 ± 0.03	0.47 ± 0.03	g g^{-1}
	0.41 ± 0.04	0.59 ± 0.04	Cmol $Cmol^{-1}$
biomass flow out of fermenter	3.0 ± 0.1	4.6 ± 0.1	μCmol s^{-1}
CO_2 production	4.0 ± 0.1	3.7 ± 0.1	μmol s^{-1}
O_2 consumption	3.7 ± 0.1	3.5 ± 0.1	μmol s^{-1}
heat production	1.67 ± 0.05	1.55 ± 0.05	W
carbon recovery	95 ± 4	105 ± 5	%
energy recovery	94 ± 6	105 ± 6	%
degree of reduction balance	94 ± 6	105 ± 6	%
heat production/O_2 consumption	453 ± 35	442 ± 35	kJ mol^{-1}

weight was determined with nitrocellulose filters (Gelman, pore width = 0.45 μm) as described by van Urk (1989). The maximum specific oxygen uptake rate was determined with several substrates in a biological oxygen monitor (BOM) according to the method described by Sweere *et al.* (1988a). For all substrates (glucose, ethanol, and acetate), the concentration in the BOM vessel was approximately 10 times the equivalent O_2 concentration to prevent substrate limitation, but still low enough to prevent substrate inhibition.

In additional to the analyses in the steady state, the dynamic behavior of the yeast, cultivated under different feeding regimes, was measured by observing oxygen uptake, heat production, and C metabolism in response to disturbances in glucose feed rate in the chemostat in two types of experiments:

Pulse Experiment. We studied the dynamics of the reaction of the yeast in the fermenter to a glucose pulse in the way described by van Urk (1989), but with lower initial glucose concentrations (5 instead of 50 mmol L^{-1}). Samples were taken from the fermenter at 5 min time intervals and afterward analyzed for glucose, glycerol, acetate, and ethanol. During the pulse experiments, the feeding pump was left running continuously ($D = 0.05\ h^{-1}$), regardless of whether the yeast was pregrown under a blockwise or a continuous feeding regime. This continuous feeding during the pulse served to compensate for medium losses due to the frequent sampling.

Prolonged Change in Feeding Regime. The ability of the yeast cultivated under the two different feeding regimes to react to prolonged changes in glucose feeding regime was tested in the fermenter by measuring the reaction of the DOT, the CO_2 and O_2 in the off-gas, and the heat production upon switching to a blockwise feed of 20 s in a total time of 200 s. This test regime was chosen because it is one-half the previously supplied continuous feeding regime or the blockwise (40 s feed in 400 s total time) feeding regime used during the two preceding steady state cultivation regimes. In short, the feeding regime was changed for both the continuous feeding regime and the 40 s in 400 s blockwise feeding regime to a blockwise 20 s in 200 s test regime.

Figure 1 shows the experimental feed sequence. The test was standardized as follows. The yeast was cultivated for at least five reactor volume changes with either a blockwise (40 s feed in 400 s total time) or a continuous feeding regime. The feed pump was then stopped for 300 s to get rid of possible residual intermediates. After this short starvation period, the feed pump was run for 300 s in continuous mode to standardize the starting levels of heat, DOT, CO_2, and O_2. After this equilibration period, the feeding regime of 20 s feeding time in 200 s total time was started. The start of this first feeding period was taken as zero time.

All experiments, including the steady states, were repeated at least twice. Steady states were characterized when the on-line measurement appeared to have reached a constant value, but at least five reactor volume changes after a switch from blockwise to continuous feeding regimes and vice versa. Between pulses, the cultures were left at least three reactor volume changes to allow them to return to steady state.

Results and Discussion

Steady States. *S. cerevisiae* and *C. utilis* were grown in continuous culture at a low dilution rate (0.05 h^{-1}) under different feeding regimes. When the on-line measurements indicated that a steady state had been reached, and after the continuous culture was run for at least five reactor volume changes, samples were taken for chemical analyses, dry mass determinations, and BOM measurements. Characteristics of the steady states for the different feeding regimes are given in Table 1. Biomass compositions were taken from Verduyn *et al.* (1991), who used the same growth medium composition at $D = 0.1\ h^{-1}$. An ash content of 6% was assumed. In some of our experiments, D was subjected to short-term variations between 0.0 and 0.5 h^{-1} with the possible formation of intermediate metabolites; however, the biomass compositions presented by Battley (1995) show only slight variations (standard deviation less than 2.5%) in the carbon content and the degree of reduction (standard deviation less than 2%) for *S. cerevisiae* grown on the different substrates that could be expected as

Table 2. BOM Measurements with Standard Deviations for *S. cerevisiae* and *C. utilis* Grown under Different Feeding Regimes

