Oxygen Transfer to Cell Culture by Membrane Diffusers

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Original scientific paper
Received: March 3, 2005
Accepted: May 25, 2005

The oxygen supply to cell cultures of *Saccharomices cerevisiae* by porous membranes has been investigated. Two diffusers with different surface areas were prepared by end sealed capillary polypropylene membranes with a nominal pore diameter of 0.2 μm. The diffusers were immersed in the cell culture kept under constant agitation. Air was fed in the membrane lumen and diffused into the cell culture without any visible large bubble formation. Bath and fed-batch cultivations were performed and the variation of cell, glucose and oxygen concentration, during the cultivation, was investigated. The results obtained by membrane diffuser were compared with those of a traditional gas sparger. The advantages of membrane diffuser over the sparger are discussed.

Key words:
Membrane diffuser, capillary membrane, oxygen transfer, *saccharomyces cerevisiae*, cell culture

Introduction

The growth rate of aerobic microorganism depends on the amount of oxygen dissolved in the culture media. Due to the low oxygen solubility in water, oxygen supply to the growing cells represents the limiting step of many fermentation processes. Gas spargers are nowadays the most common means of oxygen supply to the cultural broth, especially in the large scale production plants. Besides the obvious limitations related to the transfer rate of gas mass to the culture, the presence of air bubbles in this system can be dangerous with regards to cells that are very sensitive to shear stress. For mammal or plant cells even the simple bubble burst at the top surface of the culture is sufficient to generate stresses capable of affecting the culture itself.[1,2] Moreover, in high-density cultures, uniform mixing can become very difficult, thereby resulting in poor distribution of nutrients and oxygen, and creating anaerobic regions. In order to increase the oxygen transfer rate to the solution, it is possible to operate through the agitation velocity with the aim of reducing air bubbles size, consequently increasing gas-liquid contact area and bubble residence time in the reactor. In this way however, froth formation and cell flotation also increase considerably. To summarize, uniform mixing, oxygen transfer, and shear stress remain the most challenging areas in the correct design of industrial bioreactors. These parameters are generally correlated and a compromise, for example between oxygen transfer rate and shear stress, is necessary.

In this paper, we present the results of a preliminary study on a new bioreactor, which uses porous membranes to supply oxygen with the aim of improving, both, mixing and oxygen transfer rate, and producing at the same time less shear force, with a consequent potential benefit on the productivity of the system. Although the results obtained in this study are applicable in principle to all fermentation processes carried out by aerobic micro organisms, here we have focused on *Saccharomyces cerevisiae*, a well known yeast generally used for bread making. Yeast production for bread manufacture is carried out under limited conditions of aerobic glucose, since in *Saccharomices cerevisiae* a mixed metabolism (Crabtree effect), leading to ethanol production which lowers the biomass yield, takes place[3] when sugar mass concentration is greater than a threshold value, typically 50 – 100 mg L^{-1}.[4,5]

The main idea at the base of this work is the use of microporous membranes placed in the cell culture in order to efficiently supply oxygen even at high cell density, thus avoiding the formation of large gas bubbles and the aforementioned problems. In fact, during cultivation, oxygen in the liquid phase is continuously metabolised by the growing cells, and its concentration tends to decrease with time. Since microporous membranes possess high

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gas permeability, growing cells on the other side of the membrane can easily have all the oxygen needed. Moreover, by utilising a suitable membrane configuration such hollow fibre, capillary or tubulet, characterised by a very high surface area, it is possible to maintain high gas concentration in the medium even at high cell density. Specifically, polypropylene membranes represent a very interesting solution given their high gas permeability and their hydrophobicity, which avoids the penetration of the liquid phase in the membrane pores.

The use of gas permeable membranes in biological processes is not new. Membrane contactors have been thoroughly investigated for the biological treatment of industrial waste, in the activated sludge water treatments and in various bioremediation processes.[6–8] Although, the first quantitative studies on the transfer rate of oxygen to water solution by means of membranes are now starting to appear in the open literature,[9] knowledge on the phenomenon of oxygenation of cell cultures is still in its early days,[10] and more studies are needed before some confidence is achieved.

Experimental procedure

Yeast

Bread yeast utilised in this study is a commercial strain of *Saccharomyces cerevisiae* (produced by DSM Bakery Ingredient Italy spa), normally used in the Italian bread industrial process. It is available in cubes, at $w = 70$ % water content, and it is produced by aerobic fermentation of sugar syrup.

