# A New Tool for the Quantification of Microorganism Interaction Dynamics

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A two-reservoir, membrane bioreactor for carrying out studies of mixed population dynamics in batch fermentation is presented. Mixing requirements and design aspects for the validity of the approach are presented and discussed. Equations describing mixing times between the reservoirs are given and compared to the experimental results. The validity of the approach is demonstrated by the study of several types of situations in the bioreactor. The main validation consists of the comparison between the results obtained in an actual mixed culture and the results obtained by keeping the strains separated. Finally, this new tool is used to study the interaction kinetics between two oenological bacteria. These experiments in liquid media help to determine the seeding conditions for a chosen strain to overgrow another strain through a quantification of the interaction dynamics.

## Introduction

In typical mixed culture studies, the routine procedures used for the tracking of individual strains are time-consuming and costly. Very often, these procedures involve the isolation of microorganisms and their development into colonies, which are subsequently identified by their morphology or color, if possible. Frequently, the colonies are very similar, so a second procedure must be followed for their identification. In this case, a biochemical specificity, such as consumption of a given substrate, production of a given metabolite, or genetic differentiation, is pointed out.

Because the biomass concentration of each participating microorganism in a mixed population is necessary for the quantitative study of the interactions, these kinds of studies are limited by the availability and precision of suitable differentiation methods.

This paper presents a membrane reactor specifically designed to carry out studies of mixed population interactions. It is based on the idea of keeping the strains or species composing the mixed culture separated by way of a porous membrane, which allows the substrates and the metabolites to pass through and, therefore, the strains to interact.[1] In this way, as the different strains actually grow separated, they can be sampled distinctly and there is no need for differentiation. Further, the individual strains can be tracked with the methods used in pure culture studies.

The first part of the paper is devoted to a synthesis of the requirements for the validity of the proposal and the successive steps of validation. The second part is an example of applications of the method.

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## Materials and Methods

As the experiments in the two parts of the paper involved yeast and then bacteria, this section will be divided in the same way, after the bioreactor and membrane are described.

**Bioreactor.** A scheme of the proposed reactor is shown in Figure 1. It is composed of two jars interconnected by a hollow fiber-membrane module, which is immersed in the liquid of one of the jars. Medium flow and mixing are induced by alternately applying pressure to the headspace of each of the vessels. Compressed, filter-sterilized gas is used to apply pressure, and a system of valves controls the admission and expulsion of the gas as a function of the liquid levels, which are measured with conductivity probes. A filtration swing is required to maintain the same mean liquid volume in both reservoirs. An extra benefit of this swing is the control of fouling by backflushing. This device has been patented.[2]

Polymem (France) provided the hollow fiber module following a design specified by us. The fibers are U-shaped, and they are held together in their upper parts with an epoxy resin. The upper part of the bundle is contained in a stainless steel receptacle, and the filtering part immerses freely in the medium. The fibers are made of polysulfone and present a nominal pore size of 0.1 $\mu$m; the internal and external diameters are 0.25 mm and 0.43 mm, respectively. The water permeability of the fiberswas $3.5 \times 10^{-9}$ $m^3$ $m^{-2}$ $s^{-1}$ $Pa^{-1}$, as estimated in the module. The total filtering surface was 0.1 $m^2$, as it will be explained further (see Design Aspects).

**Validation: Yeast Fermentation.** *Salt Concentration.* Sodium chloride was used as the tracer in the mixing studies. Conductivity measurements (Metro-Ohm, Switzerland) were used to assess the salt concentration.