	S. cerevisiae dropwise $D = 0.054\ h^{-1}$	*C. utilis* dropwise $D = 0.056\ h^{-1}$
average biomass-specific oxygen consumption rate during steady state (normalized to a D of precisely 0.050 h^{-1})	1.6 ± 0.1 mmol of O_2 $gDM^{-1}\ h^{-1}$	1.6 ± 0.1 mmol of O_2 $gDM^{-1}\ h^{-1}$
substrate	$q_{O_2,max}$ (mmol of O_2 $gDM^{-1}\ h^{-1}$)	$q_{O_2,max}$ (mmol O_2 $gDM^{-1}\ h^{-1}$)
glucose	2.8 ± 0.2 (1.7 × ss value)	3.9 ± 0.2 (2.4 × ss value)
ethanol	2.4 ± 0.2	5.2 ± 0.2
acetate	1.2 ± 0.2	3.0 ± 0.2
	S. cerevisiae clockwise, $D = 0.053\ h^{-1}$	*C. utilis* blockwise, $D = 0.057\ h^{-1}$
average biomass-specific oxygen consumption rate during steady state (normalized to a D of precisely 0.050 h^{-1})	2.2 ± 0.1 (mmol of O_2 $gDM^{-1}\ h^{-1}$)	1.6 ± 0.1 (mmol of O_2 $gDM^{-1}\ h^{-1}$)
substrate	$q_{O2,max}$ (mmol of O_2 $gDM^{-1}\ h^{-1}$)	$q_{O2,max}$ (mmol O_2 $gDM^{-1}\ h^{-1}$)
glucose	4.3 ± 0.2 (2.0 × ss value)	4.0 ± 0.2 (2.5 × ss value)
ethanol	4.2 ± 0.2	5.0 ± 0.2
acetate	1.7 ± 0.2	2.9 ± 0.2

intermediates in our experiments. Other values found in the literature for *S. cerevisiae* grown on glucose (von Stockar *et al.*, 1993) are close to the values found by Verduyn *et al.* (1991). The values were used in the carbon and degree of reduction balances to ensure that no gross measurement errors were present. Table 1 also shows the actual CO_2 and O_2 conversion rates, the heat flow and the percent carbon, percent degree of reduction, and percent energy recovery calculated from carbon, and degree of reduction and energy balances during the steady states. For the energy balance the combustion enthalpy value for biomass was taken to be 560 kJ $Cmol^{-1}$ (Roels, 1983), and for glucose and NH_4^+ the values were 468.7 kJ $Cmol^{-1}$ and 296.2 kJ mol^{-1}, respectively (Larsson *et al.*, 1991). Oxygen uptake was checked with a balance on the degree of reduction. For all steady states, the quotient of heat production and oxygen consumption gave values close to the value of 460 kJ mol^{-1} found as the average in the literature (Roels, 1983).

The Crabtree-positive yeast *S. cerevisiae* showed very different characteristics for cells that were grown under the continuous regime or under the blockwise regime. Specifically, the biomass yield on substrate was about 25% lower for the blockwise regime. In agreement with this finding, the heat production, CO_2 production, and O_2 consumption were higher than those during the continuous feeding regime. The heat production per Cmol biomass formed increased from 350 to 550 kJ $Cmol^{-1}$. This increased heat production would severely limit biomass production due to the limited cooling capacity. The low carbon, energy, and redox recoveries for the blockwise regime point to the formation of an organic product. Glucose, glycerol, ethanol, and acetate were not found in samples of the culture liquid. The balance on degree of reduction shows a gap similar to that in the other balances, indicating that the degree of reduction of any product missed should be similar to those of the products that were taken into account (glucose and biomass). TOC and TC analyses showed less than 50 ppm of organic carbon in the culture supernatant, suggesting that any product missed would be volatile. Another explanation for the gap in the balances could also be a change in biomass composition. It is good to notice that all recoveries and balances were shifted the same way. This indicates that the energy balance, which is seldom used in biotechnology, gives valid results that can be used either alone or together with other measurements to provide more information with high reliability.

In contrast to the findings with *S. cerevisiae*, none of the steady state characteristics were significantly different for the Crabtree-negative yeast *C. utilis* whether it was grown under the blockwise regime or under the continuous regime. Clearly, internal control of substrate uptake and growth in this organism is not affected significantly by external substrate feeding rates.

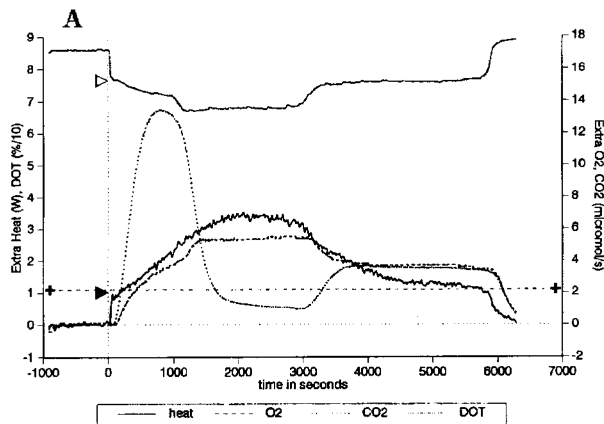
The balances for this organism all show a (too) high recovery with a surplus of recovery close to the standard deviation. Still, these values indicate that no gross measurement errors are present. It should be realized that even a slightly different biomass composition can have an important effect on all of the balances. When these balances were recalculated with a biomass composition identical to that of *S. cerevisiae*, they all closed to within 2%.

