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MICROCALORIMETRIC INVESTIGATIONS ON THE METABOLISM OF YEASTS. X. THE OCCURENCE OF A HEAT PRODUCTION PEAK LATE AFTER THE TERMINATION OF GROWTH IN LOW GLUCOSE MEDIA

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ABSTRACT

Calorimetric growth experiments with yeast in a complex medium exhibited power-time curves with a threefold structure: a main phase of substrate catabolism and growth, a thermoneutral period and an unexpected late peak of abrupt appearance (Beezer et al., 1978). The aim of this investigation was (1) to reproduce the above observations; (2) to determine the conditions for the occurence of the late peak and (3) to gain knowledge about the substrate metabolized during this period. It could be shown that the observation of the peak is independent of the pH, the aeration and the constituents of the medium, as long as the initial glucose concentration remained below 0.1 %. By comparison with other calorimetric experiments (Brettel et al., 1981), acetate becomes a favoured candidate for this effect.

INTRODUCTION

Power-time curves (P-t curves) of yeast growing in complex glucose media exhibit quite different shapes depending on the growth conditions (ref.1). Under strict anaerobic regimes or at high glucose concentrations an exponential increase of heat production to a single maximum is observed with a subsequent steep decrease to the zero line (ref.2). This behaviour is due to the fermentative catabolism of glucose down to ethanol following Michaelis-Menten kinetics (ref.3, ref.4). At lower glucose concentrations and under strong aeration of the medium the shape changes to a biphasic or multiphasic from as a consequence of consecutive (diauxic) degradations of metabolizable substrates in the medium (ref.5). These energy sources may be present in the medium at the beginning of the experiment or formed during growth as intermediate metabolites (f.i. ethanol, acetate). Such multiphasic "fingerprint" like heat profiles were supposed to be a suited tool for identification of Enterobacteriaceae (ref.6) and

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for the characterization of yeast strains used in bakery and brewery (ref.7).

In the usual experiments with yeasts growing in complex sugar media the different maxima follow one another with short temporal distances (ref.7). Beezer (ref.7, ref.8) was the first to report on a phenomenon where after the steep descend to the base line and after a long period of low heat production ("thermoneutral" period) suddenly a second exothermic effect occured with a final drop to the base line. He showed that this additional peak is not a property of the defined medium constituents and that it vanished under strong oxygenation of the culture at low glucose concentrations. He supposed this effect to be due to an oxygen deficiency in the calorimetric line (ref.8), but could not give any hint to the substrate metabolized during this peak or to the triggering effect determining the length of the thermoneutral phase.

It thus seemed worthwhile to investigate the appearance of this phenomenon, which we will call "late peak (lp)" hereafter. The main interest concerned the following questions:

- What is the typical length of the "thermoneutral" period? Is it really thermoneutral and what triggers the abrupt end of the phase?
- What correlation exists for the values of both maximum rate of heat production and total heat production between this peak and those observed for the first peak?
- Which substances could be responsible for this peak? Are they part of the medium or formed during catabolism?
- Which outer parameters (aeration, oxygenation, pH-value, medium composition, stirring of the medium, flow rate to the calorimeter, cell density etc.) influence the peak and provoke or prevent its appearance?

MATERIAL AND METHODS

Organism

All experiments were performed at 30°C with two strains of the baker's yeast Saccharomyces cerevisiae with the internal indication "211" and "RXII". 211 is a homozygote non-sticky diploid wild type (ref.9), RXII a diploid wild type (ref.10). The cells were preincubated for 24 h at 30°C in a complex medium, harvested, three times washed in a potassium dihydrogen phosphate buffer (1/20 M),

microscopically counted and set at the appropriate cell density for inoculation. Cell counts were performed during the experiments by means of a cytometer chamber or after corresponding calibration by a photometer at 560 nm. Dry weight of the crop was determined by sucking aliquots of culture liquid through a preweighted filter, drying and consequent weighing of filter plus cells.

