0273-2289/89/2021—0421$03.00

On the Mechanism of Growth of Cells (*Bacillus amyloliquefaciens*) in the Mixed Aqueous Two-Phase System

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ABSTRACT

The growth of *Bacillus amyloliquefaciens* in the aqueous two-phase system, made up of polyethylene glycol, dextran, and water, was investigated. Generally, *Bacillus* partitions in the dextran phase, but the magnitude of the separation depends largely on the overall composition of polymers in the phase system. The kinetics of growth of *Bacillus amyloliquefaciens* was studied in the polyethylene glycol-rich continuous phase, dextran-rich dispersed phase, and in the mixed phase. From the kinetic data it appears that increasing the overall polymer composition causes the cells to adsorp at the interface. On the other hand, partition measurements indicate that increasing polymer concentrations make the cell partitioning more one-sided. This anomaly is explained by studying the interfacial adsorption of cells via dynamic surface tension measurements.

Index Entries: Aqueous two-phase; *Bacillus amyloliquefaciens;* kinetics; interfacial adsorption; surface tension.

INTRODUCTION

An aqueous two-phase system is a liquid–liquid system generated by the addition of two incompatible polymers or a polymer and a salt to

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water (*1*). When particles or molecules are added to the system they preferentially distribute in one of the phases and/or at the interface. The distribution of a component between the two phases is characterized by the partition coefficient, K_i, which is defined as the ratio of concentrations of the component in the two phases. The preferential distribution of cells, cell particles, and molecules between the two phases can be effectively utilized in both bioseparation and fermentation technology.

Aqueous two-phase systems have become increasingly popular for the separation and purification of biomolecules during the last two decades (*2*). The two other potential areas of application of the two-phase system are in the immobilization of cells and in extractive fermentation. In the case of immobilization or extractive fermentation, the cells are retained in the dispersed phase and the products are transferred to the continuous phase to increase the overall productivity of fermentation. In order to design an appropriate phase system for immobilization or extractive fermentation, it is important to have a knowledge of the partition behavior of the cells in the liquid–liquid system (the liquid–liquid dispersed system is commonly referred to as the mixed system). In other words, it is imperative to have a clear understanding if the growth of the cells occur in the dispersed phase, continuous phase, or at the interface.

Adsorption of particles at the interface is one of the characteristics that distinguishes particle partition from molecule partition (*1*). Unlike soluble molecules like proteins, nucleic acids, and salts, separation of cells and organelles occurs mostly between one phase and the interface. The nature of cell distribution in the mixed phase, especially with respect to surface adsorption is of importance for reasons such as: surface adsorption is undesirable for extractive fermentation, and surface adsorption can actually be beneficial for liquid phase immobilization. By immobilizing cells at the interface it is possible to reduce the oxygen or other mass transfer related problems and thus make liquid–liquid cell immobilization more attractive than solid matrix cell immobilization.

The measurement of the partition coefficients of insoluble molecules or particles is problematic. To measure partition coefficients, the phase system must be mixed and allowed to coalesce and separate. The fate of the particles may be influenced by the complex nature of the phase separation process, making partition coefficient measurements difficult. For example, in a PEG–dextran–water system, cell particles destined for the lighter PEG phase may get trapped by falling between coalescing heavier dextran phase drops and may remain trapped in the dextran phase. This situation is analogous to the multiple-phase system in an oil–water system described by Carroll (*3*). The mechanism of phase separation as found to influence partitioning of cells by Raymond and Fischer (*4*). Some interesting phenomena of erythrocyte adsorption at the interface and its passage to the center of a globule immediately after coalescence have been described by Reitherman et al. (*5*).

In many of the partition measurements, phase separation is achieved by centrifuging the mixed phase (*6,7*). This may introduce an additional error in partition measurement by forcing the cells to settle at the interface or at the bottom phase. In addition, partition may be influenced by the polymer adsorption on the cell surface with changes overall surface characteristics of the cells and consequently the zeta potential (*8,9*). This polymer binding is an irreversible common feature of polymer adsorption. The cell partition may thus depend on the phase to which it is exposed first. Polymer adsorption has been seen to affect erythrocyte partitioning (*9,10*). High cell density at the interface and the resulting cell aggregation at the interface may also influence the partition coefficient of cells (*5*).

The general affinity of the cells for surface adsorption strongly depends on the available surface area. In the fermenter, agitation creates a large interfacial surface area making cell adsorption to the interface easier. In addition, the motion of fluid and fluid shear forces between phases contribute to the complexity of the problem. All these raise the question as to whether the partition value measured through gravity settling of the phases represents actual distribution of the cells in the dynamic mixed phase in the fermenter.

The primary objective of this study was to understand the mechanism of cell growth in the mixed two phase system. In other words, the intention was to know if the cells grow in the lighter PEG-rich phase, or the heavier dextran-rich phase, or at the liquid–liquid interface. To understand this, one must have a clear idea of cell partitioning in the two-phase system and how the dynamics of the system influences the partition behavior in the fermenter. In order to achieve the objective, the kinetics of growth and product formation in the mixed two-phase system and the corresponding two equilibrium conjugate phases were investigated. Similar methodology has been used to study the growth of cells in hydrocarbon fermentation by Erickson et al. (*11–14*). Their investigations, however, did not include any kinetic studies in the individual phases. Moreover, in terms of interfacial properties, the aqueous two-phase system is much different from the oil–water system. For the oil–water system, growth is essentially at the interface and invariant of oil–water compositions contrary to the case of the aqueous two-phase system. In the present study, it was presumed that if the cells were partitioned in the dextran-rich phase, the kinetics of cell growth in the two-phase system would be similar to that in the dextran-rich phase unless both phases are similar in terms of physical characteristics and oxygen transfer coefficients. Correspondingly, if the cells were partitioned in the PEG-rich phase, the kinetics in the two-phase system will be similar to those in the PEG-rich phase. In the event that the cells are not totally dispersed in one phase or the other phase, the overall kinetics is the combination of the kinetics of both phases. Cell partition measurements can then be qualitatively correlated to the kinetics of cells in the mixed two-phase system.

