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Effects of Mass Transfer and Reaction Kinetics on Serum Cholesterol Depletion Rates of Free and Immobilized *Pseudomonas pictorum*

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ABSTRACT

Pseudomonas pictorum in free or immobilized form can deplete serum cholesterol. The reaction kinetics control the global rate of cholesterol depletion during the initial part of free *P. pictorum* fermentation. Then, mass transfer takes control of the global rate. In this part of the fermentation, the global rate is first order with respect to the cholesterol concentration. The halftime was 330 min. The global rate is zero order with respect to the bacterial concentration. The activation energy was 83 kJ/mol. These results are consistent with the aqueous cholesterol diffusion model. The open pore agar microcapsule experimental effective diffusivity for lipoproteins at 37°C was 6.7×10^{-11} m^2/s. Mass transfer across the microcapsule was not the limiting factor for the global rate.

Index Entries: Cholesterol; depletion; mass transfer; lipoproteins; global rate; immobilized bacteria; open pore agar gel; *pseudomonas pictorum;* microcapsules.

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INTRODUCTION

Cholesterol is an essential part of animal cells (*1*). Lipoproteins ensure the transport of cholesterol in most higher animals. Low density lipoproteins (LDL) transport cholesterol from the liver to the cells (*2*). High density lipoproteins transport cholesterol from the cells to the liver, known as the reverse cholesterol transport. Elevated serum LDL cholesterol levels may cause the development of atherosclerosis. Lowering serum cholesterol levels could slow or stop the development of atherosclerosis (*3*).

In 1913, Sohngen found that soil microorganisms could grow using cholesterol as the only carbon source (*4*). Since then, other authors have shown that other bacterial strains could also degrade cholesterol (*5,6*). Free and cell-free preparations of *Nocardia restricta* can deplete serum cholesterol (*7,8*). We found that free and open pore agar immobilized *Pseudomonas pictorum* also can deplete serum cholesterol (*9–11*). Mass transfer and reaction kinetics are two factors that can control the global rate of reaction (*12*). The global reaction rate of free and immobilized *P. pictorum* were not significantly different (*11*). This suggested that the mass transfer resistance associated with the agar matrix was negligible compared to the total mass transfer resistance. However, this might change if the bacterial concentration inside the cells increases, resulting in a higher intrinsic reaction rate. Also, it was not clear whether the reaction rate or the mass transfer controlled the global reaction rate of free *P. pictorum.*

In this paper, we have studied the cholesterol uptake process using free bacteria. We have also analyzed the behavior of immobilized bacteria, and evaluated the mass transfer resistance associated with the microencapsulation.

MATERIALS AND METHODS

Microorganism

Pseudomonas pictorum (ATCC #23328) was used in all experiments. It was cultured first in nutrient broth (DIFCO). This was followed by harvesting and resuspension in a cholesterol media. This was to induce the production of bacterial cholesterol degrading enzymes. The composition of the media is as described elsewhere (*11*). After culturing this suspension for 15 d, the induced bacteria was used as an inoculum for further experiments. A Lab-Line Orbit Environ Shaker was used to incubate *P. pictorum* at 37°C and 150 rpm.

Medium for Biomass Production

Bovine calf serum was purchased from SIGMA. It was used in all experiments unless otherwise specified. It was also used to produce biomass for encapsulation experiments and other experiments. In this case,