*Strains and Media. Saccharomyces cerevisiae* K1 and *Saccharomyces cerevisiae* S6 (Lallemand, France) in-

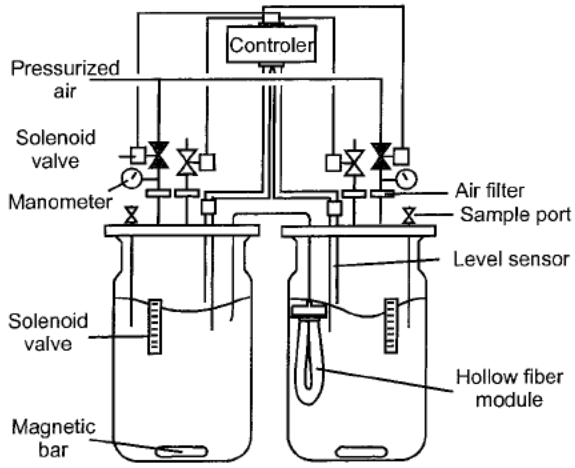


**Figure 1.** Proposed two-reservoir, hollow fiber bioreactor.

hibitory ("killer") and sensitive strains, respectively, were conserved at 10 °C in agar slants (20 g/L agar, 10 g/L yeast extract, 20 g/L glucose). Inocula and fermentation were developed in a semi-synthetic, buffered medium containing 25 g/L glucose, 0.5 g/L yeast extract, 2.5 g/L $KH_2PO_4$, 1 g/L $(NH_4)_2SO_4$, and 0.2 g/L $MnSO_4 \cdot 7H_2O$.[3] The buffer solution used to dissolve the medium components was 0.5 N citrate/phosphate at pH 4.8. The solid medium for isolation of the colonies in the actual mixed cultures and for viability measurements in simulated mixed cultures was conservation medium. For the killer test,[4] fermentation medium buffered at pH 4.8 (0.1 N citrate/phosphate) and supplemented with agar–agar at 20 g/L was used.

*Cellular Concentration.* Biomass dry weight (BDW) was determined by the classical method. Total cell number was estimated by direct enumeration using a haemocytometer. For each pure strain, the correlation between the biomass dry weight and cell number was established. In simulated mixed cultures, viability was assessed by colony enumeration. The viable BDW of each strain was computed from the correlation between cell number and BDW.

*Strain Differentiation.* In actual mixed cultures, the fraction of each strain in the population was estimated by a killer test, following Ramon-Portugal.[4] The viable BDW for each strain was then calculated from the total cell number, the fraction of each determined by the killer test, and the correlation between BDW and cell number in the actual mixed cultures.

*Glucose Concentration.* Glucose was determined with an enzymatic system using immobilized glucose oxidase (2700 Select, Yellow Spring Instruments, Yellow Spring, OH).

*Fermentation.* Inocula were prepared from slants and were used to inoculate the reactor when culture inoculum was in the exponential phase of growth. The bioreactor was seeded at 3 million viable cells per milliliter referred to the working volume of one jar. In both cases, the agitation was at 300 rpm, and the temperature was 30 °C. In mixed cultures, the sensitive strain concentration was twice the killer initial population, being 6 million and 3 million viable cells per milliliter, respectively. Both concentrations referred to the volume of one jar. For medium circulation, air was applied at 100 kPa, which led to an effective transmembrane pressure of about 35 kPa. The transferred volume was about 130 $cm^3$. In this way, the duration of the period of filtration was on the order of magnitude of one to several minutes, depending on the fouling of the membrane.

**Application: Bacterial Interactions.** *Strains and Media.* The strains used in this study were *Oenococcus oeni* (Lallemand Inc.), maintained and cultivated at 30 °C in MRS medium (Biokar Diagnostic).[5] The inhibitory and sensitive strains were coded 21A3 and 17A3, respectively, or 19A3 and EQ77 for both examples of interactions.

*Cellular Concentration.* The total cell number was estimated from the optical density at 600 nm, using a correlation previously established between direct enumeration using a haemocytometer and the optical density.

*Substrate Concentration.* The L-Malic acid concentration was assayed with an enzymatic method from Boehringer Mannheim.

