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Influence of Dissolved Oxygen Concentration on Intracellular pH and Consequently on Growth Rate of *Aspergillus niger*

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Summary

With the results presented we would like to emphasize the influence of dissolved oxygen concentration in a medium on intracellular pH values and consequently on overall metabolism of microorganisms. Intracellular pH of mycelium grown under different concentrations of dissolved oxygen in the medium was determined by recently developed cryo ^{31}P-NMR method. Sensitivity of proteins toward proton concentration was well recognised, therefore pH influences on the activities of some key regulatory enzymes of *Aspergillus niger* were determined at pH values similar to those detected in the cells grown under lower dissolved oxygen concentrations. The results have shown significantly reduced specific activities of hexokinase, 6-phosphofructokinase and glucose-6-phosphate dehydrogenase in more acidic environment, while pyruvate kinase was found to be relatively insensitive toward higher proton concentration. As expected, due to reduced specific activities of some key regulatory enzymes under more acidic conditions, overall metabolism should be hindered in media with lower dissolved oxygen concentration, as confirmed by detecting reduced specific growth rates.

Key words: *Aspergillus niger*, intracellular pH, dissolved oxygen concentration, specific growth rate, metabolism

Introduction

In microbial processes an understanding of the physiology of productive microorganism is of outmost importance. Recently, with the availability of complete nucleotide sequences in increased number of microorganisms functional genomics is becoming the prevailing method in the studies of microbial physiology. However, for an understanding of the regulation of metabolic pathways and the environmental factors that affect metabolic fluxes, the information on gene expression and protein function is not sufficient.

In biotechnological processes engineers often try to achieve higher productivity by increased biomass concentration, however, under such conditions it is difficult to maintain sufficient oxygen concentration in bioreactors and therefore the corresponding specific productivity. Namely, quite often the influence of dissolved oxygen concentration in the medium on the intracellular pH is ignored. Thus, the effect of dissolved oxygen concentration on changes in enzymatic activities and perturbations in overall metabolism are ignored as well.

There are some reports on sensitivity of intracellular pH to hypoxia in filamentous fungi (*1*,*2*) that refer to reduced activity of plasma membrane H^+-ATPase, which has been shown to be involved in maintenance of intra-

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cellular proton concentration by extrusion of protons from the cytoplasm at the expense of ATP (*3*). Namely, at reduced oxygen availability, ATP synthesis is slower and cannot cope with the requirements of proton pumps any more. One of the reasons for less interest for the effects of intracellular pH is a lack of simple and accurate method for pH_i detection in filamentous fungi. There are some reports referring to the determination of intracellular pH values by ^{31}P-NMR technique in perfused systems in *Neurospora crassa* (*1*) and by a NMR airlift bioreactor in *Aspergillus terreus* (*4*) where growth conditions were difficult to maintain with respect to dissolved oxygen concentration. An advanced technique was developed applying immobilised *Aspergillus niger* cells in a well controlled perfusion system where oxygen concentration could be monitored (*5*). Recently, a method for determination of intracellular pH in frozen cells was developed in the authors' lab using cryo-NMR technique that enables fairly accurate measurements of pH_i values in samples with quenched metabolism taken directly from a bioreactor.

With the results presented we have tried to emphasize the often overlooked influence of dissolved oxygen concentration on intracellular pH values and consequently on overall metabolism.

Materials and Methods

A. niger (MZKI A60) spores were harvested from 7-day-old wort agar slants and then suspended in 25 mL of sterile 0.1 % Tween 80 solution. A suspension of spores was used to inoculate chemically defined medium of the following composition: deionized water 1 L, glucose 10 g, KH_2PO_4 5 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 0.5 g. Glutamic acid or $(NH_4)_2SO_4$ were used as a nitrogen source. pH was adjusted to the value of 6.5 with 0.1 M HCl.

For obtaining the mycelium, the fermentation runs in a stirred glass tank bioreactor (Infors, Bottmingen, Switzerland) of 5-liter working volume were performed. The medium was inoculated with approximately 10^9 spores. The temperature was kept at 30 °C, and the medium was aerated with 5 litres of air per minute. Dissolved oxygen concentration was monitored with a sterilisable pO_2 electrode (Mettler-Toledo). For maintaining different levels of dissolved oxygen concentrations in the fermentation broth, the volume of the inlet air was dosed by a mass flow controller (5850 Brooks) connected with a computer and pO_2 electrode.

