

## Cell Physiology of Baker's Yeast in Chemostats Subjected to Lactic Acid Perturbations

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### Summary

Knowledge of microbial nutrition has become more relevant in recent years with the increased use of micro-organisms and their products as food additives and supplements. In this paper, we describe the effect of lactic acid on the steady state growth of baker's yeast (*Saccharomyces cerevisiae*). This organism is frequently used by food producers as a flavour enhancer in various yeast extract preparations. *Sacch. cerevisiae* is propagated industrially from sugar cane and/or beet molasses which is often of low-quality, being a waste product from the sugar refining process. The presence of lactic acid in batches of molasses from various sources may lead to subsequent loss of yeast biomass yield following industrial propagations. This study was aimed at defining the physiological effects occurring in the yeast cells subjected to perturbations, or »spikes«, of lactic acid when grown in glucose-limited chemostat culture. The results of analysis of yeast biomass, cellular protein, glycogen, trehalose and RNA obtained showed a differential response of cells to elevation of lactic acid levels which was dependent on yeast growth rate. At sub-optimal growth rates the effect was devastating to the cells, resulting in loss in biomass, and significant changes in intermediary metabolism, possibly due to a cytotoxic effect of lactic acid diffusing into the cells. However, the yeast cells at supra-optimal growth rates responded with much less deviation compared to steady-state control cultures in the chemostat.

*Key words:* baker's yeast, lactic acid, steady-state perturbation

### Introduction

*Saccharomyces cerevisiae* is the most extensively utilised yeast, being exploited by the alcoholic fermentation, baking and food industries. All three sectors need to be supplied with large quantities of yeast, provided as living biomass for bakers and brewers, or extracted for use as food additives. The process by which yeast is produced has given rise to companies whose speciality is yeast biomass production. Yeast is produced using aerobic fed-batch technology with molasses as growth substrate (1). Molasses is a by-product of the sugar cane and beet refining industry, and can provide most of the nutrients required for yeast growth as it contains about 50 % (w/v) total sugars (2), mainly in the form of residual sucrose. Moreover, due to its status as an industrial waste, molasses costs much less than synthetic alterna-

tives, such as glucose syrups. Unfortunately, problems arise with molasses in that its constituents can vary wildly from batch to batch and from season to season. Even transportation can cause the same supply to differ depending on the methods of transport and storage. This makes it difficult for yeast producers to supply yeast of consistent quality to meet the demands of individual customers. Lactic acid can occur in molasses naturally but may also accumulate through metabolism of contaminant lactic acid bacteria during storage and shipping. The presence of lactic acid in molasses may reduce the overall yeast yields because fed-batch processes can result in significant levels of lactic acid being dosed into growing yeast through molasses feed. Reduced yield in an aerobic fed-batch cultivation of yeast

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tends to signal that metabolic energy has been used by the cells for some purpose other than biomass production, most noticeably, alcoholic fermentation.

The aim of this research was to study the effect of lactic acid on the physiology and metabolism of a baker's yeast strain of *Saccharomyces cerevisiae* grown aerobically in a chemostat with a glucose-limited defined medium.

## Materials and Methods

### Organisms, media and culture conditions

Baker's yeast (*Sacch. cerevisiae* GB4918) obtained from Quest International, Menstrie, UK was stored on malt extract agar slopes and kept at 4 °C until required. All chemostats (modified 2 L fermenter vessels from Electrolab, Tewksbury, UK) were operated at 30 °C with 300 rpm agitation, air supplied at 2 v/v/min aeration and without pH adjustment. The medium used in the chemostat cultures was a modified EMM3 (3), suitable for yeast propagations with a glucose-limiting concentration of 0.1 % (w/v).

### Analytical methods

Yeast culture samples for protein and free amino nitrogen (FAN) determination were digested in 1 M NaOH for 1 h at 60 °C. The digested samples were then diluted 1:1 with 1 M HCl to neutralise the cell hydrolysates. Protein was determined using the Pierce Coomassie Protein Assay Kit, with bovine serum albumin (BSA) as standards prepared in 1:1 mixtures of 1 M NaOH and 1 M HCl. FAN was determined using the ninhydrin method described by Lie (4) with glycine as a standard and made up in the same 1:1 mixture of acid:alkali. RNA was determined by a modified Schmidt and Tannhauser method (5). Trehalose and glycogen were extracted from cell pellets by boiling for 10 min in a constantly boiling water bath. Determination of both carbohydrates and extracellular metabolites was carried out by HPLC using a Bio-Rad Aminex HPX-87H column at 65 °C and with differential refractometry to determine levels of sugars, organic acids and ethanol. Yeast dry weights were determined by filtering and washing 5 mL samples before drying using a Mettler IJ16 moisture analyser.