Media

Two differnt microbial growth media and a glucose buffer were used throughout the experiments with changing concentrations of the constituents. The usual complex medium applied for microbiological investigations had the following composition: 10 g Yeast Extract (DIFCO/Detroit), 5 g peptone (DIFCO/Detroit), 20 g glucose per 1,000 ml deionized water. The pH after autoclaving was approximately 6.0. The glucose concentration in these media was chosen to be less than 1 g per liter. In most of the experiments described only the glucose concentration was reduced, the amounts of the other constituents were kept constant. Besides the complex medium a minimum medium with the following composition was applied: 10 g Yeast Nitrogen Base (DIFCO/Detroit) and 20 g glucose per 1,000 ml deionized water. For investigations of lp the glucose concentration was reduced to less than 1 g/liter. The glucose buffer consisted of 1 g glucose in 1,000 ml 1/20 M KH_2PO_4 buffer (pH 4.5).

Glucose concentration in the medium was determined by the GOD-Perid method (enzymatic kid 124028, BOEHRINGER/Mannheim), ethanol by an UV test (enzymatic kid 123960, BOEHRINGER/Mannheim) and acetate by an UV test (enzymatic kid 148261, BOERHINGER/Mannheim).

Calorimeter

An LKB flow calorimeter (type 10 700, LKB/Bromma) was used for these investigations (ref.11). The sensitivity of the instrument amounted to 64.4 μV/mW, the volume of the measuring spiral to 0.587 ml. The medium with the cells was sucked through the calorimter with a speed of 32.4 ml/h and recycled to the fermentor. The calorimeter signal was recorded by a four-channel-potentiometer (BD5 + BA5, KIPP & ZONEN/Delft) together with the output of the oxygen monitor (Portable O_2 and Temp.Meter, BECKMAN INSTRU-

MENTS/Irvine) or the pH meter (type 71, KNICK/Berlin).

The calorimeter was connected to a 1l-fermentor (FE 007, BIOTEC/ Bromma) equipped with a magnetic stirrer, an aeration canule, a polarographic oxygen electrode or a pH sensor, exit and entrance of the flow line and a canule to sample specimens for cell count and enzymatic determinations of substrates and products. In aerated cultures air passed to the fermentor at a rate of 1.2 l/min through a washing bottle and a sterile filter to avoid evaporation of the medium and contamination of the culture.

RESULTS AND DISCUSSION

Figure 1 shows a typical P-t curve of the growth of S.cerevisiae in an aerated complex medium at low glucose concentration. After inoculation of the medium and a short lag phase without growth, the rate of heat production increases in an exponential

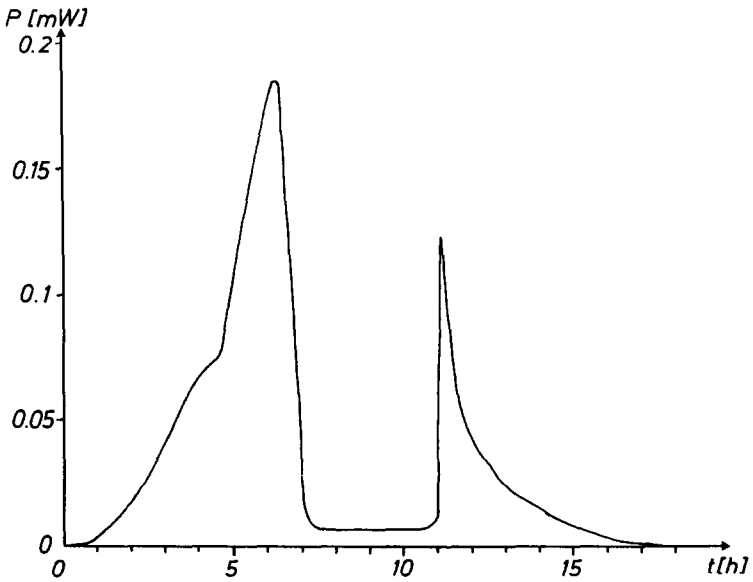

Fig.1 Power-time curve of an aerated culture of Saccharomyces cerevisiae strain RXII growing in a complex medium of 0.05% (2.5 mM) glucose. For further explanation see text.

manner, attains the maximum and then sharply drops to a quite low value. This behaviour reflects the consumption of the energy substrate which follows a Michaelis-Menten kinetics (ref.3, ref.4). After a several hours period of nearly constant heat output a

sharp increase in energy dissipation occurs with a subsequent slow return to the base line. Depending on the initial glucose concentration there may be another peak riding on the left flank of the graph which is due to the maximum of aerobic fermentation of glucose to ethanol before catabolism switches over to respiration of ethanol.