Interestingly, this situation is further complicated by the growth at the interface. To understand the affinity of cells for the liquid-liquid interface, the concept of dynamic interfacial tension was applied. Dynamic interfacial tension is widely used to study the adsorption behavior of surface active agents (*15–18*). Such studies are used for understanding interfacial phenomena such as solubilization, capsule formation, emulsification, foam stability, detergent action, and flotation (*15*). The kinetics of cell adsorption at the liquid-liquid interface were studied by measuring the dynamic tension of the interfaces in the presence of the cells. In addition to this, a separate method was used to obtain quantitative data on cell adsorption at the interface.

The kinetic data of cell growth and the interfacial adsorption data suggest that increasing the polymer concentration moves the cells to the interface. On the other hand, measurements of the partition coefficient by gravity settling of the phases have indicated that increasing polymer concentration makes the cell partitioning more one-sided. This anomaly between the two results is caused by the influence of phase separation on the cell distribution. The present investigation also explains this anomaly.

MATERIALS AND METHODS

Microorganism and Medium

The α-amylase producing strain, *Bacillus amyloliquefaciens* F (ATCC 23350), was used for this study. The fermentation medium used was the same as Alam et al. (*19*).

Phase System

PEG 8000 and dextran 70 were used as phase forming polymers in water. The phase diagram for the PEG 8000-dextran 70 system, adopted from Alam (*20*), is given in Fig. 1. The phase systems contained all the salts used to make the fermentation medium. Details of the physical properties of these polymers have already been discussed elsewhere (*20*). In the present investigation, the partition of *Bacillus amyloliquefaciens* was studied in two separate phase systems. The first system had an overall composition of 9.0% (w/w) PEG and 2.75% (w/w) dextran. The second one had an overall composition of 14.75% (w/w) PEG and 3.25% (w/w) dextran. These systems will be referred to as low polymer and high polymer systems throughout this manuscript. The composition of conjugate phases in equilibrium can be read directly from the phase diagram given in Fig. 1. The low and high polymer systems were chosen such that each has a phase volume ratio of approximately eight. In both systems, the continuous phase, which is mostly water and PEG, is referred to as the PEG phase. On the other hand, the smaller dispersed phase, mostly containing water and dextran, is referred to as the dextran phase.

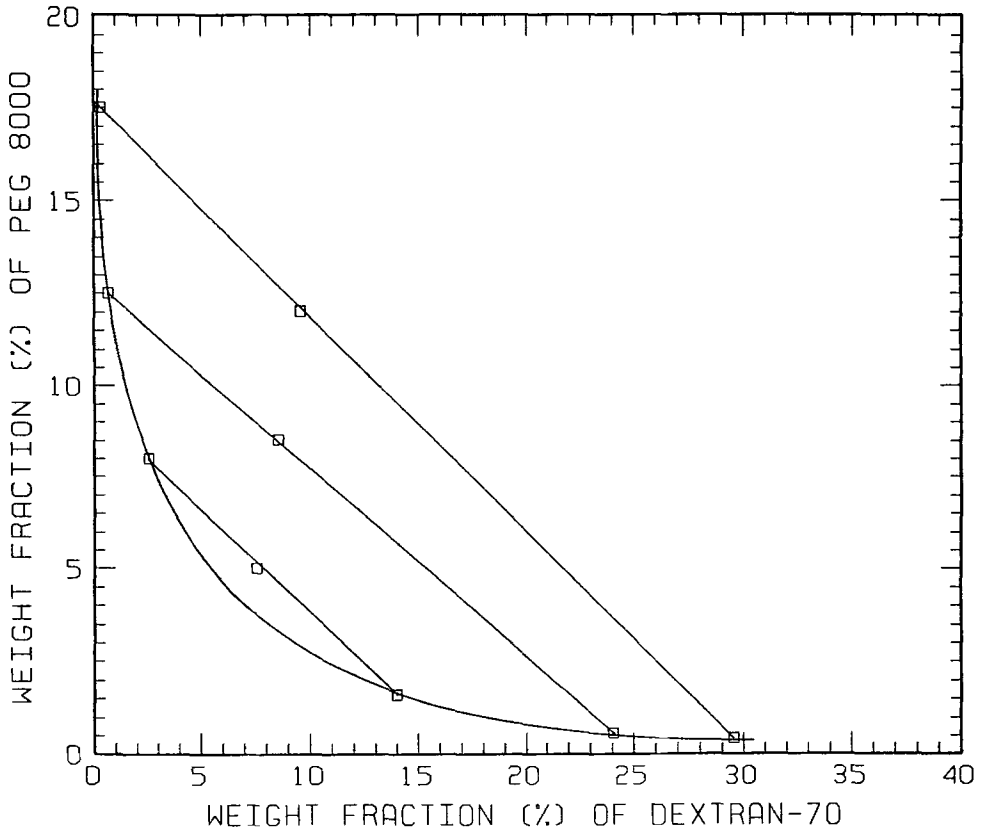

Fig. 1. Phase diagram of PEG 8000 and dextran 70 at 25°C.