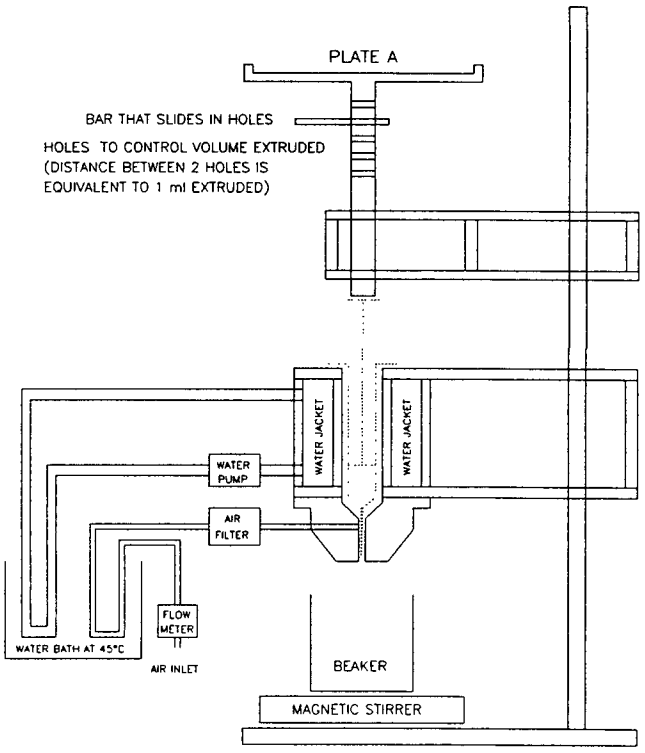

Fig. 1. Apparatus used to make open pore agar microcapsules. *See* Materials and Methods for description.

serum was inoculated with the bacteria and cultured for 36 to 40 h. Then, bacteria was harvested by centrifugation at 2000*g* for 15 min.

Microencapsulation

Open pore agar beads containing *P. pictorum* were prepared. The details of the immobilization procedures appear elsewhere (*11*). Briefly, a 2% agar, 2% alginate solution was prepared, autoclaved for 15 min, and cooled to 45°C. *P. pictorum* was suspended in saline at the desired bacterial concentration. To 10 mL of agar alginate solution at 45°C, we added 1 mL of the bacterial suspension described above. The agar alginate suspension was extruded through a syringe at 45°C. The drops were collected in cold 2% calcium chloride and allowed to harden to form beads. After 15 min, the supernatant was discarded, and the beads were resuspended in 2% sodium citrate for 15 min. Then, they were washed and stored in 0.9% saline at 4°C.

When control beads were prepared, the bacterial suspension was replaced by water, and all the other steps were kept the same. The apparatus used for this paper was modified to control the diameter of the microcapsules (Fig. 1). A water jacketed tube was designed to hold the syringe in a vertical position right above the beaker. The lower end of the tube was

connected to a 2 mm diameter tube designed to house the needle. Compressed sterile air at 45°C flowed in this tube, and around the needle. This created an annular air flow around the needle. The flow rate of the air controlled the size of the microcapsules. The higher the flow rate, the smaller the microcapsules. We found that the rate of extrusion did not affect the shape or size of the microcapsules if the air flow rate was kept constant. For this reason, the agar-alginate suspension was extruded by placing a weight in plate A. The variation in diameter was within ±10%. The water jacket temperature was 45°C±0.1°C. Water came from a recirculation bath. There were four advantages of this design. First, the dead volume was reduced since the needle was directly attached to the syringe. Second, the temperature control was better and more reliable. Third, we could control the microcapsule diameter. Finally, the volume of microcapsules extruded could be set to 1, 2, 3, or 4 mL. This eliminated the inaccuracies associated with measuring volumes of microcapsules.

EXPERIMENTAL PROCEDURES

Samples

100 μL of sample were withdrawn using aseptic techniques. The sample was placed in a 1 mL Eppendorf centrifugation tube and was frozen at −20°C. At the time of analysis, it was thawed and centrifuged at 15,600*g* for 2 min. Cholesterol levels were not affected by this procedure.

Total Cholesterol

An enzymatic color reaction (Allied Instrumentation laboratory) was used to measure cholesterol levels of the supernatant. Analysis was performed with a computerized centrifugal analyzer (MULTISTAT PLUS III), that gave standard errors of 4%.

Absorbance

The sediments were resuspended in 500 μL of water and centrifuged at 15,600*g* for 2 min to wash the cells. They were then resuspended in 500 μL of water. The automatic sampler from the centrifugal analyzer diluted this suspension 6.85 t. The analyzer then measured the absorbance at 690 nm using a 5 mm path cuvet. A calibration curve showed the linearity of the method in the experimental range used. Dry wt analysis was correlated to the method: 1 mg of dry wt/mL of original sample gave 0.0388 units of absorbance.

Incubation

Flasks were incubated in temperature controlled shakers (Lab-Line Orbit Environ Shaker) at 37°C. The temperature control was ±1°C. The speed was 150 rpm.

