

WASHOUT, BIOFILMS AND THE HUMAN COLON

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Introduction.

The concept of washout is familiar to every student of fermentation technology or biochemical engineering. It is well-known that in continuous culture in a perfectly-mixed fermenter there is a critical dilution rate above which operation is impossible. At this point the removal rate of product exceeds the maximum possible production rate of cells so that all the cells are washed out of the fermenter. With a sterile feed this condition is (approximately) given by:

$$D = \mu_{\max} \quad (1)$$

where μ_{\max} is the maximum specific growth rate and D , the dilution rate, is defined as F/V or the reciprocal of the mean residence time t . In practice, this means that, for a perfectly mixed vessel of volume V , there is a maximum volumetric flowrate, F , for which steady operation is possible. At the other extreme, if there is no back-mixing of the cells at all, i.e. plug flow, continuous operation with a sterile feed is impossible. The critical dilution rate is 0.

In practice, real systems, whether engineered or natural, rarely meet the idealised conditions of zero or perfect mixing. We can make an (incomplete) list of reasons why the conditions are not achieved:

1. Deviations from perfect mixing: e.g. large scale stirred fermenters,
2. Deviations from plug flow: e.g. flow in pipes (turbulent or laminar),
3. Biofilms: caused by cells sticking to, and growing on, solid surfaces etc.,
4. Gravitational effects: flocs or pellets of cells move relative to the liquid phase,
5. "Solid substrate" systems.

In this paper I shall discuss some of these issues, drawing on some of our recent research on modelling the human gut or colon, continuous solid substrate fermentations, and waste water treatment using a membrane reactor. These examples each show the importance of at least one, sometimes more, of the "non-idealities" listed above. To simplify the discussion I shall focus primarily on two non-ideal situations: cases 1 & 2 and case 3 from the list above.

Deviations from perfect mixing and plug flow

Much recent modelling work into mixing in large scale fermenters has been based on the idea that the flow field can be represented as a set of interacting areas or zones [1]; these methods can be traced back to approaches to mixing where processes are modelled as sequences of stirred pots, plug flow elements and recycles. An alternative approach uses the methods of computational fluid dynamics (CFD). These methods have advantages and disadvantages. One danger with CFD-based approaches is that the fluid mechanical models embedded in the codes may not describe correctly the complex multiphase interactions between the moving liquid, bubble and solid phases.

A complementary approach, well-known to chemical engineers, is to represent the flow field by a dispersion model. This has the advantage that the same simple model can capture behaviour over the whole spectrum from perfect mixing, where dispersion effects are very large and dominant, to plug flow where dispersion is zero. However, dispersion models are actually much better at describing deviations from plug flow than deviations from perfect mixing.

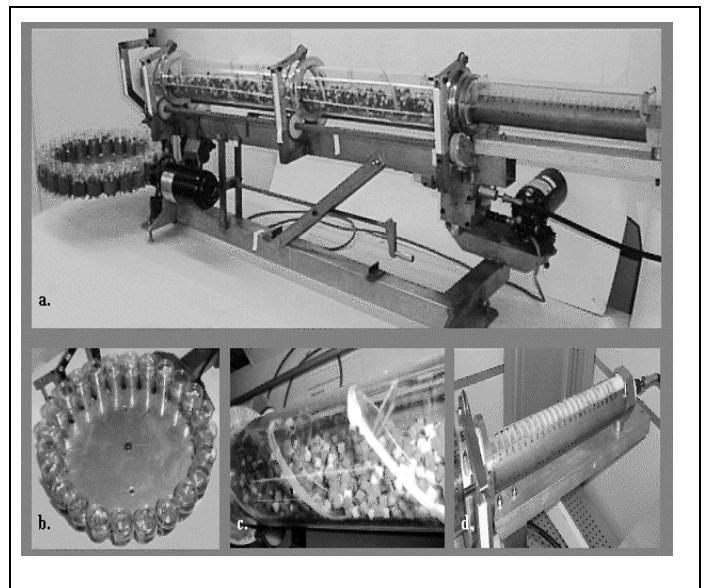


Fig. 1. Solid substrate fermenter

Our own interest in this problem has been motivated by two pieces of research. The first is the development of a

continuous solid substrate fermenter (work carried out with support from the European Community in collaboration with colleagues at UAM-Iztapalapa). [2,3]. Figure 1 shows a picture of a novel fungal fermenter in which the solid substrate enters and is transported continuously through the reactor, using either a central Archimedian screw, or by rotating the cylindrical fermenter about its axis.

