Application of pH and pO_2 Probes During *Bacillus caldolyticus* Fermentation: An Additional Approach for Improving a Feeding Strategy

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My colleague Wolf-Dieter Deckwer and I spent very productive time as PhD students in the Institut für Technische Chemie, Technische Universität, Berlin, Germany between 1971 and 1976 working together on modelling of hydrodynamics, mass and heat transfer in bubble columns. During this time we published some very good papers especially in the area of absorption and reaction of isobutene in sulphuric acid. Later, we both moved into Biotechnology and met frequently at diverse meetings and conferences. Wolf-Dieter's analysis and discussions during these politically turbulent times in West-Berlin were distinctive for me and for the whole generation of PhD students in our institute at this time. Friendly contacts with him and his family lasted over 30 years. I am glad and proud to belong to this group of (formerly) young scientists.

M. K. Popovic

In this paper, three different feeding strategies are presented and compared for fed-batch operation of bioreactor for production of α-amylase by *Bacillus caldolyticus*: constant volumetric specific feed rate, variable feed rate based on responses of pO_2 probe when subjected to short pulses in feed rate, and exponentially increasing feeding rate supported by pH and pO_2 probes. The best strategy was found to be the exponential feeding supported by pH and pO_2 probes giving a specific activity of 15.7 $U_{\alpha\text{-amylase}} \cdot g^{-1}_{\text{dry weight}}$ as well as a productivity of 2.3 $U_{\alpha\ \text{amylase}} \cdot g^{-1}_{\text{dry weight}} \cdot h^{-1}$ and negligible acetate formation. Each of the probes offers information concerning different regions of metabolic state. Using the responses of these two probes, a feed medium with balanced ratio of starch and casitone was obtained. This medium composition resulted in increased titre as well as productivity of enzyme even with the simple exponential feeding strategy.

Key words:
Bacillus caldolyticus, α-amylase, fed-batch, exponential feeding, pO_2 and pH feed-back strategy

Introduction

Thermostable enzymes are highly desirable in practice due to increasing reaction rates with temperature.[1] One such enzyme is α-amylase that is routinely used in starch industry.[2] A number of other thermostable enzymes are also in various stages of development[3] and their applications will potentially increase manifold with improved methods of production and purification of the enzymes. Fed-batch cultivations are commonly used to produce these enzymes at high titres. At the same time, complex production media are also the norm.[4] For the operation of fed-batch reactors, several feeding strategies have been proposed.[5–14] These span from adaptive feed-forward to totally feed-back control algorithms. Model-based adaptive-control strategies have often been suggested and explored.[15] However, operating practices tend to gravitate towards well designed feed-back control, mainly because of the issues related to robustness of the models, difficulties in estimation of model parameters, and implementation of the feed-forward control strategies. In this respect, the feed-back probing strategies based on dissolved oxygen concentration (pO_2), proposed by Akesson et al.,[5–7] stand out. These probing strategies involve making small temporal changes in feed rate of carbon source to the reactor and utilizing the response of a dissolved oxygen electrode to determine the metabolic state of the cells and thus guide the feeding rate while preventing formation of undesirable metabolites such as

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acetic acid. The goal is to cause the disruptions frequently enough so that the system operates near a predetermined metabolic state of cells and results in high growth rate while minimizing formation of acetic acid. This strategy has been known to run into problems in case of complex media.[16] As a result, Wiffin et al.[17] have proposed using a off-on probing strategy coupled with feed-back response of dissolved oxygen electrode to determine the directional changes in feeding rate of carbon source; the rationale being that it is thus possible to keep cells permanently in a state of carbon limitation and thus avoid acetate formation. Even with this strategy, the authors[17] had to resort to temporal changes in response time in order to reduce acetate formation.

In a previous paper,[18] we described the use of Akesson's substrate feeding strategy in controlling the feeding rate of starch solution to a fed-batch reactor for production of thermostable α-amylase by a thermophilic microorganism, *Bacillus caldolyticus*. This strain is an excellent producer of thermostable α-amylase[19] but acetate production by the cells is a barrier in achieving high activities of the enzyme. The feeding rate of a medium containing starch and casitone was determined on the basis of responses of dissolved oxygen electrode to transient shifts in feeding rates. Additionally, the metabolic state of cells was inferred at different stages of operation by the analysis of proteome of the cells also. As a result, it was established that the cells experienced either carbon limitation or nitrogen limitation while using the response of dissolved oxygen electrode in Akesson's feeding strategy with the complex media. During the experiments, it was also noticed that pH of the medium had a definitive relationship with the type of limitation the cells were facing at any time. Similar results have been obtained by others also; Suzuki et al.[20] utilized pH probe to monitor and control fermentation of *Protomonas extorquens*.

