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EFFECT OF PARTIAL PRESSURE OF CO_2 ON THE PRODUCTION OF THERMOSTABLE α-AMYLASE AND NEUTRAL PROTEASE BY *Bacillus caldolyticus*

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Controlling the concentration of dissolved oxygen is a standard feature in aerobic fermentation processes but the measurement of dissolved CO_2 concentrations is often neglected in spite of its influence on the cellular metabolism. In this work room air and room air supplemented with 5% and 10% carbon dioxide were used for aeration during the cultivation of the thermophilic microorganism *Bacillus caldolyticus* (DSM 405) on starch to produce α-amylase (E.C. 3.2.1.1) and neutral protease (E.C. 3.4.24.27/28). The increased CO_2 concentrations resulted in a 22% raise in activity of secreted α-amylase and a 43% raise in protease activity when compared with aeration with un-supplemented room air. There was no effect on the final biomass concentration. Furthermore, the lag-phase of fermentation was reduced by 30%, further increasing the productivity of α-amylase production. Determinations of dissolved CO_2 in the culture broth were conducted both in situ with a probe as well as using exhaust gas analysis and both the methods of quantification showed good qualitative congruence.

Amylases and proteases are widely used industrial enzymes. They are used in washing powders and detergents as well as in food, textile, and paper production. The estimated world market for these enzymes is projected to be $1.8 billion for amylases and $3.6 billion for proteases [1, 2] in 2011. Many applications of the enzymes involve operations at high temperature. The thermostable proteases and α-amylases are presently produced from *Bacillus licheniformis* or *Bacillus stearothermophilus*. We have focused our studies on the less examined thermophilic microorganism *Bacillus caldolyticus* DSM 405 to establish the conditions for optimal production of the enzymes, α-amylase and protease [3].

Carbon dioxide is produced in nearly all industrial fermentation processes. In common aerobic fermentation processes involving gas sparging, most carbon dioxide is fast stripped out of the medium by the sparged gases. In anaerobic processes, however, considerable accumulation of carbon dioxide (total concentration up to several g l^{-1}) may occur and may result in growth inhibition [4]. In several cases, however, CO_2-enhanced growth of several microorganisms has also been reported [5–8]. As far as α-amylase production is concerned, a stimulating effect of increased CO_2 level on α-amylase production by *Bacillus subtilis* was found by 2 groups of authors [9, 10]. In both the cases, biomass production recorded a decrease simultaneously. Narahara et al. [11] also reported an increase in α-amylase and protease activities during fermentation of *Aspergillus oryzae* when partial pressure of CO_2 was increased from 0.02 to 0.05 atm. Mudgett and Bajracharya [12] also found that high CO_2 pressure had a distinct influence on cell growth and α-amylase synthesis during solid state fermentation of *Aspergillus oryzae* in the rice Koji process. But there are no reports of the effect of carbon dioxide on *Bacillus caldolyticus*.

The aim of this paper was to study the influence of carbon dioxide on growth of the *Bacillus caldolyticus* DSM405 cells and on production of α-amylase and protease by this thermophilic microorganism. Furthermore, the comparative measurements of dissolved CO_2 concentrations in the cultivation medium were carried out using exhaust gas analyzer and fluorescence-based CO_2 probe.

MATERIALS AND METHODS

Strain and medium. The thermophilic bacterium *B. caldolyticus* DSM 405 used in this study was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany [13]. The growth and production medium contained (g l^{-1}): peptone from casein – 2.0, KH_2PO_4 – 0.05, $CaCl_2 \cdot 2H_2O$ – 0.1, Zulkowsky (soluble) starch – 1.0 and (mg

l^{-1}): $MgSO_4 \cdot 7H_2O$ – 250.0, $MnCl_2 \cdot 4H_2O$ – 1.57 and $FeSO_4 \cdot 7H_2O$ – 30.0.

Culture conditions and operating parameters. Inocula were prepared by preculturing 100 ml of growth medium in 500-ml shake flasks for 8 h at 70°C and 150 rpm. Exponential phase preculture was used for inoculation of the bioreactor to achieve OD_{600} of 0.08 in the fermenter at the beginning of cultivation.