The challenge is to develop a truly continuous processes with a "sterile" or un-inoculated feed. In other words we want to engineer the system such that once the fungus is established in the fermenter it will continue to grow and break down the substrate without further injection of inoculum. The technical question is: given the growth characteristics of the fungus on its substrate how much back-mixing is needed to sustain growth throughout the fermenter? Consider what happens to a "slug" of inoculated feed: in the absence of sufficient back-mixing, whilst the "slug" of inoculated solid feed passes through the fermenter, the biomass will grow as long as substrate is available but it would remain confined to the initial slug volume. Thus, the biomass concentration in this slug would change in time (and space) as if it were a batch process. Only with sufficient back-mixing can a continuously growing population of cells be built up throughout the fermenter.

We have been pursuing two approaches to this problem. One is a mechanistic approach to describe the solids mixing in its tumbling flow through the fermenter. The other, described here, is to model the flow of the solids and fungus (i.e. the biomass) using a dispersed plug flow framework. Experimental studies show that a dispersion model gives a fair picture of the solids behaviour [2]. We further assume that flow of the fungus, which adheres to the solid substrate surface, can also be characterised by the same dispersion coefficient. This assumes therefore that transfer of fungus from substrate particle to particle is not important and that spore transport can also be neglected. Both assumptions are rather doubtful.

The second piece of research [4] is concerned with the development of an "engineering model" of the human colon. The purpose of the work is to develop a test rig which can be used for *in vitro* experiments on the digestive system. This would help, for example, in studies on the effects of diet on cancer development or on the positive effects of pre- or probiotics on human health, since it could reduce or even eliminate the use of animals in testing new products. Several such models exist already. A three-stage chemostat system and this is being used extensively by Professor Glenn Gibson and colleagues in our department. An alternative system using flexible tubing and a peristaltic feed system has been developed at the TNO laboratories in Holland. To understand the nature of the challenge it is important to remind ourselves of some features of the human colon. The large colon, figure 2, is a long flexible tube which is about 1.5 metres long. It has a volume of around 0.5 litre. The partially digested material entering the colon (after passing through the stomach and the small intestine) has a water content of around 95%. As the material passes through the colon it undergoes an anaerobic fermentation in which, for

example, carbohydrates are broken down via short chain fatty acid intermediates to the end products (which include carbon dioxide and methane

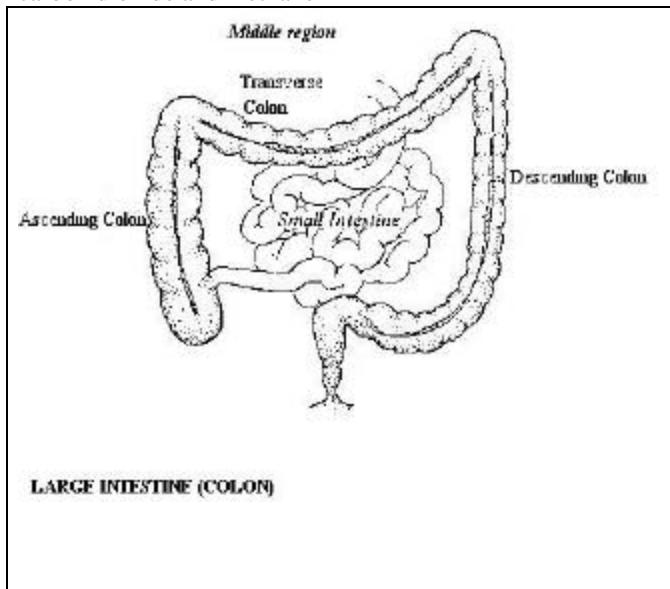


Fig. 2: The Human Colon

A very important feature of the properly functioning colon is the wide variety in microbial flora established and functioning in the gut: it depends critically on the balanced operation of a mixed culture. The colon has a very large surface area and during transit through the colon considerable quantities of water and fatty acids are removed across the wall of the colon. This has two effects: the end product typically has a moisture content of around 75 - 78%, which means that about 50% of the water entering the large colon is removed across the gut wall; transfer of fatty acids across the wall helps stabilise the pH throughout at around 5.5 - 6. In the absence of mass transfer the pH would, of course, fall dramatically. Flowrates through the flexible colon are intermittent, since we don't eat and drink continuously; residence times are of the order of 7 hours in each region of the colon, that is, around 20 hours in total. Typical mean axial velocities are therefore of order 50 mm/h, and effective Reynolds numbers are very low.