Table 2 shows the biomass-specific maximum oxygen consumption rate $q_{O_2,max}$ measured in the BOM for *S. cerevisiae* and *C. utilis* for several substrates after growth under the two feeding regimes. The values can be compared to the average oxygen uptake that the organism has during the preceding steady state in the fermenter. For both feeding regimes, the $q_{O_2,max}$ for glucose was about double the average oxygen uptake during the steady states. In addition, it is clear that the specific $q_{O_2,max}$, especially for glucose and ethanol, is much increased during the blockwise feeding regime over that during the dropwise regime. This means that the smaller amount of biomass in the fermenter has a larger total $q_{O_2,max}$ for glucose and ethanol. The extra oxygen uptake capacity allows the organism to adapt quickly to changes in the environment during the blockwise feeding regime, without the formation of external intermediate products.

From Table 2 it is clear that no significant differences are present between blockwise and continuously grown *C. utilis*. It is also clear that *C. utilis* has a high capacity for oxygen uptake, much higher (2.5 times) than is needed during the steady state in the fermenter. This high capacity and the fact that the yield is not affected by the feeding regime indicate that *C. utilis* is well equipped to respond to changes in the environment without the formation of external intermediate products.

Pulse Experiments. For all pulse experiments, carbon and energy balances were calculated over the total duration of the experiment (up to 5 h). The balances were found to close within 5%. Results of the continuous on-line measurements are shown in Figures 2A–5A. The results of the off-line chemical analyses are shown in Figures 2B–5B.

It must be noted that the feed pump was left running (continuous) during the pulse experiments to replenish the medium that was taken for the samples. The results were corrected for this steady state background, so that only the extra effects from the glucose pulse are shown.



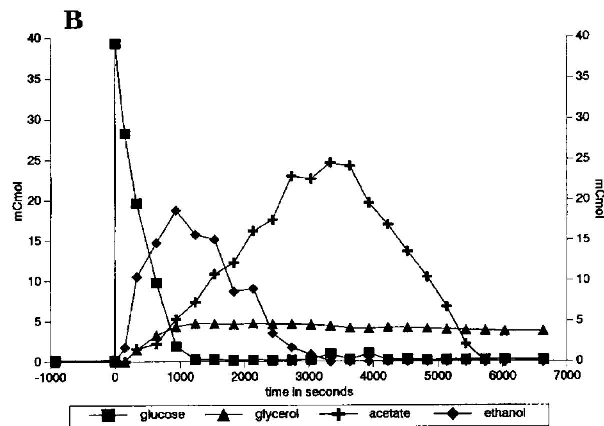
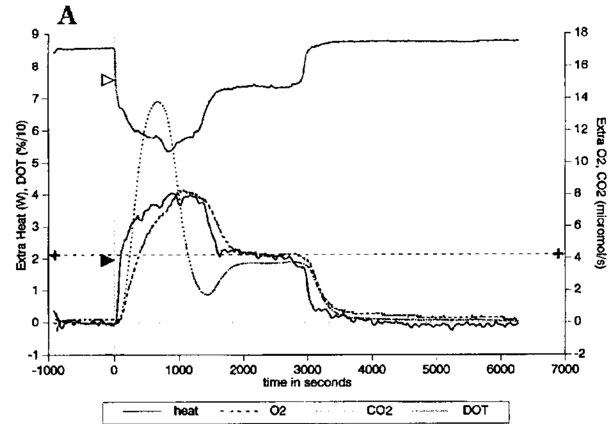


Figure 2. (A) On-line measured responses of a dropwise fed (untrained) *S. cerevisiae* culture after the addition of a glucose pulse into the fermenter. The horizontal line between the markers (+) in the figure indicates the maximal oxygen uptake possible for the total biomass present as calculated from the (glucose) BOM measurement of the biomass in the preceding steady state. The DOT level indicated by the marker (△) in the figure was calculated from the BOM measurement under the assumption that the k_La is not influenced by the glucose pulse, so that a higher O_2 uptake has a proportional effect on the DOT. The heat level indicated by the marker (▲) was calculated from the BOM measurement under the assumption that the uptake of 1 mol of O_2 is "equivalent" to 460 kJ. Both markers indicate the level that can be expected to be reached "immediately" after the addition of the glucose pulse. (B) Off-line measured responses (analyses) of a dropwise fed (untrained) *S. cerevisiae* culture after the addition of a glucose pulse into the fermenter.



