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Aeration strategy: a need for very high ethanol performance in *Saccharomyces cerevisiae* fed-batch process

Abstract In order to identify an optimal aeration strategy for intensifying bio-fuel ethanol production in fermentation processes where growth and production have to be managed simultaneously, we quantified the effect of aeration conditions—oxygen limited vs non limited culture (micro-aerobic vs aerobic culture)—on the dynamic behaviour of *Saccharomyces cerevisiae* cultivated in very high ethanol performance fed-batch cultures. Fermentation parameters and kinetics were established within a range of ethanol concentrations (up to 147 g l^{-1}), which very few studies have addressed. Higher ethanol titres (147 vs 131 g l^{-1} in 45 h) and average productivity (3.3 vs 2.6 g l^{-1} h^{-1}) were obtained in cultures without oxygen limitation. Compared to micro-aerobic culture, full aeration led to a 23% increase in the viable cell mass as a result of the concomitant increase in growth rate and yield, with lower ethanol inhibition. The second beneficial effect of aeration was better management of by-product production, with production of glycerol, the main by-product, being strongly reduced from 12 to 4 g l^{-1}. We demonstrate that aeration strategy is as much a determining factor as vitamin feeding (Alfenore et al. 2002) in very high ethanol performance (147 g l^{-1} in 45 h) in order to achieve a highly competitive dynamic process.

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Introduction

In recent years, research on improving ethanol production has been accelerating for both ecological and economic reasons, primarily for its use as an alternative to petroleum-based fuels. Ethanol fuel can be produced in two ways: the petrochemical route from the hydration of ethylene and the biotechnological route from microbial fermentation of agricultural biomass. The challenge is to identify the bottlenecks that limit industrial fermentation processes and to develop attractive high-ethanol-performance processes.

Optimising industrial performances in terms of ethanol concentration, productivity and yield necessitates the quantification of the dynamic behaviour of yeast under very high ethanol concentrations that cannot be accessible under continuous culture. By determining the extreme limit coefficients for growth and ethanol production, it will be possible to define optimal monitoring strategies. In a previous paper, we have shown the importance of the vitamin feeding strategy in reaching very high ethanol concentrations (147 g l^{-1}) in a short period of time (45 h) in fed-batch culture (Alfenore et al. 2002). According to previous works, aeration is another important control factor in growth and ethanol production of *Saccharomyces cerevisiae* during alcoholic fermentation.

In continuous cultures, the influence of oxygen supply variation on yeast growth and fermentation capability at steady state has been particularly well investigated (Furukawa et al. 1983 ; Hoppe and Hansford 1984; Ryu et al. 1984; Sweere et al. 1988; Grosz and Stephanopoulos 1990). At a given dilution rate, biomass concentration, biomass/glucose yield and viability were enhanced by increasing air supply while ethanol concentration decreased, both in oxygen-limited and oxygen-unlimited continuous cultures. Inhibition of growth by ethanol decreased under micro-aerobic conditions, where oxygen limitation occurred even though aeration was carried out, compared to anaerobic conditions where oxygen was totally absent (Hoppe and Hansford 1984). Specific ethanol productivity was stimulated when the percentage

of oxygen in the feed increased (Grosz and Stephanopoulos 1990).

Aeration conditions also have an impact on formation of by-products, such as glycerol, which has important implications in downstream steps for industrial ethanol recovery. The control of glycerol production using various oxygen levels or controlled oxygenation has been extensively investigated (Grosz and Stephanopoulos 1990; Kuriyama and Kobayashi 1993; Weusthuis et al. 1994; Costenoble et al. 2000; Franzen 2003). The presence of low oxygen levels has been clearly shown to reduce glycerol production compared to anaerobic continuous culture. Furthermore, in respiratory quotient (RQ)-controlled continuous culture, Franzen (2003) achieved very little or no glycerol production with an RQ value below 17 within a dilution rate range of 0.15–0.35 h^{-1}.

In the present paper, in order to extend the knowledge of the aeration effect on alcoholic fermentation to dynamic processes, the behaviour of yeast was investigated under conditions of very high ethanol concentration and productivity. The aim of this work was to explore dynamic yeast behaviour under different aeration conditions over a range of ethanol concentrations (up to 147 g l^{-1}), which very few studies have addressed. By determining the extreme limit coefficients for growth and ethanol production, it will be possible to define appropriate aeration strategies to intensify ethanol production. For this purpose, fed-batch cultures of *S. cerevisiae* under oxygen-limited and non-limited conditions were compared in terms of growth, ethanol and by-product formation, and cell viability.