In practice, the colon is a marvellously balanced system whose importance we usually only recognise when we have a stomach upset. It is extremely complex, and no model could ever hope - or be intended - to capture all its complexities. The chemostat model fails to capture at least two important aspects. First, mixing: the behaviour of three stirred tanks is at first sight very different from the mixing occurring in low Reynolds number flow through a tube. Second, there is no removal of water or fatty acids: the pH in each chemostat has to be controlled by addition of a base; the final product is as dilute as the feed. The TNO model approaches the slow, pulsating flow in the colon (but is very difficult to describe and model mathematically). It also fails to reproduce the concentration and acid transfer processes in the human gut.

Our intention, then, was to develop a model which realised some of the features not captured by the other models. In particular, we wanted to design a system in which the flow could be modelled easily and which approached the more realistic features of dispersed plug flow; we also aimed to achieve substantial levels of mass transfer of water and fatty acids out of the flowing system.

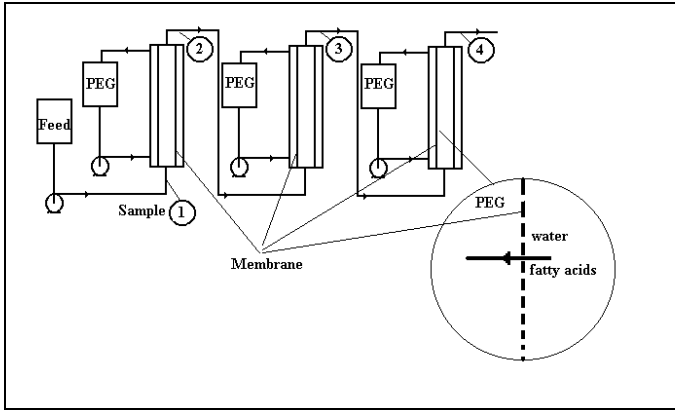


Fig.3: 3-stage membrane rig

Figure 3 shows the latest, 3-stage, version of our model. The design includes three identical tubular membrane reactors, length 50 cm, volume 92 cm³. The main flow passes through the inside of the cylindrical membrane, which has a nominal cutoff of 1000Da. The water flux across the membrane is controlled by circulating a concentrated aqueous solution of poly(ethylene) glycol, PEG, through the annulus surrounding the membrane. This effectively establishes an osmotic pressure gradient across the membrane. The water flux is driven by this gradient. Short chain fatty acids, and other molecules small enough to pass through the membrane, are driven by a combination of the concentration gradient and the convective flux of water. The PEG solution also contains minerals and salts in an attempt to minimise the loss of essential nutrients and media components from the fermentation loop.

We will return later to the experimental results achieved using this rig. Before that, however, we come back to the theme of this paper: washout. Why don't we, as human beings, normally suffer from washout, since the gut is apparently a long plug-flow reactor? There seem to be three possible (simultaneous) reasons. First, since the flow is essentially laminar, there are strong radial velocity gradients across the colon. This then gives rise to a flow field which can be characterised by an effective dispersion coefficient. This phenomenon, known as Taylor dispersion [5,6], has two components: first, mass transfer by molecular diffusion (coefficient D) driven by the radial concentration gradients and second the dispersive effect of the velocity gradient. The dispersion coefficient is $D_L = (ru)^2/48D$ where r is the tube radius and u the fluid velocity. The second factor is gravity - the contents of the colon are multiphase; the slip velocity between the liquid and solids(including the microorganisms)

may be significant in relation to the very low superficial velocities in the colon; organisms may settle under gravity in ascending regions. The third factor is that microorganisms adhere to and grow on the colon surface. The biofilm, which probably grows and detaches intermittently, plays a very important role in stabilising the system against washout. Currently we cannot estimate the contributions of these three effects to system stability and operation.

However, we can study the effect of dispersion on washout. This analysis should therefore be relevant both to the solid substrate fermenter discussed earlier and to our model of the human colon.

We assume that the mixing can be described by an axial dispersion coefficient, which can either be predicted or determined experimentally. Assuming that the microorganism and substrate travel together we use the same dispersion coefficient D_L to describe the deviation from plug flow for both biomass and substrate.