Effective Diffusivity Experiment

Control microcapsules containing no microorganisms (2 mL) were prepared and saturated in serum. Then, they were filtered, warmed to 37°C, and resuspended in water (8 mL) in a 50 mL Erlenmeyer flask at 37°C. This was placed in the temperature controlled shaker. The flask had a silicon tubing with an end filter than continuously withdrew the supernatant, using a peristaltic pump. Then, the supernatant was returned to the flask. At the exit of the pump, there was a three-way valve for sampling. This permitted frequent sampling without disturbing the system (temperature, agitation). The time delay for the sample was 20s. We corrected the data to account for this delay. Sample sizes was 100 to 200 μL.

RESULTS

Absorbance and Cholesterol Data

The absorbance and cholesterol experimental data were analyzed using the following models:

The logistic model (*13*) was used to fit the absorbance data:

$$X(t) = 1 / [X_1 + X_2 \cdot \exp(X_3 \cdot t)]$$

where X is the absorbance and t is the time. $X_1 \cdot X_2$ and X_3 are parameters estimated by nonlinear regression.

A modification of the logistic model (*9*) was also used to fit the cholesterol data:

$$C(t) = 1 / [C_1 + C_2 \cdot \exp(C_3 \cdot t)] + C_\infty$$

where C is the concentration of cholesterol at time t. C_∞ is the final cholesterol level. C_1, C_2, C_3, and C_∞ are parameters estimated by nonlinear regression.

Figures 2 and 3 show that the models describes the absorbance and cholesterol data well. To obtain the rates of change, the model was differentiated with respect to time. The value of the derivative at time zero gave the initial rate of reaction.

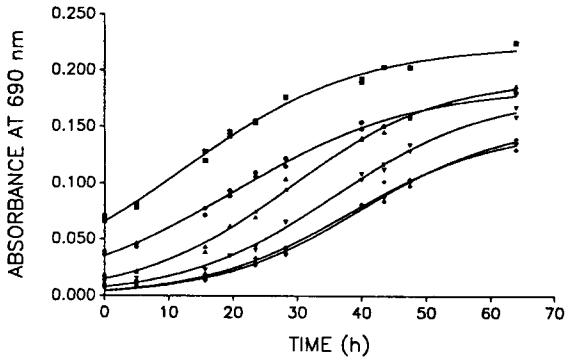

Fig. 2. Absorbance vs time for different initial bacterial concentrations (in mg dry wt/mL): ■ 0.34; ● 0.18; ▲ 0.098; ▼ 0.066; ◆ 0.044. Temperature was 37°C.

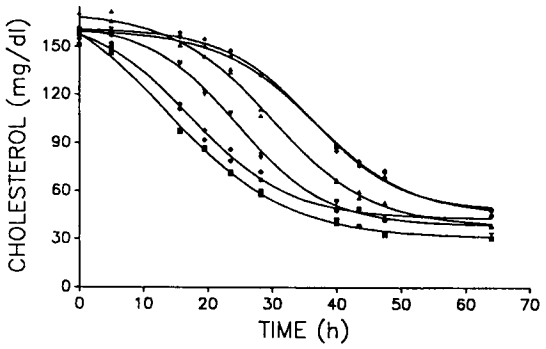

Fig. 3. Cholesterol concentration vs time for different initial bacterial concentrations (in mg dry wt/mL): ■ 0.34; ● 0.18; ▲ 0.098; ▼ 0.066; ◆ 0.044. Temperature was 37°C.

Cholesterol Depletion by Free *P. pictorum*

We grew *P. pictorum* at five initial biomass concentrations (*see* Table 1). We followed the cholesterol concentration and the turbidity during the first 64 h.

Figure 4 shows a semilog. plot of absorbance vs time. The symbols represent the experimental data, whereas the lines represent the model. Note that during the initial part of the experiment, the slopes are constant. This suggests that the growth is exponential. Also, the slopes from experiment having different initial bacterial concentrations are similar (Table 1). This is normal since the maximum growth rate should be independent of inoculum size. Towards the end of the fermentation, growth slows down, reflecting limiting substrate availability.

Table 1
Experiment Using Free Bacteria
at Different Initial Concentrations

experiment	initial bacterial concentration (mg/ml)	maximum specific growth rate (h^{-1})	first order rate constant (h^{-1})
1/1	0.34	N/A*	N/A*
1/2	0.184	N/A*	0.12
1/4	0.098	0.074	0.14
1/8	0.066	0.078	0.12
1/16	0.051	0.081	0.12
1/16	0.037	0.079	0.13
	average	0.0783	0.126
	st. dev.	0.0027	0.007

*Insufficient experimental data points in the linear portion of the curve to calculate the slope.