Materials and methods

Microorganism, medium and growth conditions

S. cerevisiae strain CBS8066 was supplied by the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Culture conditions and medium composition were as previously described (Alfenore et al. 2002).

Fermentations

Fed-batch experiments were performed in a 20 l fermentor using the Braun Biostat E fermenting system (Braun, Melsungen, Germany) with or without oxygen limitation. The temperature was regulated at 30°C, and the pH at 4 with the addition of a 14% (v/v) NH_3 solution. The fermentor was connected to a computer. Software developed in our laboratory enabled the on-line acquisition of the controlled parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen (DO), NH_3 and antifoam additions) and the monitoring and regulation of these parameters. The pressure in the bioreactor was regulated at 0.2 bar (relative pressure). Under full aeration conditions, the fermentor was flushed continuously with air through a sparger placed at the bottom of the reactor. The flow rate was 100 l h^{-1} corresponding to a maximum vvm of 0.2. The stirring rate was fixed at 400 rpm until the DO reached 20% of saturation, then increased in order to avoid any oxygen limitation in the culture. Under micro-aeration conditions, the fermentation medium was flushed with air in the headspace of the reactor at a flow rate of 100 l h^{-1}. The stirring rate was maintained at 300 rpm. No change was applied either to the flow rate or to the stirring rate during the fermentation. After inoculation, and concomitant with growth, the DO decreased to zero 2 h after inoculation and stayed at zero for the duration of the culture.

The bioreactor was also equipped with a foam detection system and controlled addition of Struktol (Schill & Seilacher, Hamburg, Germany) antifoam. The maximum amount of Struktol added was 1.2 ml. The fermentor was supplied with a sterile feed using a peristaltic pump (Masterflex). Fermentation was initiated with a glucose concentration of 100 g l^{-1}. The glucose feed concentration was 700 g l^{-1}. The mass of glucose added to the fermentor was estimated instantly by weighing. The glucose concentration in the fermentor was automatically calculated by the software based on all the inlet and outlet volumes (base addition, sampling, antifoam addition), the added mass, the density, and the glucose concentration in the feeding solution measured by the DNS (3,5-dinitrosalicylic acid) method. The density was measured by weighing a known volume.

Chemicals

Chemical products (glucose, salts, oligo-elements, orthophosphoric acid and NH_3) were provided by Prolabo, France, the vitamins from Sigma (St. Louis, Mo.) and the sodium glutamate from Merck (Darmstadt, Germany). All products were of the highest analytical grade available.

Feeding strategy

Vitamin feeding strategy

All fermentations were performed with an exponential feeding of biotin and vitamins based on the growth profile (Alfenore et al. 2002).

Glucose feeding strategy

Fermentation was initiated with a glucose concentration of 100 g l^{-1}. Whenever the residual glucose concentration fell below 20 g l^{-1}, a glucose feeding was carried out to restore a glucose concentration of 100 g l^{-1}. When the ethanol concentration was above 90 g l^{-1}, glucose feeding brought the concentration back to 50 g l^{-1} (see Fig. 1). This set-point allowed minimisation of osmotic stress of cells already stressed by the high ethanol concentration.

Analytical methods

Biomass, substrate and product concentrations were measured according to previously described analytical methods (Alfenore et al. 2002).