Assuming Monod growth kinetics and a constant yield coefficient we can write steady state balances on biomass and limiting substrate. We assume constant mean axial velocity = u. With sterile feed the biomass concentration at any point, z, in the reactor is related to the substrate concentration at that point, s, and in the feed (s_0) by $x = Y_{xs}(s_0 - s)$; a substrate balance leads to:

$$D_L \frac{d^2 s}{dz^2} - u \frac{ds}{dz} - \frac{\mu_m s(s_0 - s)}{K_s + s} = 0 \quad (2)$$

with (Danckwerts) boundary conditions:

$$\text{at the inlet } (z = 0): s_0 - s + \frac{D_L}{u} \frac{ds}{dz} = 0 \quad (3)$$

$$\text{and exit, } z = L: \frac{ds}{dz} = 0 \quad (4)$$

These equations can be put into non-dimensional form viz:

$$\frac{1}{Pe} \frac{d^2 y}{dw^2} - \frac{dy}{dw} - \frac{NO(1 - y)y}{K + y} = 0 \quad (5)$$

with boundary conditions:

$$\text{at } w = 0: 1 - y + \frac{1}{Pe} \frac{dy}{dw} = 0 \quad (6)$$

$$\text{and } w = 1: \frac{dy}{dw} = 0 \quad (7)$$

where $y = s/s_0$, $w = z/L$, $NO = L^2 \mu_m / u$, $K = K_s / s_0$ and $Pe = uL/D_L$

The dimensionless group NO is the product of the mean residence time τ ($=L/u$ or V/F where V is the reactor volume and F is the volumetric feedrate) and μ_m , the maximum specific growth rate; in other words it is the ratio μ_m/D . Pe is the Peclet number. A low Peclet number corresponds to high back-mixing; as the Peclet number increases the system tends to plug flow.

For a fermenter with sterile feed to operate steadily and continuously, it is always necessary for the mean residence time of material in the fermenter to be greater than a critical washout value which depends on the growth kinetics and the extent of mixing in the system.

For a perfectly mixed fermenter the dilution rate at washout, D_w is:

$$D_w = \frac{\mu_m s_0}{K_s + s_0} \quad (8)$$

$$\text{or: } \frac{\mu_m}{D_w} = \frac{K_s + s_0}{s_0} = NO_w = 1 + K \quad (9)$$

where subscript “w” refers to the limiting washout condition. For many microbial systems K is small so that $NO_w \approx 1$ as $Pe \rightarrow \infty$.

In pure plug flow there is no finite residence time to satisfy this condition, so the critical (washout) residence time and dilution rate are 0 and ∞ respectively. Therefore $NO_w \rightarrow \infty$ as $Pe \rightarrow 0$.

The washout conditions for a system described by the dispersion model were analysed by Fan et al [7]; for **small** dispersion coefficients (ie large Pe), and Monod kinetics, their expression for washout is:

$$NO_w = \frac{\mu_m}{D_w} = \frac{\mu_m}{D_w} = 0.25 Pe (1 + K) \quad (10)$$

It is possible to compute the washout conditions for any Pe by solving the dispersion equation (eqn 5) and its associated boundary conditions for given Pe and K and iterating to find the critical value of NO for which there is no change in substrate concentration (ie no biomass growth). Some results are shown in figure 4: computed curves are shown for $K = 0.001$ and 0.1 respectively. The computations are also compared with Fan’s approximate solution (dotted lines). The feasible operating region – that is where there is no washout – is **above** the curves. It will be seen that Fan’s predictions begin to diverge significantly from the computed curves for Peclet numbers below around 10; they are not valid for lower Peclet numbers. The numerical calculations tend to the perfectly mixed solution at low Peclet numbers. For high Peclet numbers the numerical solutions become very sensitive to Pe . As expected, higher values of K lead to slightly lower critical dilution rates (ie higher NO_w) over the whole range of mixing conditions, but the answers are not

particularly sensitive to K , particularly if the accuracy of typical kinetic data and models is taken into account. Very similar results are found if different growth kinetics (e.g. logistic growth) are assumed [2].

