**CO₂ Mediated Interaction in Yeast Stimulates Budding and Growth on Minimal Media**

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**Abstract**

Here we show that carbon dioxide (CO₂) stimulates budding and shortens the lag-period of *Saccharomyces cerevisiae* cultures, grown on specific weak media. CO₂ can be both exogenous and secreted by another growing yeast culture. We also show that this effect can be observed only in the lag-period, and demonstrate minimal doses and duration of culture exposition to CO₂. Opposite to the effects of CO₂ sensitivity, previously shown for pathogens, where increased concentration of CO₂ suppressed mitosis and stimulated cell differentiation and invasion, here it stimulates budding and culture growth.

**Introduction**

Cell-cell interactions in microbial cultures have been under particular interest and investigation for more than 80 years. The whole area includes works on the so called “mitogenetic effect” [1,2], “quorum sensing” [3,4], and rather recently discovered NH₃ signaling in yeast [5] and CO₂ sensitivity both in prokaryotes [6,7] and fungi [8,9].

The “mitogenetic effect” consists in a distant stimulation of mitosis in prokaryotic and eukaryotic cells by optical contact with other well growing cultures. The effect was shown for bacterial cultures [10], yeast [11], etc., and ultraweak ultraviolet luminescence was stated to be the mediator of these cell-cell interactions [12,13]. Altogether several hundred articles and monographies appeared in this area, mostly in 1920–1950s, both verifying [14–16] and refuting [17,18] original results. Still, the problem of mitogenetic effect remains unsolved till nowadays.

The effects of chemical cell-cell interactions in microbial cultures, most of them denoted by the notion “quorum sensing”, are proved much more unequivocally. The “quorum sensing” phenomenon (the name given in [19]) consists in cooperative “behavior” of microbial cultures depending on the population density and composition, and including gene expression, cell differentiation, antibiotic secretion, and various virulence-dealing processes, such as hyphae, biofilm and spore formation, and substrate invasion. The mechanism lies in simultaneous secretion and reception of certain species-specific or more or less universal chemicals (small peptides, alcohols, ethers etc.), which accumulate in the medium and switch on certain intracellular signaling pathways when reaching a certain concentration threshold [for reviews see [3,4,20,21]]. Besides these specific signaling factors, cell interaction can be mediated by such a “simple” molecule as NH₃ [5], which is “used” to synchronize cell differentiation and general morphology of neighboring colonies [22] and prevent them from spreading too close to each other [5].

Can CO₂ also be a factor of cell-cell interaction? CO₂ sensitivity of mammalian cells has been known for nearly 50 years [23] and investigated in detail [24]. It has also been shown for cyanobacteria [7], and pathogenic fungi [25], in which it plays the role of “host tissue sensor”. But there are practically no works dealing with CO₂-mediated cell-cell interaction [9], especially in non-pathogens (discussion of some doubtful data [26] see below). In this work a new case of yeast cell-cell interaction was shown, and the mediator of this was proved to be CO₂. Thus the observed effect turned out a new case of CO₂ sensitivity in microorganisms, and a new type of CO₂-mediated processes, where cell cycle is stimulated rather than suppressed “in favor” of cell differentiation, as it had been well shown for pathogens before.

**Materials and Methods**

**Strains**

*Saccharomyces cerevisiae*, wild-type diploid wine strain VKM J-542 (from the collection of Microb. Dep., Fac. of Biology, Moscow State University).

**Culture Preparation and Media**

Suspension cultures were grown on a rotary shaker (120 r.p.m., 30°C) in standard YPD medium (glucose 2%+yeast extract 2%+bactopeptone 1%) till the beginning of stationary phase (18–24 hours), and plated on Petri dishes (1–2×10⁸ cells per 9 cm Petri dish) with agar medium of various composition:

1. rich growth medium – YPD: glucose 2%+yeast extract 2%+bactopeptone 1%+agar 3%, pH5,5;
2. minimal medium with glucose: glucose 0,1%+(NH₄)₂SO₄ 0,1%+KH₂PO₄ 0,1%+MgSO₄ 0,05%+CaCl₂ 0,01%+NaCl 0,01%+agar 3%, pH5,5;
3. Minimal medium with acetate: CH₃COONa 0.1% + (NH₄)₂SO₄ 0.1% + KH₂PO₄ 0.1% + MgSO₄ 0.05% + CaCl₂ 0.01% + NaCl 0.01% + agar 3%, pH 5.5.