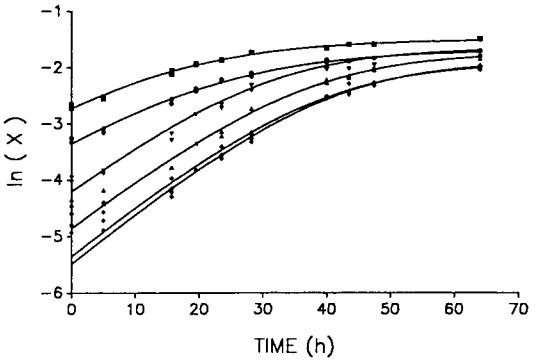

Fig. 4. Absorbance vs time in a semilog. plot for different initial bacterial concentrations (in mg dry wt/mL): ■ 0.34; ● 0.18; ▲ 0.098; ▼ 0.066; ◆0.044. Temperature was 37°C.

Figure 5 shows a semilog. plot of $(C = C_\infty)$ vs time. Here again, symbols represent data whereas lines represent the model. Note that the curves become linear after a certain lag phase. This suggests a first order cholesterol depletion kinetics with respect to the cholesterol concentration. The slope of the lines are the first order rate constants. They are similar for experiments having different initial concentration of bacteria (Table 1). This suggests that the rate constant is independent of bacterial concentration. Thus, the cholesterol depletion kinetics is zero order with respect to the bacterial concentration.

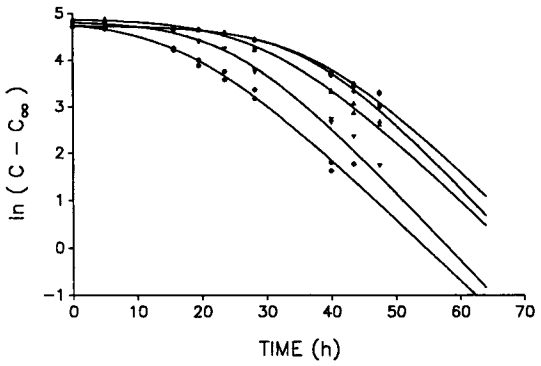

Fig. 5. $C-C_\infty$ vs time in a semilog. plot for different initial bacterial concentrations (in mg dry wt/mL): ■ 0.34; ● 0.18; ▲ 0.098; ▼ 0.066; ◆ 0.044. Temperature was 37°C.

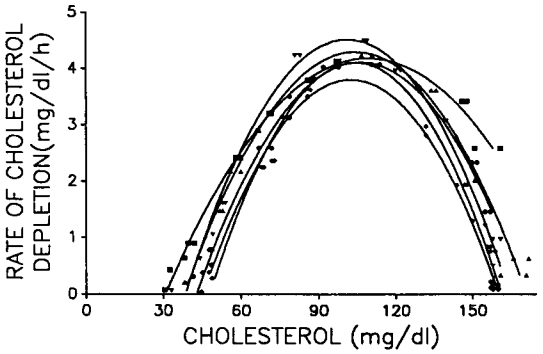

Fig. 6. Rates of cholesterol depletion vs cholesterol concentrations for different initial bacterial concentrations (in mg dry wt/mL): ■ 0.34; ● 0.18; ▲ 0.098; ▼ 0.066; ◆ 0.044. Temperature was 37°C.

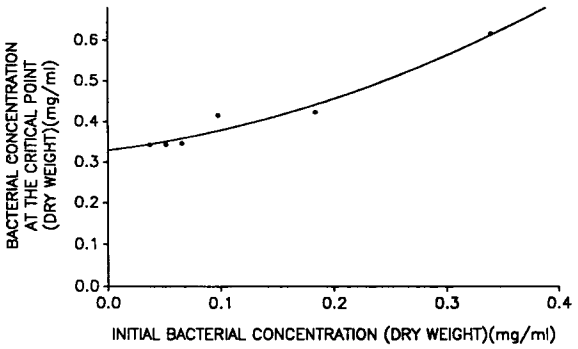

Fig. 7. Bacterial concentration when the depletion rate is maximum (critical point) vs initial bacterial concentration at 37°C.