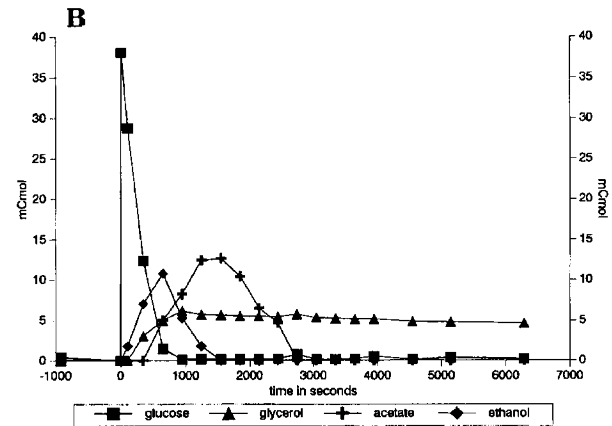


Figure 3. (A) Same as Figure 2A, but for a blockwise fed (trained) *S. cerevisiae* culture. (B) Same as Figure 2B, but for a blockwise fed (trained) *S. cerevisiae* culture.

The response of *S. cerevisiae* to a glucose pulse in the fermenter is shown in Figure 2A,B for the cultures that were grown under the continuous feeding regime and in Figure 3A,B for the blockwise feeding regime. Both experiments show the well-known response pattern to a glucose pulse for *S. cerevisiae* (van Urk, 1989). Initially there is aerobic fermentation with the formation of ethanol and CO_2 (Crabtree effect), followed by oxidation of the ethanol, first to acetate and next to CO_2. Significant amounts of glycerol are also formed.

This pattern is clearly visible not only in the off-line analysis but also in the on-line responses. The shape of both the DOT curve and the heat curve shows that there is, in all cases, an initial fast (50 s) response to the high glucose concentration up to the level of the maximal oxygen uptake that is calculated from the BOM measurement with glucose as a substrate. This initial sharp response is followed by a further, slower (50–2000 s), increase in O_2 uptake and heat production. Interpretation of the DOT and heat measurements together with the gas analysis is very difficult. The initial sharp response is not shown in the gas analysis, but it is supported by the off-line chemical analysis, which shows a very rapid decline in glucose concentration. The gas analysis does not show the fast dynamics of the yeast response because of mixing in the head space of the fermenter and in the tubing from fermenter to analyzers. The gas analysis also clearly lags behind the heat and DOT measurements because of transport phenomena. The gas analysis does show a high initial peak in CO_2 production due to the fermentation of glucose. Comparison of the DOT and heat measurements to the gas and off-line analyses shows that for both the continuous and the blockwise cultures the oxygen uptake and heat production continually increase even after the glucose in the medium is finished and the metabolism has switched to the partial oxidation of ethanol. Clearly both cultures are able to switch from glucose to ethanol as substrate and are capable of increasing their oxygen uptake capacity much faster than could be explained by growth alone, which would account for an increase of only 6% (0.6 g of glucose L^{-1} extra, with 10 g L^{-1} already present).

In fact, for the blockwise culture the total biomass-specific oxygen uptake rate reaches a value of 5.5 mmol of O_2 gDM^{-1} h^{-1}, which is 1.3 times higher than the value found in the BOM. For the continuous culture the total biomass-specific oxygen uptake rate reaches a value of 7.5 of mmol O_2 gDM^{-1} h^{-1}, which is even 2.5 times higher than the value found in the BOM. When these values are compared to the values of up to 20 mmol of O_2 gDM^{-1} h^{-1} found by Verduyn *et al.* (1992) in cultures with benzoate at $D = 0.05$ h^{-1}, it is clear that even the