Determination of cell viability

The cell viability was determined by the Methylene Blue technique (Postgate 1967). A 200 μl sterile solution of Methylene Blue (0.3 mM in 68 mM Na_3 citrate) was mixed with 200 μl yeast suspension diluted to reach an OD_{620} of 0.4–0.7. After 5 min incubation, the mixture was shaken and placed in a Thomas's counting chamber. The number of stained (non active cells) or unstained (active cells), and the number of buds, were counted in five different fields with a total of at least 200–300 cells. The percentage of viable cells was the number of unstained cells (live cells) divided by the total number of cells (stained plus unstained). Under the conditions used, the mean (m) of the viability is estimated with an accuracy of 10% (Nielsen et al. 1991). This means that the interval $m\pm0.1m$ contains the true value of viability with a probability of 95%.
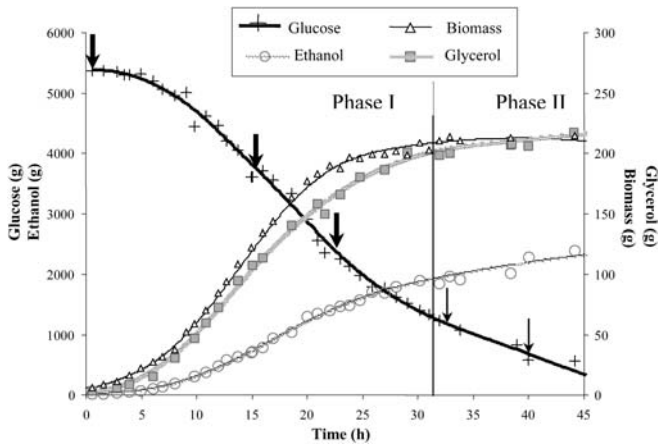
Fig. 1 Changes in the mass of glucose, ethanol, glycerol and biomass in micro-aerobic fermentation. *Thick arrows* Glucose feeding to a final concentration of 100 g l^{-1}, *thin arrows* glucose feeding to a final concentration of 50 g l^{-1}

Results

Impact of aeration conditions on fermentation parameters

To evaluate the impact of aeration on ethanolic fed-batch fermentations, two aeration conditions were tested. A first fermentation was carried out under full aeration, i.e. aeration conditions were set to avoid any oxygen limitation during the culture. The results were described in a previous paper (Alfenore et al. 2002).

The second condition, micro-aeration, was carried out in the same way as the one described above, with the exception of aeration. The aeration rate was set in order to cultivate the yeast under oxygen limitation. However, conditions of oxygen limitation were reached 2 h after inoculation. The mass of glucose consumed, and ethanol and biomass produced during the micro-aerated fed-batch culture are shown in Fig. 1. As previously described, two phases were observed: a simultaneous biomass and ethanol production phase (phase I) was followed by a decoupling of growth and ethanol production (phase II).

Growth and metabolite production parameters are summarised for the two fermentations in Table 1. Direct comparison of metabolite concentrations was valid because the final volume of the two fermentations was similar. Thus, dilution factors do not affect interpretation of the variation of the different parameters.

Full aeration led to an increase in the final biomass and ethanol concentration of 23% and 10%, respectively. Non-limiting oxygen conditions improved the global yield of growth on glucose (0.055 g g^{-1} vs 0.045 g g^{-1}) but diminished slightly the yield of ethanol production on glucose (0.43 g g^{-1} vs 0.46 g g^{-1}). Moreover, micro-aeration resulted in a 3-fold higher final concentration of glycerol as well as a 4-fold higher glycerol on glucose yield. Other by-products—acetic acid and succinic acid— were affected to a lesser extent: microaeration led to a decrease in acetate yield from 8 mg g^{-1} to 1.8 mg g^{-1} and
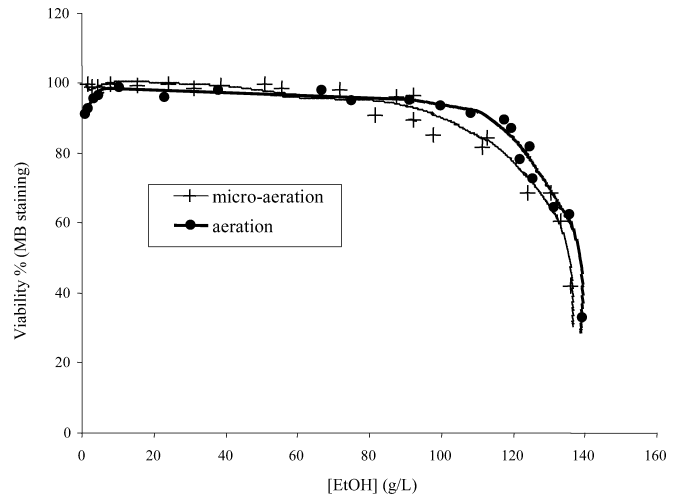