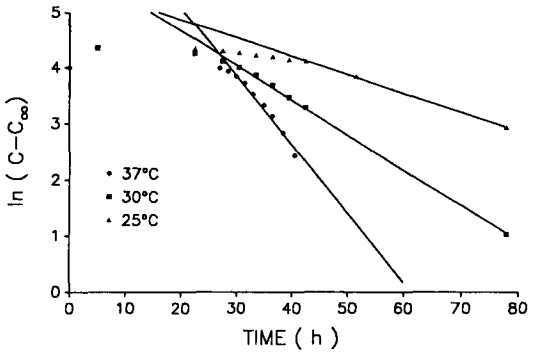

Fig. 8. $C-C_\infty$ vs time in a semilog. plot for three different temperatures.

At the beginning of the experiment, the cholesterol concentration is about 150 mg/dL in all cases. We follow the changes in cholesterol concentration during the period of the experiment. The cholesterol levels decrease, as bacteria deplete it. We analyze the relationship between the depletion rates and the cholesterol concentration during the course of the reaction (Fig. 6). The initial rate of depletion depends on the initial bacterial concentration. This will be discussed later in this paper. The rates of depletion increase as cholesterol concentrations decrease from 150 mg/dL to 100 mg/dL. This reflects the bacterial growth. However, the rates of depletion reach a maximum of about 4 mg/dL/h as the cholesterol concentration approaches 100 mg/dL. These two values are independent of the initial bacterial concentration over the range studied. We will refer to this point as the "critical point." As cholesterol concentrations further decrease, the rates of cholesterol depletion also decline. The depletion rates decrease to zero when the cholesterol concentrations reach the 30 mg/dL to 40 mg/dL range.

Figure 7 shows the relation between the bacterial concentration at the "critical point" (X_c) and the initial bacterial concentration (X_i). When X_i is less than 0.1 mg/mL, X_c is independent of X_i. However, when X_i is larger than 0.1 mg/mL, X_c start to increase with X_i.

We grew *P. pictorum* at three different temperatures: 25°C, 30°C, and 37°C. Figure 8 shows a semi-log. plot of $C=C_\infty$ against time for the different temperatures. After a lag phase of about 20 h, the cholesterol concentration start decreasing. In the semi-log. plot, the relation between the cholesterol concentration and time seems linear. The lines are the linear regression of the linear part of the curve for each temperature. The slope of these lines are the first order rate constants.

Figure 9 shows the semi-log. plot of the first rate constants found in Fig. 8 against the reciprocal of the temperature. This is known as an Arrhenius plot. The line is a linear regression, and the slope gives the activation energy for the cholesterol depletion process. We found an experimental activation energy of 83 kJ/mol.

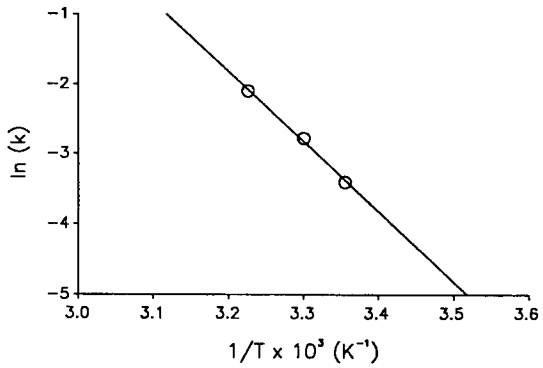

Fig. 9. Arrhenius plot for the cholesterol depletion process. First rate constants are evaluated in Fig. 8. The activation energy was 83 kJ/mol.

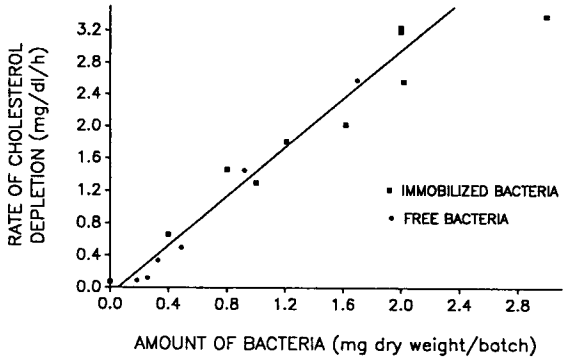

Fig. 10. Initial rate of cholesterol depletion vs initial bacterial concentration at 37°C. ● Free *P. pictorum;* ■ open pore agar immobilized *P. pictorum.*

Cholesterol Depletion by Immobilized Bacteria

Effect of Bacterial Concentration

Figure 10 shows the initial rate of reaction against the initial bacteria concentration for free and immobilized bacteria. The diameter of all the beads was about 2 mm. Results are the average of triplicates. The initial rate of reaction correlates well with the initial bacterial concentration. Also, free and immobilized bacteria gives very similar results.