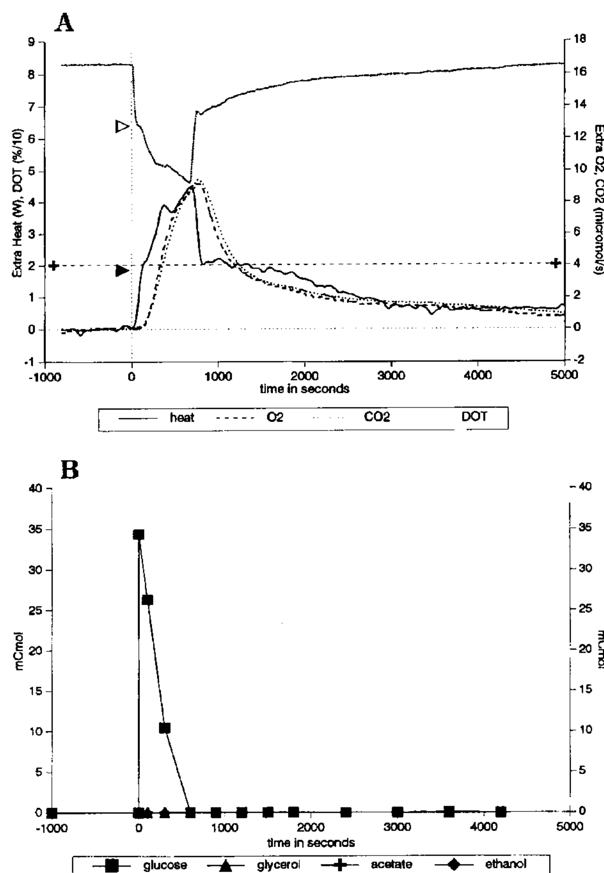


Figure 4. (A) Same as Figure 2A, but for a dropwise fed (untrained) *C. utilis* culture. (B) Same as Figure 2B, but for a dropwise fed (untrained) *C. utilis* culture.

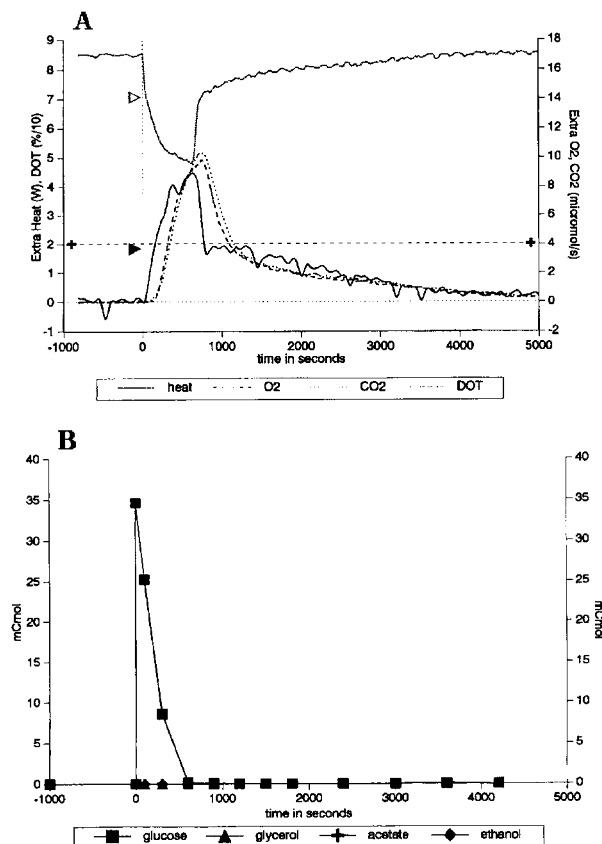


Figure 5. (A) Same as Figure 2A, but for a blockwise fed (trained) *C. utilis* culture. (B) Same as Figure 2B, but for a blockwise fed (trained) *C. utilis* culture.

increased values of the oxygen uptake rate during the pulse experiments have not yet reached their "bottleneck" values. When the ethanol in the broth is depleted, the heat production and oxygen uptake decrease. This could be expected both from the BOM measurements, since the maximum oxygen uptake capacity is lower with acetate as the substrate, and from the heat measurement as the combustion heat of acetic acid per mole of oxygen uptake is relatively low (438 kJ mol^{-1}). An interesting phenomenon is shown between 2000 and 3000 s (0.5–0.8 h) with the dropwise cultured yeast: the heat production per mole of oxygen uptake during this period is significantly higher than the well-known average of 460 kJ mol^{-1} (Roels, 1983). This could be explained if during this period the oxidation of ethanol to acetaldehyde and then on to acetic acid took place. From tables of combustion heat (Roels, 1983), it can be calculated that oxidation of acetaldehyde yields 548 kJ per mole of oxygen uptake. Overall balances over the total pulse period yielded values around 450 kJ per mole of oxygen uptake. A period with a high value ($\gg$460 kJ mol^{-1}) therefore has to be compensated by a period with a low value. This is indeed the case for the period between 4000 and 6000 s where acetic acid is oxidized. To summarize, these findings point to the possible production of acetaldehyde in the period before 2000 s and to the oxidation of acetaldehyde to acetate in the period 2000–3000 s. As mentioned before, it is difficult to interpret the slow and lagging gas analysis together with the fast heat and DOT measurements. It is interesting, however, that heat measurement together with gas analysis in this case points to a process that is not visible with the help of only conventional analysis. For the pulse experiment with the blockwise cultured yeasts, both periods follow so closely that overlap takes place.

The response of *C. utilis* is shown in Figure 4A,B for the continuous and in Figure 5A,B for the blockwise cultivated yeasts. Since this is a Crabtree-negative yeast, the response is very simple: no intermediate external products are found and glucose is directly stored or used. With the simple response found here, it is very clear that the gas analysis lags behind the heat measurement and the DOT measurement. As with the *S. cerevisiae*, the response starts with a sharp (50 s) change in DOT and heat to the levels predicted from the BOM measurements, followed by a slower (1000 s), but still rapid additional increase.

