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# Batch and fed-batch cultures of *E. coli* TB1 at different oxygen transfer rates

## Effect of stirring and oxygen partial pressures on cell growth and cytochrome b5 production

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**Abstract** Batch cultures of *E. coli* TB1/pUC13 were carried out at different oxygen transfer rates (OTR) enhanced by the increase of stirring rate and by the increase of air total pressure of the bioreactor. These two variables showed to have little effect on cell growth but a negative effect on cytochrome b5 (recombinant protein) production. However, this effect was more significant of high stirring rates than for values of pressure up to 0.4 MPa.

The effects of stirring and pressure were also investigated for fed-batch mode operation. In this type of cell cultivation high cell densities are reached, thus a high capacity of oxygen supply of the system is required. To compare the two ways of improving OTR, cell behaviour was followed in two bioreactors at different operational conditions giving the same maximum OTR value. The first one operated at a high stirring rate (500 rpm) and at atmospheric pressure (0.1 MPa) and the other one at high air pressure (0.48 MPa) and low stirring rate. The increased pressure seemed to be a better way of ensuring an adequate oxygen supply to a culture of *E. coli* TB1 cells than an increased stirring rate. For the high pressure experiment a higher cellular density was reached, as well as a higher cyt.b5 expression which led to a 4-fold increase in final productivity.

These experiments showed that bioreactor pressurization can be successfully used as a means of enhancing oxygen mass transfer to shear sensitive cell cultures.

**List of symbols**

| | | |
|---|---|---|
| $C$ | $g \cdot dm^{-3}$ | Oxygen concentration |
| $C^*$ | $g \cdot dm^{-3}$ | Oxygen solubility |
| $D$ | $h^{-1}$ | Dilution rate |
| H | $MPa \cdot g^{-1} \cdot dm^3$ | Henry's constant |
| $K_L a$ | $h^{-1}$ | Volumetric mass transfer coefficient |
| OTR | $mg\text{-}O_2 \cdot dm^{-3}\ h^{-1}$ | Oxygen transfer rate |
| $pO_2$ | MPa | Oxygen partial pressure |
| $S$ | $g \cdot dm^{-3}$ | Glucose concentration |
| $S_f$ | $g \cdot dm^{-3}$ | Glucose concentration in the feed |
| SOD | $U \cdot mg\text{-}Prot^{-1}$ | Superoxide dismutase activity |
| $X$ | g-Dry Cell Weight $\cdot dm^{-3}$ | Cell concentration |

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## 1 Introduction

Recombinant DNA technology allows faster large-scale production of important biomolecules at higher concentrations than they can be found in natural sources. Industrial success of this technological application depends on cloning techniques development, as well as on optimization of culture conditions.

Dissolved oxygen is one of the factors which dramatically affects cell growth and recombinant protein production of aerobic cultures.

For *E. coli*, as one of the most popular host, it is well known that oxygen availability affects cellular yield [1]. This is particularly important because almost all of the recombinant proteins expressed in this microorganism remain inside the cell, in spite of all the efforts being made in heterologous protein secretion research [2]. Therefore, one of the goals of cultivation conditions optimization is to reach high cell densities. It is crucial to ensure an adequate oxygen supply to the media.

There are several methods of enhancing oxygen transfer rate to a culture: increasing stirrer speed and/or air sparging rate, enriching air inlet with pure oxygen [3], increasing oxygen partial pressure by raising the total pressure of the bioreactor, etc. Yang and Wang [4] used this method to cultivate *E. coli* cells. They increased maximum dry cell weight in a batch process by headspace pressurization up to 0.27 MPa. This was also applied to *S. cerevisiae* cells. Air and oxygen partial pressure increase inhibited growth of Baker's yeast cells in a discontinuous reactor [5].

The adequate method for improving oxygen transfer rate for each case depends on the effects of the factors manipulated to ensure a certain OTR on cell growth, as well as on recombinant protein expression. The effects of oxygen on recombinant proteins production are strongly dependent of the strain/plasmid used [6].

Results of Ryan et al. [7] with *E. coli* $\beta$-lactamase producer cultivated in a batch mode at different values of air flow rate, showed that oxygen supply affects plasmid content and plasmid transcription. Plasmid stability can also be affected by oxygen insufficiency as it was found by Hopkins et al. [8]. After a dissolved oxygen shock host

cells of *E. coli*/pKN401 overtook recombinant cells in the absence of a selective pressure.