*Fermentation.* Inoculations were prepared from slants and were used to inoculate the reactor when culture inoculum was in the exponential phase of growth. The temperature was 25 °C. For medium circulation, a carbon dioxide flow (this gas is a growth factor for *Oenococcus* growth) was applied at 100 kPa, giving an effective pressure of about 35 kPa. The transferred volume was about 100 cm3, and the filtration period was about 1 min.

## Theoretical Aspects

The intended use of the proposed membrane reactor is the quantitative study of mixed microbial population dynamics. The minimum information required for such a study is the biomass evolution for each strain, as well as the evolution of substrates, major products, and any metabolite having a noticeable influence on the behavior of the microbial cells.

This approach will succeed if the behavior of the culture obtained in the reactor by keeping the strains separated is similar to that obtained when the strains are grown together. This can only be accomplished if global dynamics are governed by the reaction rates and not by the mixing between the containers, i.e., if the mixing rates are high enough compared to the reaction rates.

To keep the global dynamics of the system rate-controlled, mixing must be faster than any possible reaction involved. However, because some microbial reaction rates are rather high, commercially available devices, such as the ECOLOGEN (New Brunswick), that provide slow mixing by diffusion through membranes are not convenient. As a first proposal, we decided to provide mixing times of less than 10 min. This duration is on the order of magnitude of the lowest bacterial replication time and several times lass than typical replication times of yeast (about 1 h) and animal or vegetable cells (several days). Thus, we hope that such a mixing rate is sufficient for most cases.

**Mixing Dynamics.** As has been stated, mixing is the mean constraint for the proposal to succeed. A theoretical study is convenient for clarifying several aspects.

The proposed reactor works by periodically exchanging a given volume. Thus, the exchange rate is neither continuous nor constant. However, because the exchange period is expected to be rather small compared to the mixing dynamics, the discontinuities can be ignored, and it can be assumed that the exchange rate is continuous and constant. Let F be the net exchange

flow rate between the reservoirs, denoted A and B, and let $V$ be the liquid volume of each reservoir. $F$ is taken as the average flow through the membrane, calculated from the exchange volume and the duration of a filtration period. This concept is correct in the present case, because the exchange duration is small compared to the system dynamics, so that it can be written as a constant in the mass balance. Independently of how the medium transfer between the reservoirs is actually carried out, the mixing can be theoretically studied by using material balances. $F$, correlated with the membrane permeability and the applied pressure, is the membrane flow, continuously feeding each reservoir. Then, at any time $t$, the global material balances for the substances capable of crossing through the membrane are

$$\begin{aligned}\frac{\mathrm{d}\mathbf{c}(t)_A}{\mathrm{d}t} &= \frac{F}{V_A}[\mathbf{c}(t)_B - \mathbf{c}(t)_A] + \mathbf{r}(t)_A \\ \frac{\mathrm{d}\mathbf{c}(t)_B}{\mathrm{d}t} &= \frac{F}{V_B}[\mathbf{c}(t)_A - \mathbf{c}(t)_B] + \mathbf{r}(t)_B\end{aligned} \tag{1}$$

where $\mathbf{c}(t)_i$ is the vector of concentrations of all the solutes that can pass through the membrane and $\mathbf{r}(t)_i$ is the vector of reaction rates for each solute. The subscripts A and B indicate the reservoirs.

The flow entering and leaving each reservoir recalls the concept of dilution used in continuous flow systems; because of this similarity, we defined the ratio $F/V$ as the internal dilution rate, $D$.

*Mixing without Reaction*. In this case, $\mathbf{r}_A = \mathbf{r}_B = 0$.