Fig. 2 Comparison of the percentage of Methylene Blue-stained cells (viability %) as a function of ethanol concentration in aerobic and micro-aerobic fermentations

Table 1 Effect of aeration conditions on biomass, ethanol production and by-product formation in *Saccharomyces cerevisiae* CBS 8066 during fed-batch fermentations at 30°C. vp_{max} Maximum specific ethanol production rate, μ_{max} maximum specific growth rate, X_{max} maximum cell mass, $Y_{i/S}$ yield factors of constituent i on glucose (i: *X* biomass, *Eth* ethanol, *Gly* glycerol, *Ace* acetate, *Succ* succinate), *GL* Gay Lussac

Fermentation		Aeration	Micro-aeration
Fermentation time	(h)	45	45
Final volume	(l)	18.9	17.7
v_{Pmax}	(g g^{-1} h^{-1})	2.20	1.25
μ_{max}	(h^{-1})	0.43	0.29
X_{max}	(g)	261	212
$Y_{X/S}$[a]	(g g^{-1})	0.055	0.045
$Y_{Eth/S}$	(g g^{-1})	0.43	0.46
$Y_{Gly/S}$	(g g^{-1})	0.010	0.042
$Y_{Ace/S}$	(mg g^{-1})	8	1.8
$Y_{Succ/S}$	(mg g^{-1})	1.2	2.6
[Ethanol] final	(g l^{-1})	147	131
	(GL)	18.9	16.8
[Glycerol] final	(g l^{-1})	4.0	12.2
Average ethanol productivity	(g l^{-1} h^{-1})	3.3	2.6
Maximum ethanol productivity	(g l^{-1} h^{-1})	9.4	7.6

[a] Growth yield was calculated during the growth phase only

a slight increase in yield of succinate (1.2 vs 2.5 mg g^{-1}). Taking into account all of these metabolites, the two fermentations showed small carbon and redox balance deficits below 10%.

Cell viability was also quantified during the two fermentations. As shown in Fig. 2, no significant effect on cell viability due to aeration conditions was observed.

Impact of aeration conditions on kinetic parameters

The specific growth rate and specific ethanol production rate were plotted versus ethanol concentration (Fig. 3). At
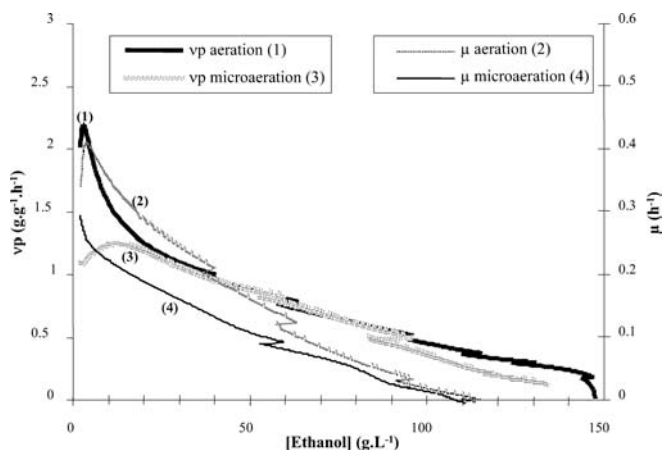
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Fig. 3 Specific growth rate (μ) and specific ethanol production rate (vp) vs ethanol concentration for aerobic and micro-aerobic fermentations

ethanol concentrations below 60 g l^{-1}, growth was favoured by the non-limiting oxygen conditions and reached a maximum rate of 0.43 h^{-1}, whereas it did not exceed 0.29 h^{-1} under oxygen limitation. In micro-aerated culture, the growth rate decreased faster with increasing ethanol titre compared to the fully aerated culture. The maximum specific ethanol production rate was higher in fully aerated fermentation (2.2 vs 1.5 g g^{-1} h^{-1})

At ethanol concentrations above 60 g l^{-1}, the profiles of growth and specific ethanol production rates were similar as a function of ethanol concentration. Decoupling occurred under both conditions at an identical ethanol concentration below 110 g l^{-1}.

The combined effect of aeration on both growth and ethanol production led to better average and maximal ethanol productivity (Table 1).