Phase Separation Studies

A system made up with 3.25% (w/w) dextran and 14.75% (w/w) PEG was used in the separation studies. Polymer solutions were prepared by dissolving each polymer in water. The polymeric solutions were then mixed together to make the phase system. Filtered cells were added to this mixture to make up a cell concentration of 1 g/L. The mixture was agitated at 400 rpm in a beaker. Ten mL of samples were taken from the beaker and poured into a 125×17 mm conical graduated centrifuge tube. The dextran phase, being heavier, settled at the bottom of the tubes. The percent of phase separation was then calculated by dividing the volume of the dextran phase at any time by the equilibrium volume of the dextran phase. The equilibrium volume of the dextran phase was determined by centrifuging the phase system at 15000 rpm for 15 min in order to obtain a complete phase separation.

Measurement of Partition Coefficients

Cells were collected from their mid-logarithmic stage and washed in 0.95% salt solution. An aliquot of washed, packed cells with a volume between 0.05–0.10 mL was introduced in a 15×125 mm test tube contain-

ing 10 mL of the phase system. The phase systems were made up of equal volumes of lighter and heavier phases. The phase systems were mixed by shaking up and down 20 times and were subsequently allowed to settle for 30 min. Samples were then collected from the top and bottom phases and then diluted (1:10) to measure the absorbance at 620 nm. For each phase, the corresponding diluted cell free phases were used as blanks for absorbance measurement.

Sedimentation Study of Cells

The cell sedimentation profiles were obtained in the continuous phase (PEG phase) of a two-phase system. Actively growing *Bacillus* cells were collected and washed in an isotonic solution. The cells were suspended in the PEG phase of a system made up of 9.0% (w/w) PEG and 2.75% (w/w) dextran and then mixed well. The mixture of cells in the PEG phase was then poured in a special test tube for measuring the absorbance. This glass test tube was covered around with a black tape leaving two windows of 1 cm×0.5 cm size located diagonally on the opposite sides of the tube. This window was made to follow the sedimentation of a band of cells in the spectrophotometer. The cell suspension was then mixed well in the tube. The change of absorbance of the cell suspension was then followed as a function of time in a Spectronic 20 spectrophotometer. Three different concentrations of cells were used for sedimentation study.

Fermentations

The fermentations were carried out in a one liter Bellco (Bellco Glass Inc., Vineland, NJ) fermentor with air flow rate of 3.6 vvm and agitation rate of 500 rpm. The fermentation volume was 750 mL. For two-phase fermentations, the salt, glucose, yeast extract, and polymer solutions were sterilized separately and were then mixed together. To obtain the conjugate two phases in equilibrium, 10 L of the two-phase medium was prepared as above and was then allowed to equilibrate in a bottle. The sterility of the system was carefully maintained. The phases were allowed to separate for at least 24 h. Each phase was then aseptically pumped out to individual Bellco fermenters. Following this technique assured the distribution of salts to be precisely the same as that in the mixed two-phases. For every fermentation, inoculums were prepared in the corresponding medium. The inoculm volumes were 10 mL. The biomass concentration (g/L) and α-amylase activity (LAU/mL) measurement methods are given by Alam et al. (*19*).

Measurement of Dynamic Interfacial Tension

The pendant drop technique was used to determine the interfacial tension between the PEG phase and the dextran phase of the two-phase system. All measurements were carried out at room temperature. First,

the phase systems were made up with the polymers and salts of the fermentation medium. Glucose and yeast extract were excluded to prevent the growth and motility of cell during surface tension measurements. The phase systems were then allowed to separate in a separatory flask for 72 h. Each phase was further centrifuged at 10,000 rpm for 20 min to remove traces of the second phase. Droplets of the lighter PEG phase were formed in the denser dextran phase from the tip (50 and 100 μm) of a microsyringe using a micromanipulator. The drops were then photographed. These photographs were projected on the screen and the necessary parameters were measured to calculate the interfacial tension (*21*). To study the adsorption behavior of the cells at the liquid-liquid interface, cells were first obtained by washing in an isotonic solution. A controlled number of cells were then dispensed in the lighter PEG phase. The cell concentrations were measured in a counting chamber under a microscope. The concentration of the cells in the PEG phases were approximately $4\times10^4-5\times10^4$ cells/mL. Droplets of the lighter PEG phase containing cells were then formed in the denser dextran-rich phase. The drops were then photographed at approximately 1-min intervals and the change in interfacial tension was calculated as a function of time.

Study of Cell Adsorption at the Liquid–Liquid Interface

The cell adsorption characteristics at the liquid-liquid interface were measured by allowing a drop (one mm diameter) of cell-free heavier dextran-rich phase to fall through the corresponding PEG-rich phase containing a known number of cells/U volume in a buret. While falling through the cell suspension, cells will adsorp at the drop surface according to their adsorption characteristics. The dextran-rich lower phase was then collected at the bottom and the cell concentration was measured by plating on nutrient agar. The adsorption curves were then developed by varying the concentrations of cells in the PEG phase. The residence time for every drop was maintained constant at 10 s by adjusting the length of the PEG-rich phase in the buret.

RESULTS AND DISCUSSION

Partition Coefficients of *Bacillus amyloliquefaciens*

Most of the studies that relate to partition measurement of cells have been for analytical purposes or for characterizing and differentiating various cells (*1,22–24*). It has also been used to identify growth cycle related surface property changes in various cell lines (*25–27*). The general problems of measuring partition coefficient have already been discussed. Andersson and Hagerdal (*28*) measured the partition coefficient of *Bacillus*