Effect of Open Pore Agar Bead Diameters

P. pictorum was immobilized in open pore agar beads of different diameter. The bacterial concentration was 1 mg of dry wt per batch in all experiments. Cholesterol concentrations vs time appear in Figs. 11, 12.

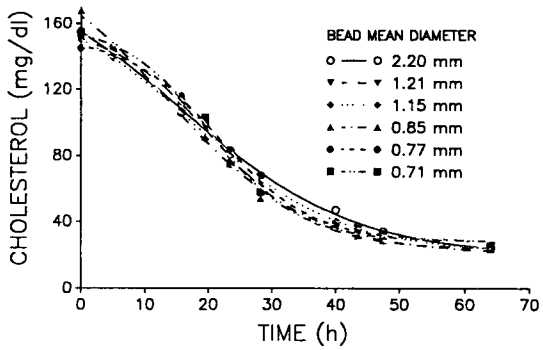

Fig. 11. Effect of microcapsule diameter on cholesterol concentration vs time for experiments containing 1 mL of microcapsules with 1 mg of *P. pictorum* in 5 mL of serum. Temperature was 37°C.

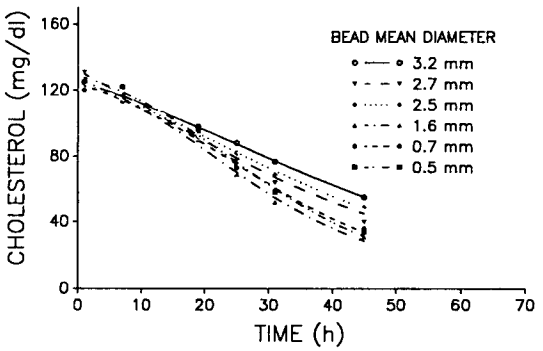

Fig. 12. Effect of microcapsule diameter on cholesterol concentration vs time for experiments containing 1 mL of microcapsules with 1 mg of *P. pictorum* in 5 mL of serum. Temperature was 37°C.

Figure 11 shows that the diameters of the microcapsules within the range of 0.7–2.2 mm, do not affect the relation between the cholesterol concentration and time. In fact, all experiment had an initial cholesterol concentration of about 150 mg/dL. During the experiment, the cholesterol concentration of the different experiments were similar. At 64 h, all experiment reached the same cholesterol concentration of about 20 mg/dL.

Figure 12 shows that larger microcapsules in the range 3.2–2.5 mm affect cholesterol concentrations. Indeed, all experiments had an initial cholesterol concentration of about 120 mg/dL. However, after 45 h, experiments containing smaller microcapsules (1.6–0.5 mm) had cholesterol levels of about 40 mg/dL. In contrast, experiments containing larger microcapsules (3.2–2.5 mm) had cholesterol levels of about 60 mg/dL.

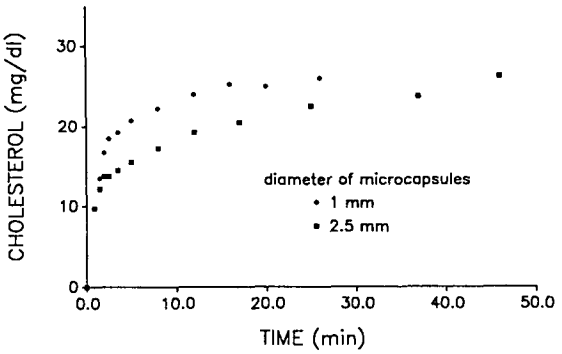

Fig. 13. Supernatant cholesterol concentration vs time. This experiment is designed to evaluate the effective diffusivity of cholesterol in open pore agar microcapsules at 37°C. *See* Material and Methods for details.