Therefore, new studies should be performed whenever a new strain or plasmid is concerned. In this work, *E. coli* TB1/pUC13 [9] was grown at different oxygen supply rates in batch cultures and the effects of OTR, stirring and pressure on biomass and r-protein (recombinant protein) cytochrome b5 production were investigated.

Cytochrome b5 (cyt.b5) of the endoplasmic reticulum of mammalian liver is a small haemoprotein of 13.6 KD which plays a central role in a variety of electron-transfer bioreactions [2]. Its successful gene coding and expression in *E. coli* TB1 cells provides an excellent way for obtaining large amounts of purified protein for many biophysical and chemical studies [9].



Fed-batch culture was also studied in order to compare the stirring and pressure effects for cases where high OTR values are needed. In fed-batch operation mode it is possible to control the oxygen uptake rate to low levels but as high cellular densities are usually reached, high OTR capacity of the bioreactor system is required. Enhancing OTR by increasing the stirring rate can present some energetical cost and cell shear stress sensitivity limitations. This work has as main aim to investigate the possibility of using increased air pressure to improve oxygen transfer to an aerobic culture and thus to increase cell growth and cyt.b5 yield. On the other hand, increase in oxygen partial pressure may cause some oxidative stress to the cells [10] thereby preventing cell growth and production. Therefore, the activity of an antioxidant enzyme (SOD) used by the cells as a defence against oxygen excess was also monitored.

## 2
## Materials and methods

### 2.1
### Strain: storage and inoculum

*Escherichia coli* TB1 cells, genetically modified with the plasmid pUC13 [8] which contains the cyt.b5 expression gene and confers ampicillin resistance to the cells, were grown in a medium composed of 1% (W/V) peptone, 0.5% yeast extract, 0.5% sodium chloride, supplemented with 100 μg/ml ampicillin and 1% glucose for inoculum and batch cultures. Cells were maintained at −70 °C in aliquots of 20% (V/V) glycerol.

Inoculum for each fermentation experiment was prepared using a glycerol stock from which a agar plate ($amp^+$) was streaked and incubated at 37 °C overnight. Pink colonies (due to the cyt.b5 accumulation) were transfered to 15 ml medium and were incubated for 16 h. This culture was used to inoculate a shaken flask with 150 ml of the same medium which was also incubated for 16 h at 37 °C. Consequently, this was used for inoculation of media in the fermenters. The same procedure was used in all experiments to ensure that the inoculum contained no plasmid-free cells.

### 2.2
### Fermentation experiments

Batch cultures: To study the effect of OTR and stirring on cell growth and r-protein expression in batch cultures, a 2 l fermenter (BIOLAB, B. BRAUN) with 1.5 l working volume was used. Experiments were carried out for 30 h at 37 °C, 1 vvm of aeration, 1 atm of pressure, at pH = 7.0 adjusted automatically with the addition of 4 N NaOH and 4 N HCl and at stirring rates from 100 to 400 rpm. Global oxygen mass transfer coefficients in Biolab fermenter were determined by the dynamic method at the middle of the exponential growth phase using a Mettler polarographic probe to measure the dissolved oxygen.

A pressurized reactor was used to perform fermentations at higher pressure values. A 500 ml stainless steel reactor (PARR 4842) was used. Experiments were carried out in the same conditions as above except that stirring rate was fixed at 200 rpm and pH was not controlled. However, to avoid great changes in pH, the medium was prepared in phosphate buffer pH = 7.0, 0.4 M. The operating pressure was set adjusting air compressed inlet pressure and regulating air outlet valve of the reactor.

Fed-batch cultures: Fermentations were carried out in both bioreactors described above. Experiments were conducted simultaneously with 10% (V/V) inoculums from the same stock. No pH control was made and all the media used were buffered.

All the conditions were the same for both bioreactors except for pressure and stirring rate. For BIOLAB fermenter working at atmospheric pressure (0.1 MPa) 500 rpm of stirring rate was used and for PARR reactor the values of pressure and stirring rate were 0.48 MPa and 200 rpm, respectively. These conditions were chosen to give identical values of OTR (870 ± 80 mg $O_2$/lh, determined by sulfite method in blank assays) for both reactors. Experiments started-up in batch mode with about 2 g/l of initial glucose concentration. Feeding of a 20 g/l glucose media was performed at constant flow rate for about 28 h, giving dilution rates varying from 0.16 $h^{-1}$ to 0.03 $h^{-1}$ and aeration from 0.67 to 1.25 vvm (constant air flow rate).

