Alteration of the Growth Rate and Lag Time of *Leuconostoc mesenteroides* NRRL-B523

Barry F. Wolf, H. Scott Fogler

Department of Chemical Engineering, University of Michigan, 2300 Hayward Street, Ann Arbor, Michigan 48109, USA; telephone: 734-763-1361; fax: 734-763-0459; e-mail: sfogler@umich.edu

Received 23 May 2000; accepted 4 October 2000

Abstract: Bacterial profile modification is an important enhanced oil recovery technique used to direct injected water into a reservoir's low permeability zone containing trapped crude oil. During water flooding, the use of bacteria to plug the high permeability water zone and divert flow into the oil-bearing low-permeability zone will have a significant economic impact. However, during the field implementation of bacterial profile modification, the rapid growth of bacteria near the injection well bore may hinder the subsequent injection of growth media so that profile modification of the reservoir occurs only in the immediate vicinity of the well bore. By slowing the growth rate and prolonging the lag phase, the onset of pore-space plugging may be delayed and the biologically active zone extended deep into the reservoir. High substrate loading, high pH values, and the addition of the growth inhibitors sodium dodecylsulfate and sodium benzoate have been used in combination to alter the growth characteristics of *Leuconostoc mesenteroides* NRRL-B523 grown in batch conditions. The highest sucrose concentration used in these studies, 500 g/L, produced lag times 12-fold greater than the slowest lag times achieved at low sucrose concentrations. When *L. mesenteroides* was grown in media containing 500 g/L sucrose, an alkaline pH value threshold was found above which bacteria did not grow. At this threshold pH value of 8.1, an average lag time of 200 h was observed. Increasing the concentration of sodium benzoate had no effect on lag time, but reduced the growth rate until the threshold concentration of 0.6%, above which bacteria did not grow. Last, it was found that a solution of 0.075 m*M* sodium dodecylsulfate in media containing 15 g/L sucrose completely inhibited bacterial growth. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **72**: 603–610, 2001.
Keywords: dextran; *Leuconostoc mesenteroides*; growth inhibition; lag time; profile modification

INTRODUCTION

Controlling the flow of water in the subsurface is essential for a variety of crude oil recovery operations. Bacterial profile modification (BPM), an enhanced oil recovery methodology, relies on bacteria that produce extracellular polysaccharides (exopolymers) to alter the porosity and permeability of the subsurface porous matrix. In the absence of profile modification, water injected into a partially oil-exhausted reservoir follows the path of least resistance through the water-flooded high permeability zone, and bypasses the oil-containing low permeability zone (Fig. 1a). The accumulation of biomass within the high permeability zone diverts injected water into low permeability regions containing crude oil to force oil out of the production well, as shown in Figure 1b (Jenneman et al., 1985; Lappan and Fogler, 1992; Vandevivere and Baveye, 1992). While the presence of bacterial cells can affect permeability in low permeability systems (Sarkar et al., 1994), the production of exopolymers augments pore blockage.

L. mesenteroides NRRL B-523 was chosen for BPM research because it produces a highly branched water insoluble dextran containing 15% to 34% α-1,3 and α-1,4 linkages (Jeanes, 1965; Jeanes et al., 1954). After injection, the bacteria quickly enter the exponential growth phase near the well bore region, the section of the reservoir containing the highest nutrient concentration. As can be seen in Figure 1c, rapid bacterial growth and exopolymer production near the well bore prevents deep penetration of bacterial cells and nutrients (Cusack et al., 1987; Jenneman et al., 1984; Taylor and Jaffé, 1990). Consequently, one of the greatest challenges of BPM is near well bore biomass plugging.

To improve BPM, this study focuses on chemical strategies that prolong the onset of biomass plug formation by reducing the growth rate and increasing the lag time of *Leuconostoc mesenteroides* NRRL B-523. The inhibition kinetics of dextransucrase, the enzyme that catalyzes the formation of dextran by *L. mesenteroides* NRRL B-523, provides some qualitative insight into the growth inhibition of this bacterium, but a quantitative understanding of the effects of inhibitors on both the enzyme and its bacterial producer are required to predict the kinetics of exopolymer production for BPM. The activity of dextransucrase from *L. mesenteroides* NRRL B-512 has been measured as a function of pH by Otts and Day (1988), and the sodium dodecylsulfate (SDS) inhibition of *L. mesenteroides* NRRL B-1416 dextransucrase was investigated by Kobayashi et al. (1985). However, the growth kinetics of *L. mesenteroides* NRRL B-523 growing in whole cell broth at a high pH value and containing SDS have not been investigated prior to this study.

A preliminary investigation of the effects of high sucrose

Correspondence to: H. S. Fogler

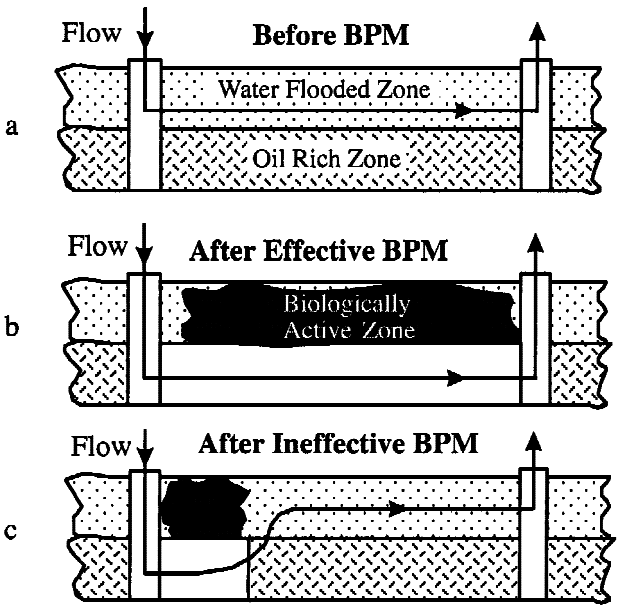

Figure 1. Ideal implementation of BPM.