Cellular mass and metabolites determination

Cell mass concentration was determined according to the following procedure. Ten mL of culture sample were filtered by using nitrocellulose filters with a porosity of 0.45 μm. Filters were then washed with deionised water and dried at 105 °C until a constant weight was reached. In order to minimise the effect of air humidity, filters were left in a desiccator at room temperature before being weighted. Glucose mass concentration in the culture broth was determined by HPLC analysis (440 Water Associates), using a ion exchange column HPX-87H Aminex (300 by 7.8 mm, Bio-Rad, Richmond, Cambridge, MA), a refractive index detector and sulphuric acid 5 mmol L^{-1} with a 0.6 mL min^{-1} flow rate, as mobile phase. Ethanol was checked by gas-chromatographic analysis (Perkin Elmer Auto-System) with FID detector and $Q = 25$ mL min^{-1} helium flow rate, with a packed column (3 m) SE-30 at 40 °C. Before the injection, cells were removed by filtration and a known amount of methanol was added.

Culture broth preparation

For the batch growth a standard culture medium was utilised, containing $w = 2$ % glucose, 2 % bactopeptone (Difco, USA), 1 % yeast extract (Difco, USA), and $\rho = 0.3$ mL L^{-1} antifoam agent SIGMA 289 (Sigma-Aldrich). The pH was set and kept at 5.0 by using 1 % phosphoric acid. The substrate was sterilised for 20 min at 121 °C before it was inserted in the fermenter. The medium cultivation for the fed-batch growth contained 1 % glucose, 2 % bactopeptone (Difco, USA), 1 % yeast extract (Difco, USA), 0.3 mL L^{-1} antifoam agent, at pH 5. After a few hours of fermentation, 12.5 g of glucose were added to the cultivation medium. Analogous medium was used for the continuous fed-batch growth; during the fermentation process a $w = 50$ % solution of glucose was added in quantities estimated necessary for the cell growth.

Bench-scale fermenter

The batch and fed-batch cultures were carried out in a laboratory-scale fermenter of 1 L volume at 30 °C and pH 4.7, using 14.7 nmol L^{-1} ammonia hydroxide as a buffer solution. 15 to 25 g of yeast, suspended in 100 mL isotonic glucose solution, was aseptically inoculated. Air was supplied with a classical sparger (flow rate 1.5 N L min^{-1}) or with a membrane diffuser (flow rate 0.7 N L min^{-1}). During cultivation the stirring speed was kept constant at $n = 600$ min^{-1} for the membrane diffuser, and 800 min^{-1} for the sparger. Dissolved oxygen concentration was continuously measured by a polarographic cell (model 025IP27, B&C electronics, Italy). At regular time intervals samples were drawn from the fermenter in order to determine cell and glucose mass concentrations.

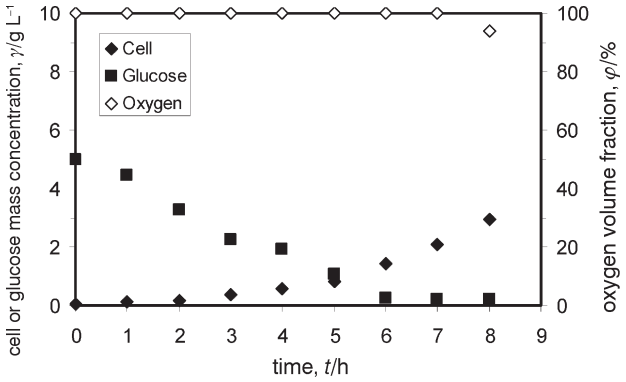
Membrane diffuser and gas sparger

Polypropylene capillary membranes (S6/2 type; 0,2 μm nominal pore diameter; 1800 μm inner diameter; 450 μm wall thickness) supplied by Membrana, Germany, and a sparger with a hole diameter of 2 mm were used in this work. The membranes were folded and jointed at both ends with epossidic resin to obtain diffusers with two different surface areas (190 and 460 cm^2).