The plated cultures were cultivated at 30°C.

Measurement
Culture density and budding were measured during experiments.

To evaluate density of agar culture, it was carefully washed off the plate with three portions of water, and optical density of the resulting suspension was measured with nephelometer at 650 nm (OD₆₅₀).

Culture budding was characterized with budding index (BI) – total number of buds divided by the total number of cells counted, in %. To calculate BI of the culture, agar sections of 4 cm² in area were cut off the plates, fixed with formalin, and microphotographed with a phase contrast microscope with 40× objective and digital 5 MP camera. The photographs were digitally processed with specially created original software [27], automatically recognizing cells and buds in digital pictures (fig. 1). No less than 1500 cells were counted for each BI calculation.

Both culture density and budding were measured periodically, to obtain growth and budding dynamics of the culture (fig. 2).

Experiment
The “induction” experiment. Two open plates with yeast cultures were fixed together, their cell layers directed towards each other, and left for 10–150 min (fig. 3A). After that, one of the plates (called “inductor”) was removed, and the other one (called “recipient”) – closed, and left at 30°C for further growth. The recipient density and budding were periodically gauged, every 15–30 min for 3–5 hours, and compared to budding and culture density in separated single control plates with identical medium and culture.

The inducing factor testing. To test whether the inducing factor was a volatile chemical, two identical plates with the
Figure 3. Scheme of experiments. A. The "induction" experiment. 1– Open plates with the "recipient" and the "inductor" yeast cultures were fixed together, their cell layers directed towards each other (1 cm distance between the cell layers), made airtight with parafilm, and left at 30°C for 10–150 min. 2– After that, the inductor was removed, and the recipient was left in a closed dish for further growth. 3– Agar sections of 4 cm² in area were periodically cut off the recipient plate, every 15–30 min, during the first 3–4 hours after the end of induction. Fixing fluid (glycerol : formalin : water, in proportion 50: 25 : 25) was spread onto the surface of these samples, imprinted on the cover glass, put on the slide, and... 4– microphotographed with a phase contrast microscope with 40× objective and digital 5 MP camera. B. Testing, whether the inducing factor is a volatile chemical. Hermetically closed airtight container is half filled with YPD-agar and plated with the "inductor" yeast culture. Two open Petri dishes with identical recipient cultures are fixed inside the container. Both recipients are equally available to volatile chemicals (possibly secreted by the inductor). Plate #1 has direct optical contact with the inductor, plate #2 is turned off the inductor and has no direct optical contact with it. Arrows show possible gas exchange. C. Induction in the presence of alkaline trap for (possibly) CO₂ (1M NaOH) in a small Petri dish, fixed inside the plate with the recipient.

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Figure 4. Budding dynamics of *S.cerevisiae* after induction and in control, on various agar media. A. rich growth medium – YPD (glucose 2%+yeast extract 2%+bactopeptone 1%-agar 3%). B. minimal medium with lowered glucose content (glucose 0.1%+(NH₄)₂SO₄ 0.1%+KH₂PO₄ 0.1%+MgSO₄ 0.05%+CaCl₂ 0.01%+NaCl 0.01%, pH5.5). C. minimal medium with acetate (CH₃COONa 0.1%+(NH₄)₂SO₄ 0.1%+KH₂PO₄ 0.1%+MgSO₄ 0.05%+CaCl₂ 0.01%+NaCl 0.01%, pH5.5). D. minimal medium with acetate, without nitrogen (CH₃COONa 0.1%+KH₂PO₄ 0.1%+MgSO₄ 0.05%+CaCl₂ 0.01%+NaCl 0.01%, pH5.5). The experiment scheme is given in fig. 3A. Duration of induction was 60 min, inductor – *S.cerevisiae* culture on YPD-agar in early stationary phase (20 hour old), recipient – *S.cerevisiae* culture 15 min after inoculation. No effect is observed on YPD (medium A). On minimal media (B–D) induction leads to BI increase, highly reliable in certain time points: B –120–165 min, *P*<10⁻²; C –210 min, *P*<10⁻³, 240–270 min, *P*<10⁻⁵; D –180–270 min, *P*<10⁻⁶.