The solution of the previous equation at $t = 0$, $\mathbf{c}_A = \mathbf{c}_{A0}$, and $\mathbf{c}_B = \mathbf{c}_{B0}$ is

$$\begin{aligned}\mathbf{c}_A(t) &= \frac{\mathbf{c}_{A0} + \mathbf{c}_{B0}}{2} + \frac{\mathbf{c}_{A0} - \mathbf{c}_{B0}}{2}\exp\{-2Dt\} \\ \mathbf{c}_B(t) &= \frac{\mathbf{c}_{A0} + \mathbf{c}_{B0}}{2} - \frac{\mathbf{c}_{A0} - \mathbf{c}_{B0}}{2}\exp\{-2Dt\}\end{aligned} \tag{2}$$

From this equation, the relative degree of homogeneity can be defined as

$$\frac{(\mathbf{c}_{A0} - \mathbf{c}_{B0}) - (\mathbf{c}_A - \mathbf{c}_B)}{\mathbf{c}_{A0} - \mathbf{c}_{B0}} = 1 - \exp\{-2Dt\} \tag{3}$$

This equation can be used to define the mixing time, which is defined as the time required to achieve a given degree of mixing. We took the value of 95% homogeneity, i.e., a value of 0.95 for the right-hand expression in eq 3. The mixing time is thus defined by the equation

$$\theta_m = -\frac{\ln(0.05)}{2D} \tag{4}$$

Therefore, the mixing time is proportional to the reciprocal of the internal dilution rate.

*Mixing with Reaction*. When a reaction takes place in one or both of the reservoirs, no general solution to eq 1 exists because the expressions needed to calculate the reaction rates can be quite complex. However, in the case where the reaction rates are constant, a solution can be obtained as follows: The concentration difference between the reservoirs is

$$\begin{aligned}\frac{\mathrm{d}\mathbf{c}_A}{\mathrm{d}t} - \frac{\mathrm{d}\mathbf{c}_B}{\mathrm{d}t} &= 2D(\mathbf{c}_B - \mathbf{c}_A) + (\mathbf{r}_A - \mathbf{r}_B) \\ \frac{\mathrm{d}\Delta\mathbf{c}}{\mathrm{d}t} &= -2D\Delta\mathbf{c} + \Delta\mathbf{r}\end{aligned} \tag{5}$$

The solution of eq 5 is

$$\Delta\mathbf{c} = \frac{\Delta\mathbf{r}}{2D} + \left(\Delta\mathbf{c}_0 - \frac{\Delta\mathbf{r}}{2D}\right)\exp\{-2Dt\} \tag{6}$$

It can be noted that, for $Dt \rightarrow \infty$

$$\Delta\mathbf{c} \rightarrow \frac{\Delta\mathbf{r}}{2D} \tag{7}$$

This means that, whenever a reaction takes place at different rates in the two reservoirs, a concentration difference will be generated. Thus, a zero difference, that is, complete mixing, cannot be achieved. This is true independently of the internal dilution rate and even if reaction rates are not constant. Then, it can be shown that, in this case, the mixing time is also given by eq 4.

## Design Aspects

The theoretical studies on mixing rate led us to the conclusion that the approach would be valid only if a very high mixing rate was used. As explained previously, we fixed, somewhat arbitrarily, a mixing time less than 10 min as our goal. From the equations presented in the preceding section, it can be shown that this requires a dilution rate higher than 8 $h^{-1}$ and given by

$$F = \frac{V_E}{t_A + t_B} \tag{8}$$

where $V_E$ is the exchanged volume in the pressurization of reservoirs A and B and $t_A$ and $t_B$ are the times required for the exchange from reservoir A to reservoir B and vice versa, respectively. $V_E$ is the volume of a complete cycle, with the flow once in each direction.

Now, the parameters in eq 8 depend on the module design. Several designs were tested, but it was concluded that the design best suited for our purposes consisted of a bundle of hollow fibers immersed freely in the liquid of one of the reservoirs (Figure 1). For such a system, if $V_D$ represents the displaced volume actually seen as a variation in the reservoirs levels and $V_M$ represents the dead volume, then the exchange volume $V_E$ and the filtered volume $V_{F,i}$ are given by

$$\begin{aligned}V_E &= 2V_D - V_M \\ V_{F,A} &= V_D \\ V_{F,B} &= V_D - V_M\end{aligned} \tag{9}$$