### 2.3
### Analytical methods

Cellular concentration was evaluated by the optical density measured at 600 nm. Glucose concentration was determined using the 3,5-dinitrosalycilic acid (DNS) method and acetate was determined by HPLC analysis.

Cyt.b5 and SOD were determined after cell disruption. *E. coli* TB1 cells were harvested by centrifugation at 4 °C and 9000 rpm, washed in phosphate buffer (pH = 7.0, 20 mM) and then frozen at −20 °C for latter disruption. Cell disruption was accomplished by ultrasounds using a procedure described before [11]. Quantification of cyt.b5 was then achieved by absorbance measurements of the clear supernatant at 410 nm, using the Lambert-Beer law with a known extinction coefficient ($\varepsilon = 130\,mM^{-1}\,cm^{-1}$) [9].

SOD was assayed by the method of McCord and Fridovich [12] and total soluble protein was determined by the Bradford's method [13].

## 3
## Results and discussion

Oxygen transfer rate (OTR) from gas phase to liquid medium is a function of several parameters and can be calculated by the following expression:

$$OTR = K_L a(C^* - C) \quad , \qquad (1)$$

where $K_La$ is the overall volumetric mass transfer coefficient which depends on reactor and media characteristics and operational conditions as stirring and aeration rates, $C$ is dissolved oxygen concentration and $C^*$ is oxygen solubility. Thus, OTR can be increased for instance by either increasing the stirring rate which increases $K_La$, and/or increasing the oxygen partial pressure ($pO_2$) in the gaseous phase. The $pO_2$ by Henry's law is going to increase $C^*$, $H$ is the Henry constant:

$$pO_2 = HC^* \quad . \tag{2}$$

To compare these two different ways of enhancing OTR, batch and fed-batch cultures of *E. coli* TB1 cells were performed at increased stirring rate and air pressure.

Figure 1 shows the results obtained for batch cultures of *E. coli* TB1 at atmospheric pressure and at increased stirring rates. As it can be seen from growth curves (Fig. 1a) the increase in stirring from 100 to 400 rpm has a small effect on biomass production.

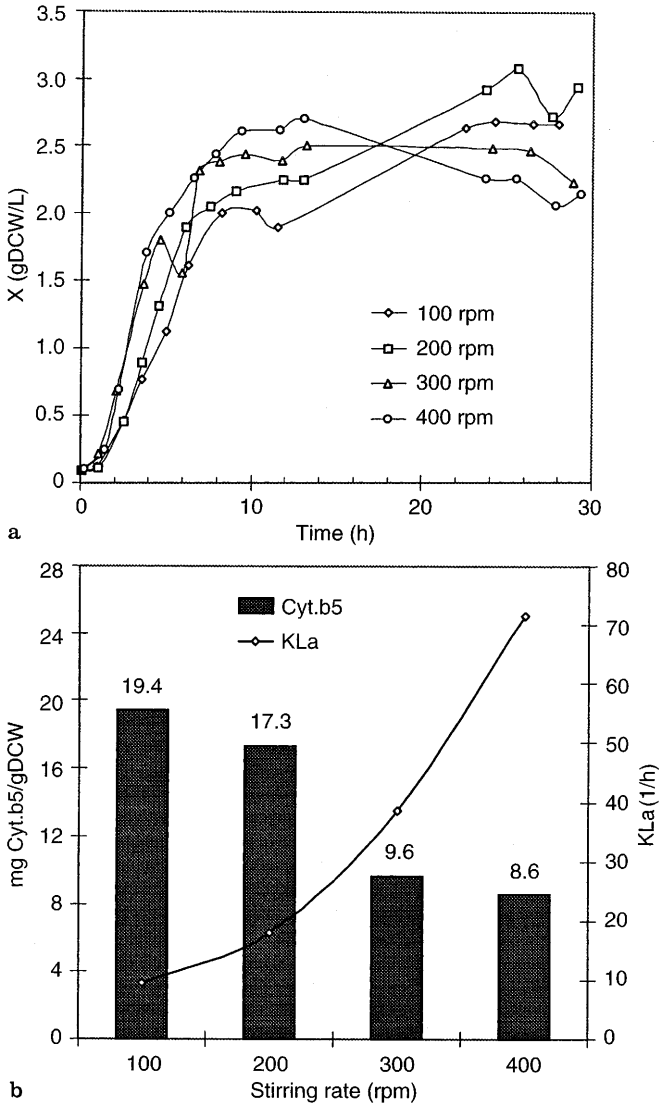


**Fig. 1.** **a** Growth curves for *E. coli* TB1 in batch cultures at atmospheric pressure, **b** Levels of cyt.b5 production for batch cultures at increased stirring rates and respective values of $K_La$

