Bioprocess Engineering 14 (1996) 281–289 © Springer-Verlag 1996

Substrate gradients in bioreactors: origin and consequences

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Abstract Gradients of glucose in time and space are shown in a 30 m^3 cultivation of Saccharomyces cerevisiae grown in minimal medium to a cell density of 20 $g\,l^{-1}$. The fed-batch concept was used with glucose as the limiting component which was fed continuously to the process. As the mean glucose concentration declined throughout the process, the level of glucose was at all times different in three sampling ports (bottom/middle/top) of the reactor. These gradients were furthermore shown to depend on the feed position. This means that if the feed was supplied in the relatively stagnant mixing zone above the top impeller, the gradients were more pronounced than by feed in the well mixed bottom impeller zone. A rapid sampling system was constructed, and continuous glucose samples of every 0.15 s were analysed from a point of the reactor. Fifty samples were collected with this system, but the amount and frequency is possible to change. The results of these series show a variance of the glucose concentration where at one stage, a peak appeared of a relative difference in concentration of 40 $mg\,l^{-1}$. The pattern of these rapid glucose fluctuations was shown to depend on the turbulence level at the location of the feed. It was shown, that the fluctuations were more pronounced when the feed was localised in a relatively stagnant area than in the well-mixed impeller area, where the deviation from the mean was negligible. The fluid flow, in the impeller (gassed and ungassed) and bulk area (ungassed) of the reactor, was characterised by turbulence measurements using thermal anemometry. These types of areas resembles well the different areas of sampling as mentioned above. The turbulent frequencies in these areas were in the range of 10^{-1} to 10^4 Hz with the highest amplitudes at low frequencies. The spectra depicts a uniform time scale for all zones, especially at the low frequencies. The dominance of low frequency, high amplitude flow variations and the observed short-time oscillations in substrate concentration support the hypothesis of substrate transport over fairly long distances without substantial mixing both in the impeller, but especially, in the bulk zone of the reactor.

Simulations with an integrated CFD and biokinetic model were performed. The predictions of the glucose gradients of this model were compared to measurements.

Received: 28 August 1995

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This project was supported by a grant from the Nordic Programme on Bioprocess Engineering under the auspices of NI, the Nordic Fund for Technology and Industrial Development and NUTEK, The Swedish Board for Technical and Industrial Development. This investigation was possible to perform due to the support from Statoil Biosentrum in Stavanger, Norway, that provided their pilot plant. Especially the cooperation with Lars Aasberg and Andreas Rag is greatly acknowledged.

1 Introduction

Gradients in bioreactors

The bioreactor performance is determined by a number of factors. These are mainly connected to the capacity of cells and their performance in the microenvironment inside a reactor. This environment is in turn created by parameters of both physical and biological origin. Among all these are, notably, a large number of factors that change throughout the process. If the microenvironment is changing, it is of importance to know what effects this has on such variables as productivity, yield and product quality.

Studies of the environment inside a specific bioreactor concerns properties of the flow as well as temperature and concentration distributions. The literature of flow measurements will not be reviewed in this paper, neither the microbial response towards a fluctuating environment, that was performed earlier [10].

The descriptions of gradient formation in literature have mostly concerned oxygen, because it is available to in situ analysis and is often critically low in aerobic cultivation. It has been shown by Manfredini et al. [1], that the dissolved oxygen tension varied by liquid height in a 112 m^3 non-Newtonian streptomyces cultivation in a stirred reactor vessel. The radial distribution of oxygen was however negligible. The oxygen gradients varied with stirrer speed, with the time of cultivation and with organism and process. Similar results are found in publications from Steel and Maxon [2] and Carilli et al. [3].

Other authors, e.g. Oosterhuis et al. [4], performed measurements in a 19 m^3 gluconic acid cultivation and demonstrated oxygen gradients in both axial and radial directions. This seems very reasonable, since bubble size distribution, pressure and turbulence varies in different parts of the reactor. From own experiments in a reactor of 30 m^3, it was also confirmed that the partial pressure of both dissolved

oxygen and oxygen in the local gas phase varied by position in the reactor. This was not only due to pressure differences at different heights [7].

Which parameters can be expected to form gradients in a reactor? The time constants of different processes appearing in the reactor, of both physical and biological origin, can be compared. If the transport constants exceeds those of the reaction, gradients are likely to appear. This situation is expected in two cases. The first is due to a low degree of mixing and limitations in the mass transport. In such cases, gradients in e.g. temperature could be promoted, especially if a rapid rise is required, as in the case of induction of recombinant protein. Also the pH and any component fed in growth limiting concentrations and/or amounts to the reactor, are hereby candidates for gradient formation. Alternatively, a high rate of reaction, as in the case of a low substrate saturation constant, will promote gradients. From the above mentioned reasons, cellular products and cell concentrations are generally not expected to form gradients. An exception of this is carbon dioxide which forms gradients by the similar reasons as oxygen.

The determination of local substrate gradients (excluding oxygen) in reactors have not been described in literature and their nature is therefore unknown. From overall measurements some conclusions about the frequencies of oscillations might be drawn. It is therefore likely, that the concept of mixing time can be of some guidance. Mixing times of seconds to minutes in large bioreactors have been reported. From a recent publication of CFD simulations of a yeast fermentation, the so called feed zone could be calculated [7]. This is a high substrate concentration area that can be considered to form close to the feed inlet due to insufficient mixing and mass transfer. It was seen that the total volume of such a freed zone, that in this case was considered as above 40 $mg\,l^{-1}$ (a possible limit for ethanol formation of Saccharomyces cerevisiae by overflow metabolism), was approximately 10%. The scale of fluctuations could therefore, if this information is combined, be in a span of fractions of seconds up to tenths of seconds.