The expression for the filtered volume is necessary for calculating the fouling of the membranes and, thus, the times $t_A$ and $t_B$. To perform an estimation of this quantity, it was assumed that, because of the very high surface area, surface fouling was small and thus the curvature effects on the cake arrangement could be neglected. Consequently, by using the cake model for dead-end filtration on flat membranes and the volumes

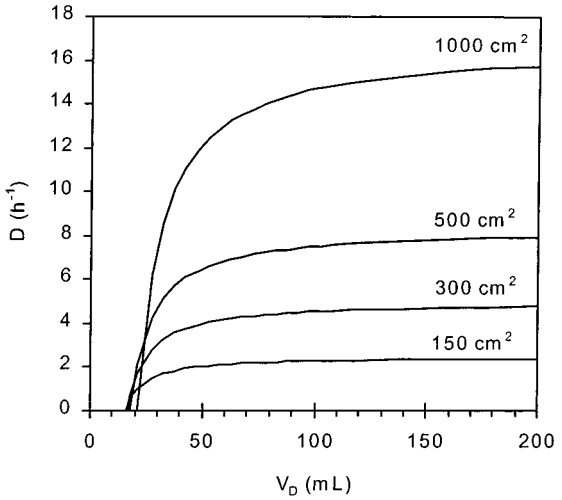


**Figure 2.** Internal dilution rate, $D$, as a function of displaced volume, $V_D$, and filtration area.

**Table 1. Parameters Used in the Estimation of *D***

| parameter | value |
|---|---|
| $V_D$ | variable |
| $S$ | variable |
| $V_M$ | variable |
| $V$ | 2 L |
| $R_m$ | $2.7 \times 10^{11}$ m$^{-1}$ |
| $\eta$ | 0.001 Pa s |
| $\Delta p$ | 50 kPa |
| $\hat{R}_{gi}$ | $3 \times 10^{12}$ m kg$^{-1}$ |
| $X_A$, $X_B$ | 5 kg m$^{-3}$ |

defined by eqs 9, the following equation for $D$ was obtained

$$D = \frac{1}{V} \times \frac{2V_D - V_M}{2\frac{R_m\eta}{\Delta p}\left(\frac{V_D - 0.5V_M}{S}\right) + \frac{\eta}{2\Delta p}\left[\hat{R}_{gA}X_A\left(\frac{V_D}{S}\right)^2 + \hat{R}_{gB}X_B\left(\frac{V_D - V_M}{S}\right)^2\right]} \quad (10)$$

Equation 10 was used for the final sizing of the hollow fiber module. In Figure 2, the expected dilution rate is presented as a function of the displaced volume and total filtering surface. The parameters used are reported in Table 1; they are average values usually met in cell filtration experiments. It can be seen that a dilution rate greater than about 8 h$^{-1}$ can be obtained with a surface area greater than 0.05 m$^2$. Thus, we fixed the final surface area at 0.1 m$^2$.

## Results

**Mixing Quality.** *Mixing without Reaction.* Experiments consisted of the mixing of a saline solution contained in one of the reservoirs with water contained in the other reservoir. A comparison of the experimental and theoretical values showed good agreement. Its value was necessary to compute the value of the dilution rate $D$. It did not change significantly for displaced volumes between 100 and 200 mL. Therefore, the value for this parameter was fixed at about 130 mL for the following experiments.

*Mixing with Reaction.* Mixing experiments with fermentation taking place in one of the reservoirs were the first performed (data not shown[6]). The observed evolutions of the consumption of the main substrate were very similar in the two reservoirs. The greatest difference observed in substrate concentration between the reservoirs was less than 1 g/L, coinciding with the maximum substrate consumption rate of about 6 g/(L h). This result is higher than expected from eq 7, but that equation is for constant reaction rates. As this was not the case, the observed difference was probably a manifestation of the cumulated effects of changes in the reaction rate. The suitability of the device was thus supported by these results.