In the present paper, online detection of feed demand during the fed-batch fermentation of thermophilic *B. caldolyticus* using a two-component complex culture medium feeding is discussed. A combined pH- and pO_2-based approach was applied to avoid the overload of respiratory capacity of the cells on one hand and starvation of the cells of *Bacillus caldolyticus* as well as the improved induction of α-amylase production on the other hand. By the correct interpretation of the probe signals, acetate accumulation in the broth could be held below the concentration of 0.06 $g \cdot L^{-1}$ during fermentation.

Materials and methods

Strain and media: The bacterial strain used in this study was *Bacillus caldolyticus* DSM 405.[19] The medium composition was: 2.0 $g \cdot L^{-1}$ casitone, 0.05 $g \cdot L^{-1}$ KH_2PO_4, 0.25 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.03 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 1.57 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.1 $g \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, and 0.5 $g \cdot L^{-1}$ Zulkowsky starch (Merck, Darmstadt, Germany). Initial pH of the medium was 7.0. $MnCl_2 \cdot 4H_2O$ and $CaCl_2 \cdot 2H_2O$ were sterilized together but separately from the rest of the salts. Starch was also sterilized separately. Feed solutions contained starch and casitone; compositions of feed solutions have been reported in the text below.

Culture conditions: A 7-liter BIOSTAT® E-Fermenter (Sartorius BBI Systems, Melsungen, Germany) with an initial operating volume of 3 liters was used for fed-batch fermentation. At the start, the stirring speed was 500 rpm, and airflow rate was 0.5 vvm (volume gas per volume liquid per minute). pH was controlled at 6.9 ± 0.1 using 1N KOH or 1N phosphoric acid solutions. Temperature was controlled at 70 °C. Stirrer speed and airflow were increased during fermentation to keep pO_2 above 50 %. The maximum solubility of oxygen calculated using an expression proposed by Paul[21] at the fermentation temperature of 70 °C is 3.6 $g \cdot L^{-1}$. Thus the minimum dissolved oxygen tension in the broth was 1.8 $mg \cdot L^{-1}$. Silicon oil was used to control foam. Each experiment started as a batch fermentation using medium composition stated above and feeding was initiated when glucose was depleted as indicated by an increase in dissolved oxygen concentration. Feeding rate was controlled by an Amersham peristaltic pump P-1. This pump was operated by a PC-interfaced controller and software that permitted ±20 % modifications of the feeding rate for pulse durations mentioned below. A digital interface [SERAI 8-12 USB; AK MODUL-BUS Computer GmbH] with custom software was used for recording online cultivation parameters every 5 seconds.

Three different feeding strategies were utilized in this work. The first one was feeding of a solution of 10 $g \cdot L^{-1}$ starch at a constant volumetric specific rate of 0.1 g starch $\cdot L^{-1} \cdot h^{-1}$. This feed rate was determined on the basis of extended batch fermentations conducted in shake flasks. The second strategy was based on a pO_2-probing feed-back control proposed by Akesson et al.[5] extended by interpretation of pH probe signals. Operating strategy for this method (identified as the "Akesson strategy") was to initiate feeding of solution containing 10 $g \cdot L^{-1}$ starch and 40 $g \cdot L^{-1}$ casitone at a rate of 0.1 g starch $\cdot L^{-1} \cdot h^{-1}$, and then make changes in feeding rate based on response of pO_2 probe to transient changes in feeding rate. As per Akesson et al.[5], acetic acid production in cells is related to respiratory system overload which can be determined by superimposing short