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recipient culture were fixed inside a big airtight container (20×20×4 cm) half filled with agar medium, and plated with inductor (fig. 3B). Both recipients were equally available to volatile chemical factors from the inductor (arrows in fig. 3B show possible gas exchange), and while one of the recipients had normal optical contact with the inductor, the other one was turned away from it. In fig. 3C a modification of the standard induction experiment is shown. A small Petri dish with 1M NaOH was fixed inside the recipient plate to partially absorb CO2 from the atmosphere inside.

**CO2 inducing experiments.** To check the inducing capacity of CO2, recipient cultures were put into hermetically closed containers (20×20×4 cm), and atmosphere with various concentrations of CO2 (0.1–4%) was created inside by injecting the needed volume of 99.99% CO2 into the container, through an airtight rubber stopper.

**Reproducibility**

Altogether more than 500 budding curves were registered at various conditions: media content, age of the recipient and inductor cultures, and duration of induction. Each point on the budding curve was obtained by automatic counting of 1500–2000 cells in microphotographs. Each particular experiment was repeated no less than 7 times; some experiments were repeated up to 20 times.

The main experimental data obtained in our work, were budding curves of yeast cultures, which (although looking like standard S-shaped functions), could not be correctly approximated by functions of a single family. Thus we preferred to compare values of budding index in individual time points on the curves. According to our experimental scheme, each experiment had its own control, and thus criteria for dependent samples could be used. As not all the data were always distributed normally, we preferred to use nonparametric Wilcoxon T-test for dependent samples to calculate the data confidence. Intervals given in tables are effective 99% confident intervals calculated from normal approximation.

**Results**

1. **Distant Stimulation of Budding and Growth**

The “induction” experiment, as shown in fig. 3A, was performed under various conditions – age of the inductor and the recipient cultures, their medium content, and the induction length. Under particular conditions (see below) the experiment led to stimulation of budding and growth in the recipient culture (compared to adequate control).

The main conditions for the induction effect are listed below.

The **“recipient” cultures can be stimulated only on weak media.** Plated onto rich growth media (YPD or 2–18° beer worth), the recipient cultures didn’t react to induction, i.e. both
budding and growth dynamics of the “induced” culture coincided with control (fig. 4A, growth not shown).

Plated onto minimal medium with 0.1% glucose, yeast showed a slower (suppressed) dynamics of budding (compare control lines in fig. 4A and B). On this medium induction led to budding stimulation, the culture achieving maximal BI ~1 hour earlier than in control (fig. 4B). Plated onto minimal acetate-containing medium, control culture showed practically no budding (BI<10%) up to 270 min after inoculation (fig. 4C), and the induced culture achieved maximal BI of ~50%, which was 5–10 times higher than in control at the same time (see 210–270 min period in fig. 4C, P<10^-5). Budding-stimulation on minimal media led also to growth stimulation (fig. 5). Still, the budding stimulation effect could also be observed even on extremely weak media lacking nitrogen, where subsequent growth was impossible (fig. 4D, growth not shown).

When on similar media, with malate, succinate or fumarate as the only substrate (instead of glucose or acetate), or with no substrate at all, the recipient culture showed no budding either in control or after induction (data not shown).

The recipient cultures can be stimulated only during the lag period of budding, with the induction lasting from 15 to 150 min. The recipient cultures could be stimulated only during the first ~2 hours after inoculation, and the earlier the recipient was subjected to induction, the higher was the observed effect (fig. 6). Notice that the effect of induction exhibited ~2 hours after the beginning of induction, and 1–1,5 hour after its end.