Shortly after the glucose in the supernatant is used up, the heat and DOT return to the values predicted by the BOM measurement. The reason for the short delay could be an artifact in the glucose measurement, but it could also point to the swift uptake of glucose by the yeast, followed by a slower processing of the glucose. Only after a long period do all measurements return to the steady state values they had before the pulse. Since no intermediate products were found in the culture supernatant, this prolonged period of higher activity must be completely sustained by the consumption of internal (storage) material. The on-line gas analysis in these cases suggests a very gradual process, while the fast heat and DOT measurements both show that very rapid response changes take place. The independently measured DOT and heat curves are very similar, especially

in this phase of the transient. Together they prove convincingly that the rapid changes are indeed present. The off-line chemical analysis, showing a very steep decline in glucose concentration, provides further proof that glucose uptake is among the rapid changes that are present.

A minor, but interesting phenomenon can be found in the heat production with the blockwise cultured *C. utilis*: small dips in the heat production with a period close to 500 s. This period is close to the block feeding cycle time of 400 s. It could be speculated that the feeding cycle synchronizes a physiological clock that is still running during the pulse experiment. As before, no fine structure is visible in the gas analysis; the small dips in this case also are not found in the DOT. An explanation for this could be that the ratio of heat production to oxygen uptake is not uniform for all processes.

Prolonged Change in Feeding Regime. For the feed regime change experiments the results are shown in Figures 6A–7B. The horizontal lines between the markers (+) indicate the average levels of DOT, CO_2, or heat production or O_2 consumption during the preceding (dynamic) steady states. Liquid samples were not taken because the routine sampling procedure disturbs and complicates the process, and calculations showed that the buildup of intermediate products during a 200 s block period would be too small to be analyzed with conventional methods.

The responses that were obtained when the feeding regime was changed from either continuous or 40 s in 400 s to the test regime of 20 s in 200 s are shown in Figure 6A,B for *S. cerevisiae* grown under the continuous feeding regime and the blockwise feeding regime, respectively. Even with the standardization of the test the initial conditions for these experiments differ slightly, since the biomass yield during the blockwise regime is lower so that less biomass is present at the start of the experiment (cf. Table 1). Figure 6A shows that the continuous cultured, "untrained" *S. cerevisiae* has significant problems to deal with in the sudden change in the feeding regime, whereas Figure 6B shows that the blockwise cultured, "trained" *S. cerevisiae* is completely capable of doing so, even though less biomass is present.

The trained *S. cerevisiae* initially responds to the 20 s addition of glucose with an increase in heat production and an increased oxygen uptake, resulting in a decreased DOT. During the 180 s period following the addition, the activity of the yeast gradually returns to the original value at time 0 and even lower because the glucose is depleted before the addition of the next feeding block. This cycle is repeated every 200 s. Heat and DOT values vary around the values of the preceding dynamic steady state. Gas analysis shows no fine structure and the average values are slightly lower than that of the preceding steady state, probably because of the 300 s starvation period before the start of the experiment.

The untrained *S. cerevisiae*, however, initially responds to the new feed regime of the 20 s addition of glucose with only a slight increase in heat production, and the decrease in DOT is also minor. The result of this slow response is probably that the substrate is not completely used up during the 180 s period following the addition. This process is repeated during the following cycles and completely disturbs the uptake of the substrate, even to the point (around 1000 s) where gas analysis suggests the onset of ethanol formation (since CO_2 production exceeds O_2 uptake) and (around 1400 s) where gas analysis and heat production suggest the complete combustion of all glucose to CO_2 and H_2O (biomass yield should then become zero, but prolonged experiments