Results and discussion

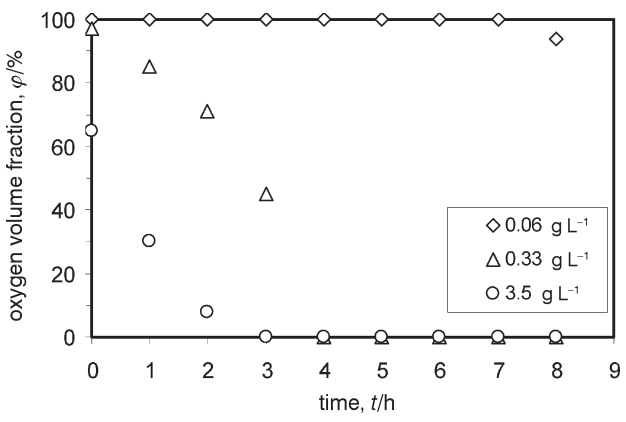
The influence of the oxygen distribution on the biomass growth rate was first studied by using the simplest arrangement, i.e. a batch system where all

nutrients and biomass were charged into the agitated bioreactor at the start of the run, and oxygen was supplied with a sparger. This first run was carried out starting with a very small quantity of cells, 0.06 g L^{-1}. In this case glucose and biomass concentrations followed the very typical behaviour reported in Fig. 1. Cells mass concentration grew with time while glucose mass concentration decreased, as it was used as carbon source for cell growth. As measurements of oxygen volume fraction in the liquid phase demonstrated, oxygen supply was more than enough, its concentration being always close to that of oxygen saturation in water.

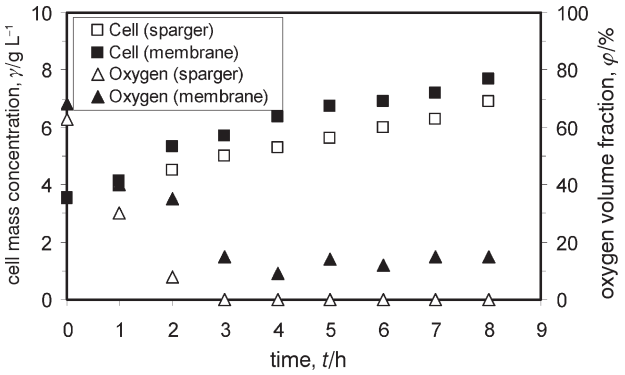


F i g . 1 – *Cell, glucose mass concentration and oxygen volume fraction as a function of time for the sparger during cultivation carried out in a batch bioreactor at initial cell mass concentration of 0.06 g* L^{-1}

The picture changed substantially when the initial cell concentration was progressively increased from 0.06 to 3.5 g L^{-1}, as depicted in Fig. 2. It is evident from the figure, that the oxygen supplied to the system was nowhere near enough to sustain cell growth, when large quantities of initial biomass were charged in the bioreactor. As such parameters increased the oxygen was depleted and in the batch bioreactor 3 to 4 h after the growth process had started it was practically absent. The limitation of the present oxygen supply system, i.e. a standard sparger, was therefore quite evident. To overcome this problem the sparger was then replaced by the membrane diffuser, and firstly the diffuser with the lowest surface (190 cm^2) was tested. The results, in terms of cell mass concentration and oxygen fraction, are reported in Fig. 3. The analysis of this figure reveals that, although, the cell mass concentration was very similar in the two arrangements, oxygen level was always higher in the case of membrane diffuser and never dropped to zero; this is even more significant if we take into account that in the case of membrane diffuser only half of the oxygen was supplied (0.7 N L min^{-1} compared to 1.5 N L min^{-1} for the sparger) and the agitation speed was much lower (n = 600 min^{-1} with respect to n = 800 min^{-1} for the sparger).



F i g . 2 – *Oxygen volume fraction as a function of time for the sparger during cultivation carried out in a batch bioreactor at different initial cell mass concentration*

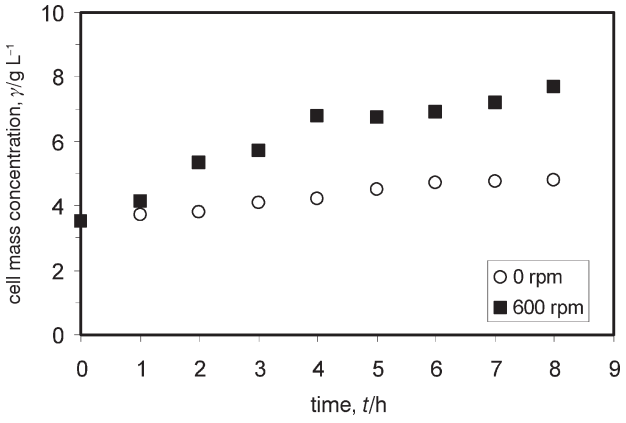


F i g . 3 – *Cell mass concentration and oxygen volume fraction as a function of time for the sparger and the membrane diffuser (190* cm^2 *membrane surface) during cultivation carried out in the batch bioreactor at initial cell mass concentration of 3.5 g* L^{-1}