The possible range of the substrate gradients in a large reactor is from the feed solution concentration (for glucose usually 600 $g\,l^{-1}$) to complete exhaust. While the high concentrations are formed because of insufficient mixing alone, the low concentrations are due to the relative rates of mixing and the biological reaction. This means that cells could be circulating in an environment that theoretically is fluctuating between values of these extremes. The cells could hereby respond to a range of concentrations giving situations of feast to famine. The metabolic changes to these situations were described many times in literature (see e.g. 14) although these results often stem from investigations of the natural habitat and other cases where fluctuations are of longer time scales.

Most industrial processes use the concept of fed batch technique i.e. a substance is fed to the process in limited amounts with time to avoid such problems as oxygen deficiency and limited cooling capacity. A reason can also be the avoidance of overflow metabolism in glycolysis or catabolite repression that may appear during unlimited growth. The fed-batch technique could be used very differently, according to literature, see for example the review of high cell density cultivation of Yee and Blanch [13]. A feeding scheme promotes generally the maximum cell productivity ($g\,l^{-1}\,h^{-1}$) over the process time, if that is not contradictory to a high product formation. The feed is hereby exponential until the point of maximum productivity, whereafter a constant feed profile allows the cell concentration to rise in expense of the growth rate. The substrate concentration, at the point of constant feed, is often below the saturation constant value for the organism and substrate in question. For some common organisms as Escherichia coli and Saccharomyces cerevisiae, this value is about 5 and 150 $mg\,l^{-1}$, respectively, for growth on glucose. After the switch point to constant feed, the substrate concentration is slowly declining throughout the process and will approach zero. During this process, the physiology of the cells will constantly change and a number of rearrangements to the shifting conditions can be postulated to appear. How this effects production is rarely known.

The substance fed to the reactor during a fed batch process is often in a concentrated form to avoid a too high volume build up. This means that for example a glucose concentration of 600 $g\,l^{-1}$ in the feed solution is a common figure. This is often fed in one point of the reactor, a point which sometimes can not be, or is not, optimally chosen from a transport point of view. Also the transport of the glucose solution inside the reactor, could imply a mixing problem since the viscosity difference is quite large [6]. Often the energy source is chosen as limiting and fed to the process. The rates of addition of concentrated solutions is quite slow and could many times be seen as "dripping"into the reactor. Discontinuous feeding of methanol to a methanol limited chemostat was described in literature to cause a high drop in biomass yield depending on the frequency [5] of the addition.

A model of velocity and mixing properties, integrating the biokinetics and using computational fluid dynamic (CFD) procedures for prediction of the resulting local conditions of concentrations of substrate, oxygen, microbial metabolites etc., is a powerful tool for the simulation of bioreactor performance. It has also the capability to predict local physical properties as velocity, turbulent diffusivity, concentration etc.. It cannot however resolve the short time variations previously mentioned, as a time averaging procedure is applied to cope with complex features of the turbulence (through turbulence models) that are created in the reactor. Thus velocities, diffusivities, concentrations and so on, are mean values over time periods of some tenths of seconds, or more precisely, the time required to average the effects of the turbulence. Any time-dependent change over this range can however be considered in a conventional time integrating approach. Simulations give therefore an anticipation of the velocity and concentration gradients locally in the reactor.

The aim of this investigation is to confirm the predictions of glucose fluctuations during large scale industrial conditions. This requires sampling of glucose in different parts of the reactor. It was therefore necessary to develop a method for large number sampling also at high frequencies. The effects of a change in the feed position is chosen as a variable. The gradients should also be compared to turbulence data from the reactor where measurements should be taken in the same type of mixing zones as the sampling positions. Finally, the measurements should be compared to mathematical models of different complexity.

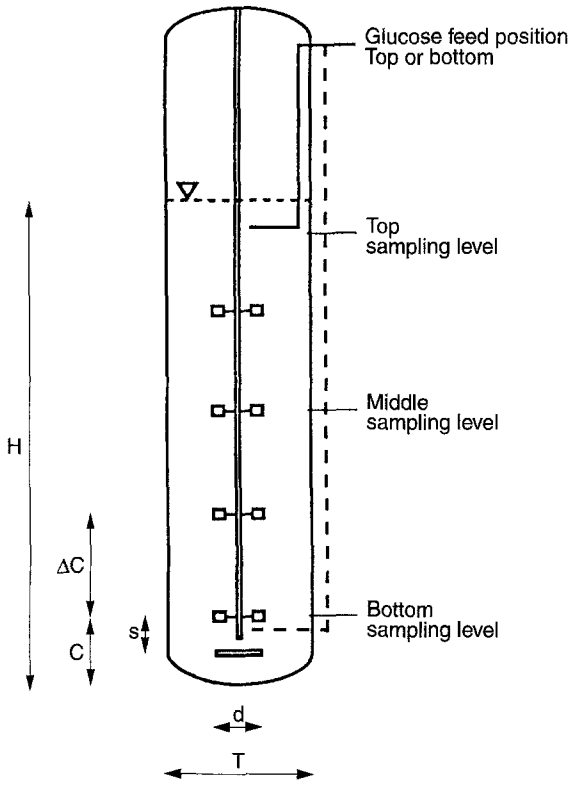

Fig. 1. The geometry of the 30 m^3 stirred tank reactor where $T=2.09$ m, $d=1/3$ T, $\Delta C=0.70$ T, $C=0.50$ T, $s=0.31$ T and H is approximately 3.5 T

2 Materials and methods

2.1 Reactor geometry and operating conditions

A reactor with a total volume of 30 m^3 was used. The geometry is presented in Fig. 1. The tank was equipped with four equally spaced Rushton turbines and four baffles, 90 degrees apart. The total liquid volume was 19.8–22.3 m^3. The volume increased due to feed of glucose solution. The impeller rotational speed was 2.22 s^{-1}, and the air flow rate 0.182 m^3 s^{-1}. From liquid velocity measurements it was concluded that these conditions did not cause any flooding of the lower impeller (results not shown).