Lipoprotein Effective Diffusivity in Open Pore Agar Microcapsules

We used control open pore agar microcapsules containing no microorganisms to evaluate the effective diffusivity of open pore agar. One experiment had microcapsules of about 500 μm in diameter. The other had microcapsules of about 2.5 mm in diameter. The microcapsules were saturated in serum. When placed in water, cholesterol diffused out of the matrix increasing the supernatant concentration. Figure 13 shows the relation between the supernatant cholesterol concentration and time. During the first minute, the cholesterol concentration increased sharply with time. This resulted from some serum present outside the microcapsules. Then, the cholesterol concentration increased at a slower rate, reflecting the diffusion of cholesterol from the inside of the microcapsule. After some time, the cholesterol concentration reached an equilibrium value of about 25 mg/dL. The experiment containing smaller microcapsules reached the equilibrium value faster than the other one. We used the method presented by Crank (*14*) to calculate the effective diffusivity from this data. The effective diffusivity found was 6.5×10^{-11} m²/s for 2.5 mm diameter and 6.8×10^{-11} m²/s for 500 μm diameter.

DISCUSSION

Cholesterol Depletion by Free *P. pictorum*

The removal of cholesterol by *P. pictorum* is a complex process, since it includes many mass transfer resistances. *A priori,* it is not clear whether the rate of mass transfer or the reaction rate controls the global rate. Previous work (*11*) has shown that about 20% of the cholesterol was not exchangeable. So, we added the C_∞ term in the model for cholesterol.

Results show that the growth rate is exponential for the initial part of the experiment (Fig. 4). In this part, reaction rate seems independent of cholesterol concentration and dependent on bacterial concentration (Fig. 10). Results also show that after a lage phase, the rate of cholesterol depletion is first order with respect to cholesterol levels (Fig. 5). In this part of the experiment, the rate of cholesterol uptake is zero order with respect to the bacterial concentration. All this indicates that reaction kinetics controls the global rate in the initial part of the experiment. Then, mass transfer takes control.

Many authors suggested that the lipoprotein cholesterol transfer rate is first order with respect to the cholesterol content in lipoproteins (*15*). We found an average first order rate of 0.126 h^{-1}. The half time (ln(2) divided by the first order rate constant) was 330 minutes. This value is much higher than the 8 to 14 min reported for human lipoproteins (*16,17*). However, the rate is highly dependent on lipoprotein structure or size (*15*). For example, a halftime of 100 min was reported for rat HDL particles. These are much larger than the human ones (*18*). In our experiments, we used bovine calf serum.

The cholesterol depletion rate of bacteria in the initial part of the experiment is low. Thus, lipoproteins can deliver the cholesterol required by the bacteria. As bacteria grow, the net cholesterol depletion rate also grows. Also, both the cholesterol concentration and lipoprotein transfer rate decrease. At the "critical point," cholesterol becomes limiting, and the mass transfer rate takes control of the global rate. At this particular point, the cholesterol depletion rate is at its maximum, and is about 4 mg/dL/h.

We found that the bacterial concentration at the "critical point" was not constant (Fig. 7). It appears as if the cholesterol activity of the biomass present in the inoculum is low. As bacteria grow in serum, specific cholesterol activity increases. New generations of bacteria have higher activity. If the growth is exponential, the specific activity should reach a maximum. The bacterial level at the "critical point" reaches a minimum when the specific activity is at its maximum. From Fig. 7, the minimum bacterial level at the "critical point" seems to be close to 0.34 mg of dry wt/mL. This corresponds to a maximum specific activity of about 0.12 mg of cholesterol/mg of dry wt/h. As the inoculum size increases, the bacterial level at the "critical point" increases reflecting a lower specific activity.

After the "critical point," mass transfer controls the global rate since cholesterol depletion is independent of bacterial concentration. The mass transfer process is quite complex. Two models have been proposed to describe the mass transfer of cholesterol between lipoproteins and cells:

1. The collision model (*19*). This model considers that the transfer of cholesterol occurs via "transient collision complexes."
2. The aqueous diffusion model (*20*). Cholesterol would transfer by diffusion through the aqueous phase. Phillips et al. (*15*) reviewed in detail both models.

McLean and Phillips (*21*) summarized four criteria that characterized the aqueous diffusion model.

1. The rate of cholesterol exchange is first order with respect to cholesterol concentration.
2. This rate is zero order with respect to the acceptor concentration.
3. The transfer is strongly temperature dependent. Activation energy is close to 75 kJ/mol.
4. The interfacial flux of cholesterol is six times lower than that of phosphatidylcholine.