Samples for intracellular pH determination were collected and prepared as reported previously (*2*).

^{31}P-NMR spectroscopy was accomplished on a Varian Unity Plus-300 spectrometer (Palo Alto, USA), operating at 121.384 MHz. For a typical spectrum 500 scans were accumulated using 45° pulses with a repetition time of 0.585 s. Chemical shifts were related to 85 % H_3PO_4 (0 ppm) contained in a capillary tube. Condensed and cooled mycelium was placed in 10 mm NMR tube and poured with cooled buffered methanol. Finally 10 % of D_2O was added. During the spectroscopy the sample was kept at (–40.0 ± 0.1) °C by ventilating the sample with nitrogen pre-cooled on dry ice. Intracellular pH values were determined from a calibration curve by comparing the chemical shift of the ortho-phosphate peak, as reported previously.

Dry weight of biomass was determined gravimetrically after 250 mL of fermentation broth was taken from the bioreactor, filtered over a pre-dried and weighted filter paper, rinsed extensively with three-fold volume of cold distilled water and dried in the microwave oven to the constant weight. Specific growth rates and doubling times were determined according to Pirt (*6*).

Specific enzyme activities were determined in cell-free homogenate, which was prepared as reported previously (*7*).

Enzyme activities (V_{max}) were determined by following NADH-NAD^+ or $NADP^+$-NADPH transformation kinetics in a DU660 spectrophotometer (Beckman, Fullerton, CA, USA) at 340 nm and were performed at 30 °C. They were measured in 50 mM phosphate buffer or 50 mM imidazole buffer of indicated pH values in the presence of 5 mM $MgCl_2$ and 100 mM KCl. Each specific assay mixture contained in 1 mL the following substrates and auxiliary enzymes which were purchased by Sigma (St. Louis, MO, USA) or Boehringer (Mannheim, Germany). For hexokinase: 10 mM glucose, 2 mM ATP, 0.4 mM $NADP^+$ and 3U glucose-6-phosphate dehydrogenase; for 6-phosphofructo-1-kinase: 10 mM fructose-6-phosphate, 1 mM ATP, 0.2 mM NADH, 5 mM DTT, 1 U aldolase, 50 U triosephosphate isomerase and 15 U glycerol-3-phosphate dehydrogenase; for piruvate kinase: 1 mM phosphoenolpiruvate, 2 mM ADP, 0.2 mM NADH, 15 U lactate dehydrogenase; for glucose-6-phosphate dehydrogenase: 0.5 $NADP^+$ and 1 mM glucose-6-phosphate.

Specific enzyme activities were determined after dissolved proteins were measured in the crude enzyme preparation according to Bradford (*8*) with crystalline bovine serum as a standard.

Results

It is known that oxygen concentration in the fermentation broth influences the intracellular pH value. To check how pH_i values are changed during the *A. niger* growth under different concentrations of dissolved oxygen, the mycelium was initially grown in a 5-L bioreactor for 70 hours at maximal aerating conditions to obtain sufficient biomass. After 70 hours the computerised control of aeration was switched on to maintain the level of dissolved oxygen at 80 % of saturation. Afterwards, every 2 hours the dissolved oxygen concentration (DOC) level was reduced for additional 20 %. Meanwhile mycelial samples were taken for pH_i determination. The analysis has shown that *A. niger* cells are relatively resistant to reductions of DOC in the range of 80 to 60 % of saturation with respect to acidification. Intracellular pH dropped for only about 0.04 units. However, by further 20 % reduction of oxygen concentration in the broth, pH_i dropped to the value of about 6.78 ± 0.01. Even more pronounced accumulation of protons in the cells could be observed at 20 % of oxygen saturation exhibiting the reduction of intracellular pH value to 6.58 ± 0.03 units (Fig. 1).