Cultivations were performed in a fed-batch mode. The feed position was either at a top level, at a height 6.5 m above the bottom of the tank close to the stirrer shaft, or at the bottom level, 0.9 m above the bottom of the tank, just below the disc of the lowest impeller (Fig. 1). The head space pressure was 1.29 bar. Throughout the cultivation the dissolved oxygen tension was above approximately 30% of air saturation. It was measured at a height 3.90 m above the bottom of the vessel at a distance of 0.10 m from the wall. The overall volumetric mass transfer coefficient was approximately 180 h^{-1} as calculated from an oxygen balance. The feed rates, F, glucose feed concentrations, C_{sf}, initial reactor volumes, $V(0)$, and initial concentrations of biomass, C_x, glucose, C_s, and ethanol, C_e, are given in Table 1. The temperature was 30 °C and pH 5.0 was controlled by additions of NH_4OH.

Table 1. Feed characteristics and initial concentrations for the two cultivations (runs) in the 30 m^3 reactor with the feed rates (F), glucose feed concentration (C_{sf}), initial reactor volume ($V(0)$), and the initial concentrations of biomass (C_x), glucose (C_s), and ethanol (C_e)

	Run 1	Run 2	Unit
Feed characteristics			
C_{sf}	597	528	kg m^{-3}
$F(0)$	1.26×10^{-2}	1.42×10^{-2}	m^3 h^{-1}
F	$F(0)\times e^{0.2\times t}$	$F(0)\times e^{0.2\times t}$	m^3 h^{-1}
F_{max}	9.0×10^{-2}	10.2×10^{-2}	m^3 h^{-1}
Feed location	top	bottom	
Initial volume and concentrations			
$V(0)$	20.8	19.8	m^3
$C_x(0)$	0.15	0.18	kg m^{-3}
$C_s(0)$	2.43	1.11	kg m^{-3}
$C_e(0)$	0.78	1.29	kg m^{-3}

2.2 Microbial strain, medium and inoculum

The cultivations were performed with Saccharomyces cerevisiae CBS 8066. The minimal medium contained per litre tap water; $(NH_4)_2SO_4$ 20 g, $MgSO_4*7H_2O$ 2 g, KH_2PO_4 1.2 g, biotin 0.2 mg, calcium-pantothenate 4 mg, nicotinic acid 4 mg, inositol 100 mg, thiamin*HCl 4 mg, pyridoxin*HCl 4 mg, *p*-amino benzoic acid 0.8 mg, EDTA (disodium) 60 mg, $ZnSO_4*7H_2O$ 18 mg, $MnCl_2*4H_2O$ 4 mg, $CoCl_2*6H_2O$ 1.2 mg, $CuSO_4*5H_2O$ 1.2 mg, $Na_2MoO_4*2H_2O$ 1.6 mg, $CaCl_2*2H_2O$ 18 mg, $FeSO_4*7H_2O$ 12 mg, H_3BO_3 4 mg, KI 0.4 mg and 30% silicone antifoam emulsion 0.1 ml. The vitamin solution was sterile filtered and the trace element solution was autoclaved. These solutions were added to the medium after the reactor sterilisation.

The inoculum was grown batch-wise in a series of two reactors with a volume of 0.1 m^3 and 1.0 m^3 with 40 kg m^{-3} glucose initially present. The cells were in exponential growth phase upon the transfer to the 30 m^3 reactor which gave some initial amount of ethanol in the large reactor. The maximum growth rate was measured to be 0.40 h^{-1}.

2.3 Estimation of growth

The growth was followed by cell dry weight measurements. These measurements were determined in triplicate; three samples of 5 ml were pipetted into pre-weight test tubes, centrifuged for 5 minutes at 2250 g and washed once with distilled water. The test tubes were dried at 105 °C overnight. The growth was also followed by optical density measurements.

2.4 Glucose sampling and the rapid sample collector

The sampling system, which includes magnetic valves and time relays, was developed and described in previous work [9]. The sampling time of the relay was variable from 0.01 s–99 h. A sample collector was constructed, where the test tubes were mounted in the periphery of a device, that minimised the distance between the test tube walls. The capacity was chosen

to 50 test tubes per cycle, but the number can be changed if necessary. The sample collector was mounted on a pump head shaft in order to have a variable rotational speed. This sample collector was used for series with a sampling period of 0.15 seconds per sample. A conventional sample collector was used for sampling of series with longer sampling intervals (7.5–9 s).

Three series of samples were taken with different sampling periods; the time course of the cultivation was followed with a sampling interval of 45 minutes, two intermittent series of 50 samples with a sampling interval of 7.5 and 9 seconds, and four continuous series of 50 samples with a period of 0.15 and 0.18 seconds. The different sampling time periods were selected to cover a wide range of time scales since the frequencies of the fluctuations were not known a priori. The time course of the cultivation was followed at the three positions indicated in Fig. 1. These positions were at the heights 0.97, 3.90 and 6.35 m. The continuous and intermittent series were taken at the top and bottom position i.e. in a zone of low and high turbulent intensity.

2.5
Glucose analysis

A method was used for cell inactivation and analysis that was earlier modified for evaluation of extracellular low glucose concentrations [9]. The cells were hereby inactivated by an instant decrease in pH to 0.7. This was obtained by mixing the sample with a 0.66 M perchloric acid in a volume ratio of 1 : 1.

The glucose concentration was measured with the combination of hexokinase and glucose-6-phosphate dehydrogenase. This enzymatic method was earlier modified for evaluation of low concentrations. An analysis protocol was used, that is based on microtitre plates, developed in order to make it possible and convenient to analyse a large number of samples [9]. These results were determined by this method with a 95% confidence limits of the mean ($n = 4$).

Due to the large number of samples (> 2400) it was not possible to neutralise these at the same time. They were instead stored overnight in a refrigerator before neutralisation. It was earlier shown that such a performance did not change the analysis results [9].