In Figure 2 several P-t curves are compared after normalizing to the same heigth and time of the main maximum. They were run at different glucose concentrations in aerated cultures. One is taken from the original paper of Beezer et al. (ref.7) to show that the same effects appear in their as well as in our cultures in spite of using two different yeasts.

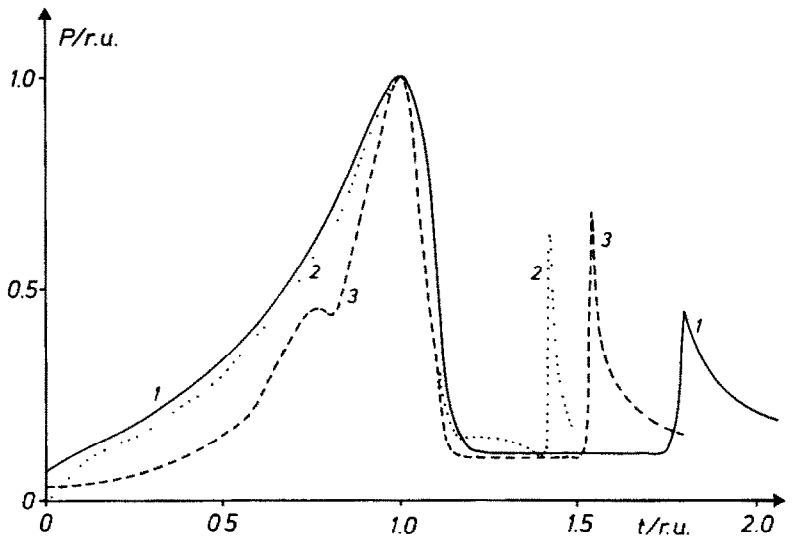

Fig.2 Comparison of different power-time curves with lp peaks after normalizing to the same time and heigth of their maxima. 1: Original slope reported by Beezer et al.(ref.7) at 2.2. mM glucose for a culture of Kluyveromyces fragilis under oxygenation. 2: Growth of S.cerevisiae RXII in an aerated culture (1.0 mM). 3: Growth of S.cerevisiae 211 in an aerated culture (5.0 mM).

The size and the form of the lp is most easiliy compared with those of the first peak. In the mean its maximum corresponds to 67 ± 3% of that of the main peak and its area to 28 ± 13%. From the original paper of Beezer et al. (ref.7) one obtains 44% and 10%, resp. As it is demonstrated in Fig.3, a semilogarithmic transformation of the heat production rate renders a straight

line indicating a reaction with first order kinetics. The rate of heat production in the thermoneutral phase is not always zero but amounts to 10.7% of the main maximum in the original (ref.7) and to 16 ± 12% in these investigations. For this calculations only P-t curves with a pronounced thermoneutral period were considered.

The lp was observed in complex and minimum medium of glucose concentrations below 0.1%, but never in glucose buffer of the same concentration. If the lp is due to catabolism of a metabolic product it should be detectable in the heat profiles of maintenance metabolism in glucose buffer, too. Corresponding experiments with a 0.05% glucose buffer never rendered any hint as to a lp. However, the fact that lp was observed in a complex medium as well as in a minimum one demonstrates that the substrate of the lp is not a constituent of the medium itself but must be formed during proceeding metabolism. Presumably this is during the aerobic fermentation of glucose or the incomplete oxidation of ethanol.

Not in all cases did the lp appear in the expected time. The time gap between the main maximum and the lp peak varied considerably (see Fig.2) without any direct explanation. Under the chosen simple experimental conditions with a continuous recycling of the medium from the calorimeter to the fermentor the culture could not be kept sterile for much more than two days. It might well be that the lp could have shown up at a later time.