## Results and Discussion

Chemostats were established in which *Sacch. cerevisiae* was grown at the following steady-state growth rates: 0.12 h<sup>-1</sup> termed *sub-optimal*, and 0.22 h<sup>-1</sup>, termed *supra-optimal* (the optimal growth rate for this industrial yeast strain propagated under fed-batch conditions was 0.18 h<sup>-1</sup>). Under conditions of a supra-optimal growth rate, it was noted that the cultures were still operating below the respiratory capacity of the cells. The dilution rate which switched the yeast from entirely respiratory to respiro-fermentative metabolism was ascertained to be around 0.24 h<sup>-1</sup>. This compares quite favourably with the value of 0.28 h<sup>-1</sup> recently reported for another baker's yeast strain of *Sacch. cerevisiae* (6).

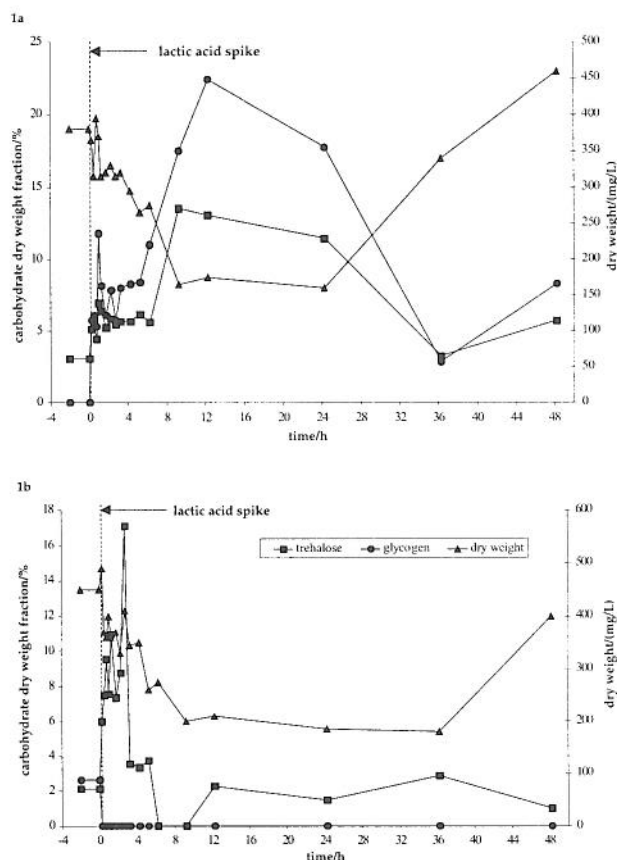


Fig. 1. Effect of a volume fraction of 2 % lactic acid spike on yeast storage carbohydrates. *Sacch. cerevisiae* was grown continuously under glucose-limitation in a chemostat at: (a) sub-optimal growth rate, 0.12 h<sup>-1</sup>, and (b) supra-optimal growth rate, 0.22 h<sup>-1</sup>.

Lactic acid »spikes« were introduced into steady-state chemostat cultures by sudden elevation of lactic acid to a final volume fraction of 2 %. From results shown in Figs. 1a and 1b, it is evident that the major effect of a lactic acid spike is on yeast biomass accumulation. Yeast dry weight reduced to 45 % of the steady-state within 10 h, where it remained until after 24 h when a recovery process began leading to a biomass overshoot to 360 mg/L. This overshoot was eventually eliminated and biomass levels returned to steady-state levels by 72 h (data not shown). The recovery of yeast biomass appeared consistent with a reduction in extracellular lactic acid to a volume fraction below 0.05 %. This effect on yeast dry weight was consistent in both the sub-optimal and supra-optimal growth experiments, although the recovery in the supra-optimal culture was delayed.

The most dramatic event that occurred in sub-optimally grown cells was the accumulation of large amounts of glycogen. Under normal aerobic steady-state conditions, the cells contained almost no glycogen, but within 1 h following the lactic acid spike the level reached around 7.5 % of cell dry weight and this continued to increase to around 27.5 % after 12 h. Glycogen was quickly utilised, falling to around 17.5 % dry weight by 24 h coincident with cell growth recovery.