2.6
Integrated CFD and biokinetic model

The approach is in principle the same as previously described [12]. This implies a control volume differencing scheme for solving the differential equations [11] and boundary conditions taking into consideration the presence of baffels and the pumping capacity around the implellers. The consumption rate of the substrate (glucose) was calculated by Monod type kinetics, assuming that the maximum specific substrate uptake rate is constant ($1.7\ g\,g^{-1}h^{-1}$) and not varying with e.g. process time and that the saturation constant is $180\ mg\,l^{-1}$.

2.7
Flow and turbulence measurements

Measurements of turbulent liquid velocities were performed in water at two levels in the 30 m^3 reactor, in the impleller zone at the third impeller from the bottom and in the bulk zone above the third impeller. These regions may well represent the two different types of sampling sites for glucose determination.

The experimental technique for liquid flow was thermal anemometry, using a two-channel CTA 56 system from Dantec, Skovlunde, Denmark, which is suitable for turbulent velocity measurements. Data was simultaneously collected with a sampling rate of 25 kHz from the two channels of the split fibre probe (Dantec, 55R, special for water). The probe was oriented perpendicular to the main flow direction and calculation of velocities was made in two directions: in and perpendicular to the main flow direction.

Apart from statistical methods, spectral analysis was used to examine the influence of the turbulent part of the flow. A result of this is the power density spectral function, which was obtained by Fast Fourier Transform of the fluctuating part of the velocity.

3
Results

3.1
Measurements

The glucose analysis at the three different positions in the reactor shows that a gradient is formed along the axis (Fig. 2). This figure shows also the feed profile as a dotted line. Gradients are formed both when the feed was in the bottom and the top. The gradients so formed were however different depending on the location of the feed. The distribution was hereby more narrow in the bottom fed process than in the one with top feed. There was also a difference in the scattering of the glucose values closest to the feed point. Especially during the late exponential period, from 5–10 hours, the variance in the top fed case was much higher. From the figure is seen that the values taken at each 45 min. decline according to fed batch theory.

The more rapidly taken values of glucose in a fixed point of the reactor are shown in Fig. 3 for two different situations. When the feed was in the top, a rapid sample series was taken both at the top and at the bottom sampling locations. Correspondingly, a series was taken with bottom feed and sampling both in the bottom and at the top position. Each series contains fifty samples, giving between 0.15–0.18 seconds between each sample. The results of all series show certain variation around the mean. This variance was always less when the feed was close to the impeller (bottom position), with a value of $21.3 +/- 0.9\ mg\,l^{-1}$ calculated at the bottom position. The variance in this case is very close to the accuracy of the analytical method for glucose. During feed in the top stagnant zone, a large variation was seen in the top, where also a peak of an amplitude of $40\ mg\,l^{-1}$ and a duration of 1.2 seconds was shown. It is likely that this is not an artefact since five independent analyses depict this elevation. In the other positions, no major deviations were found during the short time interval of continuous sampling (7–9 seconds).

The results of the two series with a sampling interval of 7–9 seconds are shown in Fig. 4. The series in this figure show a deviation of the mean that is generally larger in the top than in the bottom. Although a scattering is present, no elevations of the amplitude as seen in the short time series were found. Neither of these measurements admits a reliable statistical

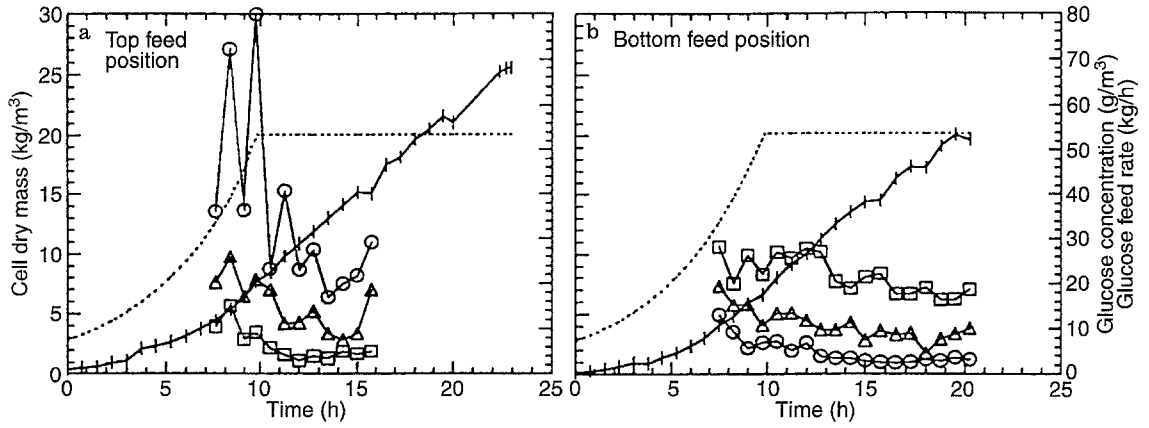

Fig. 2a, b. The effect of the feed position on the glucose gradient formation during the time course of a fed-batch cultivation. Sampling positions are indicated in the figure by the symbols: circle: top sampling position, triangle: middle sampling position and square: bottom sampling position. The dotted line shows the feed curve. **a** Feed in the bulk zone in the top of the reactor; **b** Feed in the bottom impeller zone