The minimal length of induction that produced any budding-stimulation effect was found to be 10–15 min. The effect was increasing to maximum with the induction length rising up to 60–90 min, and remained constant for longer inductions (table 1).

Yeast cultures used as inductors must be alive and growing on rich media. Cultures grown on minimal media didn’t produce reliable budding stimulation effect (on any recipient cultures) with induction lasting either 30 or 90 min (data not shown). Yeast of the same strain grown on rich growth media (YPD or beer worth) produced budding stimulation effect (on proper recipient cultures) from exponential phase to the beginning of stationary phase (4–30 hours old – see sect. 4.1.1 and 4.1.2, table 2). Under these conditions, both agar and suspension cultures were good inductors. Lag-period cultures had a much lower induction capacity comparing to older inductors (table 2). Dead (boiled) cultures didn’t stimulate budding at all (data not shown).

The Induction Effect is Caused by a Volatile Chemical Factor that can be Absorbed by Alkaline Solution

To test whether the inducing factor was a volatile chemical, we separated the inductor and the recipient with metal, glass and quarts plates, and the budding-stimulating effect disappeared in any case (data not shown). The effect was also missing if the atmosphere between the inductor and the recipient was being constantly renewed during the experiment (data not shown). Two identical recipient plates fixed inside a big container with inductor, equally accessible to volatile chemicals, but oppositely located...
towards the inductor (fig. 3B), showed standard budding stimulation, identical for both recipient plates (fig. 7). Thus the induction effect was definitely caused by a volatile chemical factor secreted by the inductor.

We then tested if the inducing chemical was alkaline or acidic, by fixing a small Petri dish with 1 M NaOH solution inside the recipient plate, as a trap for acidic chemicals from the air (fig. 3C). Addition of this trap utterly abolished the stimulation effect at 30 min induction (fig. 8) and decreased the effect more than twice at 120 min induction (data not shown). A separate set of experiments showed that addition of such a trap (Petri dish with 1 M NaOH) didn’t influence budding or growth curves of non-stimulated (control) cultures (data not shown).

Thus the budding stimulation effect was caused by a volatile chemical factor, secreted by yeast cultures from early exponential phase to early stationary phase, and absorbed by alkaline solutions.

Exogenous Carbon dioxide Mimics the Induction Effect

The only known chemical secreted by yeast and corresponding to all the data obtained, is CO₂, NH₃ and the known quorum sensing factors – tryptophol and phenilethanol – are not absorbed by alkaline solutions. Besides, the quorum sensing factors are not volatile, and NH₃ is not secreted by yeast colonies till rather late stationary phase (4–10 days old [5]).

Measured with infrared CO₂ sensor, the rate of CO₂ production by our inductor cultures was found ~0.1 micro-mole/sec from 1 cm² of agar medium (V. Ptushenko, unpublished data). This rate of CO₂ production leads to accumulation of ~1% CO₂ in 10–20 min, in the atmosphere between the recipient and the inductor. To check the inducing capacity of CO₂ the recipient cultures were put into hermetically closed containers, and atmosphere with various concentrations of CO₂ (0.1–4%) was created inside. Exogenous CO₂ stimulated budding in the recipient at least in the concentrations from 0.1% to 4% (table 3), and at induction length more than 10 min (table 4). These conditions generally corresponded to the amount of CO₂ secreted by the inductor culture.

Thus the effect of budding stimulation, observed in S. cerevisiae cultures on specific poor media, when in contact with another actively growing yeast culture, was caused by CO₂ secreted by the latter, and exerting the stimulating influence in concentrations 0.1–4% in the atmosphere, and at the induction length ≥10 min.

Some Evidences for Signaling Action of CO₂

Table 3. Budding index of agar cultures of S. cerevisiae, 270 min after inoculation – in control and after induction with CO₂ of various concentration (see fig. 4D for the whole budding curve on this medium).

| CO₂ concentration | Budding index, % |
|-------------------|------------------|
| control           | 5% ± 4%          |
| 0.13%             | 17% ± 7%         |
| 0.25%             | 36% ± 9%         |
| 0.5%              | 45% ± 16%        |
| 1%                | 37% ± 12%        |
| 2%                | 30% ± 10%        |
| 4%                | 25% ± 7%         |

1. medium acidification,
2. heterotrophic fixation of CO₂ [9,28,29],
3. signaling action (through adenylyl cyclase [7,8]).