Subsequently, glycogen levels continued to fall and then fluctuated up to 48 h. A similar trend was observed with the stress carbohydrate, trehalose, which accumulated to around 17.5 % dry weight from an initial level of 2.5 % dry weight. At this sub-optimal dilution rate, trehalose was present in yeast possibly due to the carbon-limiting growth conditions prevailing in the chemostat. Under supra-optimal growth conditions, yeast cell physiology behaved very differently following a lactic acid spike. For example, glycogen failed to accumulate and trehalose levels oscillated and eventually returned to a pre-perturbation level at 12 h.

In Figs. 2a and 2b, the effect of lactic acid on the main nitrogen containing fractions of the yeast was studied. The major effect appeared to be a reduction in cellular protein, with little effect observed on RNA and free amino nitrogen (FAN). After initial oscillations for the first few hours, RNA and FAN reached steady-state levels but cellular protein levels were significantly reduced compared with steady-state levels (17.5 % to 12.5 %). Levels were assumed to return to steady-state values as biomass reached its steady-state value, although cellular protein was not determined beyond 48 h.

Figs. 3a, 3b, 4a and 4b reveal information on the extracellular environment of yeast cultures during lactic acid spikes. Yeast cells appeared to take up lactic acid

relatively quickly after the first 6 h compared with levels expected if lactic acid was removed from the chemostat solely through physical washout (Figs. 4a and 4b). These results also indicate that considerable amounts of succinate were released from the cells when spiked with lactic acid. This is consistent with a shift in metabolism, which requires less throughput from the citric acid cycle (7). Under supra-optimal conditions, yeast cells excrete less succinate compared to sub-optimally cultured cells. Significant amounts of citrate were also released, above that normally found in the medium (results not shown). Another notable effect was the rise in both residual glucose and ethanol levels during a lactic acid spike (Figs. 3a and 3b). Ethanol rose markedly in the first 2 h before being consumed by 6 h in the sub-optimally cultured cells. This was then followed by another production/consumption cycle that finished after 12 h. Levels of ethanol appeared to remain relatively high until 48 h when it was completely removed. In the case of the supra-optimally cultured cells much less ethanol was produced although there was a long period (12–36 h) when the level appeared to remain unchanged. Ethanol again was completely removed by 48 h. Throughout oscillating ethanol production, residual levels of extracellular glucose rose to significant levels at 12 h (approx. 25 % added glucose

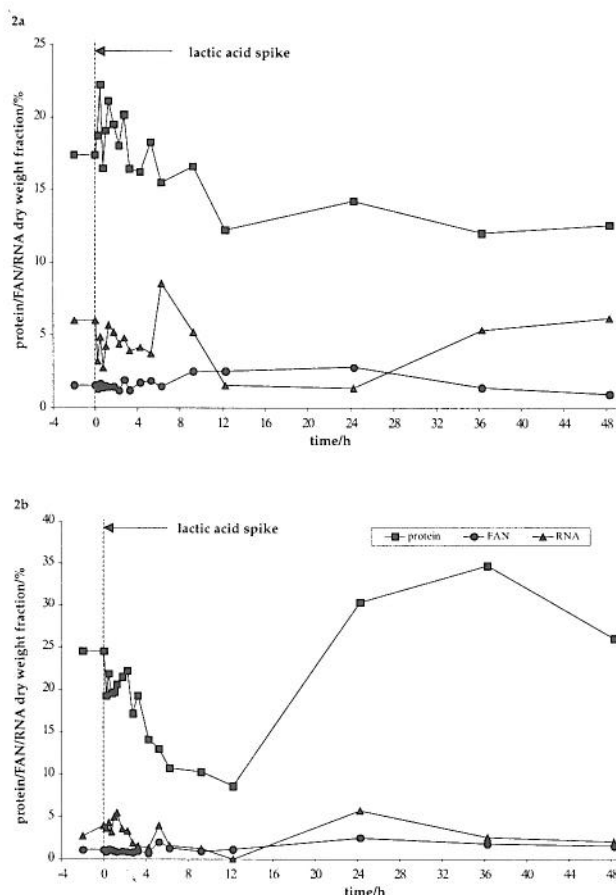


Fig 2. Effect of a volume fraction of 2 % lactic acid spike on yeast protein, FAN, and RNA. *Sacch. cerevisiae* was grown continuously under glucose-limitation in a chemostat at: (a) sub-optimal growth rate,  $0.12 \text{ h}^{-1}$ , and (b) supra-optimal growth rate,  $0.22 \text{ h}^{-1}$