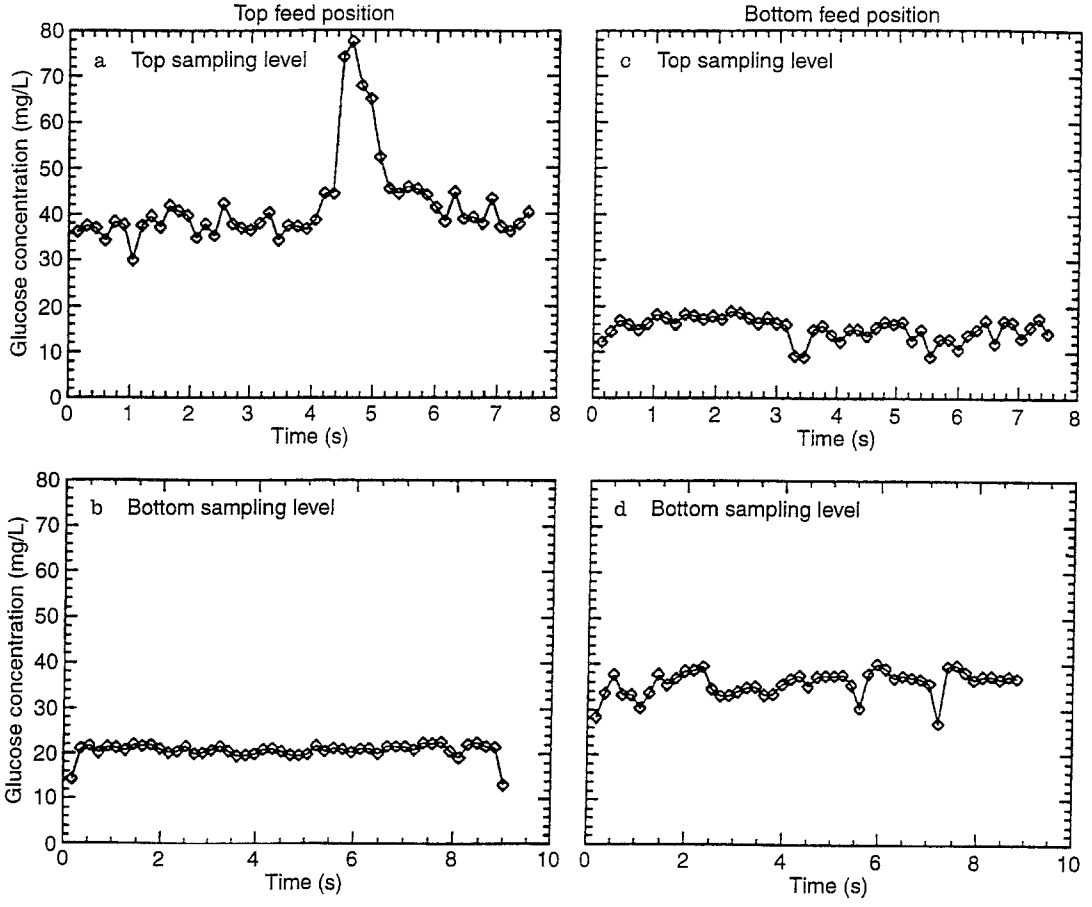

Fig. 3a–d. Local substrate concentrations in the reactor. Four continuous sampling series consisting of 50 samples taken in 7–9.5 seconds. The effect of two different feed positions is shown by sampling at two different locations in the reactor. **a** Feed position at the top, sampling at the top; **b** Feed position at the top, sampling at the bottom; **c** Feed position at the bottom, sampling at the top; **d** Feed position at the bottom, sampling at the bottom

analysis, evaluating regularities in the fluctuation pattern. However, the results show that it is likely that the glucose concentration is randomly distributed.

Turbulence measurements were also performed in different parts of the reactor during this investigation. Three turbulence frequency spectra are combined in Fig. 5, and they are normalised towards the measured variance, expressed by the root mean square (rms, as shown in the figure). The spectra represents the amplitude of the fluctuations of flow at different frequencies both gassed and ungassed. At low frequencies

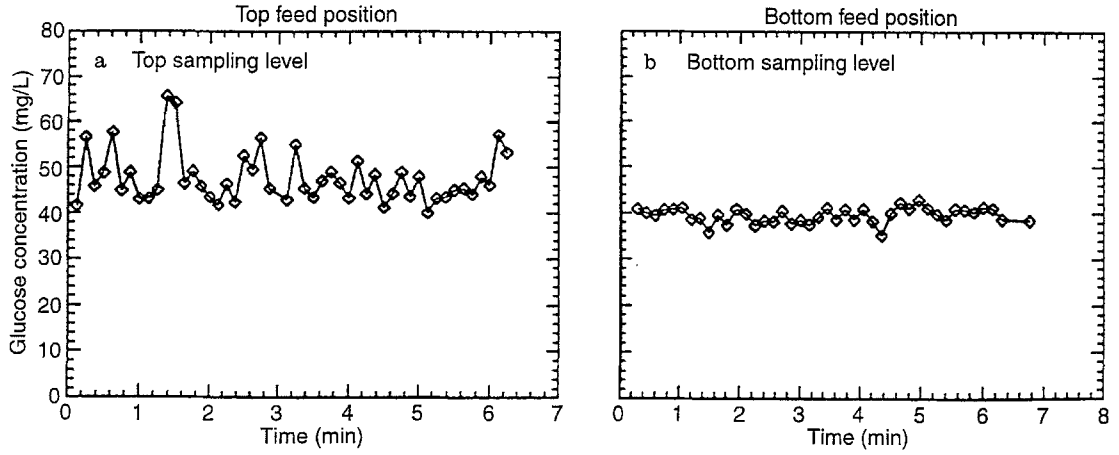

Fig. 4a, b. Local substrate concentrations in the bioreactor. Two intermittant sampling series. **a** 50 samples during 6.25 minutes with a sampling interval of 7.5 seconds. Feed from top reactor position with sampling at the top; **b** 50 samples during 6.75 minutes with a sampling interval of 9 seconds. Feed from bottom reactor position and sampling at the bottom