The lp occured in aerated cultures as well as in non-aerated ones. To keep homogeneous conditions in the fermentor and to avoid sedimentation of the cells it is necessary to maintain a fixed speed of stirring by which a slight aeration of the culture always happened. It was never tried to study complete anaerobic conditions in this connection. In non-aerated but intensively stirred minimum media the lp appeared after 12 to 14 h. Simultaneous measurement of the pH of the medium showed a reduction in the pH from 5.4 to 3.6 with the strongest decrease just after the maximum of growth. Nearly no change occured during the thermoneutral period and no detectable alteration at the lp, the latter of which appeard at pH values between 2.6 and 3.6. Because of the higher buffer capacity of the complex medium the initial pH amounted to approx. 6.5 to 7.0 and did not change more than one unit at glucose concentrations below 0.1%. In this case the pH decrease continued till the thermoneutral phase and then transformed to a quicker increase before the onset of lp. The lp normally showed

up at pH values around 6.5 during or near to the end of the increase. In no case was there a sign of a dramatic change corresponding to the abrupt rise of heat production (Fig.3). This occurence of lp at different pH values underlined that it did not depend on a special pH or was triggered by a pH threshold.

Cell counts throughout the whole experiment, i.e. from exponential growth over the thermoneutral phase till the lp, rendered no further increase in cell density beyond the steep decline after the main maximum, neither in the thermoneutral period nor during lp, in contrast to the results of Beezer et al. (ref.8) and Brettel et al. (ref.5). This cannot be due to other limiting factors in the medium, as its composition offered sufficient growth conditions up to glucose concentrations of 2%. Comparing heat dissipation during lp and during the first period underlines that a corresponding growth must easily be detectable.

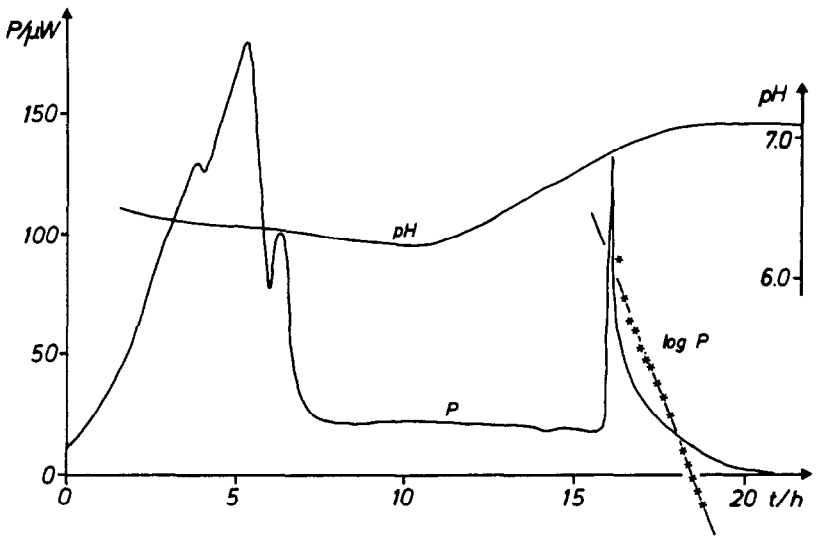

Fig.3 Power-time curve (P) of an aerated culture of S.cerevisiae RXII in a complex medium (2.5 mM), logarithmic transformation (log P) of the lp to demonstrate the first order kinetics, and change of pH value during the experiment.

Aerobic metabolism of glucose by baker's yeast is performed by respiration and additionally by a considerable aerobic fermentation. This aerobic fermentation yields an excess of breakdown products from the Embden-Meyerhof-Parnass pathway which leave the pathway when the subsequent citric acid cycle is saturated as a

consequence of the limited oxygen transfer system (ref.12). Glucose concentration has to be decreased below 0.1% before the aerobic fermentation becomes negligible and pure respiratory conditions are predominant in the fermentor. It is only under these conditions that the lp occurs: above 0.1% glucose we were not able to detect lp in any of the media.