The advantage of using the membrane diffuser over the sparger can be explained by keeping in mind, that the mass transfer rate of a component from a gas to a liquid phase is directly proportional to the interfacial area, and the use of membrane results in a very large increase of the mass transfer rate. In a gas-liquid system the interfacial area is directly proportional to the gas volume fraction and inversely proportional to the mean Sauter diameter of the bubbles, i.e. the mean bubble size of the dispersed gas. Gas volume fraction was estimated for, both, membrane and sparger by simply noting the increase in the liquid height in the vessel, compared to the level at no gas flow. For example, by using a gas flow rate of 1.5 N L min^{-1}, gas volume fraction was estimated to be 1.1 % when the sparger was used, and 2.4 % when membrane diffuser (A = 190 cm^2 membrane surface) was utilised. Mean bubbles sizes were not directly measured in this work; however it is well known that bubbles are produced

with a size proportional to the hole diameter of the distributor to the power of 0.33.[11] Therefore in our case, given that gas sparger has holes of 2 mm and the membrane diffuser has pores of 0.2 μm, we could expect the bubble diameter produced by the membrane to be smaller by a factor of about 20, compared to the bubble diameter produced by the sparger (4.5 mm compared to 0.2 mm). Visual observation of the transparent bioreactor confirmed this estimate, whilst in the case of the sparger gas bubbles were quite visible with the naked eye and were of the order of 5 mm in diameter, when membranes were used it was difficult to individuate single gas bubbles, the largest being definitely smaller than 1 mm, but a much finer dispersion of the gas was evident. Although, bubble coalescence and stirring interference are bound to have some effects on the eventual bubble size, we can still expect the interfacial area for the membrane diffuser to be much higher than that for the gas sparger.

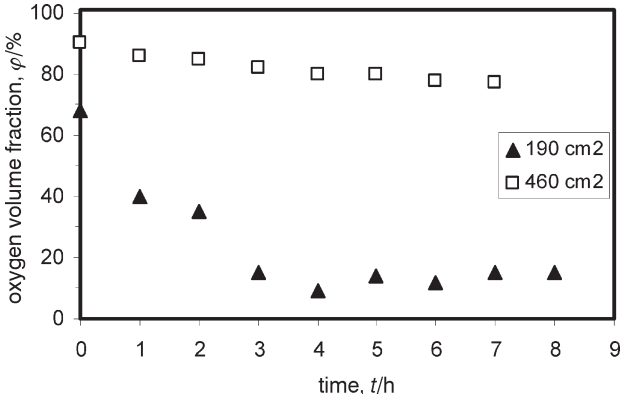
It is worth observing that by using the membrane diffuser agitation was only needed in order to keep the cells suspended, rather than to also favour oxygen diffusion. Fig. 4 represents the fact that when no agitation was present the cells just sedimented at the bottom of the vessel where any growth was basically stopped.



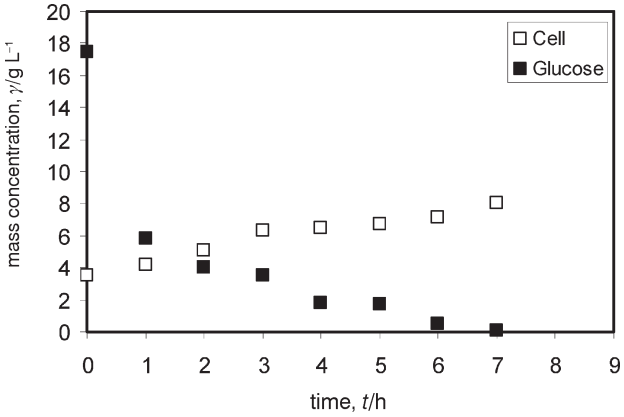
F i g . 4 – *Cell mass concentration as a function of time for the membrane diffuser (190 cm² membrane surface) during cultivation carried out with no agitation and with agitation in the batch bioreactor at initial cell mass concentration of 3.5 g L⁻¹*

Oxygen volume fraction in the culture broth increased as the membrane surface of the diffuser increased. The difference in the oxygen concentration was quite dramatic and this is evident from Fig. 5, which shows that, for the greater contact surface diffuser, the concentration stayed very close to saturation values for the whole time the run was carried out.