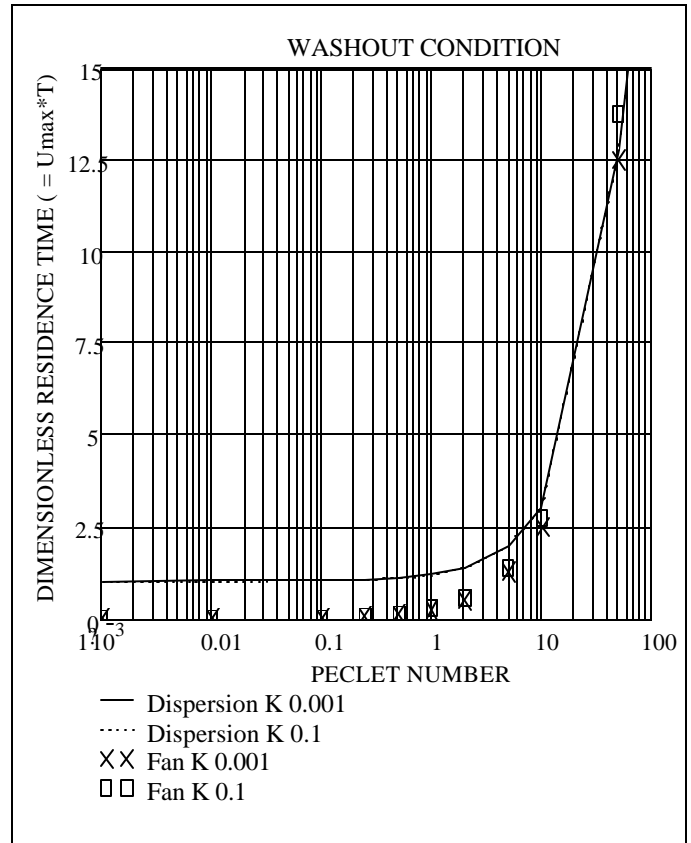


Fig. 4: Washout versus Peclet number

Thus we can now predict the critical conditions to ensure continuous operation in any dispersed plug flow system. It is interesting to note that in laminar flow the dispersion coefficient is inversely proportional to the diffusion coefficient; this would imply that in a liquid environment the Taylor dispersion coefficient for microbial cells is higher than the dispersion coefficient for the solute. At high Reynolds numbers the Peclet number (based on the tube diameter) tends to a finite value around 2; at low Re , the situation is far more complex, but in general the Peclet number decreases rapidly [6] - thus we have the perhaps surprising result that the flow regime in the colon is probably quite well-mixed, even though the Reynolds number is very low. Thus it becomes easier to understand why the human gut is so stable, even without considering the role of the biofilm.

There are very few experimental studies on the washout limits in the two situations discussed so far. Typical profile along a continuously operating solid substrate fermenter show moderate agreement with the dispersion model [2]. It is clear from this that more work needs to be done.

Figures 5 and 6 show some results from the 3-stage human colon model [4].

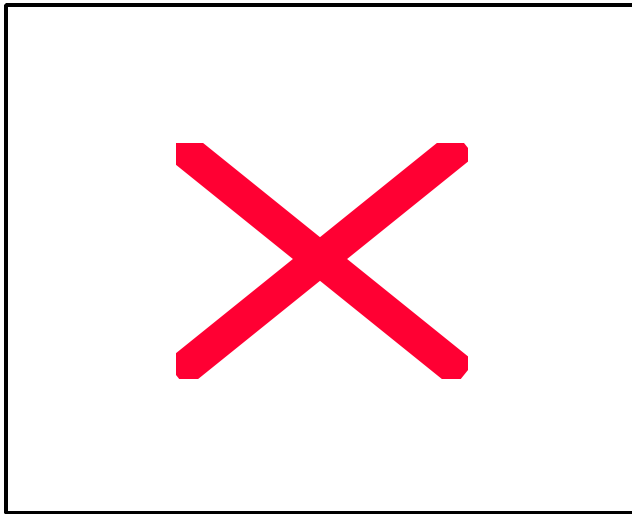


Fig 5: Colony counts

Figure 6 shows carbohydrate levels along the membrane model as a function of time.