To test the first opportunity, we performed the main budding stimulation experiments on media with different pH. Budding stimulation, both by inductor yeast cultures, and exogenous CO₂, was equally observed (on appropriate minimal media – see section 4.1.1) at pH from 4.5 to 6 (data not shown). The medium pH in the recipient culture after the end of induction was equal to pH in the control culture (and not changed comparing to initial pH of the medium). Thus, the stimulation effect was not connected to any CO₂-induced change of the medium pH.

Metabolic CO₂ fixation is for the greatest part taking place in reactions of phosphptroyse carboxylation, generating oxaloacetate (OA) and “supporting” the Krebs cycle [30]. This way is important on media with glucose, and practically useless on media with acetate, as all OA is generated through glyoxylate bypass.

Figure 7. Budding dynamics of S. cerevisiae after 30 min induction in a big airtight container with inductor (the experiment scheme is given in fig. 3B). Induction chem+light – plate No 1 in fig. 3B, induction chem – plate No 2 in fig. 3B (no optical contact with the inductor). Inductor – S. cerevisiae culture on YPD-agar in early stationary phase (20 hour old). Medium content: CH₃COONa 0.1%+KH₂PO₄ 0.1%+MgSO₄ 0.05%+CaCl₂ 0.01%+NaCl 0.01%, pH5.5. doi:10.1371/journal.pone.0062808.g007

Figure 8. Budding dynamics of S. cerevisiae after 30 min induction with and without alkaline trap for acidic volatile chemicals (the experiment scheme is given in fig. 3C). Inductor – S. cerevisiae culture on YPD-agar in early stationary phase (20 hour old). Medium content: CH₃COONa 0.1%+KH₂PO₄ 0.1%+MgSO₄ 0.05%+CaCl₂ 0.01%+NaCl 0.01%, pH5.5. doi:10.1371/journal.pone.0062808.g008
Table 4. Budding index of agar cultures of S.cerevisiae, 270 min after inoculation – in control and after induction with S.cerevisiae culture, or with 1% exogenous CO₂ (see fig. 4D for the whole budding curve on this medium).

| Length of induction, min | Budding index, % (age of recipient –270 min) | Yeast induction | 1% CO₂ induction |
|-------------------------|---------------------------------------------|----------------|------------------|
| control                 |                                             | 6%±3%          | 5%±4%            |
| 3                       |                                             | 4%±4%          | 7%±5%            |
| 10                      |                                             | 21%±14%        | 14%±7%           |
| 30                      |                                             | 34%±7%         | 23%±5%           |
| 90                      |                                             | 44%±10%        | 33%±6%           |
| 150                     |                                             | 47%±10%        | 37%±12%          |

Inducers: S.cerevisiae culture in early stationary phase (20 hour); 1% exogenous CO₂. Various duration of induction. Medium content: CH3COONa 0,1%+KH2PO4 0,1%+MgSO4 0,05%+CaCl2 0,01%+NaCl 0,01%+agar 3%, pH5,5.
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Table 5. Budding index of agar cultures of S.cerevisiae on different media with and without oxaloacetate – in control and after induction with 4% CO₂.

| Medium | Age of recipient, min | Budding index, % | ΔBI (induction – control), % |
|--------|-----------------------|------------------|-----------------------------|
|        | Control               | 4% CO₂ induction |                               |
|        | 150                   | 15%±6%           | 40%±8%                       |
| Medium A+oxaloacetate | 26%±10%               | 58%±7%           | 25%, P<10⁻⁴                 |
| Medium B                   | 5%±5%                      | 25%±7%           | 20%, P<10⁻⁵                 |
| Medium B+oxaloacetate     | 15%±5%                     | 47%±10%          | 32%, P<10⁻⁵                 |
| Medium B+oxaloacetate     | 5%±4%                      | 30%±10%          | 25%, P<10⁻⁵                 |
| Medium B+oxaloacetate     | 30%±5%                     | 61%±10%          | 31%, P<10⁻⁵                 |