However, it seems that at the late exponential phase, initial time of stationary phase, higher stirring rates are advantageous for the biomass formation, but have an adverse effect on the late stationary phase. This may decrease considerably the final productivity of cyt.b5, an intracellular protein, which is produced in its holoform only at the stationary phase, when cells grow at low rates. Gallagher et al. [14] showed that cyt.b5 accumulated predominantly in a haem-deficient apoform in the batch growth because cells were unable to synthetise the haem prosthetic group.

Figure 1b shows the results for cyt.b5 measured at the end of each experiment. Increase in stirring rate enhanced greatly $K_La$, but had a negative effect on the cyt.b5 (holoform) production. As this r-protein is produced at the stationary phase, when cells grow slowly with low oxygen uptake rates, the increase of OTR may not be necessary and the consequent increase in shear stress can cause some cell damage and consume some energy resources needed for the cyt.b5 expression. On the other hand, dissolved oxygen may have itself a direct effect on cyt.b5 production if the heterologous protein is used by the cells in electron-transfer reactions involving oxygen.

Stanfield et al. [15] reported a 3-fold indution of native cyt.b5 in *S. cerevisiae* cells in chemostat cultures by reducing the dissolved oxygen concentration from 70% to 15%. This observation was explained by the fact that cyt.b5 supplies electrons to oxygen-requiring reactions such as sterol and lipid desaturation and it is possible that an accumulation of substrate due to lack of oxygen would cause feedback induction of this enzyme.

From the results obtained for batch cultures in the pressurised reactor (Fig. 2) it seems that pressure up to 0.4 MPa is less injurious to r-protein production than stirring. The effects of pressure on biomass formation (Fig. 2a), acetate production and glucose consumption (data not shown) are practically negligible. The decrease in cyt.b5 levels in the final culture (Fig. 2b) was smaller than it was observed for cultures at increased stirring rates. Thus, pressure may be used as a means of enhancing OTR for high density cultures with high oxygen demands, which is the case of cultures produced in fed-batch operation mode. This operation mode is commonly used for r-protein production and usually it is necessary to blend pure oxygen with air in the sparge gas [16].

Figure 3 shows the results obtained for fed-batch experiments. As it can be seen from Fig. 3a, *E. coli* TB1 cells grew better in the pressurized reactor (P) than in the Biolab (B) fermenter at atmospheric pressure and high stirring rate. In the latter case, after about 8 h of feeding, the cell concentration began to decrease (Fig. 3a). Glucose feeding was the same in both reactors and the glucose concentration profile with time observed was similar for both reactors (Fig. 3b), but acetate and probably production of other acids (pH increased with time in Biolab fermenter Fig. 3c) was considerably lower in atmospheric pressure bioreactor. Thus, glucose specific uptake rate for cells in this reactor was significantly higher than in the pressurized reactor probably due to a higher substrate consumption for cell maintenance.

Acetate production in the pressurized reactor was due to the high glucose feeding rate at the start-up of the experi-