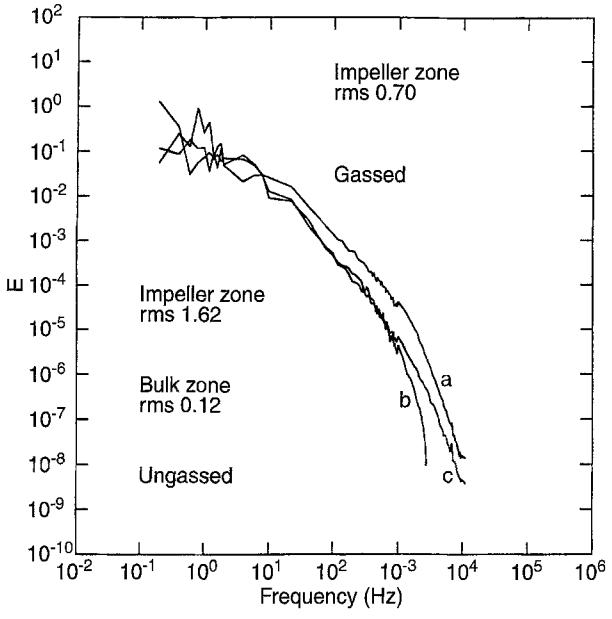

Fig. 5a–c. Three energy-frequency spectra from **a** gassed impeller zone; **b** ungassed bulk zone; **c** ungassed impeller zone, representing the amplitude of the flow fluctuations. The spectra are normalized with the variance. The corresponding turbulence intensities are: 0.60, 0.93 and 0.41, respectively

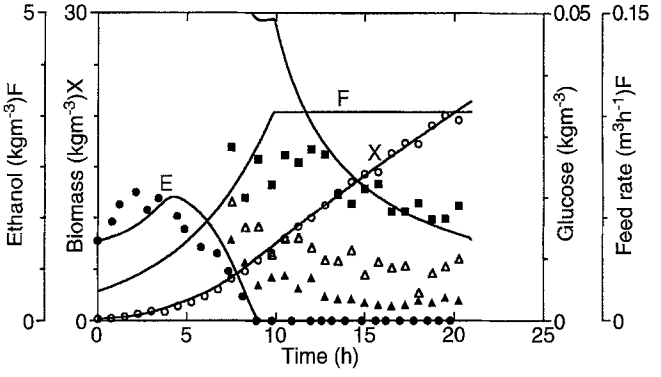

Fig. 6. Simulation (solid lines) and experimental data of a 30 m^3 fed-batch yeast cultivation with feed from the bottom. The simulations assume homogeneity and the following constants: Specific glucose consumption rate = 1.7 $g\,g^{-1}\,h^{-1}$ and saturation constant = 0.180 $g\,l^{-1}$. Included in the figure are the results of glucose measurements at different levels of the reactor (bottom/middle/top) as described in Figure 1. The symbols indicates the following: open circle: biomass (X), filled circle: ethanol (E), filled square: glucose, bottom sampling position, open triangle: glucose, middle sampling position, filled triangle: glucose, top sampling position. The continuous heavy line indicates the feed rate (F)

there is a high energy content in the fluctuations which influence the flow more, than the high frequencies where the energy content is low. One spectrum represents the bulk zone and two the impeller zone, of which one is measured during gassed conditions. The two spectra which more or less fall on top of each other, are the ones measured during ungassed conditions. The impeller spectrum measured during gassed conditions deviate from the other two. The bulk zone and the impeller zone are very different when analysed with statistical methods usually applied for turbulent flow. The impeller zone has a wide range of velocities and large rms values whereas the bulk zone has lower velocities with similar rms both for the main flow direction but also for the flow perpendicular to it.

3.2 Simulations

A simulation of the fed-batch yeast process is shown in Fig. 6, where also measured glucose values from the process with feed from the bottom of the reactor are plotted. This simulation is based on material balances over a homogeneous reactor and Monod kinetics is used for the sugar uptake. As is seen from the figure, the simulated glucose concentrations (solid lines) as well as the measured values, declines during the constant feed period according to the theoretical approach. The measured concentrations are levelled by the positions in the reactor which these simulations naturally cannot depict. The measured glucose concentrations at the bottom sampling position are, in this case, best resembled by the simulated glucose concentration. These results are however very sensitive to the values of the maximum glucose uptake rate, as well as the

saturation constant. The figure shows that this type of simulation can not well describe certain parts of the progress of the cultivation.

Simulations of the 30 m^3 reactor, were therefore also performed by use of an integrated CFD approach that takes into account both the kinetic data of the organism as well as the fluid properties. These results are given in Fig. 7a–d. The figure shows a glucose gradient formed according to a change in the point of feeding and due to different cell concentrations, which refers to two different times of the process. The glucose concentration is high near the feed inlet and declines further away. The effect of the feed position appears as a generally higher mean concentration with the top feed compared to the bottom (43.5 compared to 38.8 $mg\,l^{-1}$ for a cell mass of $10\,g\,l^{-1}$).

The measured values are also shown in Fig. 7. The comparison shows the same general picture as the simulation, with declining values away from the feed point. The range of values are however not depicted by the simulation as the values do not decline at the same rate in the simulations. The discrepancy increases at higher cell concentrations. Assuming that the biokinetic model (Monod) correctly describes the glucose uptake rate, the results obtained indicate that the CFD model predicts a too low axial mixing. On the other hand, it must be pointed out that the predicted axial concentrations are also here strongly influenced by e.g. the values of the maximum substrate uptake rate and the saturation constant.