The main question still remains as to which substrate is metabolized during the lp. Beezer et al. (ref.8) supposed a limiting substrate such as ethanol or an organic acid. In our opinion, ethanol has to be excluded as the complete respiration of ethanol formed from glucose during the first phase should give a considerably higher heat output than found by the authors. If only part of the ethanol was left for the lp the question remains open why such a rest appears. Moreover, they recorded a strong decline in oxygen concentration in the calorimetric line and a zero oxygen tension during part of the thermoneutral period. Just prior to the lp oxygen concentration increased again and attained 70% of saturation in the moment of the peak. In the oxygen slope no indication can be seen for an augmentation of oxygen consumption during lp as it should be for the degradation of ethanol.

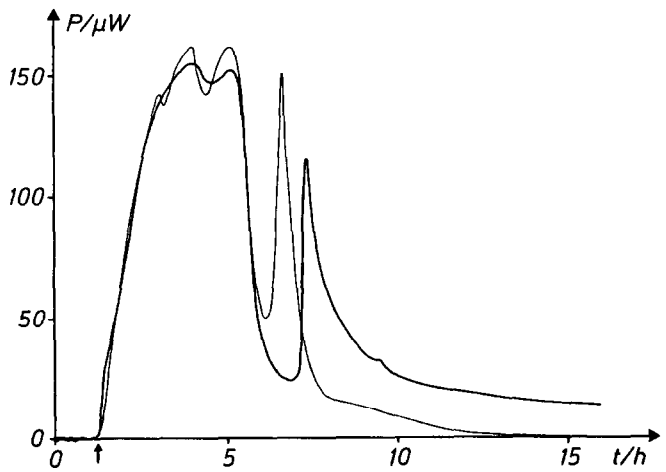

Fig.4 Power-time curve of two aerated cultures of S.cerevisiae RXII in a minimum medium (2.5 mM).

Brettel et al. (ref.5) observed a pronounced third maximum just after the peak of maximum ethanol degradation in the P-t curves

of yeasts growing in complex media with glucose concentrations of 50 mM or higher (1%). From an energy balance, a carbon balance, oxygen consumption in the first period and a drop in pH the authors concluded that this third peak must be due to the degradation of acetate which was formed by incomplete oxidation of ethanol before. In those experiments under comparable conditions there was no longer temporal distance between ethanol and acetate metabolism and no thermoneutral period at all. But as the gap between the main growth period and the lp varied considerably and even made the thermoneutral period disappear (Fig.4), acetate is a favoured candidate for the lp. On the other hand there are heat profiles, e.g. in Fig.3, which demonstrate the third "acetate" peak and lp as in the investigations of Brettel et al. (ref.5). Since the third peak is not determined in Brettel's experiments there is no strong argument against acetate being the substrate for the lp. The drop in pH underlines this supposition although acetate as well as ethanol needs oxygen for its further degradation. At the chosen low glucose concentrations it is difficult to establish an energy balance as in other systems (ref.5). Under these conditions the energy contents of glucose and yeast extract are in a ratio of 1 to 20 in the medium and an ill-defined unknown amount of energy is taken from yeast extract by the cells. Brettel et al. (ref.13) observed that appr. 25% of the energy content of yeast extract was utilized by yeasts in a continuous culture of a complex 0.2% glucose medium.

While investigating the substrate of the lp several "possible" substances were added to the culture medium just after the decline of the lp. Pyruvate was readily metabolized rendering as even slimmer peak than lp with a time constant mainly determined by that of the calorimeter. The heat output corresponded to complete dissipation of energy without any storage in energy rich compounds (see ref.12).

The observation of an oxygen consuming process during the thermoneutral phase (ref.8) is confusing. One would expect oxygen concentration in the fermentor and even more so in the calorimeter line to drop during the main first period of intensive growth and respiration right up to the steep decrease in heat production and then to increase again. In the experiments of Beezer (ref.7) the oxygen consumption continues till a short time before the lp.

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