Cultivation of bacterial cells for enzyme production was carried out in a lab-scale stirred tank bioreactor (Biostat E, Sartorius Stedim Systems GmbH, Germany) equipped with a dissolved O_2 (DO) probe, a dissolved CO_2 probe, and controllers for pH, temperature, agitation, and foam. Total broth volume in the reactor was 3.3 l. pH was controlled at 7.0 ± 0.1 using 20% (w/v) KOH or 1.0 M HCl solution. Polypropyleneglycol P2000 was used to control foam. Temperature in the bioreactor was controlled at 70°C. Flow rate of inlet air was fixed at 1 vvm (volume of air per volume of fermentation broth and minute) and the concentration of dissolved oxygen in the medium was kept above 50% saturation by gradually increasing the speed of agitation.

Experiments were conducted with room air and with room air supplemented with CO_2 to 5 and 10% (v/v) for aeration. At least 3 experimental runs were made with each inlet concentration of CO_2. The concentrations of cells, starch, α-amylase, and protease were monitored in each experiment.

Analytical procedures. α-amylase activity was determined by a modified method developed by Manning and Campbell [13]. A mixture of 40 μl of culture supernatant, 40 μl of 1% starch solution, and 40 μl of 1.0 M sodium acetate buffer (pH 5.4) was incubated at 70°C for 10 min. Subsequently 1 ml of cold water and 30 μl of iodine solution (30 g l^{-1}) were added to the incubated mixture on ice, and absorbance (OD_{620nm}) was measured. As a reference, 40 μl fresh medium was used instead of 40 μl of supernatant. The enzymatic activity was calculated using equation (1).

$$A = \frac{\Delta E_{620\,\mathrm{nm}} \cdot VF \cdot V_T \cdot 1000}{t_{\mathrm{Ink}} \cdot V_R \cdot m \cdot MW}, \qquad (1)$$

where A is the enzymatic activity (U l^{-1}), $E = E_{\mathrm{reference}} - E_{\mathrm{sample}}$ is the difference between absorbances (AU) in the reference and the sample at 620 nm, VF is the dilution factor for the sample (–), V_T is the total volume (ml), V_R is the reaction volume (ml), t_{Ink} is time of incubation (10 min), m is the slope of calibration curve (4.7646 ml mg^{-1}), and MW is the molecular weight of anhydroglucose (162 g mol^{-1}).

Activity of neutral protease was determined by a modified method developed by Strydom et al. [14]. 200 μl of the sample was mixed with 200 μl of 2% azocaseine solution dissolved in 50.0 mM Tris-HCl buffer (pH 7.0) containing 5.0 mM $CaCl_2$ and incubated for 30 min at 70°C. 400 μl of 1.5 M $HClO_4$ was added and the mixture was cooled on ice for 30 min to complete precipitation. After centrifugation at 8.000 g, 400 μl of the supernatant was mixed with 400 μl 1.0 M NaOH, and optical density (OD_{440}) was measured. One unit of the enzyme was defined as 1 mmol of azocasein cleaved per minute; the calculation of enzymatic activity was conducted using equation 2.

$$A = \frac{\Delta E_{440\,\mathrm{nm}}}{\varepsilon d} \cdot \frac{V_T}{V_R} \cdot \frac{1000 \cdot VF}{t_{\mathrm{Ink}}}, \qquad (2)$$

where ε is the extinction coefficient of azocasein (38 AU l mol^{-1} cm^{-1}) and d is the thickness of cuvette (1 cm).

Cell density was monitored as OD_{600} with Philips PU 8625 UV/VIS spectrophotometer (Philips GmbH, Germany). OD was converted into cell dry weight (DW) by using equation 3.

$$DW = OD_{600} \cdot 0.33, \qquad (3)$$

where DW is cell dry weight (g l^{-1}) and OD_{600} is optical density of broth at 600 nm.

Starch concentration in cell-free broth was analyzed by adding 750 μl DI water and 15 μl 4% iodine solution in water to 250 μl sample supernatant and measuring OD_{620} using a UV/VIS-Photometer. Starch concentration (g l^{-1}) was calculated using a calibration curve prepared from solutions of known concentrations of starch (equation 4).

$$c_{\mathrm{St}} = \frac{E_{620} - 0.053}{0.9965}, \qquad (4)$$

where c_{St} is the concentration of starch (g l^{-1}).

Glucose concentration was quantified with a glucose-kit (Roche Diagnostik, Germany: Kit-No. 10716251035). Acetate concentration was determined in the supernatant using the acetate-kit (Roche Diagnostik, Germany: Kit-No.: 10148261035). Cooled and dried exhaust air was analyzed with a multi-component gas analyzer (Sidor, Sick Maihack GmbH, Germany).