Results of the free bacteria experiments are consistent with three of these four criteria. First, the rate of cholesterol exchange is first order with respect to the concentration of cholesterol in serum (Fig. 5). Second, the Arrhenius plot gives an experimental activation energy of 83 kJ/mol (Fig. 9). This is consistent with the strong temperature dependence discussed by McLean and Phillips. Third, cholesterol depletion is independent of the bacterial (acceptor) concentration (Fig. 6).

Cholesterol Depletion by Immobilized Bacteria

Immobilization of bacteria adds a new mass transfer barrier: the diffusion of lipoproteins or cholesterol in the porous matrix. Increasing the concentration of bacteria or the diameter of the microcapsules could lead to diffusion limited depletion rates.

Effect of Bacterial Concentration

Increasing the initial bacterial concentration would increase the depletion rate. This is analogous to a spherical porous catalyst. In this case, the effectiveness factor decreases with increasing catalytic activity when mass transfer is limiting (*12*). Results show that immobilized and free bacteria have similar initial rate of cholesterol depletion. This is true on a wide range of bacterial concentrations (Fig. 10).

Effect of Microcapsule Diameter

Here again, the analogy with a spherical porous catalyst holds. The diameter of the particle strongly affects the effectiveness factor (*12*). In fact, the larger the microcapsule, the higher the concentration gradient inside the microcapsule. Results show that levels of cholesterol are independent of microcapsule diameters of up to 2.2 mm (Fig. 11). As the microcapsule diameter further increases, the activity drops (Fig. 12). However, this drop is too small to calculate the effective diffusivity. Also, this calculation needs a well-defined simple kinetics, that is not the case here. The drop in activity may also be on account of method of encapsulation. The time that the agar-alginate microcapsules spend in sodium citrate was 15 min. For larger microcapsules, this might be too short a time to completely

leach out the alginate. In this case, the core of the microcapsule could have smaller pores that might prevent the diffusion of lipoproteins. More experiments should clarify this point.

Lipoprotein Effective Diffusivity in Open Pore Agar Microcapsules

We found that the diffusivity of lipoproteins in open agar was about 6.7×10^{-11} m²/s. This is a very high diffusivity. Figure 13 shows that over 80% of cholesterol bound to lipoproteins diffuses out of 2.5 mm microcapsules in one hour. This rate is much larger than the global rate of cholesterol depletion by bacteria. This indicates that lipoproteins can diffuse in and out of the microcapsule. In fact, using this diffusivity with 2.5 mm microcapsules, a concentration gradient of less than 10 mg/dL could develop between the surface and the core of the bead (*12*). This is assuming a first order rate constant of 0.12 h^{-1}, and a bulk cholesterol concentration of 100 mg/dL. The effect of this concentration gradient on the global rate is probably negligible.

General Discussion

Results discussed in this paper give an understanding of the cholesterol depletion process by immobilized bacteria. Our previous publication (*11*) identified the need to increase the global rate. Thus, it is important to determine what controls the global rate. Results suggest that mass transfer of cholesterol between lipoproteins to the aqueous solution are limiting the rate of depletion. Patients suffering from hypercholesterolemia have levels of cholesterol over 250 mg/dL (*22*). We can extrapolate our results to predict what would happen when the initial cholesterol concentrations are higher. Extrapolation indicates that the rate of depletion should increase proportionally. According to this, the rate of cholesterol transfer for serum having 250 mg of cholesterol/dL should be around 10 mg/dL/h. This rate can further increase if we consider that we used bovine serum, and that human serum has higher rates of transfer. Thus, further research has to concentrate in this area.

The open pore agar microcapsules should give minimal mass transfer resistance even if the global rate becomes many times higher. This is assuming that the diffusivity will stay constant with concentration changes.

CONCLUSION

This paper identified the factors that control the global rate of cholesterol depletion by *P. pictorum* at 37°C. In the initial part of the fermentation, reaction rate controls the global with respect to the cholesterol concentration, and zero order with respect to bacteria concentration. Results are consistent with the aqueous diffusion model.

The results for both immobilized bacteria and free bacteria are similar. The initial rate of reaction is proportional to bacterial concentration. The diameter of the microcapsules do not affect activity if under 2.2 mm. Finally, we found an effective diffusivity of lipoproteins in open pore agar of 6.7×10^{-11} m^2/s. Using this diffusivity, we found that the concentration gradient inside the microcapsule should be negligible.

ACKNOWLEDGMENTS

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