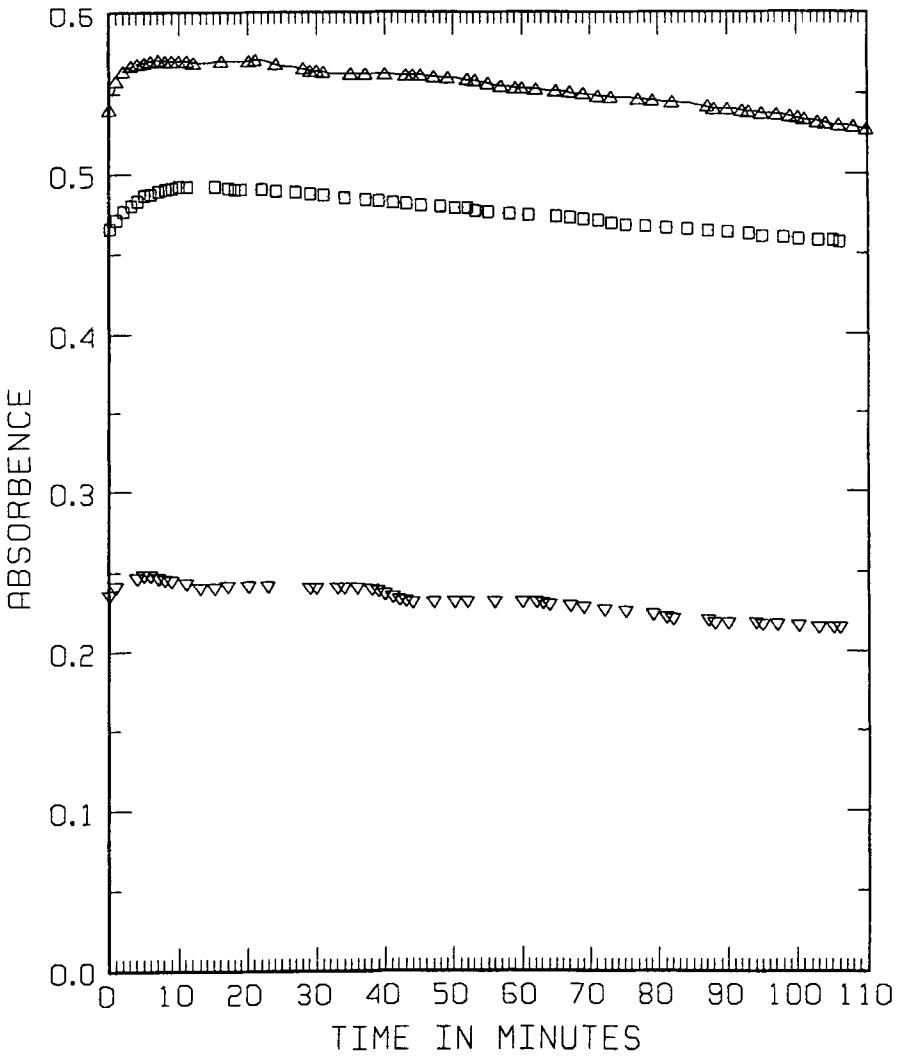

Fig. 2. Sedimentation profile of *Bacillus amyloliquefaciens* in PEG phase of a PEG 8000-dextran 70 system.

subtilis in the polyethylene glycol-dextran system. They found that most of the cells have the tendency to locate on the dextran side of the interface.

As mentioned earlier, the separation of the two phases is generally achieved by centrifuging the mixture or by gravity settling the phases before measuring the cell concentrations in each phase. During phase separation, cells that favor the dextran phase will settle to the bottom with the heavier dextran phase. In addition to this, a part of the cells that remain suspended in the PEG phase may also sediment with time during phase separation and thereby influence the partition measurement. Hence, sedimentation characteristics of the cells in the PEG phase were investigated first. Figure 2 represents the sedimentation profiles measured at three different cell concentrations. It appears that only 3–7% of the cells