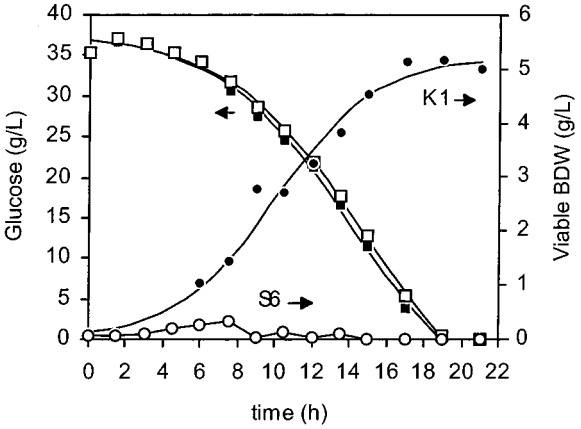


**Figure 3.** Evolution of glucose and estimated evolution of *S. cerevisiae* K1 and *S. cerevisiae* S6 in an actual mixed culture.

**Validation: Yeast Fermentation.** As stated previously, the proposed bioreactor can be considered adequate only if the behavior of a pair of strains is the same when they are grown in the same reservoir (actual mixed culture) and in different reservoirs (simulated mixed culture).

Two heterogeneous cultures exhibiting two kinds of interactions[7] were used to test the validity of the bioreactor. The results obtained with the first of these cultures, composed of *Kluyveromyces marxianus* ATCC 28912 and a *Saccharomyces cerevisiae* strain competing for the substrate, are reported elsewhere.[6] Essentially, the expected similarity between the actual and simulated mixed cultures was found. Minor differences, probably due to small differences in the inoculation ratio, were observed. As the interaction took place only through small molecules, such as glucose and ethanol, we decided to test the validity with a protein-mediated interaction. Thus, the second mixed culture used for validation and reported here was the so-called "killer interaction" between two strains of *Saccharomyces cerevisiae*.

The killer phenomenon is established between a yeast strain producing a killer protein and a yeast strain sensitive to this protein.[8] Several killer proteins have been identified.[9] The particular strain used in this work produces a toxin of type K2, which has been identified as a glycoprotein of about 16 kDa.[9] A period of time from a few minutes to several hours is observed between the adsorption of the protein and the loss of viability.

The killer protein activity is very sensitive to the pH. Therefore, we used buffered media during our studies to keep the pH values between 4.5 and 4.8, near the pH of maximum activity reported by Ramon-Portugal[4] for the strain used.

**Actual Mixed Culture.** The results of the reference fermentation, i.e., in the actual mixed culture, are shown in Figure 3. This fermentation was carried out in the proposed reactor with both strains growing in

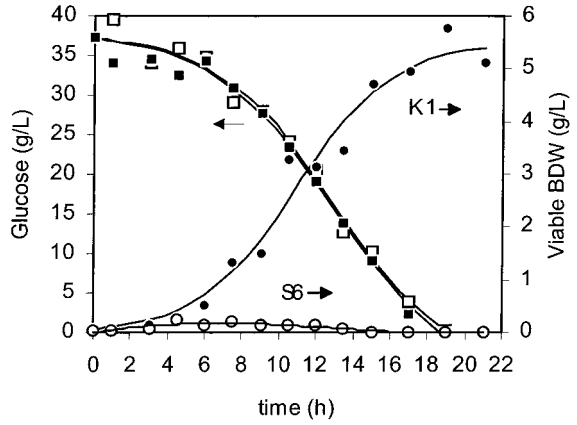


**Figure 4.** Evolution of glucose and estimated evolution of *S. cerevisiae* K1 and *S. cerevisiae* S6 in a simulated mixed culture.