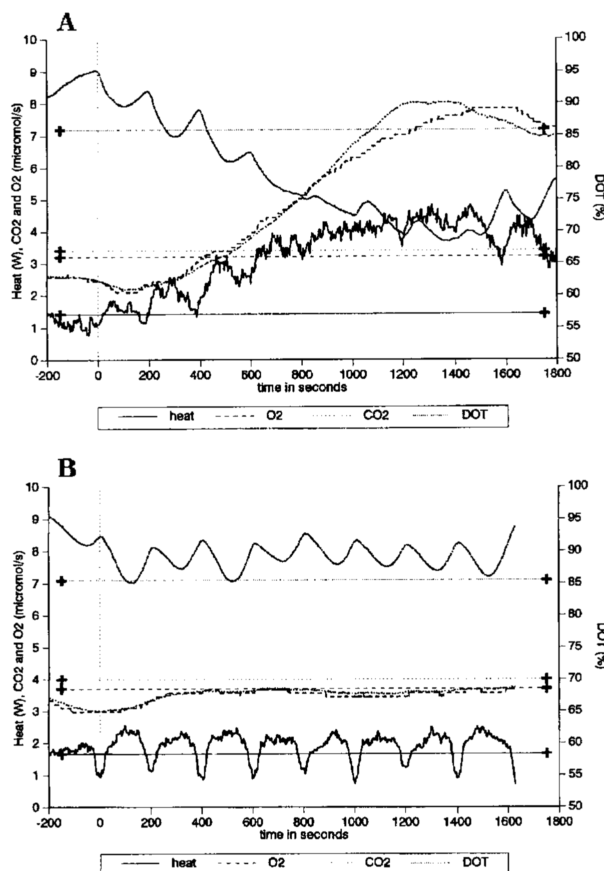


Figure 6. (A) Response of a dropwise fed (untrained) *S. cerevisiae* culture upon a regime change. The horizontal lines between the markers (+) indicate the level of the preceding steady state. (B) Same as panel A, but for a blockwise fed (trained) *S. cerevisiae* culture.

showed that the yeast adapts to the new regime after several hours so that is not diluted out significantly). It should be remembered that the average flow of glucose is the same as that during the preceding (continuous) steady state. On-line gas analysis shows that the O_2 consumption increases after 1400 s to a level of 7.5 μmol s^{-1}, which corresponds to the average glucose flow into the fermenter. At this time the RQ dips below 1, which indicates the consumption of ethanol that is formed during the preceding time.

The blockwise feeding test regime started at zero time clearly has dramatic effects on this continuous cultivated untrained yeast culture. This change of regime experiment could not be prolonged, since measurements were taken and stored with high frequency to detect fine structure; the computer memory put a limit on the experiment time. Other experiments showed that it took *S. cerevisiae* several hours to finally adapt to this kind of change in feeding regime.

Figure 7A,B shows the responses for *C. utilis* after continuous and blockwise regimes. They are very similar. Clearly even an untrained *C. utilis* is very able to cope with sudden variations in the glucose supply. As with the trained *S. cerevisiae*, both the trained and the untrained *C. utilis* respond to the 20 s addition of glucose with an increase in heat production and an increased oxygen uptake, resulting in a decreased DOT. Again, during the 180 s period following the addition, the activity of the yeast gradually returns to the original value at time zero. Especially with the trained *C. utilis*,

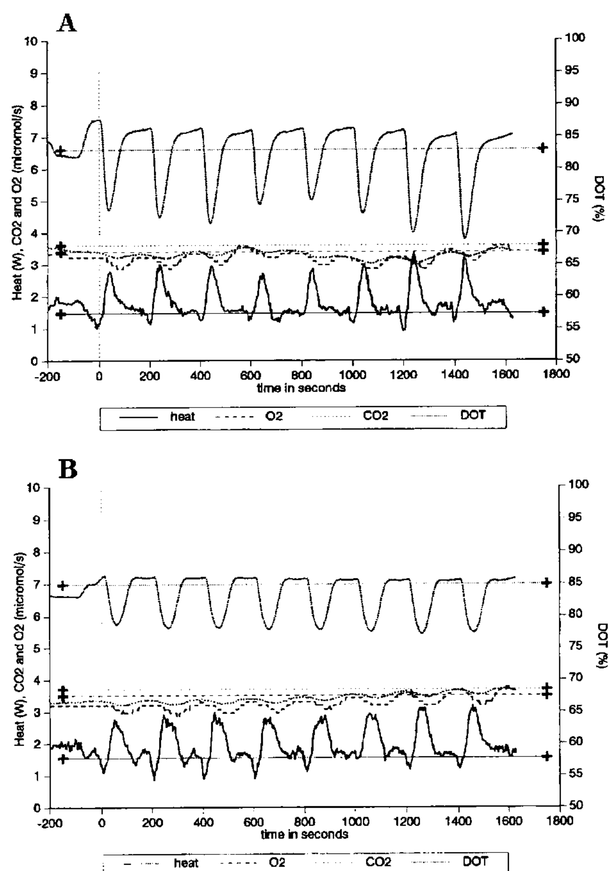


Figure 7. (A) Same as Figure 6A, but for a dropwise fed (untrained) *C. utilis* culture. (B) Same as Figure 6A, but for a blockwise fed (trained) *C. utilis* culture.

a fine structure in this reproducible transient is visible in the heat production. Since this fine structure is repeated every 200 s, it probably is not an artifact, although it is not visible in the DOT. Further independent and fast measurements would be required to analyze the biochemical basis of this transient response. Heat and DOT values vary around the values of the preceding steady state. Gas analysis shows no fine structure: the average values are slightly lower than that of the preceding steady state, again probably because of the 300 s starvation period before the start of the experiment.