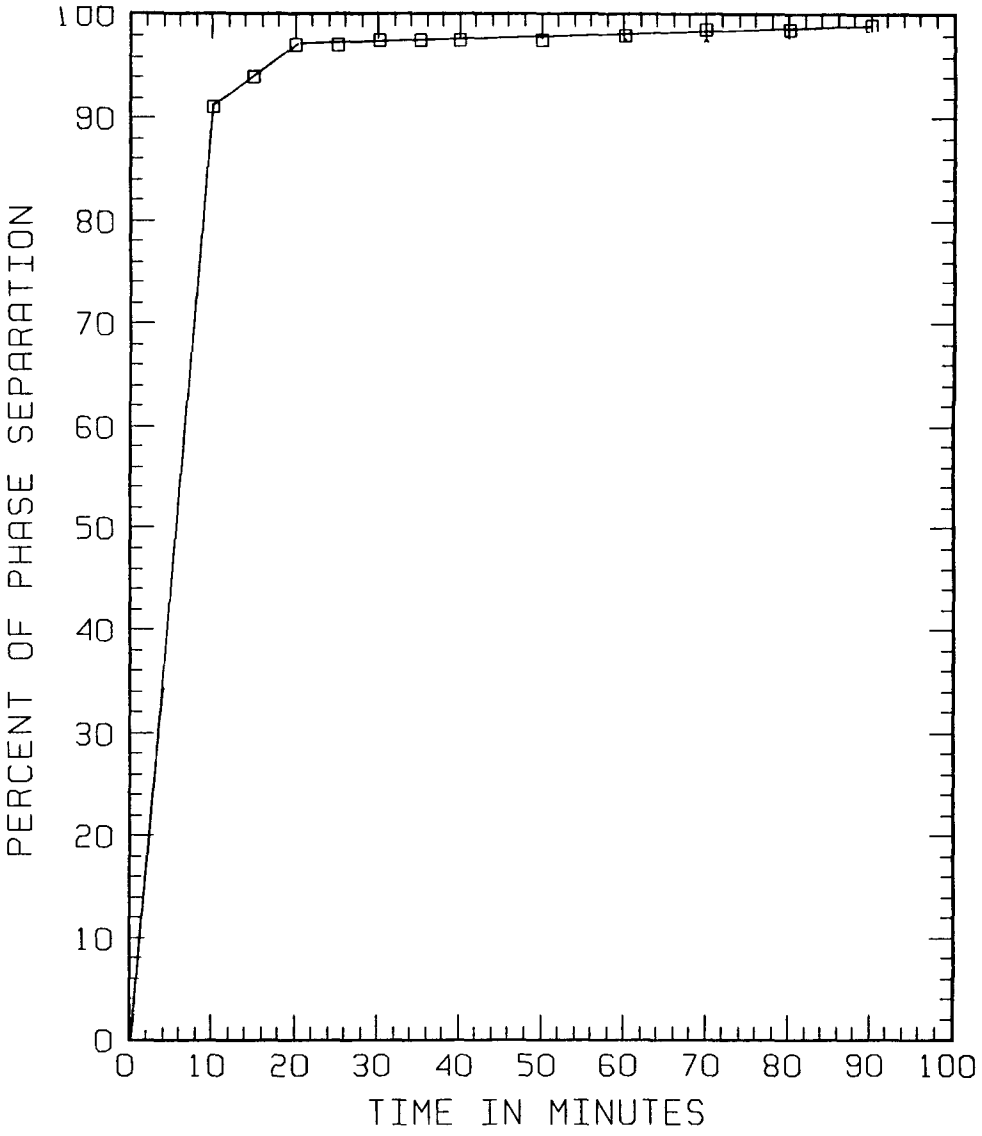

Fig. 3. Phase separation characteristics of PEG 8000-dextran 70 system.

sediment within 60 min. Increasing the cell concentration slightly decreases the sedimentation rate. This may be because of increased steric stabilization caused by the adsorption of polymers on the cell surface (*29*). Raymond and Fischer (*4*) also observed about 2–5% sedimentation of erythrocytes in the top phase of PEG–dextran system. On the other hand, even mild centrifugation of these cells suspended in top phase resulted in a considerable amount of sedimentation. The phase separation time for the PEG–dextran system is between 30–60 min. Generally, the separation time decreases with increasing polymer concentration. The phase separation characteristics of the low polymer system are shown in Fig. 3. It appears from Fig. 3, that over 97% phase separation can be achieved within 30 min. Since appreciable sedimentation of cells does not occur in 30 min

and a good separation of phases is obtained within that time, it was therefore decided to adapt gravity settling for 30 min as a method for phase separation in partition measurement. The partition coefficients for *Bacillus amyloliquefaciens* measured by this technique were found to be 0.10 in low polymer and 0.008–0.01 in high polymer systems.

Microscopic Observation

Very low concentration of cells were dispensed in the two-phase system for microscopic studies (1240X magnification). A droplet of mixed phases was poured in a Howard cell (a microscopic slide with a concave surface with depth size in microns) and was covered with a plastic cover slip. The plastic cover was used to reduce the coalescence rate of the drops. Use of a Howard cell ensures that the drops are not smeared between the two flat plates. However, because of the low interfacial tension of the systems, the dispersed drops coalesced very rapidly in the Howard cell. Our observation indicated that most of the cells appear on the dextran side of the interface. But in a two-dimensional field, it is very difficult to estimate the exact location of the cell, as the cells that are located at the liquid–liquid interface, and those inside the dispersed droplet would appear as if they are at the interface. The problems of such measurements have already been discussed elsewhere (*28*). It should also be noted that the difference in refractive index between the two conjugate phases in PEG–dextran system is very small.

Kinetics of Cell Growth

The cell growth and α-amylase production characteristics in the continuous phase, dispersed phase, and the mixed phase of the low polymer system are given in Fig. 5. Similar results in the high polymer system are given in Fig. 6. A schematic description of these fermentations is shown in Fig. 4. It appears from Figs. 5 and 6 that the growth in the dispersed phase of both the low polymer and the high polymer system are lower than the corresponding continuous phase. In addition, the α-amylase production is severely affected in the presence of a high concentration of dextran in the dispersed phases. When the growth kinetics of the mixed phases are compared to the corresponding continuous phase a striking difference can be observed between the low polymer and high polymer systems. The growth in the mixed phase of the low polymer system is lower than the corresponding continuous phase but higher than in the dispersed phase, as shown in Fig. 5. This is caused by the fact that the cells are distributed between the two phases and hence the overall kinetics of growth are the combination of these two growth kinetics. On the other hand, for the high polymer system, the growth of the mixed phase is very similar to that in the continuous phase. However, according to the partition coefficient measurements, most of the cells in high polymer sys-

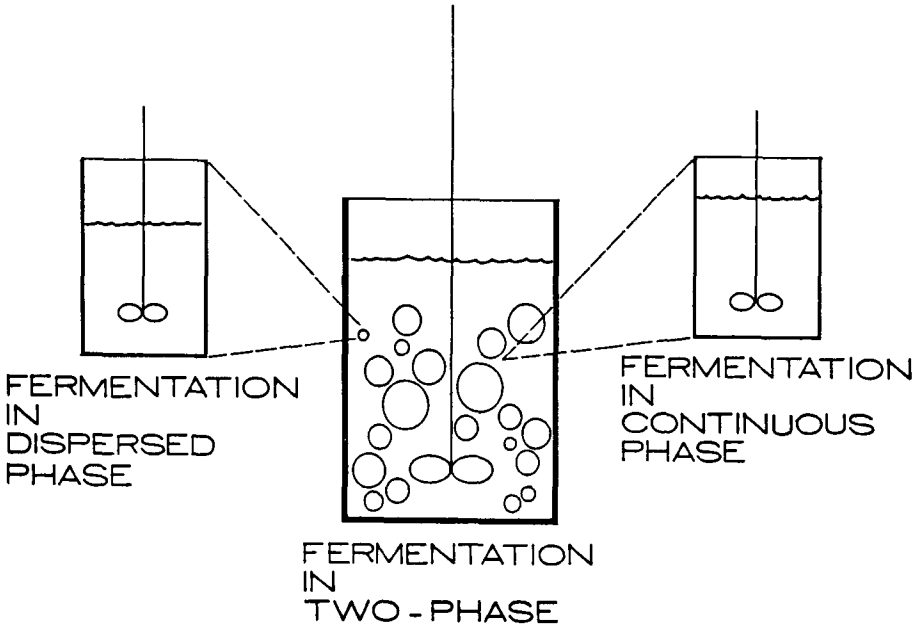

Fig. 4. Schematic description of different fermentations.