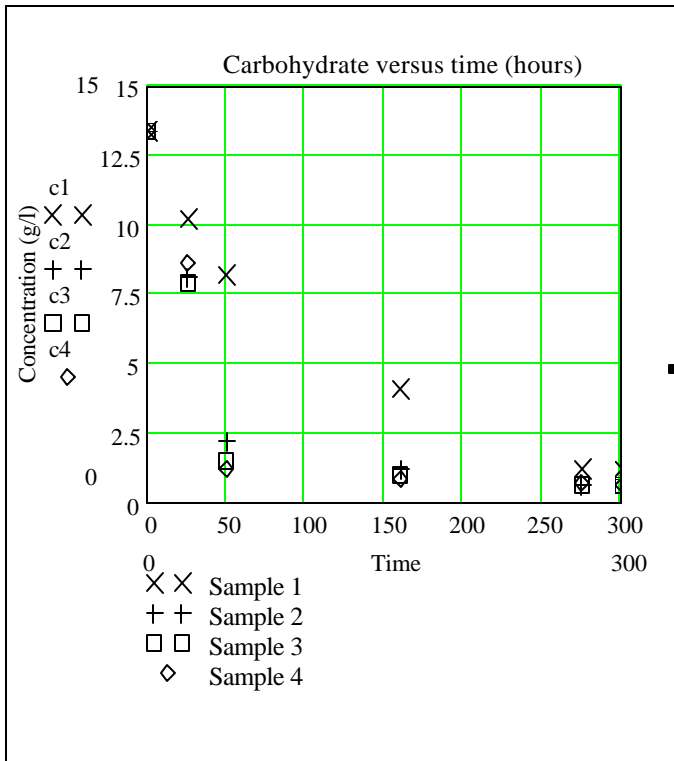


Fig. 6: Carbohydrate profiles

Figure 5 shows measured colony counts at the four sampling points (the entry and the ends of the three modules - positions 1,2,3,4). Note that the colony counts at the entrance are high, and that the drop along the modules is not as pronounced as would be expected in pure plug flow. However, the results are consistent with the arguments above about the role of Taylor dispersion. The figure also shows colony counts from the wall of the membrane in each module (positions 5,6,7): the values are very high and

illustrate the significance of the biofilm. The figure also shows two other results, for comparison. Colony counts from the 3-stage chemostat (positions 8,9,10) are all significantly lower than from the 3stage membrane system. It is very encouraging to note that the microbial levels in the membrane design approach those in the human colon (positions 11,12).

In these experiments the main carbon source was a carbohydrate feed. Whilst levels at the inlet are always higher than in the rest of the model, the values at other sampling points along the system do not vary very much between themselves (because of back-mixing etc); they all decline with time to achieve a steady state. Under these conditions the water removal rate was around 25%; the pH was maintained virtually constant at around 5.5 by circulating a 300g/l solution of 3350 Da PEG.

Clearly, there is a long way to go in developing a robust experimental model of such a complex system as the human colon, but the results are extremely encouraging and illustrate the challenges to biochemical engineers in contributing to the design and analysis of in vitro models.

Biofilms

Biofilms are ubiquitous: many organisms have evolved the capacity to adhere to, and grow on, surfaces either to gain competitive advantage or to survive stress. It is known that microorganisms in the colon adhere to the colon surface. If the shear stresses on the biofilm from the moving contents of the colon do not completely remove the film and if the mass transfer rate of nutrients is adequate the microorganisms will continue to grow and function. The biofilm therefore provides a very important mechanism to limit washout, that is to allow the digestive system to operate over a wide range

Biofilms are also exploited industrially: examples of conditions.

fermentation processes reliant on biofilms include waste treatment systems, ore leaching etc. The last couple of decades has also seen the development of membrane-based processes where microbial biofilms play a key role. Livingston and his colleagues have published extensively on membrane bioreactors [8,9a,9b,10]. The idea is to separate the hostile waste water from the biodegradation side of the process by a semi-permeable membrane. Transfer of the pollutant (e.g. phenol) across the membrane provides the carbon source to the microbial colony on the other side of the membrane. Under many typical conditions the microorganisms will then grow in the form of a biofilm on the surface of the membrane. Typically, as the biofilm grows, it provides increasing resistance to mass transfer, so that the flux of pollutant declines with time. Also, as the film increases in thickness, resistance to transfer of nutrients in the biofilm increases, leading potentially to starvation of

parts of the biofilm. Oxygen transfer, in particular, can become a problem, as illustrated schematically in figure 7.