The concentration of dissolved carbon dioxide in medium was calculated from the exhaust air composition and dissolved oxygen probe reading using the procedure of Royce and Thornhill [15] (equation 5).

$$c_L^{\mathrm{CO_2}} = \frac{(P - p_w)\, x_g^{\mathrm{CO_2}out}}{H^{\mathrm{CO_2}}} + \frac{1}{0.89}\left(\frac{(P - p_w)\, x_g^{\mathrm{CO_2}out}}{H^{\mathrm{O_2}}} - c_L^{\mathrm{O_2}}\right), \qquad (5)$$

where c_L is the concentration of dissolved gas component in fermentation medium (mol m^{-3}), P is the overhead pressure (Pa), p_W is the partial pressure of water in the bioreactor overhead space (Pa), x_g is mole frac-

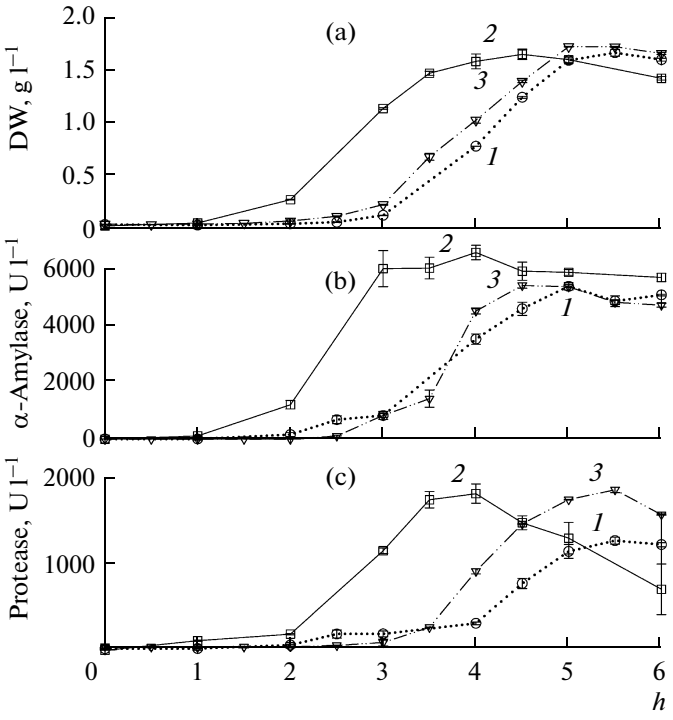

Fig. 1. Dry weight (DW, a), α-amylase (b) and protease (c) activities during the fermentations of *B. caldolyticus* aerated with room air (*1*); air supplemented with 5% CO_2 (*2*) and air supplemented with 10% CO_2 (*3*).

tion of the component in gas phase (–), H is Henry′s law constant for the gas (Pa m³ mol^{-1}).

The Henry′s law constants for 70°C were calculated using equations (6) and (7) [16, 17].

$$H^{CO_2} = e^{\left(11.25 - \frac{395.9}{T - 175.9}\right)} \text{ and} \quad (6)$$

$$H^{O_2} = e^{\left(12.74 - \frac{133.4}{T - 206.7}\right)} \quad (7)$$

Here, T is temperature of medium (K).

The concentration of oxygen in medium ($c_L^{O_2}$) was calculated from the response of dissolved oxygen probe using the following equations:

$$c_L^{O_2} = \frac{c^*_{O_2} \cdot p_{O_2}}{100}. \quad (8)$$

Here $c^*_{O_2}$ is the solubility of oxygen in medium at 70°C (mol m^{-3}), p_{O_2} is the dissolved oxygen probe signal (%).

$$c^*_{O_2} = \frac{(P - p_W)\, x_{O_2}}{H^{O_2}}, \quad (9)$$

where x_{O_2} is mole fraction of oxygen in inlet gas which was also used to calibrate the DO probe response to 100%.

In situ measurement of dissolved CO_2 was also done with a fluorescence-based YSI 8500 CO_2 Monitor (YSI Inc., USA).