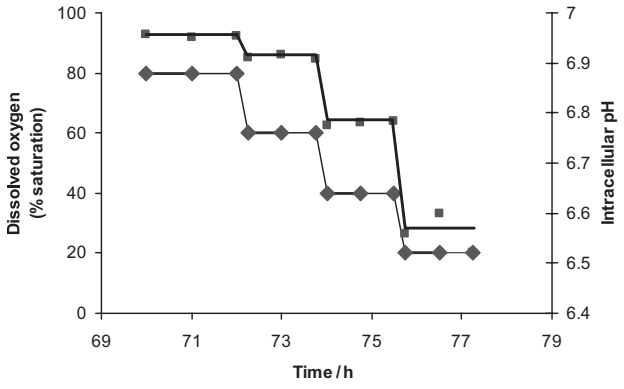

Fig. 1. Intracellular pH (■) dependence of *A. niger* cells on stepwise dissolved oxygen concentrations decrease (◆) during the growth in a chemically defined medium with ammonium ions as a sole source of nitrogen

Within the range of DOC used the intracellular pH dropped for nearly 0.5 units, which must affect the activity of specific enzymes and influence the overall metabolism. In order to find out more about the sensitivity of some key regulatory *A. niger* enzymes at different proton concentrations, we determined specific V_{max} activities in buffers with different pH values in the presence of substrates in excess. The specific activities of some key regulatory enzymes (hexokinase, 6-phosphofructo-1-kinase, piruvate kinase and glucose-6-phosphate dehydrogenase) were determined in two buffers and are shown in Figs. 2–5. Apart from piruvate kinase, where minor influence of different pH values on activity was recorded (Fig. 4), all other tested enzymes have shown remarkable sensitivity toward more acidic conditions. In the pH range of 6.5 to 7.0, which was determined in the cells during the incubation at DOC values between 20 and 80 % of oxygen saturation, hexokinase specific activity was reduced for about 60 % in both buffers (Fig. 2). Even more pronounced decrease in activity in the same pH range was recorded with 6-phosphofructo-1-kinase but interestingly more expressed reduction was observed in phosphate buffer (from about 285 mU/mg to only 70 mU/mg) than in imidazole buf-

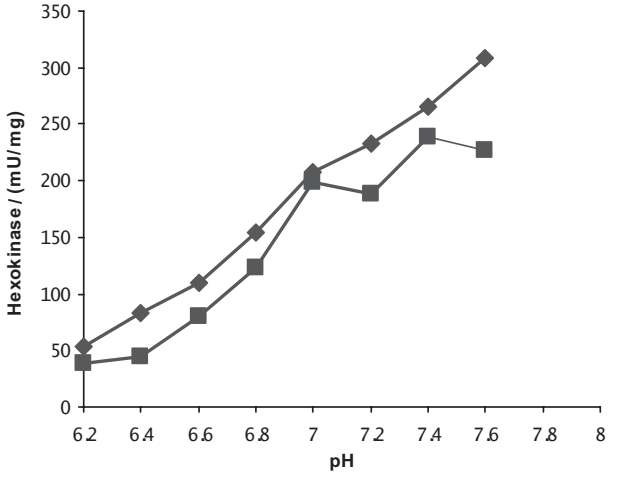

Fig. 2. Maximal specific activities of *A. niger* hexokinase measured at different pH values in phosphate (■) and imidasole (◆) buffer

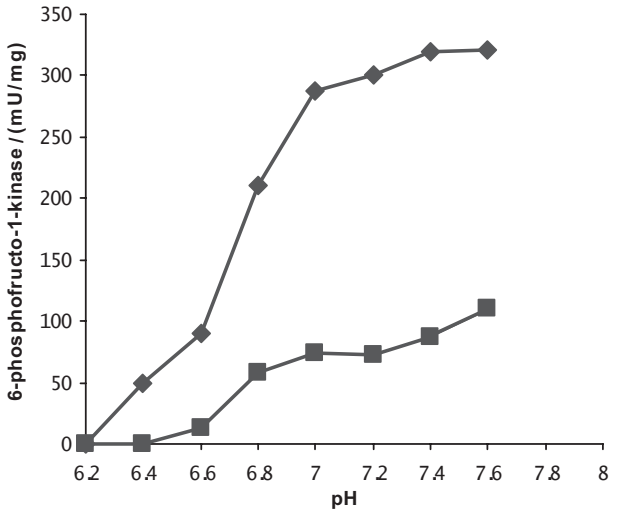

Fig. 3. Maximal specific activities of *A. niger* 6-phosphofructokinase measured at different pH values in phosphate (■) and imidasole (◆) buffer