(1 minute) pulses (up as well as down) on the feed rate. If the dissolved oxygen probe responded downward to pulse-up of feed rate and upward to pulse-down of feed, it implied no overload in cells under the operating conditions and no acetic acid production. In this case, the feeding rate was increased linearly. The respiratory metabolism was supposed to be just saturated (point of 'onset of production of acetic acid') when the probe responded to pulse-down but not to pulse-up of feeding rate. In this case, feed rate was left unchanged. Failure of the probe to respond to either of the pulses showed overloaded respiratory system and acetic acid production by the cells. In this case, the feed rate was decreased. During this experiment, the composition of starch and casitone in the feed solution was also manipulated (10 $g{\cdot}L^{-1}$ starch + 40 $g{\cdot}L^{-1}$ casitone to 10 $g{\cdot}L^{-1}$ starch + 20 $g{\cdot}L^{-1}$ casitone to 15 $g{\cdot}L^{-1}$ starch + 10 $g{\cdot}L^{-1}$ casitone). Rationale for these changes was discussed by Bader et al.[18]

In the third strategy – exponential feeding supported by pH and pO_2 probes – feeding of solution containing 30 $g{\cdot}L^{-1}$ starch and 20 $g{\cdot}L^{-1}$ casitone was started beginning at the moment of glucose depletion in the fermentation broth with a feeding rate of 80 $ml{\cdot}h^{-1}$ (0.8 g $starch{\cdot}L^{-1}{\cdot}h^{-1}$). The ratio of starch to casitone in the feed solution was thus identical to that found best in the second strategy. Feeding rate was increased exponentially according to

$$F_t = F_0 \exp(\mu t) \quad (F_0 = 1.3 \text{ ml min}^{-1},\ \mu = 0.32 \text{ h}^{-1}) \tag{1}$$

The initial feeding rate of starch (0.8 g $starch{\cdot}L^{-1}{\cdot}h^{-1}$) and the specific growth rate (μ = 0.32 h^{-1}) were based on the experiments conducted by Schwab[22] who found these values to be optimal for production of α-amylase by *Bacillus caldolyticus*. During the fermentation, metabolic state of the cells was determined according to the method of Akesson et al.[5] using 1-minute pulses in feeding rate. However, the probe responses were not used to affect any change in the exponentially increasing feeding rate.

Analytical methods: Cell density was monitored as optical density at a wavelength of 600 nm with Philips PU 8625 UV / VIS spectrophotometer. Starch concentration in cell-free broth was analyzed by adding 700-μl water and 15 μl of iodine-solution (4 % w/v) to 300 μl of the sample supernatant and measuring light absorption at 600 nm using a UV-VIS-Photometer. Blanks were prepared by adding 300 μL medium (without starch) in stead of cell-free broth. The starch-concentration in samples was calculated using the following calibration prepared from solutions of known concentrations of starch:

$$\gamma = -0.034 + 0.8\,\mathrm{E} \tag{2}$$

Where γ is starch concentration in $g{\cdot}L^{-1}$ and E is light absorption at 600 nm.

Glucose concentration was measured with a Glucose-kit [Roche Diagnostik, Mannheim Kit-No. 0 716 251]. α-amylase activity was analyzed according to Manning and Campbell[23] with some modifications: 20 μl culture supernatant + 20 μl starch solution (1 % w/v) + 20 μl buffer were incubated for 10 min at 70 °C, and 1 ml water + 15 μl iodine solution (30 $g{\cdot}L^{-1}$) were added after cooling on ice. Extinction was measured at 580 nm. As a reference, 20 μl fresh medium was used instead of 20 μl supernatant. Acetate was assayed with the Acetate-Kit [Roche Diagnostik, Mannheim, Kit-No. 10 148 261 035].

Results and discussion

In the complex medium containing a mixture of casitone and starch, starch provides saccharides that can be used as carbon source and induce amylase expression. Casitone can be used by the cells as nitrogen source as well as a carbon source. Accumulation of starch causes acetate formation that has to be avoided as it interferes with amylase production. Utilization of casitone as a carbon source does not contribute to production of amylase. Furthermore, utilization of casitone as a carbon source interferes with interpretation of responses of pO_2 probe during the up- and down-pulses of feeding rate of starch per Akesson's strategy. To avoid any accumulation of substrate components, a well adapted ratio of casitone and starch as well as an optimal feeding rate has to be found.