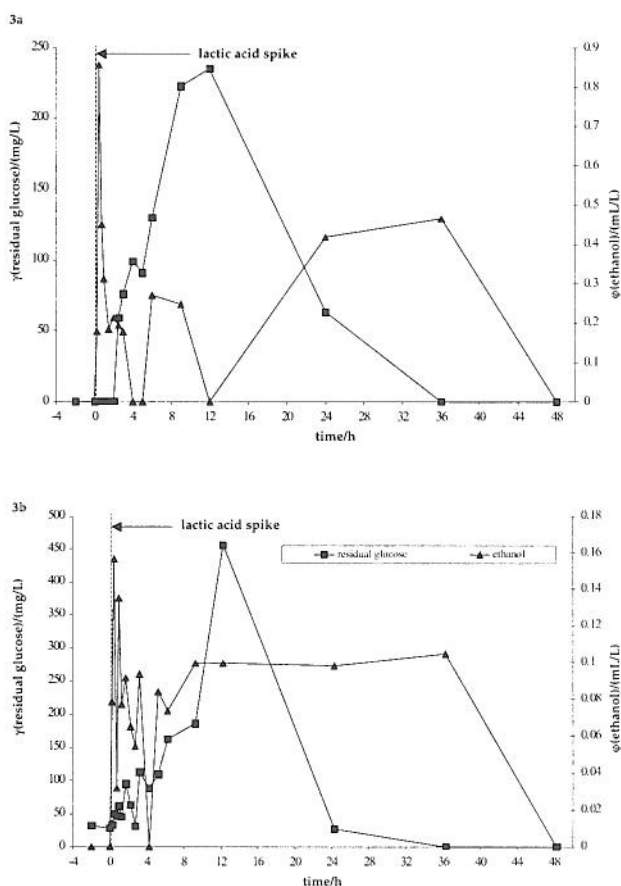


Fig 3. Effect of a volume fraction of 2 % lactic acid spike on yeast glucose metabolism. *Sacch. cerevisiae* was grown continuously under glucose-limitation in a chemostat at: (a) sub-optimal growth rate,  $0.12 \text{ h}^{-1}$ , and (b) supra-optimal growth rate,  $0.22 \text{ h}^{-1}$

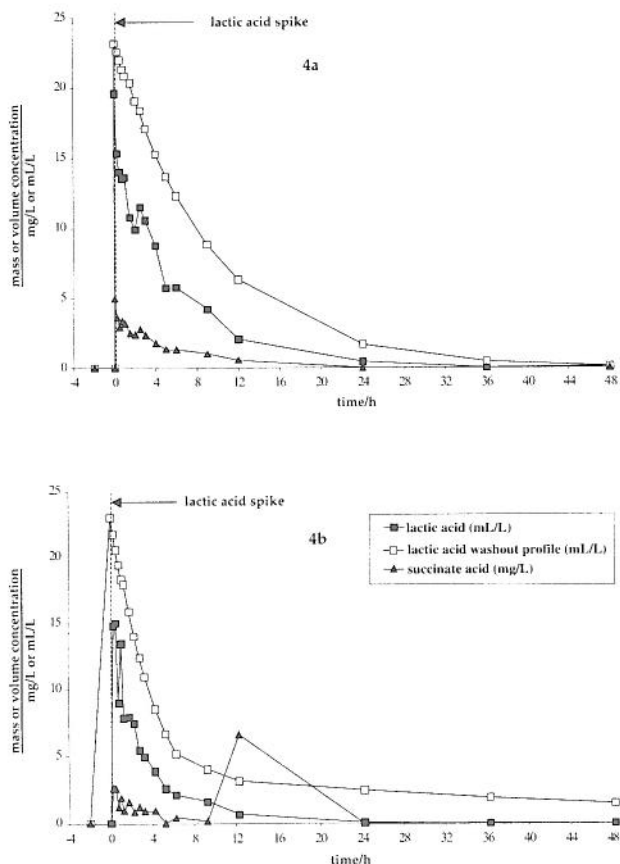


Fig 4. Effect of yeast on washout of a volume fraction of 2% lactic acid spike from a chemostat. *Sacch. cerevisiae* was grown continuously under glucose-limitation in a chemostat at: (a) sub-optimal growth rate,  $0.12 \text{ h}^{-1}$ , and (b) supra-optimal growth rate,  $0.22 \text{ h}^{-1}$

still remained in culture media compared with 0% during normal steady-state conditions). Residual glucose levels significantly reduced by the time biomass recovered at 24 h. These results suggest that the changes in metabolic rate may be due to cells switching from a fully respiratory state to a respiro-fermentative one (8). This may be due to a decrease in oxygen uptake by the cells which would explain the rapid production of ethanol observed. However, oxygen electrode studies of respiring yeasts subjected to a volume fraction of 2% lactic acid spike showed no change in respiratory function over a 15–30 minute period (results not shown).