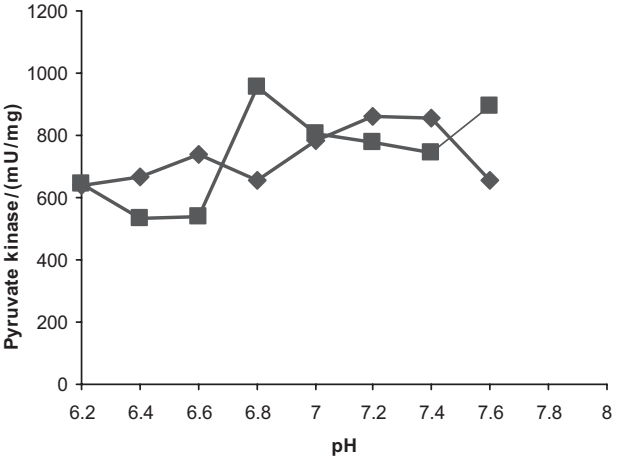

Fig. 4. Maximal specific activities of *A. niger* pyruvate kinase measured at different pH values in phosphate (■) and imidasole (◆) buffer

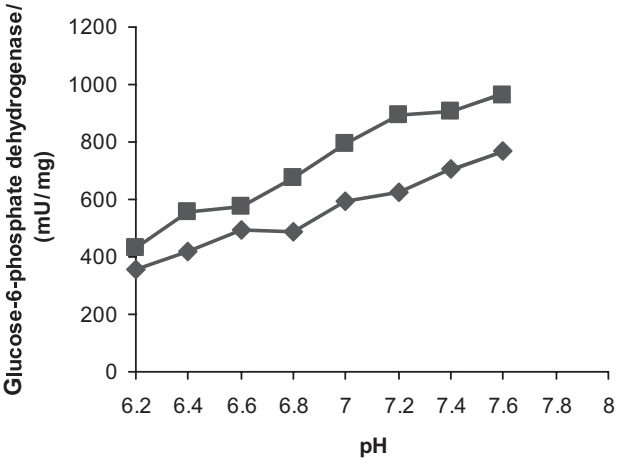

Fig. 5. Maximal specific activities of *A. niger* glucose-6-phosphate dehydrogenase measured at different pH values in phosphate (■) and imidasole (◆) buffer

fer, where PFK activity seemed to be relatively insensitive to changes between pH value of 7.0 and 6.8 (Fig. 3). Some influence of pH on the activity was observed also with glucose-6-phosphate dehydrogenase. However, specific activities of this enzyme were reduced for about (25 ± 3) % in the range of physiological pH changes (Fig. 5).

From the data obtained by measuring specific activities of some key regulatory enzymes in different pH environment, significant reduction in metabolic fluxes could be expected under lower dissolved oxygen concentrations, which must affect the specific growth rate of the fungus in the same medium.

The increase of dry weight of biomass was monitored from 24 to 36 hours in batch fermentation runs under defined dissolved oxygen concentrations (Fig. 6). In glucose medium with ammonium ions as a sole nitrogen source a transition from lag phase to exponential phase took place before 24 hours of fermentation regardless of the level of dissolved oxygen. However, distinct correlation between oxygen availability and specific growth rate could be observed later. In the medium with 80 % of oxygen saturation the cells grew most rapidly with μ value of 0.212 h^{-1} having a doubling time of 3.26 hours. Negligible difference in specific growth rate (μ=0.211 h^{-1}) and doubling time (t_d=3.28 hours) was recorded in the same medium with 60 % of oxygen saturation. In the medium with further reduction of dissolved oxygen (40 % of saturation), dry weight doubled in 3.5 hours (μ=0.198 h^{-1}). Even slower growth was recorded with 20 % of dissolved oxygen saturation giving the doubling time of 3.91 hours and specific growth rate of 0.177 h^{-1}, respectively.