Constant volumetric specific feeding

When a constant specific feeding rate of 0.1 $g{\cdot}L^{-1}{\cdot}h^{-1}$ starch with a feed medium containing 10 $g{\cdot}L^{-1}$ starch was applied, an amylase activity of 8 $U{\cdot}mL^{-1}$ was obtained after 410 minutes of operation. The corresponding amylase productivity was only 0.9 $U{\cdot}g_{cell}{}^{-1}{\cdot}h^{-1}$ while accumulating 12.1 $mg{\cdot}L^{-1}$ glucose and 23.1 $mg{\cdot}L^{-1}$ acetate in the broth (Table 1).

Extended pO_2 probing feed-back control per Akesson et al.[6]

The course of a fed-batch fermentation of *B. caldolyticus* with feeding medium containing starch and casitone as per Akesson et al.[6] is shown in Fig. 1.[18] Phase I represents the batch operation which is followed by fed-batch operation. During fed-batch operation, perturbations in feeding rate according to Akesson *et al.*[6] were used to infer the

Table 1 – *Comparison of different fermentation strategies of B. caldolyticus after similar feeding durations; number 1: linear feeding; 2: feeding according to Akesson with varied ratios of casitone and starch; 3: exponential feeding with 20 g·L^{-1} casitone and 30 g·L^{-1} starch.*

No.	Time [min]	Productivity [U·g^{-1}·h^{-1}]	Volumetric activity [U·mL^{-1}]	Specific activity [U·g^{-1} (Biomass)]	Total activity [kU]	Glucose [mg·L^{-1}]	Acetate [mg·L^{-1}]
1	420	0.9	8	6.2	27.2	12.1	23.1
2	410	1.6	44	11.2	175.0	7.7	0
3	440	2.3	83	15.7	327.7	13.8	2.2

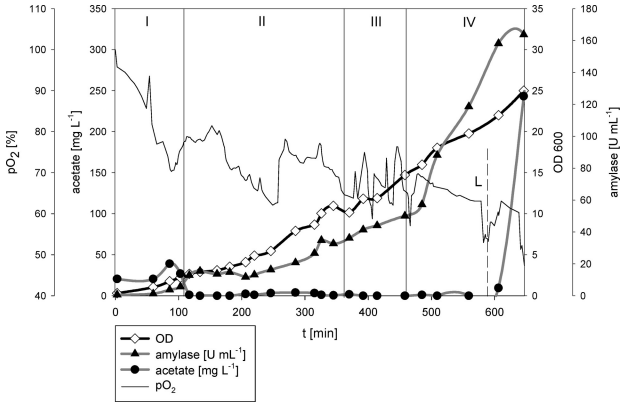


Fig. 1 – *Production of α-amylase by Bacillus caldolyticus in fed-batch Biostat fermentor with dissolved oxygen and pH control. "L" marks onset of casitone limitation in phase IV (Akesson strategy).*

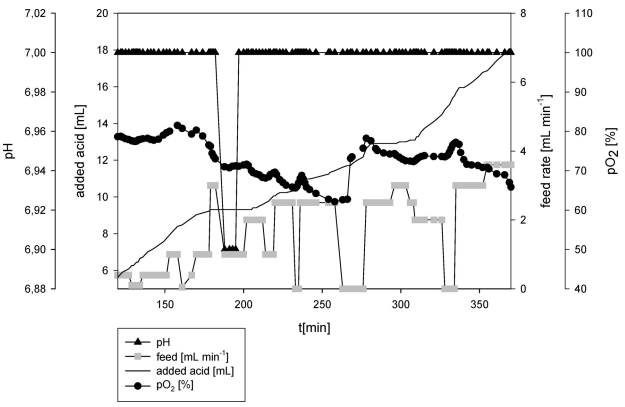
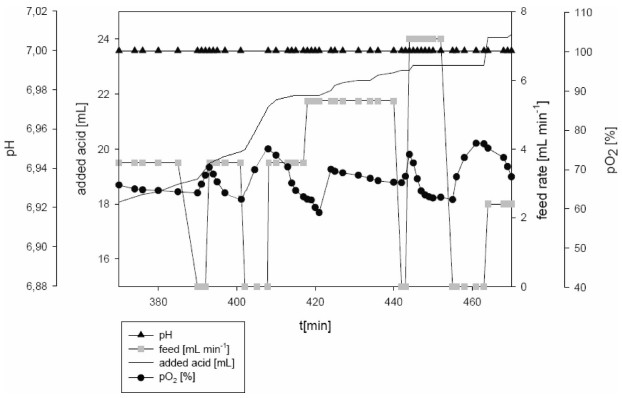