Discussion

For the purpose of intensifying ethanol production, the aim of our investigation was to quantify the dynamic behaviour of *S. cerevisiae* under aerobic and micro-aerobic conditions in very high performance fed-batch cultures characterised by very high ethanol titres (147 g l^{-1}) reached in a short fermentation time (45 h). The effects of oxygen-limited and non-limited conditions on biomass and metabolite concentrations, cell viability, yields and specific rates were evaluated in a dynamic process reaching very high ethanol titres up to 147 g l^{-1}.

In the two fermentations, the specific growth rate decreased with increasing ethanol concentration and stopped at 110 g l^{-1} ethanol. Within the range of ethanol concentrations from 0 g l^{-1} to 60 g l^{-1}, the inhibitory effect of ethanol on growth was significantly lessened under aerobic conditions. This observation agrees with reported studies on chemostat cultures at different degrees of micro-aeration (trace levels of DO) and at different
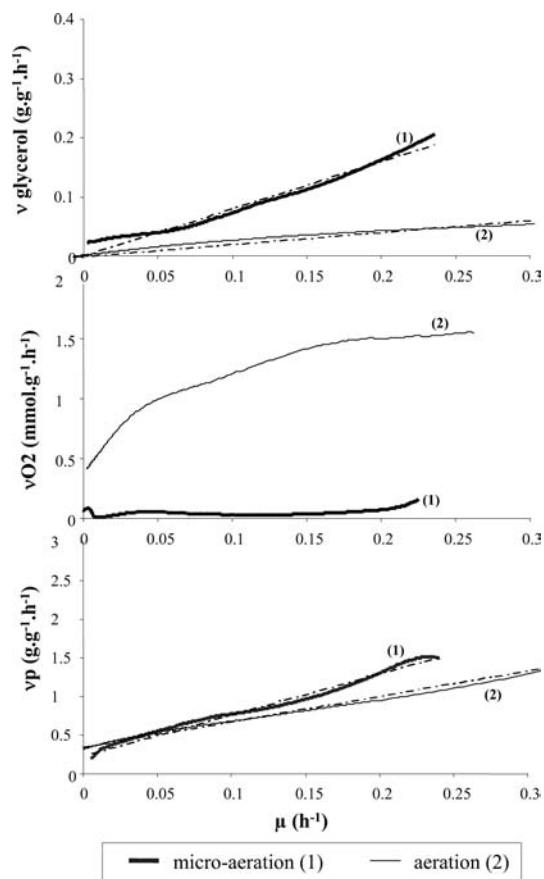

Fig. 4 Specific glycerol production rate ($v_{glycerol}$), specific oxygen consumption rate (v_{O2}) and specific ethanol production rate (vp) vs specific growth rate (μ) for aerobic and micro-aerobic fermentations

dilution rates (Hoppe and Hansford 1984) and is extended in this study to dynamic fully aerated cultures.

The growth yields on glucose were determined from our data and confirmed the well-known beneficial effect of aeration (Table 1) (Furukawa et al. 1983; Ryu et al. 1984; Grosz and Stephanopoulos 1990), which is commonly explained by the fact that aeration leads to an increased participation in more energetically oxidative metabolism.

As a result of increases in both the growth specific rate and yield on glucose, the cell mass reached at the end of growth was 23% higher in the oxygen-unlimited culture compared with the micro-aerobic culture.

Glycerol production was coupled to growth in the two fermentations as shown in Fig. 4. However, it can be observed that micro-aeration strongly favoured glycerol production, leading to an approximately 4-fold higher glycerol-biomass yield. The increase in glycerol production in response to lower aeration is generally correlated to the amount of excess NADH to be oxidised (Van Dijken and Scheffers 1986; Kuriyama and Kobayashi 1993; Weusthuis et al. 1994; Costenoble et al. 2000; Franzen 2003). Oxygen limitation indeed led to lower respiration as shown in Fig. 4. Therefore, glycerol

production contributed to restoring the redox balance in the cells.

The specific rate of ethanol production was also coupled to the specific growth rate in both fermentations (Fig. 4). As a result, the higher maximal growth rate reached under full aeration conditions led to a higher maximal specific ethanol production rate. However, micro-aerobic conditions favoured the ethanol on biomass yield, calculated from Fig. 4 as 5.5 and 3.3 g ethanol [g dry cell weight (DCW)]$^{-1}$ under micro-aerobic and aerobic conditions, respectively.