In spite of the obvious difference in oxygen fraction observed for the two membrane diffusers,



F i g . 5 – *Oxygen volume fraction as a function of time for the two membrane diffusers during cultivation carried out in the batch bioreactor at initial cell concentration of 3.5 g L⁻¹*

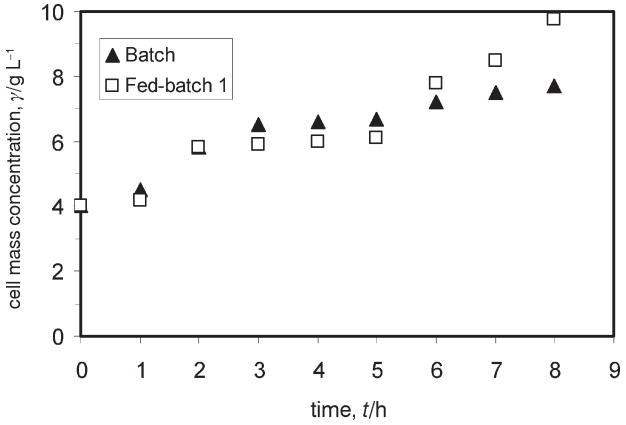


F i g . 6 – *Cell and glucose mass concentration as a function of time for the membrane diffuser (460 cm² membrane surface) during cultivation carried out in the batch bioreactor at initial cell concentration of 3.5 g L⁻¹*

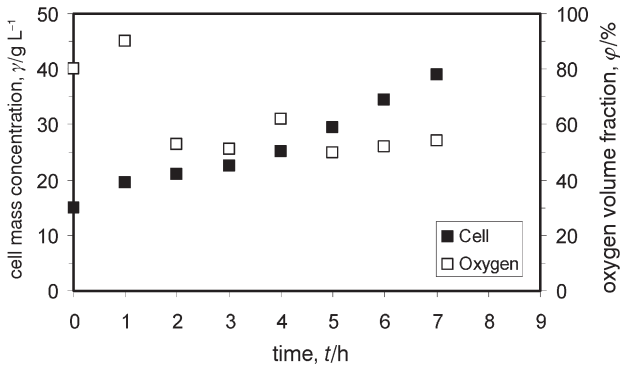
cell growth did not show any appreciable change. This was due, in this particular case, to the fact that nutrients were not able to sustain any further growth as they were almost completely depleted after four hours, as clearly shown in Fig. 6 (i.e. glucose rather than oxygen was the limiting factor for the biomass growth).

The full potentiality of the membrane diffuser could therefore be made clearer by supplying the reactor system with enough nutrients. This was achieved in two further runs: the first, in which glucose was reintegrated into the vessel every three hours (fed-batch 1), and the second where the glucose was supplied every hour (fed-batch 2). The results are depicted in Figs. 7 and 8.

Figure 7 shows the superiority of the fed batch system in terms of cell production when compared to a batch system, when all the other parameters (such as oxygen supply system and initial cell concentration) were kept constant. Above all, the results reported in Fig. 8 (semi-continuous system) are worth stressing out. The initial cell mass con-



F i g . 7 – *Cell mass concentration as a function of time for the membrane diffuser (460 cm² membrane surface) during cultivation carried out in the batch and in the fed batch 1 bioreactors at initial cell concentration of 4 g L⁻¹*



F i g . 8 – *Cell mass concentration and oxygen volume fraction as a function of time for the membrane diffuser (460 cm² membrane surface) during cultivation carried out in the fed-batch 2 bioreactor at initial cell concentration of 15 g L⁻¹*

centration was set to a very high value, 15 g L^{-1}. As opposed to all the other situations investigated in this study, cell growth was very positive and a concentration of ca. 40 g L^{-1} was measured at the end of the run. More importantly, thanks to a very efficient membrane diffuser, oxygen concentration never dipped below 50 %. This result should be compared, for example, with those reported in Fig. 2, where an initial cell mass concentration of 3.5 g L^{-1} was enough to deplete all the oxygen when a sparger was used.

Conclusions

The results of this study demonstrate that membrane diffuser can be successfully applied for supplying oxygen to cell cultures. With respect to a traditional gas sparger, membrane diffuser allows gas transfer to the liquid phase without formation of large bubbles that cause cell damage. Moreover, a higher gas-liquid interfacial area for the membrane diffuser determinates an increase of the mass transfer rate from the gas to the liquid phase and, consequently, a substantial improvement of the bioreactor performance.

ACKNOWLEDGMENT

The authors thank Dr. G. Dondo, Analisi e Controlli, Genova for his help in the biotechnological part of this work.

List of symbols

A – surface area
d – pore diameter, μm
n – agitation speed, min^{-1}
Q – volume flow rate, L min^{-1}
t – time
T – temperature, °C
w – mass fraction, %
γ – mass concentration, g L^{-1}
ρ – volume fraction, mL L^{-1}

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