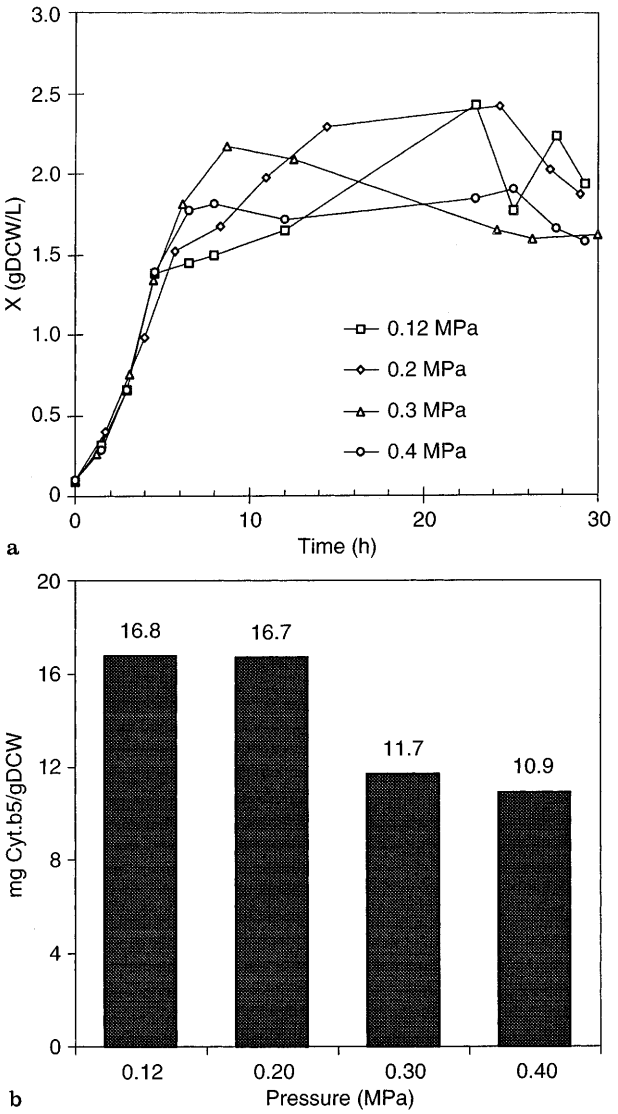


**Fig. 2.** **a** Growth curves of *E. coli* TB1 in batch cultures at increased air pressure, **b** Effect of pressure on levels of cyt.b5 obtained in batch cultures

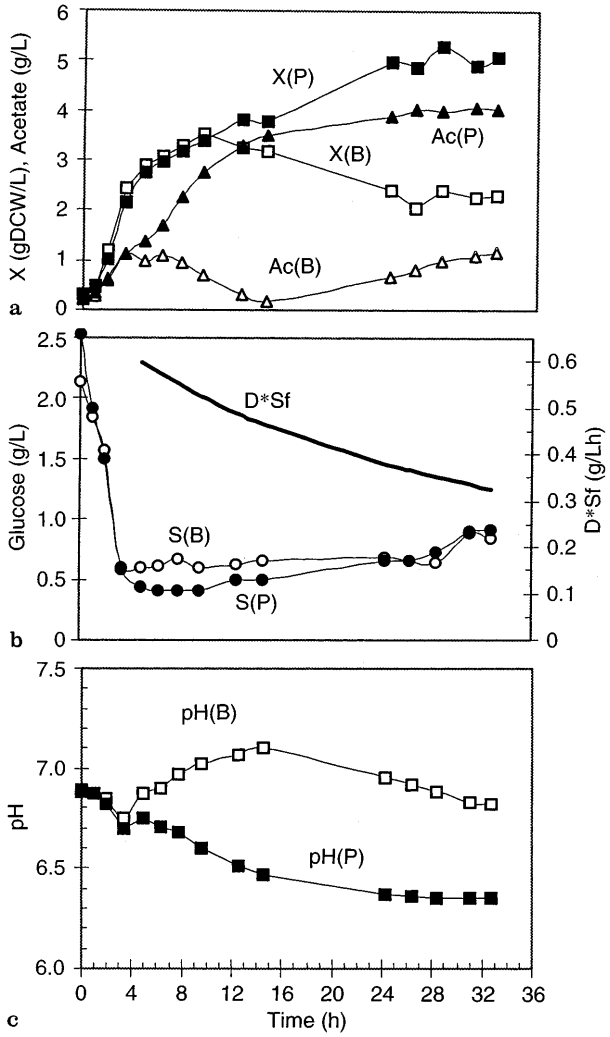


**Fig. 3.** **a** Growth curves (X) for *E. coli* TB1 and acetate (Ac) production in fed-batch cultures in the Biolab fermenter (**B**) and pressurized reactor (**P**) **b** Glucose consumption and substrate feeding rate variation with time, **c** pH evolution for both reactors

ment, which probably exceeded the respiratory capacity of the cells. As expected acetate secretion rate decreases with time because glucose feeding rate $(D^*S_f)$ also decreased. Thus acetate production was not due to oxygen limitation which was prevented by setting an adequate value of OTR for the cell concentration at the start time, according to the oxygen uptake capacity values found in literature [17].