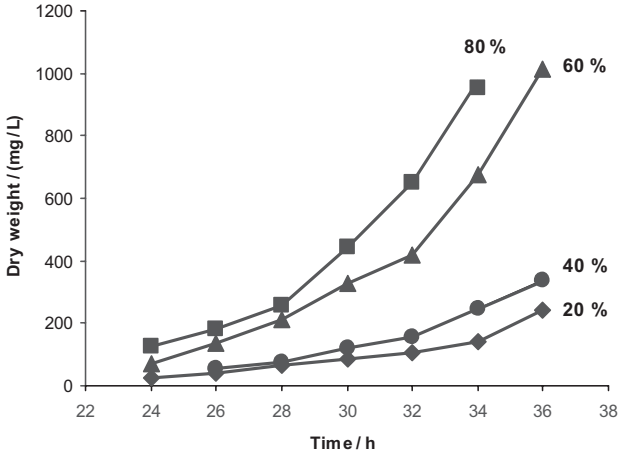

Fig. 6. Growth curves of *A. niger* mycelium grown in a chemically defined substrate with ammonium ions as a sole nitrogen source at different concentrations of dissolved oxygen, shown as percents of saturation

More sensitive were *A. niger* cells toward lower dissolved oxygen concentrations in the medium where ammonium ions were replaced by glutamic acid (Fig. 7). Again the most rapid growth rate was recorded at higher dissolved oxygen concentrations although the specific growth rate values were lower and μ value of only 0.19 h^{-1} was detected in the medium with 80 % of oxygen saturation. The doubling time was accordingly extended to 3.64 hours. As expected, more reduced specific growth rate was determined in the medium with dissolved oxygen concentration of 60 % of saturation value with doubling time of 3.74 hours and μ value of 0.185 h^{-1}. Markedly slower growth with specific rate of 0.139 h^{-1} and doubling time of 4.9 hours were observed in the substrate with 40 % of oxygen saturation. In the medium with only 20 % of dissolved oxygen the increase of biomass could not be followed adequately.

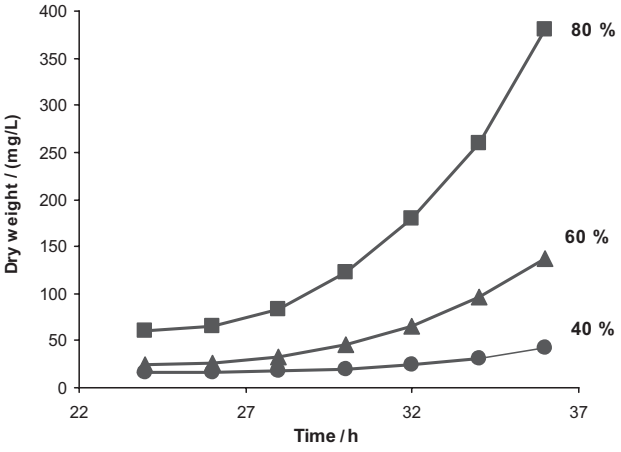

Fig. 7. Growth curves of *A. niger* mycelium grown in a chemically defined substrate with glutamic acid as a sole nitrogen source at different concentrations of dissolved oxygen, shown as percents of saturation

Discussion

The influence of dissolved oxygen concentration on the intracellular pH in fungal cells has been reported several times (*1,2*). However, most measurements with ^{31}P-NMR technique were performed in perfused system in a low volume NMR tubes, where growth parameters were difficult to follow. By introducing the method that quenches the metabolism of the cells taken directly from a fermenter and measuring the ^{31}P-NMR spectra at low temperatures a tool for determination of pH_i values in the mycelium taken from better controlled environment was available (*2*). For the first time this technique was developed for application on microbes, while similar cryo ^{31}P-NMR method was designed previously for measuring ATP and ADP levels in mammalian cells (*9,10*).

The measurements of intracellular pH in *A. niger* cells grown at different oxygen concentrations revealed relative insensitivity of pH_i changes toward decreased oxygen concentration to about 60 % of oxygen saturation, while further decrease of oxygen concentration caused more rapid accumulation of protons in the cells. These data are in accordance with the theory of H^+-ATPases proposed by Serrano (*3*) that plasma membrane located enzymes play a crucial role in cellular pH regulation, although their activity depends on ATP availability and is diminished by reduced ATP synthesis under oxygen li-

mitation. The recorded values of intracellular pH of A60 strain in a well aerated system presented in the present paper are about 0.5 units lower than those detected in NW131 strain of the same species (*5*). A60 strain is known to be a good citric acid producer able to convert up to 80 % of sucrose into citric acid within 7 days under optimal conditions (*11*), while strain NW131 is a less efficient producer (*12*). Its intracellular citric acid concentration measured at 24 hours was 2.5 mM (*13*), which is about 4-fold less than that detected in A60 strain (*14*). At neutral pH values citric acid is present in ionic form (pK'=3.13) releasing one or more protons, therefore higher acid concentrations in the cells most probably contribute to more acidic conditions in cytosol.