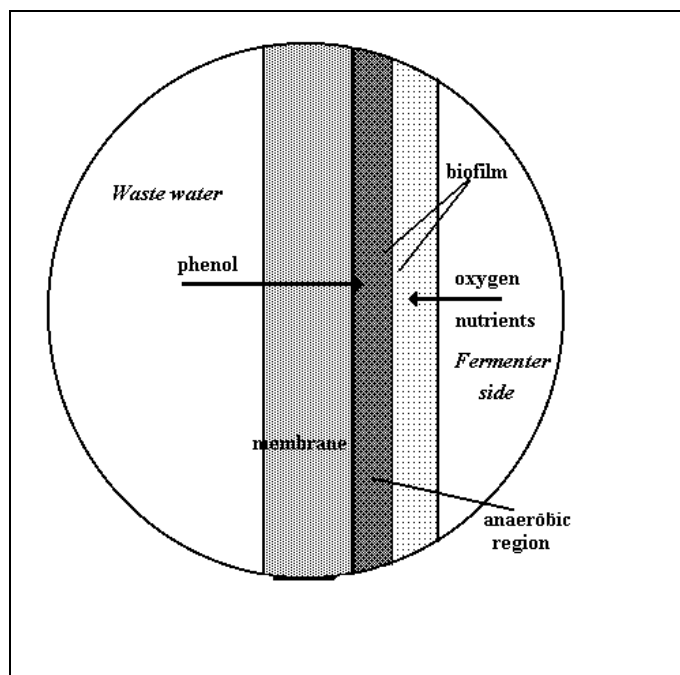


Fig 7: Principle of membrane system

Many of the applications discussed in the literature involve the use of mixed cultures. Here we report some current work using a pure culture - a facultative strain of *Pseudomonas* [11]- to degrade phenolic waste water. When the organism is grown aerobically in conventional batch culture with phenol as carbon source, growth is inhibited at concentrations above around 250 mg l⁻¹. The organism will also grow on phenol under anaerobic conditions, but at a reduced growth rate.

The membrane reactor loop used in these studies is shown schematically in figure 8. A silicone rubber tubular membrane, 3mm internal diameter, wall thickness 0.3 mm, with working length 28 cm is used. The hydrophobic membrane is permeable to phenol but not to water. A 1-litre fermenter is connected to the external side of the membrane. Liquid flowrates on both sides of the membrane were chosen so that further increases did not lead to a significant improvement on the overall mass transfer coefficient between the feed and fermenter sides. Under these conditions the overall mass transfer coefficient was around 3×10^{-7} m/s; most of the resistance to transfer is due to the membrane itself. The system is inoculated on the fermenter side using an inoculum grown on mineral medium in batch culture. Thereafter, no further inoculum is added. The rig shown can be run in repeated batch or continuous culture. In the first option, mineral medium is added from time to time to avoid depletion of key nutrients. In continuous mode, sterile nutrient solution is added continuously at a defined flowrate F and a corresponding flow is removed from the

fermenter to maintain constant volume V . The dilution rate is thus F/V .

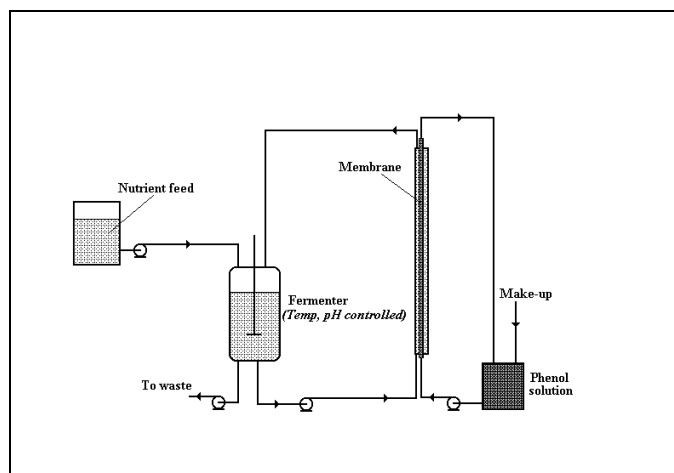


Fig. 8: Schematic of membrane reactor system.

Early experiments started with a phenol concentration = 250 mg/litre in the feed tank. Under these conditions the phenol in the fermenter loop rapidly falls from its initial concentration and a biofilm of the *Pseudomonas* begins to form on the outside of the membrane. Growth of the biofilm was relatively slow; the film appeared within hours but grew slowly thereafter. Moreover, the biofilm has an uneven surface and this remains typical of its morphology. Unlike other mixed culture systems it proved impossible to measure biofilm growth rates directly, so that quantitative estimates of growth rates and yield coefficients are very difficult. Subsequently the phenol concentration was raised to 1500 mg/litre; growth and phenol uptake continued without any problem over several week's semi-batch operation [11]. Interestingly, the growth of the biofilm had no sustained measurable effect on the overall mass transfer coefficient: under these conditions, with a pure culture and substantial resistance to the transfer of phenol in the membrane itself, continuing operation did not lead to a drop in phenol flux, as had been expected. The structure of the biofilm and, in particular, the composition of the exo-polysaccharide (EPS) responsible for the film are clearly important in this regard and part of our current research is investigating these aspects. Perhaps most significant is that throughout it was impossible to detect any phenol in the fermenter loop; however some film detachment occurred leading to a small increase in the biomass concentration in the fermenter loop. Before any detachment occurred it was clear that all the phenol degradation took place in the biofilm. However, once biomass was detected in the fermenter loop it was impossible to know whether all the degradation took place in the biofilm or whether the suspended cells also contributed to phenol breakdown. At this point the operation was switched to continuous mode - that is, a constant feed of nutrients was added to the system at a defined dilution rate.