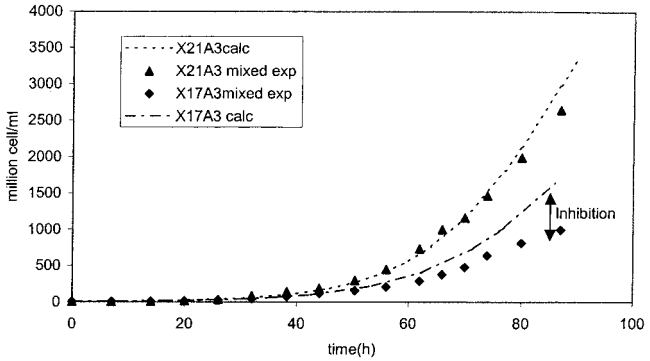


**Figure 5.** Comparison of the biomass concentrations of strains in pure and mixed cultures 21A3−17A3.

only one of the reservoirs but with the second reservoir connected and containing fermentation medium. This was necessary to distribute the substrates and products in the same way as they distribute when the strains are grown separated and, thus, to avoid any possible differences in the behavior of the culture due to the solute concentrations. The viable BDW of each strain was calculated from the total cell number, the percentage of S6 determined by the "killer test", and the correlation between cell number and BDW.

**Simulated Mixed Culture.** The results obtained for the strains grown separately in both reservoirs are shown in Figure 4. Viable BDW was calculated from colony counting of diluted aliquots of the medium contained in each of the jars. This was done to maintain similar analytical methods for the actual and simulated mixed cultures. The percentage of S6 was thus calculated from colony counts and not from killer tests.

Similar evolutions were observed for the two fermentations for the different parameters. The evolutions of the two strains were also very similar. Glucose was also depleted at roughly the same time, and the evolutions of the total viable count and percent S6 were likewise similar. A discussion has been developed elsewhere;[6] given some details on the interpretation of these results, it was concluded that the apparatus is suitable for the study of an interaction dynamic.

**Application: Bacterial Interactions.** The second part of this paper is an example of the use of the membrane reactor described and validated above. The industrial background is wine making. Two fermenting steps are required, occurring in nonsterile medium.[10] To control the production rate and also the quality of the final product, a seeding with selected microorganisms must be performed in order to ensure the implantation of the convenient strain.[11] This study deals with lactic bacteria involved in the second fermentation of wine making. It has been pointed out that the strain *Oenococcus oeni* involved here is a producer of an inhibitory substance for other bacterial strains.[12]

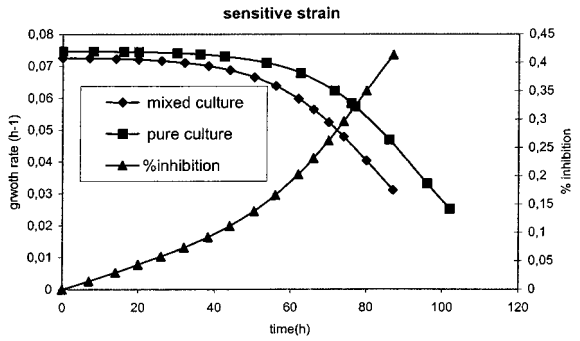


**Figure 6.** Quantification of the inhibition by comparing growth rates in pure and mixed cultures.

**Pure Culture.** The first experiments consisted of cultivation of the pure strains in order to obtain the kinetics under these conditions. The experimental results are the biomass concentration and the consumption of the substrate versus time. The experiments were stopped when there was no more substrate in the medium (L-malic acid). The logistic equation[13] gives analytical expressions for the biomass (eq 11) and the growth rate (eq 12) as functions of time. By parametric identification with the Excel solver, $\mu_{max}$ and $X_f$ were obtained with good agreement for the pure cultures.