RESULTS AND DISCUSSION

When room air (without CO_2 supplementation) was used to aerate the batch fermentation broth, the onsets of logarithmic growth phase and α-amylase formation were observed after approximately 2 h of inoculation. In the subsequent 3–4 h, cell dry weight concentration peaked at 1.7 g l^{-1}, and α-amylase and protease activities rose up to 5432 U l^{-1} and 1296 U l^{-1} respectively (Fig. 1). When the volume fraction of CO_2 in the inlet-air was increased to 5%, the lag-phase reduced considerably. In this case the final cell dry weight concentration was not affected by CO_2 fraction in air but the concentrations of α-amylase and protease increased to 6634 U l^{-1} and 1853 U l^{-1}, respectively, by 4.5 h. This amounted to 22% increase in amylase activity and 43% increase in protease activity over the highest levels achieved with room air only. When the inlet-air CO_2 fraction was increased further to 10%, however, the maximum amylase levels decreased (Fig. 1) even though the maximum protease activity increased slightly again to 1899 U l^{-1}. In all the cases, the activities of both the enzymes recorded some drop beyond the maximum, suggesting that harvesting needs to be done at the right time to prevent losses (Fig. 1). It is noticeable that the activity of protease has the potential for more significant drop than the activity of α-amylase. Similar effect of CO_2 on the duration of lag phase has been reported by Gaffney [16], who explained the effect by the improved synthesis of oxaloacetate in the tricarboxylic acid (TCA) cycle. Gandhi and Kjaergaard [9] also revealed similar effect of carbon dioxide on amylase production by *B. subtilis*. These authors observed the highest amylase production at 6% CO_2 volume fraction in the sparged air and hypothesized that CO_2 influence is exerted possibly through reduced rate of metabolism of glucose. To emphasize the effect of CO_2 fraction in air on product formation by *Bacillus caldolyticus*, the maximum values of cell dry weight concentration and activity of enzymes at different CO_2 fractions in air are listed in Fig. 2.

A link between dissolved CO_2 concentration and enhanced secretion of enzymes, especially that of protease, was also observed by Stretton and Goodman [17], who suggested that high CO_2 concentrations affect production of enzymes involved in improving growth conditions and in secretion of toxic components that suppress competing microorganisms. It is also possible that increased CO_2 concentration alters intracellular pH in cells even though the fermentation was conducted under controlled pH conditions. Another explanation for the increased concentrations of

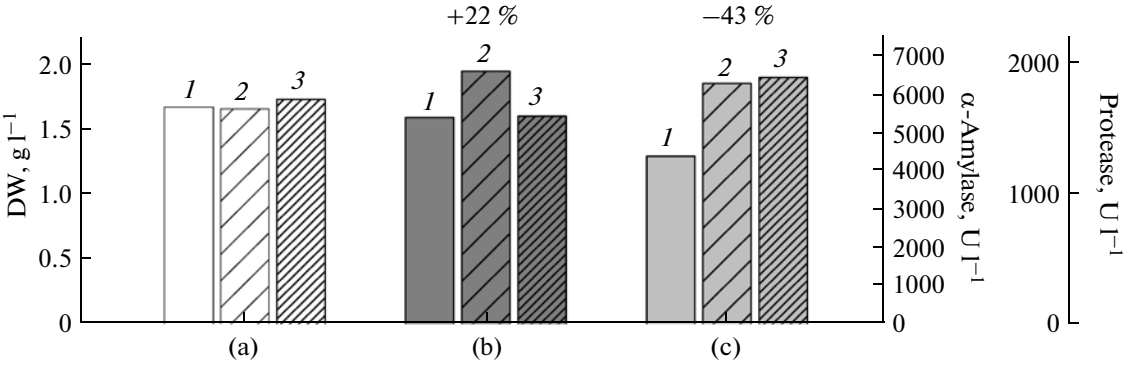

Fig. 2. Comparison of biomass concentration (DW) (a) and the maximum activities of α-amylase (b) and protease (c) enzymes during the fermentation of *B. caldolyticus* aerated with room air (*1*); air supplemented with 5% CO_2 (*2*) and air supplemented with 10% CO_2 (*3*).

the secreted enzymes might be a regulatory influence of CO_2 on transcriptional level, as reported for *B. anthracis* by Drysdale et al. in 2005 [18].