tem reside in the dispersed phase, and so the overall kinetics of growth in the mixed phase of the high polymer system should be similar to that in the dispersed phase. This anomaly between the kinetics of growth in the high polymer system and partition coefficient measurement can be explained as follows. The kinetics of growth and product formation in the mixed phase of the high polymer system were similar to that in the continuous phase because most of the cells were actually at the interphase. Because of high cell density at the interface, the interface lost its usual characteristics. Presence of cells at the interface eliminated the oxygen transfer problem and thereby sustained a good growth. In order to provide justification for this, the adsorption characteristics of the cells at the interface were studied.

Adsorption of Cells at the Liquid–Liquid Interface

Dynamic surface tension measurements have been used to study the kinetics of adsorption of surface active agents at the liquid–liquid interface (*15–18*). The term dynamic surface tension refers to a surface tension not corresponding to the equilibrium state of the surface of the liquid under consideration (*30*). The transient deviation from equilibrium is due to the adsorption of surface active agents. This parameter has been widely used in understanding interfacial phenomena such as emulsification, solubilization, and in enhanced oil recovery. Of several methods used, the pendant drop technique is a suitable method of determining the tran-

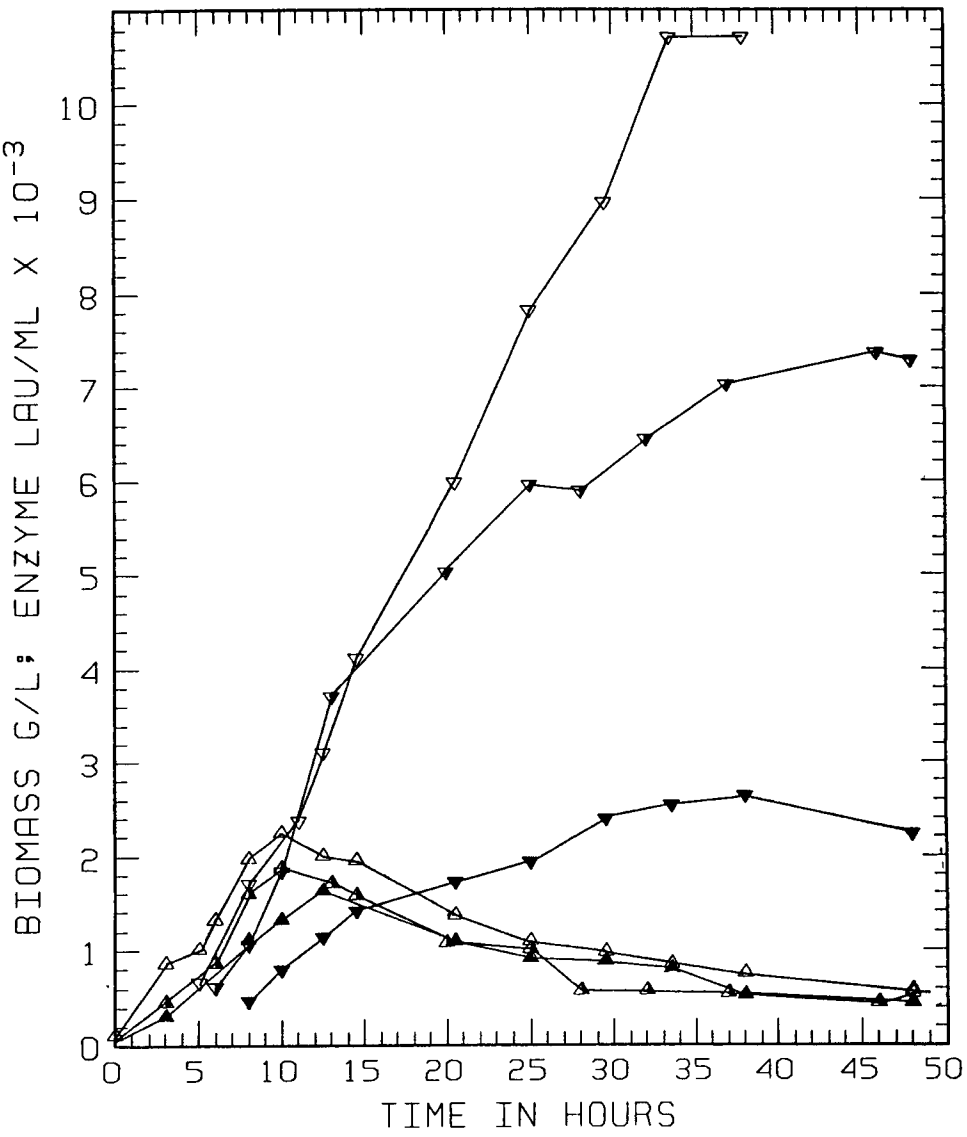

Fig. 5. Time course of *Bacillus amyloliquefaciens* fermentation in the low polymer system. Filled symbols: Fermentation in dispersed phase; open symbols: Fermentation in continuous phase; half-filled symbols: Fermentation in mixed phase. Biomass (△ ◭ ▲); enzyme (▽ ▼ ▼).