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \frac{X_o}{X_f}[1 - \exp(\mu_{max} t)]} \quad (11)$$

$$\mu = \frac{\mu_{max}\left(1 - \frac{X_o}{X_f}\right)}{1 - \frac{X_o}{X_f}[1 - \exp(\mu_{max} t)]} \quad (12)$$

**Mixed Cultures.** For the mixed culture, to limit the effect of competition for the substrate, the ratio between the initial seedings of the two strains was calculated by comparing the maximum values obtained for the biomass growth rates from logistic eq 12. Then, the mixed culture was performed in the membrane bioreactor, and the same experimental parameters were collected, i.e., biomass and substrate concentration. These results are reported in Figure 5. The experimental results, represented by the plain dots, are compared to the growth of the pure culture under the same conditions of initial seeding, given by the above-defined equation (eq 11) and represented by the dotted lines.

In Figure 5, the inhibition appears clearly, as the biomass concentration of the sensitive strain increases more slowly and reaches a lower level in the mixed culture than in the pure culture. At the same time, Figure 5 allows us to verify that the dynamics of the inhibitory strain was not affected by its growth in the mixed culture.

Figure 6 reports the specific growth rate of the sensitive strain in the pure and mixed cultures. A comparison of the two curves shows that the inhibitory

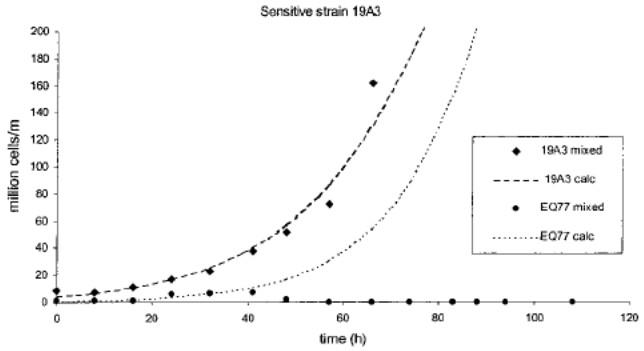


**Figure 7.** Comparison of pure and mixed cultures 19A3−EQ77.

effect was mainly pointed out after the first 30 h of fermentation.

Given the percentage of inhibition in eq 13, Figure 6 shows an increase of inhibition to 45%.

$$\% \text{ inhib} = \frac{X_{\text{pure}} - X_{\text{mixed}}}{X_{\text{pure}}} \tag{13}$$

For this example, it can be concluded that the seeding proportion has not been conveniently chosen to totally eliminate the growth of the sensitive strain.

Another pair of *Oenococcus Oeni* strains was studied, coded 19A3 and EQ77. Figure 7, representing the biomass concentrations of the two strains versus time, in the pure and mixed conditions of culture, shows that the seeding proportions were adequate to allow for the implementation of the inhibitory strain seeding 19A3.

## Conclusion

This paper presents in three parts the design and validation of an original device for the study of the dynamics of mixed populations of microorganisms, as well as an example of its use. This device is based on the ability of membranes, on one hand, to isolate microorganisms, and on the other hand, to allow solutes to permeate through. As membranes are hollow fibers, the filtration area is easily adaptable to the volume required to ensure convenient mixing. In fact, this device is probably useful for a large variety of microorganisms interactions studied in liquid media.

## Nomenclature

$\mathbf{c}$ = vector of solute concentrations (kg m$^{-3}$)
$D$ = internal dilution rate (h$^{-1}$)
$F$ = flow exchange rate (m$^3$ h$^{-1}$)
$\mathbf{r}$ = vector of reaction rates (kg m$^{-3}$ h$^{-1}$)
$p$ = pressure (Pa)
$R_m$ = membrane hydraulic resistance (m$^{-1}$)
$\hat{R}_{gi}$ = specific cake resistance in side $i$ (m kg$^{-1}$)
$S$ = filtering surface area (m$^2$)
$t$ = time (s, min, or h)
$V$ = medium volume (m$^3$)
$X$ = total biomass concentration (kg m$^{-3}$)

*Greek Letters*

$\eta$ = medium viscosity (Pa s)
$\theta_m$ = mixing time (min)

*Subscripts*

A, B = reservoirs
D = displaced
E = exchanged
F = filtered
M = dead

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