4 Discussion

Sampling during a fed-batch process in a stirred reactor where turbulent conditions prevail, is by far not a trivial procedure. Today, some factors limit our possibilities of resolving the effects of rapid flow fluctuations in an infinitesimal volume of the reactor. The first factor is the sample volume. If the micro-scale analysis of glucose samples is used [9], it is possible to reduce this volume to 400 µl in order to have a sample within a 95% confidence interval (4×100 µl). This is therefore the lower volume limit, which means that the concentration will be a mean value for a zone of this volume in the reactor. Another parameter is the sample flow rate. Depending on the geometry of the sampling device, the overpressure of the reactor, the local hydrostatic pressure, the proximity of the impellers and gas inlet together with the gas content (both air and evolved carbon dioxide), different volumes will be obtained at the opening of the reactor sample valve. In the case of our measurements in the 30 m^3 reactor, the sample rate was twice as high in the bottom of the reactor as in the top. The sampling time of the equipment was therefore designed to give a mean of 2 ml per sampling (± 0.3 ml). The linear out-flow of the different sampling points will therefore deviate and will not be correlated to the linear fluid flow inside the vessel, which may become important [15]. Furthermore, the flow rate is fluctuating inside the vessel due to the turbulence. The result of this mismatch is an uncertainty of the exact location of the sampling position and a reduced capacity of resolving the phenomena in an adequate manner. Further analysis of the nature of fluctuations will give guidance to this problem. In biological systems, the consumption rate of the gradient forming substance, has furthermore to be considered in the choice of sampling time. In this case, the maximum substrate consumption rate was 1.7 g glucose g cells^{-1} h^{-1}. First of all, this means that an acceptable sampling time depends on the concentration of substrate and cells. If the cell concentrations is 20 $g\,l^{-1}$, the maximum of the present process, the cells consume approx. 10 $mg\,l^{-1}\,s^{-1}$. The sampling rate and cell inactivation has then to be completed in 0.1 s if a resolution of 1 $mg\,l^{-1}$ is desired. Now, in fed-batch cultivations, the growth rate is restricted, but even if we reduce the rate to half of the maximum, the sample has to be taken in parts of a second. In order to obtain a glucose mean value, it is probably useful to take the sample at the same rate as the mean flow rate in the vessel and to take this sample over a period of time.

Glucose gradients in time and space were measured in this investigation. The time scale and origin of these fluctuations are due to three phenomena. Firstly, the long declining phase of the fed-batch cultivation with constant feed, reduces the substrate concentration in the tens of hours that the process spans. This is only dependent of the cultivation technique. Secondly, the circulation pattern, due to the macromixing of the reactor as well as the mass transfer on a micro scale, influences the gradient formation. This gives levels of substrate in the reactor which in turn gives the cells an oscillatory pattern of seconds to minutes, depending on the circulation time distribution of the reactor. Thirdly, superimposed on these events, are local rapid fluctuations of seconds of parts of seconds. The reason for these rapid fluctuations are given in the information from the turbulent velocity spectra (Fig. 5). These depict a uniform time scale, both for the bulk and the impeller zone, shown by the fact that the spectra are rather alike in the low frequency range. This seems to be valid for the gassed situation too, a situation that is more comparable to an actual fermentation, even though the spectra deviate in the high frequency range. The conclusion is therefore that there probably exist low frequency, high amplitude phenomena in the reactor. In the bulk zone, where the turbulent intensity is comparatively low, these have the capability of transporting the limiting substrate that is fed to the process, over fairly long distances without substantial mixing. This hypothesis is supported both by the shape of the turbulence spectra (Fig. 5) and by the observed short time oscillations of the substrate concentrations (Figs. 3 and 4). The location of the feed point will also influence the gradient formation depending on the mixing characteristics of the feed area. From the combined information of the turbulence and gradient spectra it seems like feeding in the bulk zone makes the glucose droplets stay intact for a longer period of time. This may be due to the comparatively low degree of high amplitude eddies.

The results of this investigation raise questions about the consequences of gradients in bioreactors and the strategy of dealing with them. One question raised, concerns the control of a process with on-line determinations of the limiting substrate. Depending on the position and the response time of this equipment, different results will be achieved. This is a situation that is comparable to the use of dissolved oxygen measurements for control of bioprocesses. Signals from such measurements, in large scale, should be interpreted in the headlight of the outcome of experiments here described.