The concentrations of glucose, starch, and acetate in broth at different CO_2 fractions in air are presented in Fig. 3. Note that starch was consumed the fastest when inlet air contained 5% CO_2. Starch hydrolysis leads to formation of polysaccharides and ultimately to glucose which is metabolized by the cells. As expected, a higher rate of starch hydrolysis was accompanied with a higher level of glucose in the broth. In general, the concentration of glucose above a threshold triggers overflow metabolism and results in production of acetate, a growth inhibitory chemical [3]. Concentration profiles of acetate in the different experiments are shown in Fig. 3. It is interesting to note that although starch was hydrolyzed very fast in the fermentation with 5% CO_2, less acetate accumulated in the broth than in the fermentations with 0 and 10% CO_2 in aeration air. This suggests that glucose metabolism is influenced by carbon dioxide in a manner such that glucose is consumed by the cells without entering the carbon overflow pathway, indicating a more effective utilization of the carbon source. A possible explanation may be the enhanced activity of phosphoenole pyruvate carboxylase and pyruvate carboxylase under elevated concentration of CO_2 [19]. Both enzymes catalyze formation of oxaloacetate from their substrates and oxalocetate enters the TCA. Hence less pyruvate is available to enter the enzymatic pathway leading to acetate formation. On the other hand, the activities of α-amylase and protease increased much faster in the experiments with 5% of CO_2 than with other concentrations (Fig. 1).

In all our experiments, a dry weight concentration of 1.7 gl^{-1} was achieved (Fig. 2). This observation, that the different CO_2 fractions in the aeration air did not influence the maximal biomass concentration in the experiments, are in disagreement with the results presented by Gandhi and Kjaergaard [9] who reported a growth inhibiting effect of CO_2 even with much smaller increases of CO_2 in inlet air. On the other hand, our observations of increased amylase and protease activities with increased CO_2 level in feed air are in agreement with the observations of Gandhi and Kjaergaard [9]. The extent of positive effect of CO_2 in the aeration air depends, however, on the specific enzyme system.

Measurement of dissolved CO_2 in fermentation broth. The observed influence of CO_2 on the secretion

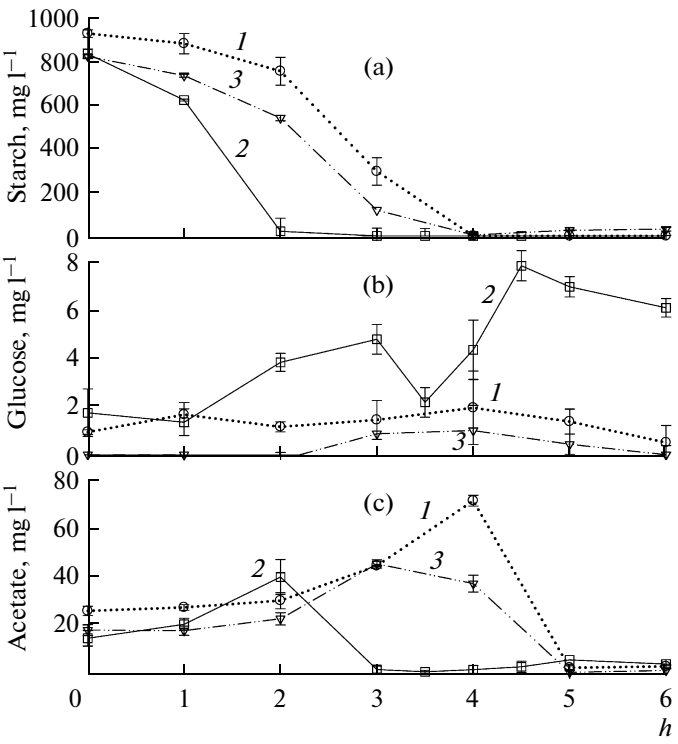

Fig. 3. Starch (a), glucose (b) and acetate (c) concentrations during the fermentation of *B. caldolyticus* aerated with room air (*1*); air supplemented with 5% CO_2 (*2*) and air supplemented with 10% CO_2 (*3*).