Previous research (9) has suggested that yeast cells growing continuously in the presence of glucose take up to 24 h to start utilising lactate as a sole carbon source. Jones (9) also showed that the respiratory capacity (assessed by manometry) of yeast grown on glucose reduced as dilution rate increased, when supplied with lactate as a sole C-source.

Results in this study have revealed that a volume fraction of 2% lactic acid perturbation caused yeast cells to react at both a cell cycle and at a metabolic level. The cells clearly stop growing and reproducing, but it also appears that their predominant metabolic pathway changes from a respiratory state to one which relies on

respiro-fermentation. Alternatively, results from oxygen electrode studies performed in this research and by Jones (9), indicate that a high level of lactic acid presented to the yeast cells may diffuse into the cell in sufficient amounts to alter the metabolic flux in favour of ethanol production, without detrimentally effecting oxygen uptake. Transport of lactate and other short length organic acids has been described in the respiratory yeast, *Candida utilis* (10). Lactate transport in cells grown on glucose was shown to be by passive diffusion of the undissociated acid across the cell membrane rather than mediation *via* a lactate transporter (10). Diffusion across the cell membrane increases with increasing external pH suggesting that the permeability of the membrane to undissociated lactate decreases linearly with increasing extracellular proton concentration. Similar observations have also been made in *Sacch. cerevisiae* (11). This suggests that uptake of undissociated lactic acid would increase due to increased undissociated acid under low pH conditions. However, diffusion across the membrane would be inhibited due to decreased permeability of the membrane to undissociated lactic acid at the same low pH.

## Conclusion

The effect of lactic acid spikes on glucose-limited chemostats of *Sacch. cerevisiae* has been investigated and results have shown that the severity of the effect of lactic acid is dependent on the growth rate of the cells. Yeast cells growing at growth rates close to the critical dilution rate for respiratory metabolism appeared to be much less susceptible to lactic acid perturbations, an effect that was shown to be not merely due to the quicker rate of removal of lactic acid from the chemostat at higher dilution rates. At both growth rates the lactic acid spike was detrimental to yeast biomass yield. These findings are therefore pertinent to yeast producers wishing to optimise growth of *Sacch. cerevisiae* on molasses feedstocks.

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## Fiziologija stanica pekarskog kvasca u kemostatima pod utjecajem mliječne kiseline

### Sažetak

Poznavanje ishrane mikroorganizama posljednjih je godina sve značajnije jer je povećana primjena mikroorganizama i njihovih produkata kao dodataka i nadomjestaka hrani. U radu je opisan učinak mliječne kiseline na rast u dinamičkoj ravnoteži pekarskog kvasca (*Saccharomyces cerevisiae*). Taj organizam često koriste proizvođači hrane za poboljšanje mirisa u raznim pripravcima ekstrakata kvasca. *Sacch. cerevisiae* se industrijski uzgaja na šećeru od trske i/ili melasi od repe koja je često slabe kakvoće jer je otpadni proizvod u procesu rafinacije šećera. Prisutnost mliječne kiseline u pojedinim šaržama melase iz raznih izvora može dovesti do naknadnoga gubitka biomase kvasca tijekom industrijskog uzgoja. Svrha je ove studije bila odrediti fiziološki učinak dodatka mliječne kiseline na stanice kvasca kada rastu u kemostatu u glukozom ograničenoj kulturi. Rezultati analize biomase kvasca, staničnih proteina, glikogena, trehaloze i RNA pokazali su da na povećanu koncentraciju mliječne kiseline, ovisno o brzini rasta, stanice različito reagiraju. Pod suboptimalnim brzinama rasta utjecaj je bio uništavajući za stanice, rezultirajući gubitkom biomase i značajnim promjenama u intermedijarnom metabolizmu, vjerojatno zbog citotoksičnog učinka mliječne kiseline koja je difundirala u stanice. Nasuprot tome, stanice kvasca pri supraoptimalnim brzinama rasta imaju puno manje devijacija u usporedbi s kontrolnim kulturama koje su rasle u dinamičkoj ravnoteži u kemostatu.