Sensitivity of proteins toward proton concentration is well recognised and the pH effects on activity of key regulatory enzymes and metabolic pathways have been studied previously (*15*). No such data were presented for *A. niger* enzymes so far, in spite of the fact that this is one of the most important commercial microorganisms. Interestingly, pH optima of all enzymes tested were in slightly alkaline region but declined rapidly under the pH value of 6.8. The activity of pyruvate kinase alone was relatively insensitive towards more acidic environment. These data are in accordance with the measurements of specific growth rates and intracellular pH values under different dissolved oxygen concentration, where slower growth rate was recorded at values below 60 % of saturation while pH_i values were determined to be below 6.8. The specific growth rate that reflects the efficiency of overall metabolism was less affected by dissolved oxygen concentrations above 60 % of saturation and again it fits the data about the changes of intracellular pH values that were less affected by decreased oxygen concentration in this range. The pH dependence of enzymatic activities was determined in phosphate and imidazole buffer. The enzyme 6-phosphofructo-1-kinase showed significantly higher specific activities in phosphate buffer while with other enzymes the values measured in both buffers were comparable. It is known that PFK of A60 strain is activated by phosphorylation and it seems that phosphate ions present in buffer prevent dephosphorylation and deactivation of the activated form.

It is important to realise that enzymatic activities were measured with substrate concentrations in excess giving V_{max} values, which are comparable to those previously detected in *A. niger* (*16*). Substrate levels *in vivo* are, however, much lower and adequately decreased levels of specific enzymatic activities could be observed that would cope with calculations of metabolic fluxes (*17*).

Recently the activation of plasma H^+-ATPases by the addition of ammonium ions to the medium was described in *A. niger* (*18*). Glutamic acid on the other hand showed no such effect. It was found that H^+-ATPase is activated by phosphorylation, which is triggered through the phosphatidyl-inositol signalling pathway. Since plasma membrane proton pumps play a crucial role in the regulation of intracellular pH, it was expected that under the conditions of inactivated mechanism for pH_i control the specific growth rate would be more sensitive toward the decreased dissolved oxygen concentration. The measurements of specific growth rate in less aerated media with glutamic acid as a sole nitrogen source actually confirmed our expectations.

Conclusion

Considerable changes in intracellular pH values were recorded in *Aspergillus niger* cells during the growth in a medium with decreased oxygen concentration. More acidic conditions affect the activity of some key regulatory enzymes resulting in changes of overall metabolisms that are reflected as altered specific growth rate.

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Utjecaj koncentracije otopljenog kisika na intracelularni pH i na brzinu rasta *Aspergillus niger*

Sažetak

Prikazanim rezultatima istaknut je utjecaj koncentracije otopljenog kisika u mediju na intracelularne pH-vrijednosti i s tim u vezi na ukupni metabolizam mikroorganizama. Intracelularni pH micelija, uzgajanog pri različitim koncentracijama otopljenog kisika u mediju, određen je nedavno razvijenim postupkom krio-^{31}P-NMR. Poznata je osjetljivost proteina na koncentracije protona. Stoga je određen utjecaj pH na aktivnosti nekih ključnih regulatornih enzima u *A. niger* pri pH-vrijednostima sličnim onima otkrivenim u stanica što su rasle pri smanjenim koncentracijama otopljenog kisika. Rezultati su pokazali bitno smanjenu specifičnu aktivnost heksokinaze, 6-fosfofruktokinaze i glukoza-6-fosfat dehidrogenaze u jače kiseloj sredini, dok je piruvat kinaza relativno neosjetljiva na veće koncentracije protona. Očekivalo bi se da će u medijima sa smanjenom koncentracijom otopljenog kisika cjelokupni metabolizam biti ometen zbog smanjenih specifičnih aktivnosti nekih ključnih regulatornih enzima u kiselijim uvjetima. To je i potvrđeno jer su utvrđene smanjene brzine specifičnog rasta *A. niger*.