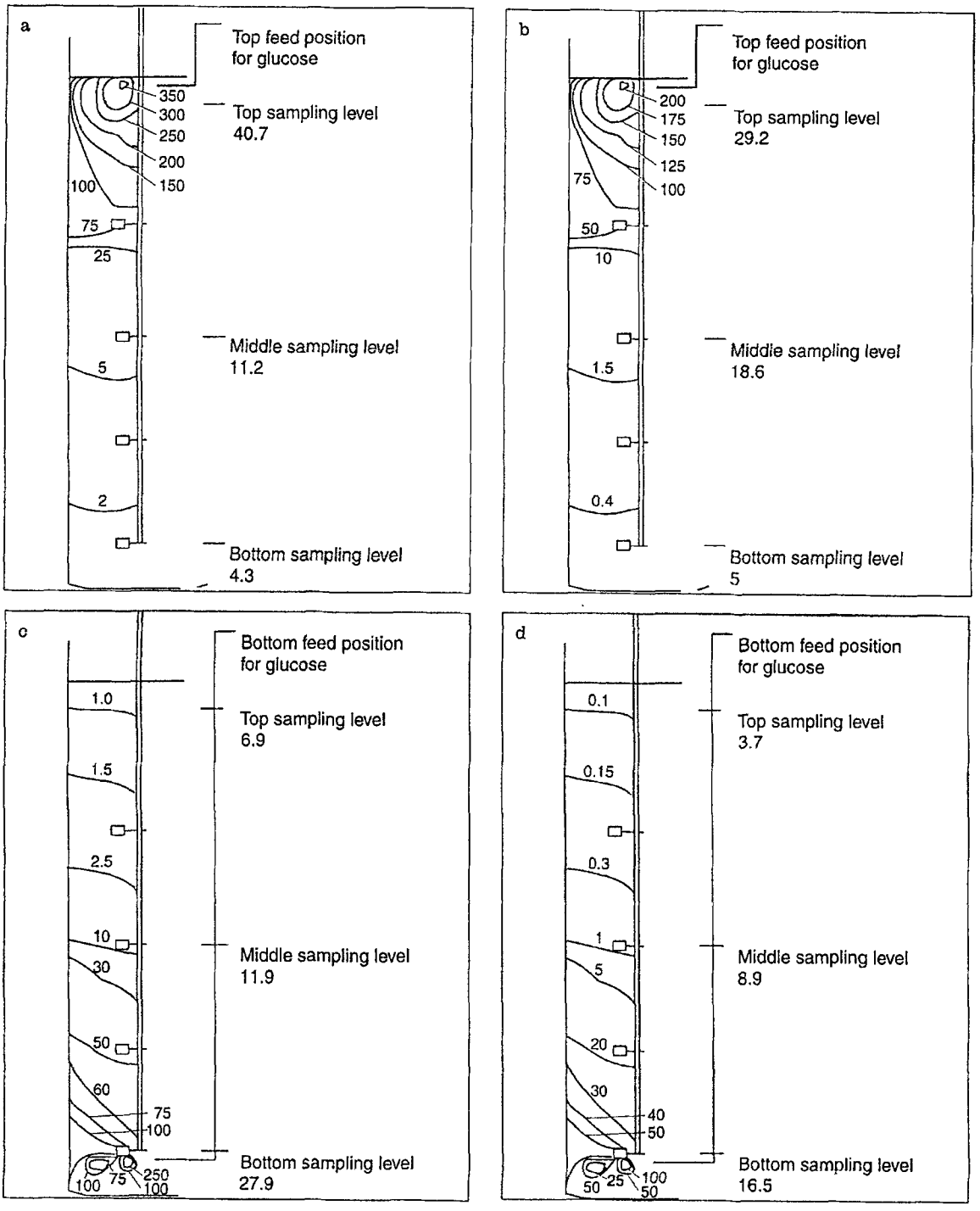

Fig. 7a–d. CFD simulation of concentration gradients in the reactor of Figure 1. The simulations are based on the integrated physical-biological model described in the text. Measured values are in $mg\,l^{-1}$ and are also indicated to the right in the figure. **a** Cell concentration $= 10\,g\,l^{-1}$ feed from top position. Mean substrate concentration $= 43.5\,mg\,l^{-1}$; **b** Cell concentration $= 15\,g\,l^{-1}$, feed from top position. Mean substrate concentration $= 26.1\,mg\,l^{-1}$; **c** Cell concentration $= 10\,g\,l^{-1}$, feed from bottom position. Mean substrate conc. $= 38.8\,mg\,l^{-1}$; **d** Cell concentration $= 20\,g\,l^{-1}$, feed from bottom position. Mean substrate concentration $= 16.7\,mg\,l^{-1}$. Feed rate at both positions $= 53.7\,kg\,h^{-1}$

If one considers the range of the concentrations in the reactor, gradients could of course be larger than those measured, since the feed solution was $600\,g\,l^{-1}$. This concentration would probably be possible to measure if sampling was performed closer to the feed inlet. Local areas of complete exhaust can also be anticipated. Hence, cells are subjected to oscillating glucose concentrations while they are transported in the reactor. An obvious biological effect of the gradients in the space of the reactor, is that the specific glucose consumption rate will oscillate if the cellular response time is instant. As an example from one time event of the studied process, when concentrations were 4, 10 and 44 $mg\,l^{-1}$ at different levels of the reactor, the uptake rates will be 0.07, 0.17 and $0.53\,g\,g^{-1}\,h^{-1}$. Thus, the energy metabolism would be

more than seven times higher for a cell at the top of the reactor, than for one in the bottom. Furthermore, under these conditions ethanol will be produced in the top, in the region of higher glucose concentration, but may be consumed again in the bottom. This will happen if the cells react to the concentration differences immediately. However, such information is not present in literature at the moment. It is known that different strains of microorganisms e.g. E. coli have different possibilities to assimilate the acetic acid formed during overflow metabolism at high glucose concentration. Both in this case and during acetic acid formation with simultaneous formation of carbon dioxide, a reduced yeild of cells will result.

For very low concentrations in the reactor or complete exhaust, another class of biological reactions could be important to study. These are the microbial stress responses. These are known to be rapid and involves such important features as protein transport, proteolysis and folding of the cellular and probably also recombinant formed proteins. Especially the stringent response and the carbon stress are likely to be affected by the short term fluctuations. How all these microbial responses in turn affect a specific product formation is not known.

In order to evaluate the biological effects, new scale-down equipment must be designed for parts of the findings in this work. The large elevations of glucose concentration measured in parts of the reactor and the turbulence spectra together with the gradient characteristics indicate very rapid random fluctuations. No equipment permits today such evaluations. It is clear that this equipment must be based on fluid dynamic studies of the reactor.

In order to deal with control of processes by measurements of the limiting substrate or the prediction of scale-up performance, where the biological responses to gradients formed in large scale is considered, there is a need for more information of the local microenvironment in the bioreactor. It will however, be almost impossible to perform hydrodynamic measurements for all cases of cultivation and for the different reactors used. There is therefore a need for proper pedictions of the effects of a certain change in the process and in the process equipment. A suitable strategy for such evaluations are simulations with a physical-biological model. The properties of an integrated model are briefly described in this paper where the model is challenged by different feed positions. Although there is a qualitative agreement, the question whether the quantitative discrepancy between the measured and predicted concentration profiles are due to shortcomings of the CFD or the biokinetic model or both, is still open. However, if a concordance with the simulations should be proven, the time averaged simulation values should be compared to mean values from the reactor. In our case, the measured concentrations are instant values that are determined by the turbulent fluctuations.

Depending on the source of gradient formation, actions can be taken on both the microbial and on the mixing side to reduce the gradients and the effects thereof. On the physical side one may reduce mixing problems by use of multiple inlets in well-mixed zones. A use of diluted feed solutions of glucose gives a higher volume build up, but will probably reduce the mixing time since the density and viscosity differences are reduced. On the biological side, a microorganism with a higher saturation constant for the substrate can be chosen. For organisms with multiple uptake systems, the high affinity part could be deleted. The result would be an organism that is less gradient sensitive. However, it can be anticipated that oscillations can have a positive effect on production as well and might therefore enhance the process output as was earlier described for the gassing power of the yeast of the Baker's yeast process [8].

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