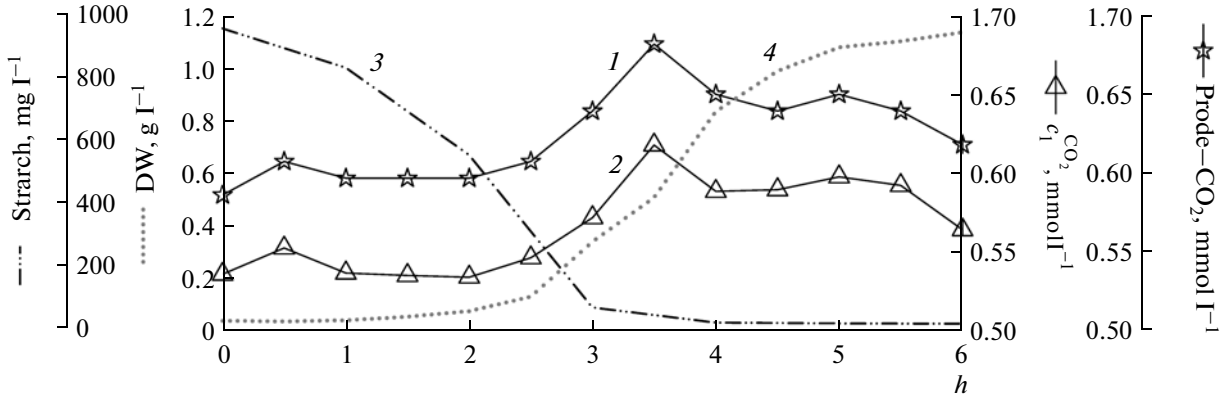

Fig. 4. Comparison between the CO_2 concentration measured directly with the in situ CO_2 probe (*1*) and the concentration calculated using data from the exhaust gas measurement (*2*); starch concentration (*3*) and growth of the culture as dry weight (*4*).

of amylase and protease (both hydrolases) indicates the importance of a fast and reliable CO_2 measurement in the bioreactor at 70°C. Two methods for estimation of dissolved CO_2 concentrations during thermophilic production of amylase and protease by *B. caldolyticus* were utilized and compared in this work. The first one involved an in situ measurement by CO_2 probe that offers potential for direct real time measurements of non-ionic forms of CO_2 in culture broth. The second method involved analysis of exit gas composition. This method, however, requires removal of water vapors from gas phase before exhaust gas analyses. The composition of dissolved CO_2 was then determined using equations (5 to 9) suggested by Royce and Thornhill in 1991 [15].

The measured and the calculated concentrations of dissolved CO_2 during an experiment with room air containing 5% CO_2 are presented in Fig. 4. Note the congruence between the two methods of measurement of dissolved CO_2. Still, the dissolved CO_2 values obtained from the probe signal are considerably higher than those predicted from exhaust gas composition. This observation is in qualitative agreement with those of Dahod [20] who also reported that probe-measured dissolved CO_2 concentrations in high cell density fermentation were higher than the values calculated using exhaust gas analyses. Dahod [20] found that the measured concentrations of CO_2 were 90% higher than those calculated using the exhaust gas analysis by the procedure suggested by Royce and Thornhill [15]. These differences could be a result of the factor of 0.89 attributed to the ratio of k_La for CO_2 mass transfer and $k_L a$ for O_2 mass transfer in equations (1 to 5). Another potential explanation can be the effect of medium constituents on Henry law constants (equations 6 and 7).

In our knowledge, the application of a fluorescence based CO_2 probe at high temperatures has been not published before. It is often suggested the fluorescence-based in-situ CO_2 probes have low temperature tolerance. The observations of this work that the measurements by the in situ fluorescence-based probe and the calculations based on exhaust gas analyses are congruent, however, suggest that the probe is a reliable and cost efficient method of monitoring the dissolved carbon dioxide concentration even under temperature as high as 70°C.

A positive influence of carbon dioxide was observed on the formation of α-amylase and neutral protease during batch fermentations of *B. caldolyticus*. Increasing the fraction of carbon dioxide in inlet air from 0.038% to 5% (v/v) resulted in a 22% increase of α-amylase activity in culture broth, although no change in the biomass production was revealed. When the content of CO_2 was increased further to 10% (v/v), the cell growth remained unchanged but the enhancement in amylase production was lost. On the other hand, protease activity continued to increase as the CO_2 fraction was increased. A major positive effect observed during the fermentations with 5% CO_2 was reduction of lag-phase by more than 1 h resulting in even higher enzyme productivity. Further investigations are necessary to explain the observed positive influence of CO_2 on metabolic level. In addition, a fluorescence-based in situ probe was found to stably measure dissolved CO_2 concentration even at 70°C. To our knowledge, such application of fluorescence based probes for the reliable in situ measurement of dissolved carbon dioxide at a fermentation temperature of 70°C has been reported here for the first time.

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