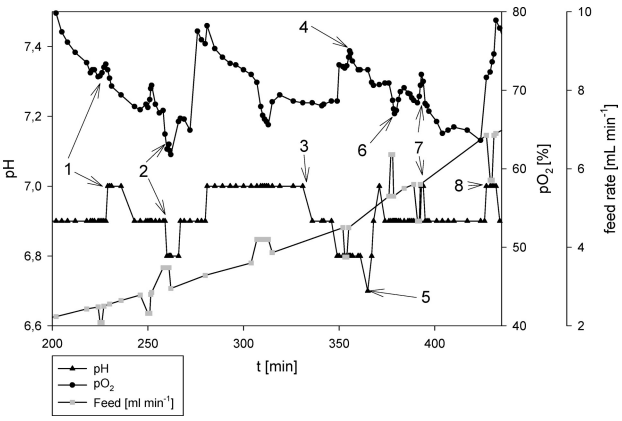
Fig. 2 – *pH, pO_2, feed rate, and phosphoric acid consumption between t = 120 min and t = 370 min (Phase II, Fig. 1, Akesson strategy)*

metabolic state of the microorganisms and feeding rate was adjusted accordingly. The ratio of starch and casitone was modified several times based on the response of pH probe. During phase II (between t = 120 min and t = 370 min), the concentrations of starch and casitone in the feed medium were 10 g·L^{-1} and 40 g·L^{-1}, respectively. pH, pO_2, feed rate, and amount of phosphoric acid added during phase II are plotted in Fig. 2. No accumulation of starch and acetic acid was noted during this period in spite of the fact that feed rate was increased from 0.5 mL·min^{-1} to 3.6 mL·min^{-1}. pO_2 probe responded to downward changes in feeding rates but only very weakly to upward changes (presented in Fig. 2) showing the limitations of pO_2-probing feed-back control. Feeding rate and dissolved oxygen concentration during phase II (between t = 120 min and t = 370 min) are separately plotted in Fig. 2. Another observation during this period was that pH was consistently at the high end of the pH control bracket (6.9 ± 0.1) and phosphoric acid was continuously added to keep it from rising further. As indicated earlier, the pH response indicated utilization of casitone as carbon source during this phase of operation. Hence, the composition of feed medium was changed to 10 g·L^{-1} starch and 20 g·L^{-1} casitone in phase III (between t = 370 min to t = 470 min). pH, pO_2, feeding rates, and phosphoric acid consumption during this phase are shown in Fig. 3. It is clear that the reduction of casitone concentration in feed medium resulted in a much stronger response of dissolved oxygen probe to both up- and down-pulses of feed rate in phase III. Still, pH of the medium continued to be controlled at the upper limit of the pH control band (6.9 ± 0.1) and phosphoric acid addition continued to control pH.

Casitone can be used by cells as a carbon source after deamination of the proteins in it. The resulting release of ammonia causes increase in pH value and, hence, a demand for acid to control pH. Still, increasing feeding rates (from 3.6 mL·min^{-1} to 7.2 mL·min^{-1}) in phase III resulted in no accumulation of starch and acetic acid, again implying that (a) starch was not overloading the metabolism and (b) casitone was still being provided in excess and it was being used as a carbon source. The lack of phosphoric acid addition for a short while after t = 400 min (fig. 3) is explained by the fact that feeding was disrupted for a short while during this period. This was reflected in increased pO_2 values also. In this strategy, α-amylase activity after 410 minutes



F i g . 3 – *pH, pO_2, feed rate, and phosphoric acid consumption between t = 370 min and t = 470 min (Phase III, Figure 1, Akesson strategy)*



F i g . 4 – *Feed rate, pO_2, and pH during the exponentially feeding in fed-batch reactor operation*

of operation was 44 $U \cdot mL^{-1}$ and the average enzyme productivity was 1.6 $U \cdot g_{cell}^{-1} \cdot h^{-1}$. Glucose concentration in the broth at this time was 7.7 $mg \cdot L^{-1}$ but no acetic acid could be detected in the broth. Up- and down-pulses of feeding rate during this period reported a strong response of pO_2. The pH probe response was congruous to that of dissolved oxygen probe.