If the cell concentration at the time t_0 of switching over to continuous operation is x_0 , then, in the absence of detachment from the biofilm and growth of the suspended cells, the cell concentration in the fermenter loop should follow:

$$x(t) = x_0 \cdot \exp(-D(t - t_0)) \quad (11)$$

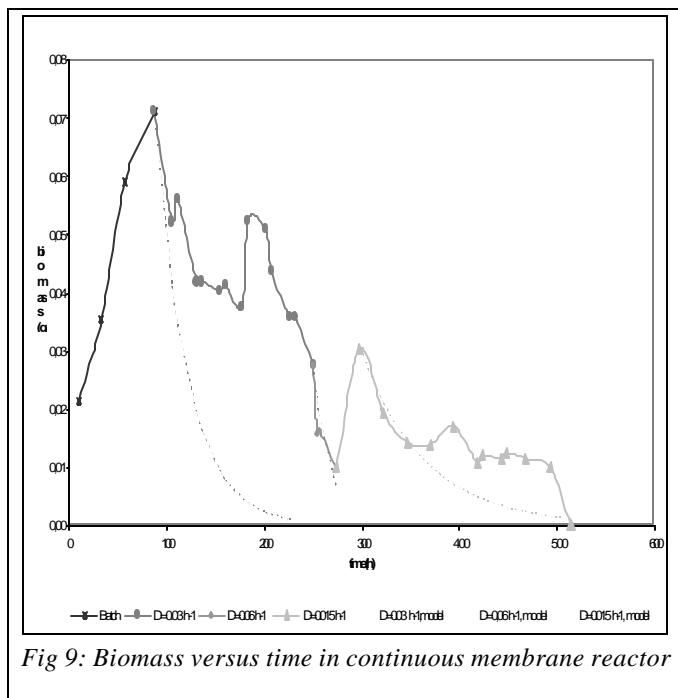


Fig 9: Biomass versus time in continuous membrane reactor

Figure 9 shows the biomass in the fermenter loop as a function of time.

Operation was switched from batch to continuous mode ($D = 0.03 \text{ h}^{-1}$) after 100 hours; operation was switched to different dilution rates ($D = 0.06 \text{ h}^{-1}$ and 0.015 h^{-1}) after 250 and 275 hours respectively. The biomass concentration is compared with the prediction from eqn (11); it will be seen that, whilst in each case the biomass concentration fell, as predicted, the fall in concentration was always lower than the prediction because of intermittent detachment from the growing biofilm. After 500 hours the biomass was completely washed out from the fermenter loop, but phenol continued to transfer and to be consumed by the biofilm. This shows very clearly that considerable biological activity can be maintained in the film and that this continues even when washout of the suspended cells has occurred. There was an initial fall (in the first 50 hours) in the measured phenol flux; the effective overall mass transfer coefficient fell from around 10^{-3} m/s to around $4 \cdot 10^{-4} \text{ m/s}$ and this was then maintained for more than 500 hours continuous operation [12]. At this time the biofilm was around 2-3 mm maximum thickness.

It was very noticeable that the colour and morphology of the biofilm appeared to change. It was suspected that this could

be due to the development of an anaerobic region close to the membrane since theory [10,12] suggests that this should occur once the membrane was thicker than around 200 - 300 μm . Recent experiments have therefore been carried out under anaerobic conditions; the biofilm morphology became less rigid and more fragile; whilst phenol uptake continued, this was at a considerably lower rate than under aerobic conditions. Moreover, the biomass concentration in the fermenter loop itself increased, because of regular detachment of the fragile anaerobic film. These results show that under anaerobic conditions the nature of the biofilm changes dramatically - the expression of EPS is considerably lower than under aerobic conditions and the film is readily detached from the membrane surface. They also suggest that the anaerobic zone shown schematically in figure 7 plays a very important role in biofilm detachment even when aerobic conditions are maintained in the fermenter loop. Finally, they also show that biofilm growth does not necessarily correlate with reduced substrate flux.

Summary and Conclusions

In this paper I have attempted to show how two features of the "real" world - dispersion and growth of biofilms - can produce important effects in fermenting systems. In particular, I have shown how washout may be postponed so as to allow operation at conditions outside those predicted by simple textbook theory. All the examples chosen to illustrate the paper are active research areas. Biochemical engineering is not dead yet!

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