sient surface tension of very low magnitude measured in seconds (*18*). To the knowledge of the authors, this is the first time that this technique has been used to study the adsorption kinetics of cells at an interface. The dynamic surface tension of the high and low polymer phase systems in the presence of *Bacillus* are presented in Fig. 7. For the high polymer system, the surface tension rapidly decreases with time and then remains more or less constant indicating a strong adsorption of cells at the liquid-

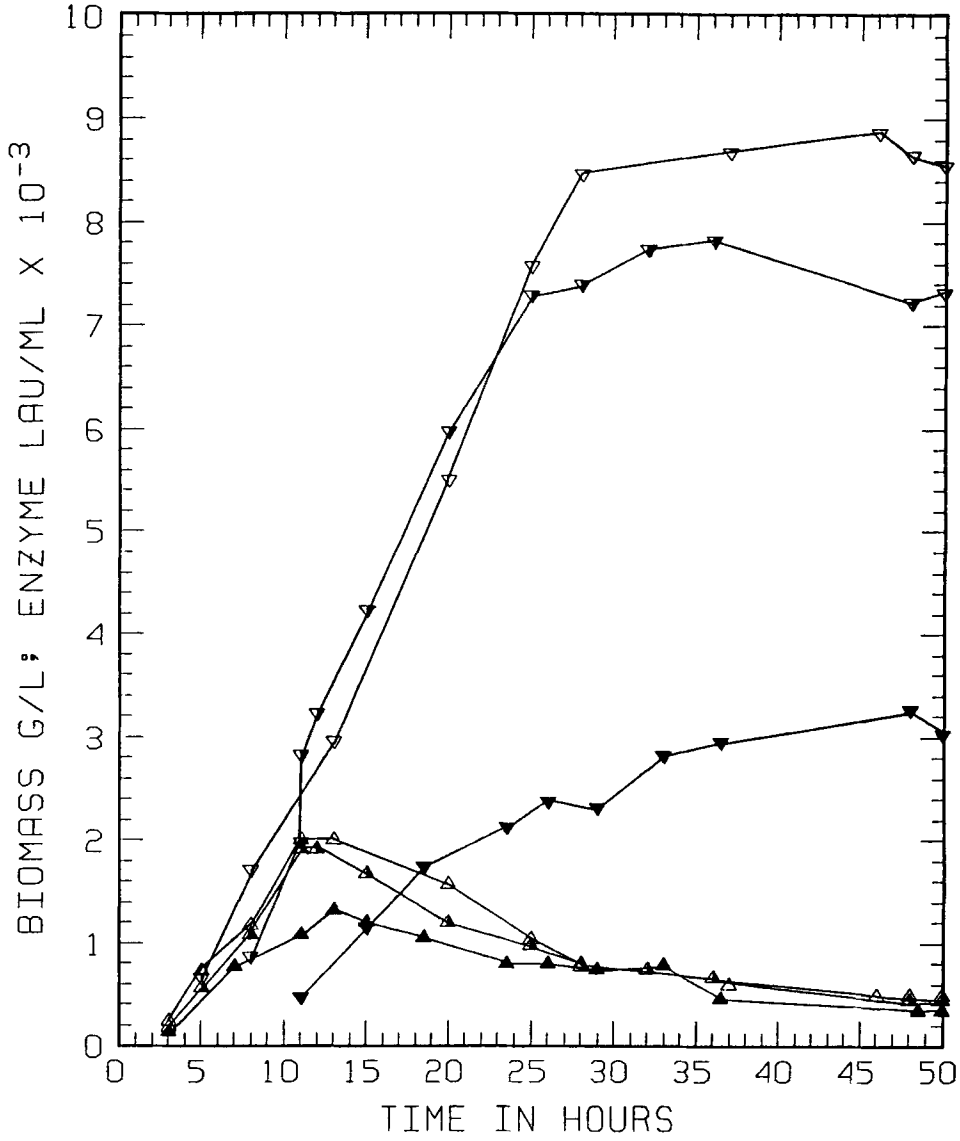

Fig. 6. Time course of *Bacillus amyloliquefaciens* fermentation in the high polymer system. Filled symbols: Fermentation in dispersed phase; open symbols: Fermentation in continuous phase; half-filled symbols: Fermentation in mixed phase. Biomass (△ ◭ ▲); enzyme (▽ ▼ ▼).

liquid interface. On the other hand, for the low polymer system, the surface tension passes through a minimum and then slowly rises indicating a phenomena of surface adsoption followed by a slow rate of desorption of cells. These results are then compared with the quantitative surface adsorption data obtained by making drops of dispersed phase to fall through a corresponding continuous phase containing various concentrations of cells. Figure 8 shows the behavior of cell adsorption at the interface in the