Cyt.b5 production was also higher in the pressurized reactor (Fig. 4a). Cyt.b5 final productivity was considerably greater in pressurized reactor than in the non-pressurized one, being 2.4 mg cyt.b5/lh and 0.6 mg cyt.b5/lh, respectively. Cyt.b5 expression started immediately after the beginning of the substrate feeding which is in accordance with the hypothesis that holoform cyt.b5 production is related with the specific growth rate. Other r-protein expression in *E. coli* are specific growth rate dependent, such as for instance, α-amylase produced by *E. coli* JM107 [18]. The acetate concentration at the levels reached in our experiments did not inhibit r-protein expression. It must however be emphasized that an acetate concentration $> 10\,g \cdot dm^{-3}$ may cause inhibition of r-protein production in *E. coli* cells as reported by Shimizu et al. [19].

Figure 4b shows the results obtained for SOD analysis and as it can be seen there is little difference in SOD levels between the results obtained for Biolab fermenter and for the pressurized reactor. In the latter reactor, SOD is slightly induced at the end of the experiment. Induction of SOD by pressurized oxygen was reported by Taniguchi et al. [20] for *Streptococcus lactis* cells. Induction of this enzyme by increased oxygen partial pressure is probably caused by the stimulation of the superoxide radicals $(O_2^-)$ formation. This reactive oxygen species is the substrate for SOD. Therefore, bioreactor pressurization may also be used as a way of inducing high activity levels of antioxidant enzymes like SOD, which might have potential applications on dairy and pharmaceutical industry.

## 5 Conclusion

*E. coli* TB1 cells showed to be more sensitive to high shear stress caused by stirring than to air total pressure up to

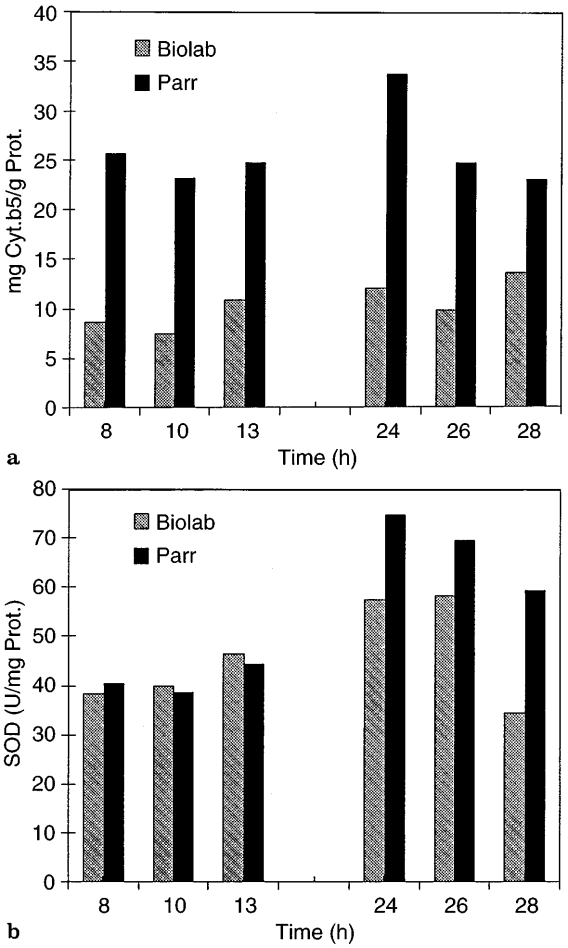


**Fig. 4. a** Levels of cyt.b5 and SOD **b** on *E. coli* TB1 in fed-batch cultures at high stirring rate (Biolab) and high pressure (Parr)

0.4 MPa in batch growth. For this cultivation mode the increase in $K_La$ had a negative effect on the cyt.b5 expression.

In fed-batch cultures pressurization of the bioreactor seems to be more adequate to ensure a certain OTR value than stirring. Furthermore, OTR values can be controlled easily by simply manipulating the inlet air pressure. For *E. coli* TB1/pUC13 cells a 4-fold increase in the cyt.b5 final productivity was achieved by an air pressure increase to 0.48 MPa as compared with an increase in the stirring rate to 500 rpm.

These results showed that for shear sensitive cells, pressure could successfully be used to enhance oxygen transfer rate to a culture. For the pressure range studied, this could be easily accomplished by introducing a regulatory valve at the air outlet line, without a significant increase in capital costs, because the vast majority of the actual bioreactors used in the biotechnology industry are constructed of materials that can withstand the pressure values used in this work.

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