General Discussion. From the results it is clear that the physiological behavior of the Crabtree-positive yeast *S. cerevisiae* can be influenced in a major way if, during continuous culture, the medium is not added dropwise and as continuously as possible, but rather in larger amounts and with several minutes between feeding blocks. The change to a blockwise feed for *S. cerevisiae* in the fermenter leads to a change in metabolic capacity with two time constants: (1) A near-immediate response saturating the existing maximum O_2 uptake capacity and (2) a slower response (1000–2000 s) where the maximum O_2 uptake capacity increases significantly from about 2.5 to about 7.5 mmol of O_2 gDM^{-1} h^{-1}.

On the other hand, we found a decrease in biomass yield with the blockwise fed *S. cerevisiae*. Both effects are of interest for the production of baker's yeast in large industrial fermenters, since circulation times in industrial scale reactors can be on the order of minutes. As a consequence of this imperfect mixing, during fed-batch cultures the yeast is exposed to periodically changing glucose concentrations similar to those applied in these experiments.

Sonnleitner and Käppeli (1986) propose that increased oxygen uptake capacity increases the glucose concentration at which aerobic fermentation sets in. Predictions of oxygen uptake capacity made from measurements with cells grown in well-mixed and smoothly fed continuous cultures in the laboratory can be in error if they are applied to the large scale industrial fermentations where *S. cerevisiae* is trained by the varying environment to a higher maximum oxygen uptake capacity.

A marked difference was found between the Crabtree-positive *S. cerevisiae* and the Crabtree-negative *C. utilis*. A possible explanation might be found in the very different residual glucose concentrations in continuous fed cultures of these yeasts. Values of 110 μmol L^{-1} for *S. cerevisiae* and 5 μmol L^{-1} for *C. utilis* were reported by van Urk (1989) for continuous cultures at $D = 0.1$ h^{-1}. In the experiments shown, the amount of glucose added during one 40 s feeding block (roughly 300 μmol L^{-1}) is in the same range as the residual glucose concentrations for *S. cerevisiae*. The amount present in a drop (0.05 mL) of medium (2.8 μmol) is comparable to the residual glucose concentration for *C. utilis*. A dropwise fed culture of *S. cerevisiae* would then experience a glucose concentration that varied by only a few percent around the residual 110 μmol L^{-1}, but a dropwise fed culture of *C. utilis* would experience a glucose concentration that varied *relatively* much more around the residual 5 μmol L^{-1}. Following this approach, even a dropwise fed culture of *C. utilis* is then well trained. It must be added that *S. cerevisiae* showed fewer different responses to the various tests in experiments (not published) where the block period (and thus the variation in glucose concentration) was smaller (10 s feeding in a 100 s period). From these findings it is suggested that the physiology of the yeasts is influenced by glucose variations in the order of the residual concentration found in continuous (dropwise) cultures. However, the experiments we did with *C. utilis* in which the glucose was added as smoothly as possible directly into the liquid to eliminate variations in glucose concentration did not show an untraining effect. Work of a more physiological nature is needed to elucidate these findings.

One of the aims of our experiments was the evaluation of heat measurements as an analytical tool for fermentation processes in biotechnological research compared to more conventional methods. The usefulness of the different methods that we applied to determine the dynamic reaction of yeasts to variations in glucose concentration could be compared: (1) Traditional off-gas analysis lags behind DOT and heat measurements, as shown in the data from the pulse experiments. (2) Fine structure in the dynamic responses is lost in off-gas analysis and also partly in the DOT measurements. This is visible in the pulse experiment with a blockwise fed *C. utilis* and in the regime change experiments, where the heat measurement shows a periodic and repeating fine structure that is not present in the DOT measurement. (3) The long-term stability of the heat signal is better than that of the DOT signal. This is not shown in the results presented, but it was clear from recalibrations during the several months the experiments lasted. Recalibration of the DOT during starvation periods yielded differences of up to 15%. For the heat measurement this value was lower, typically less than 5%. (4) The response speed of the heat measurement was comparable to that of the DOT signal in our experimental setup. The manufacturer indicates a response time of 45–90 s for this type

of DOT electrode. Averaging of the heat signal was done in a time window of 32 s. (5) The heat signal can be used together with other measurements to give an indication of the physiological process that is going on. A good example is found in the pulse experiment with the continuous fed *S. cerevisiae*, where different phases clearly have a different ratio of heat production and oxygen uptake.

Conclusion

The physiological response of *S. cerevisiae* to carbon feed disturbances, especially the oxygen uptake capacity, the excretion of byproducts, and the biomass yield, can be strongly influenced by the feeding regime in continuous culture. The measurement of heat production provides a valuable extra method to study microbial response capacities on-line with respect to the dynamics of substrate utilization. Since the heat measurement is completely independent from other measurements, not only can it provide new data but combination with other methods can also give reliable confirmation of the results of other methods.

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