At minute 470 the composition in feed solution was changed to 15 $g \cdot L^{-1}$ starch and 10 $g \cdot L^{-1}$ casitone.[18] This resulted in a strongly increased α-amylase production (Fig. 1), indicating an improved feed composition. This starch to casitone ratio (1.5:1) was used in the third strategy.

Exponential feeding using pO_2 and pH probing strategy

In third strategy, the fed-batch fermentation was conducted with feed containing 30 $g \cdot L^{-1}$ starch and 20 $g \cdot L^{-1}$ casitone with the aim of showing that this ratio of starch to casitone (1.5 : 1) was indeed a balanced one. To study the potential of using a single probe response (as per Akesson[5–7]) for control of reactor operation with a balanced medium, the two-component feed solution was fed into the broth according to an exponentially increasing feeding rate. At different times, the feed rate was pulsed up and down (as per Akesson[6]) but without making any adjustments in feeding rate as a result of probe responses. The responses of the pO_2 and pH probes in this experiment between t = 200 and 440 min are shown in Fig. 4.

During the up- and down pulsing (labels 1 and 2 in Fig. 4) of feed rate, a congruent response of pH and pO_2 probes was observed. This indicated a balanced nutrient composition in broth. Beyond t = 280 min, solution pH was controlled at the upper range of control point, suggesting utilization of casitone as carbon source. However, pH started to decrease at t = 330 min. This suggested slowing metabolism of the cells and accumulation of substrate components (hence glucose) and formation of acetic acid. Since no changes were made in the feeding strategy or in feed composition, other parameters must have influenced the decreased metabolism of the culture. With this reasoning, the aeration rate was increased at minute 350 which resulted in an increase in dissolved oxygen concentration. After the regulation of the pH value to pH = 7.0 (label 5, Fig. 4), the pH and pO_2 probes again showed a strong reaction to up- and down-pulses of feed rate (labels 6 to 8, Fig. 4). The fermentation was stopped at minute 440 as a result of pump failure. Amylase activity in the broth at the end of this experiment was 83 $U \cdot mL^{-1}$, about twice that obtained with Akesson's strategy at the end of similar feeding period (410 min). Based on these results, it may be stated that a congruent response of pH and pO_2 probes indicates a balanced ratio of the C and N sources in the feed medium.

The amylase activity, average enzyme productivity and other pertinent parameters from the three feeding strategies are presented in Table 1. It is clear that the best productivity, volumetric activity and specific activity was achieved with exponentially feeding supported by interpretation by pO_2 and pH probes. The acetate production was reduced to a negligible 2.2 $mg \cdot L^{-1}$. The improved ratio of starch to casitone (1.5 : 1) resulted in enhancements in the final activity as well as productivity even without resorting to manipulation of the feeding rate in response to the probe signals. Since the balanced feed composition results in congruent responses in the probe signals, the feeding strategy of Akesson[6] can be perhaps be used again. It is to be noted that the primary parameters used for the control of fed-batch fermentations are pH[20] and dissolved oxygen.[5–7] The concentrations of glucose[24] or acetate[25] are seldom used due to difficulties of

their online measurement even though these are more direct measure of metabolic state of the cells. The combination of both pO_2 and pH probe, presented in this paper, enables identification of balanced composition of critical components in feeding medium.

Conclusions

Probing pO_2 pulse technique coupled with interpretation of pH probe signal enabled identification of imbalances in the composition of complex feed medium in fed-batch fermentations. Analytical techniques discussed in this paper helped us to identify a balanced medium composition for feed solution to be used in production of α amylase from thermophilic *B. caldolyticus*. Use of the balanced feed medium resulted in congruent responses of the two probes to up- and down-pulses in feeding rate and achievement of two-fold increase in activity of α-amylase activity in broth even without the use of probing pO_2 pulse technique for controlling the feeding rate.

List of symbols and abbreviations

E – light bsorption at 600 nm
F – feed rate, $g \cdot L^{-1} \cdot h^{-1}$
h – hour
min – minute
OD – optical density
pO_2 – dissolved oxygen concentration, %
t – time, min
U – unit
w/v – weight per volume
γ – starch concentration, $g \cdot L^{-1}$ (eq. 2)

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