The coupling effect between ethanol production and growth can be explained by energetic considerations. With the hypothesis that the five main reactions involved in the energetic balance are oxidative-phosphorylation, and production of ethanol, glycerol, succinic acid and acetic acid, theoretical yields of production/ATP consumed were calculated as:

$Y_{O2, ATP} = 2\times$ P/O mol ATP formed per mole of O_2 consumed, $Y_{ethanol, ATP} = 1$ mol ATP formed per mole of ethanol produced, $Y_{glycerol, ATP} = -1$ mol ATP formed per mole of glycerol produced, $Y_{succinic, ATP} = 2$ mol ATP formed per mole of succinic acid produced, $Y_{acetic, ATP} = 1$ mol ATP formed per mole of acetic acid produced. The catabolic ATP specific production rate is then evaluated from the following equation:

$$v_{ATP} = \sum_i \left(\frac{Y_{i,ATP} \times v_i}{MM_i} \right) \quad (1)$$

with i corresponding to O_2, ethanol, glycerol, succinic or acetic acid, MM molecular mass (g mol^{-1}) and v specific rate (g g h^{-1}). P/O was fixed within the range 1–1.2 as reported in Vanrolleghem et al. (1996).

The global ATP/biomass yield ($Y_{x,ATP}$), calculated as (μ the specific growth rate), is slightly higher under limited-aeration conditions compared with unlimited-aeration conditions [ranging from 12 to 13 depending on the P/O ratio in full aeration compared to 9.5 g DCW (mol ATP)$^{-1}$ under oxygen limitation]. These results confirm previous data on the beneficial effect of aeration on $Y_{x,ATP}$, varying between 7.5 and 12 g DCW (mol ATP)$^{-1}$ (Grosz and Stephanopoulos 1990). Although acetate and succinate were taken into account in the calculation of $Y_{x,ATP}$, it should be noted that their contribution was negligible. These data suggest that when oxidation of glucose is limited in micro-aeration culture, the energetic requirement for yeast growth is mainly fulfilled by the increase in ethanol production. However, the different values of $Y_{x,ATP}$ calculated could be explained by the following: If the ATP requirement for anabolism—strictly related to the chemical biosynthesis pathways—is assumed to be identical for the two aeration conditions, the energetic efficiencies of non-chemically dependent processes are likely to vary. For transport systems energetically related to the proton gradient, the cost in "equivalent" ATP may be higher in the absence of an external electron acceptor. Such mechanisms include transport of metabolites and salt between the cytosol and the external medium, and also between intracellular compartments.

We have previously shown the importance of obtaining viable cells in order to reach high ethanol performances (Alfenore et al. 2002). In this study, we quantified cell viability during microaeration fermentation. No significant effect was observed on cell viability due to aeration conditions when compared with the fully aerated culture (Fig. 2). This suggests that the vitamin feeding strategy is the key parameter in maintaining cell viability at a high level (90%) up to an ethanol concentration of 115 g l^{-1}, regardless of the aeration conditions.

In conclusion, the beneficial effect of non-limiting oxygen conditions for production of ethanol in a dynamic process such as a fed-batch culture was demonstrated in this study. Fully aerated culture allowed higher viable cell mass to be achieved due to the increase in growth rate and yield, and was sufficient to counteract the decrease in ethanol production yield on glucose. The latter is the key factor in obtaining high ethanol productivity. The second beneficial effect of aeration is better management of by-product production since production of glycerol, the main by-product, was reduced.

We showed that nutrient feeding and aeration strategies both act as determining factors in very high ethanol performance (147 g l^{-1} in 45 h) and are critical in achieving a highly competitive process compared to performances reported in the literature: 162 g l^{-1} in 20 days with *Saccharomyces sake* (Hayashida and Ohta 1981), 159 g l^{-1} in 175 h using very high gravity technology (Thomas and Ingledew 1992; Thomas et al. 1993), and 134 g l^{-1} in a multi-stage continuous fermentation system (Bayrock and Ingledew 2001). These results are of practical importance in the development of high cell density and high ethanol production fermentation processes where it is crucial to manage biomass production, cell viability, ethanol production and by-product minimisation.

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