Medium content:
A – Minimal medium with glucose (glucose 0,1%+(NH₄)₂SO₄ 0,1%+KH₂PO₄ 0,1%+MgSO₄ 0,05%+CaCl₂ 0,01%+NaCl 0,01%, pH5,5).
B – Minimal medium with acetate, without nitrogen (CH₃COONa 0,1%+KH₂PO₄ 0,1%+MgSO₄ 0,05%+CaCl₂ 0,01%+NaCl 0,01%, pH5,5).
Inductor – 4% CO₂, length of induction –120 min. Recipient – S.cerevisiae culture 15 min after inoculation.
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[30]. To test whether the budding stimulation effect was connected to metabolic CO₂ fixation, we performed our main experiments on glucose and acetate containing minimal media (see fig. 1B and C), with addition of 0,1% oxaloacetate. This led to increase of both control and CO₂-stimulated budding dynamics on both media (comparing to identical media without OA – see table 5), but didn’t decrease the culture sensitivity to CO₂. Absolute increase of budding, caused by CO₂, was equal or even slightly higher on media with OA than on identical media without OA (table 5, column ΔBI). Thus, OA was used as additional substrate, important for the culture budding (table 5) and growth (data not shown), but didn’t “substitute” exogenous CO₂. Besides, CO₂ action on yeast was equally high on glucose and acetate containing media, with or without additional OA.

Thus, the CO₂-induced budding stimulation effect in our experiments was not connected to non-specific stimulation of metabolism through CO₂ fixation, and remained as high (or even higher) on media containing excessive amount of oxaloacetate, the key product of CO₂ fixation.

Discussion

In the last decade a number of works appeared, showing CO₂ sensitivity for a vast number of microorganisms [9]. Two general mechanisms of CO₂ action on the cell are known: (1) metabolic – heterotrophic fixation, and (2) regulatory – participation in signaling pathways. Heterotrophic fixation of CO₂, long known for S.cerevisiae [30], Schizosaccharomyces pombe [29], and other species, is essential for culture growth on minimal media, mainly by supporting Krebs cycle through phosphotriose to oxaloacetate carboxylation. This way is not active when the culture is grown on rich media, or on minimal acetate-containing media, as all the needed oxaloacetate is produced in Krebs cycle (rich media) or glyoxylate bypass (acetate-containing media).

Regulatory action of CO₂ goes through class IIIb (soluble or cytoplasmic) adenylyl cyclases by direct binding with their catalytic domain. This way is shown for mammals, cyanobacteria [7], and pathogenic fungi [25], in which it stimulates cell differentiation and virulence [31]. Regulatory pathway of CO₂ sensitivity was also supposed for S. pombe [29], and argued for S.cerevisiae [26], but disproved by the same authors in [32], where they showed that the effect of HCO₃⁻ stimulated spore formation, observed in their work, was caused by alkalization of the medium [33].

The present work is the first to show significant effects of CO₂-mediated interaction of cells on S.cerevisiae. We cannot make any direct statements concerning mechanisms of our effect yet. Still we can conclude that (1) it is not connected to the medium pH shift, and (2) it is not connected to heterotrophic fixation and metabolic use of CO₂. This allows us to suppose the budding-stimulation effect going through regulatory, rather than metabolic pathways.
Besides, the effect is observed 1.5–2 hour later than the interaction is finished.

The main difference of our results from the effects of CO$_2$ action, known for pathogenic fungi, is that here CO$_2$ increase stimulates cell division, rather than mitosis block and cell differentiation [9,31].

Anyway, the effect of distant CO$_2$-mediated interaction of S. cerevisiae cultures, shown in this work, can be interpreted as cell-cell interaction, regulating cell behavior according to the culture density, i.e. a quorum sensing effect.

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Author Contributions

Conceived and designed the experiments: IV RI. Performed the experiments: IV RI. Analyzed the data: IV. Contributed reagents/materials/analysis tools: IV EK. Wrote the paper: IV.

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