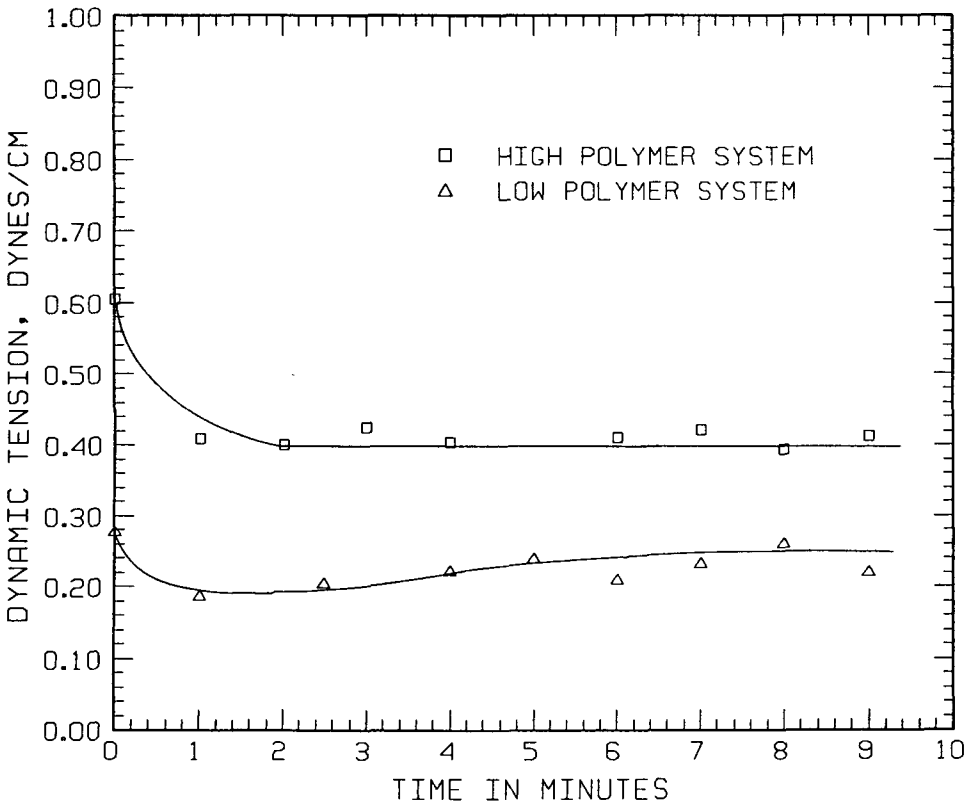

Fig. 7. Dynamic surface tension of low polymer and high polymer systems in the presence of *Bacillus amyloliquefaciens*.

two systems under investigation. It appears from Fig. 8 that the cells have higher adsorption characteristics at the interface in the high polymer system than that in the low polymer system.

CONCLUSIONS

Traditionally, partition coefficients of insoluble particles are determined by mixing particles with a phase system and then separating the phases to measure their concentrations in each phase. The phase separation process is a complex phenomenon. The phase separation process itself can influence the partition values measured for insoluble components and, therefore, these values may not represent actual distribution of these components in a mixed phase system. Understanding of cell distribution in the two-phase system is essential for designing phase systems for extractive fermentation or immobilization. Generally, increasing polymer concentration causes cells to adsorp at the interface. This is characterized by a lowering of the surface tension of the system. The high cell accumulation

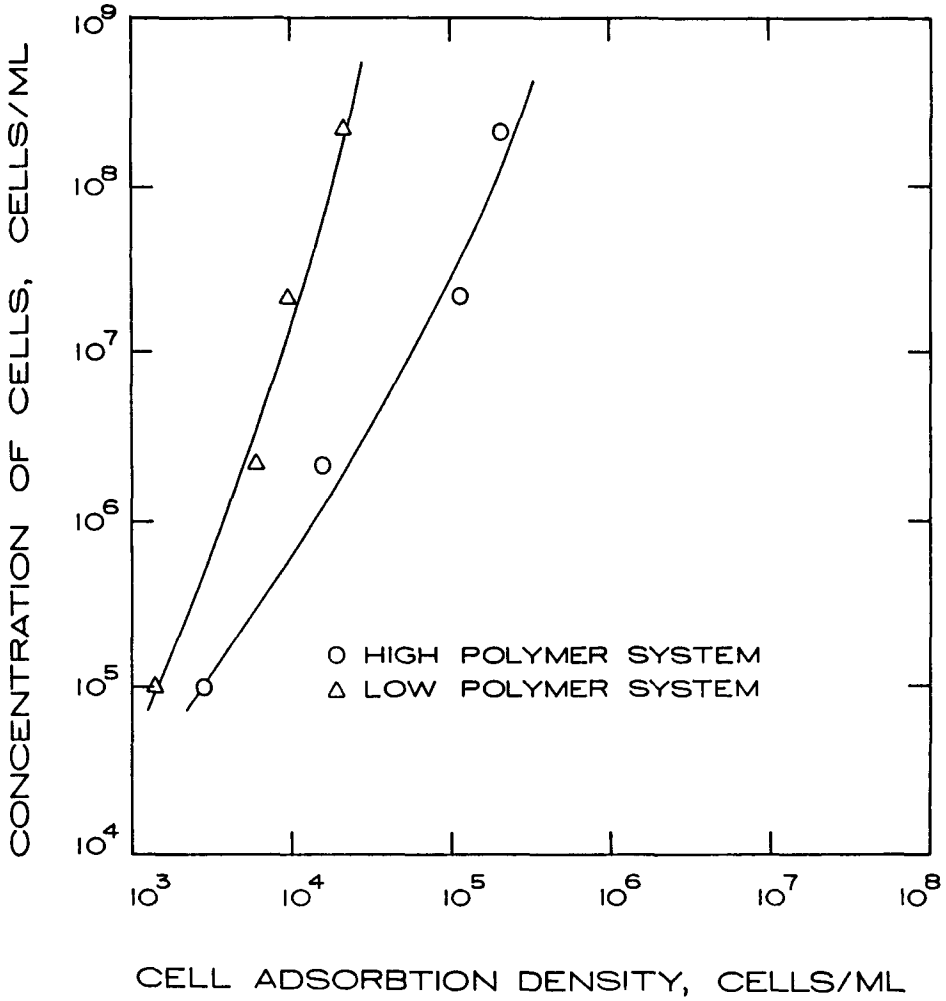

Fig. 8. Cell adsorption characteristics for low and high polymer systems.

at the liquid-liquid interface during two-phase fermentation decreases the problem of reduced oxygen transfer. The growth and metabolic product formation characteristics under such conditions are very close to that in the continuous phase itself. For extractive fermentation, these situations would be highly undesirable and detrimental to the primary objectives of such fermentations. Therefore, care should be taken in designing a polymer system to obtain the best partition coefficient for the cells and products formed.

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