concentrations on the growth of aerobically cultured *L. mesenteroides* was conducted by Holló and László (1971). They qualitatively compared the growth rates of *L. mesenteroides* (strain was not specified) for different sucrose concentrations and determined that 50 g/L sucrose produced the greatest final cell density and fastest growth rate. Unfortunately, growth rates and lag times were not quantified and no attempt was made to extend the lag time or to reduce the growth rate. Consequently, further study is required to provide a quantitative analysis of the effects of high sucrose concentrations on *L. mesenteroides* NRRL-B523 growth inhibition under anaerobic conditions.

Although sodium benzoate has been studied as an inhibitor of microbial growth, previous studies have concentrated on its effects in an acid medium. Furthermore, the effect of sodium benzoate on the growth of *L. mesenteroides* NRRL-B523 has not been investigated. Hence, the present study was undertaken to determine the feasibility of using sodium benzoate to extend the lag time of *L. mesenteroides* NRRL-B523.

MATERIALS AND METHODS

Bacteria

Leuconostoc mesenteroides NRRL-B523 (ATCC 14935) was obtained from the American Type Culture Collection (Rockville, MD) and placed in a −80°C freezer for long-term storage. Every 30 days an aliquot was removed from the freezer, streaked out onto agar plates prepared using Difco Lactobacillus MRS broth, grown for 18 h, and stored at 4°C. Streak plates were discarded after 30 days of 4°C storage.

Growth Conditions

L. mesenteroides inoculum was prepared by transferring one colony from the streak plate into media containing 7.9 g/L glucose, 7.9 g/L fructose, and 10 g/L Difco yeast extract solution diluted in a mineral salt solution. The mineral salt solution contained 0.067 *M* sodium acetate, 0.075 *M* sodium chloride, 0.061 *M* ammonium chloride, 6.1×10^{-3} *M* $K_2HPO_4 \cdot 3H_2O$, 3.1×10^{-3} *M* ascorbic acid, 4.1×10^{-4} *M* $MgSO_4 \cdot 7H_2O$, 4.0×10^{-5} *M* $MnSO_4 \cdot H_2O$, and 4.0×10^{-5} *M* $FeSO_4 \cdot 7H_2O$. After 22 h of growth at 30°C, 10 mL of inoculum was aseptically transferred to a 1000-mL batch reactor containing 550 mL of the desired growth medium. The initial cell concentration was $7.5 \times 10^6 \pm 0.1 \times 10^6$ cells/mL. The growth medium contained 10 g/L Difco yeast extract, varying concentrations of sucrose, and varying concentrations of inhibitors, all of which were dissolved in the mineral water solution just described. For the hydroxide ion inhibition experiments, the medium initial pH value was fixed using 4 *M* sodium hydroxide; the medium pH value was not controlled after inoculation. The batch reactor pH value at inoculation was 6.4 ± 0.15 for all other experiments. Throughout this study, the term "initial pH" refers to the growth medium pH value immediately following inoculation.

The reactor contents were not modified during the course of the reaction except for the withdrawal of liquid samples. All batch reactions were conducted at a constant 30°C with constant mixing using a 1.5-in. stir bar rotating at 120 rpm. Sealed batch reactors provided anaerobic growth conditions.

Sampling and Analysis Techniques

During the reaction period, liquid samples were aseptically taken from the reactor upon inoculation and at intervals ranging from 10 min to 24 h. The total cell count was measured using a model ZF Coulter Counter fitted with a 30-μm-diameter aperture tube. Viable colonies were enumerated using the spread plate technique, with phosphate-buffered saline used as a diluent. Dextransucrase activity was determined by measuring the rate of production of fructose. One unit of dextransucrase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose per minute in a 20 m*M* sodium acetate buffer (pH 5.4) containing 100 g/L sucrose, 0.05 g/L $CaCl_2$ and 1 g/L NaN_3. Fructose was measured using the dinitrosalicylic acid assay.

For dextran analysis, 3-mL samples were withdrawn from the reactor. Water insoluble dextran (WID) and cells were separated from water-soluble dextran (WSD) by centrifugation. WSD was then precipitated from the supernatant using 66% v/v ethanol. WSD precipitates were weighed after filtration through a 47-mm 0.2-μm Poretics track-etched polycarbonate membrane. WID was separated from cellular material with potassium hydroxide, decomposed into monomers using hydrochloric acid, and assayed for total sugar content by the phenol-sulfuric acid assay (Ko-

bayashi et al., 1985). A more detailed procedure for dextran analysis has been described by Kim and Fogler (1999).

RESULTS

Sucrose, hydrogen ion, sodium benzoate, and SDS concentrations were varied over a physiologically significant range to slow the growth rate and increase the lag time of *L. mesenteroides.* A typical growth curve of *L. mesenteroides* is shown in Figure 2.

The cell concentration data from batch growth experiments were fit to the modified Gompertz equation using the Levenberg–Marquardt method, as shown by the solid line in Figure 2 (Press et al., 1992; Zwietering et al., 1990). The modified Gompertz equation is:

$$y = A \cdot \exp\left[-\exp\left(\frac{\mu \cdot e}{A}(\lambda - t)\right) + 1\right] \qquad (1)$$

where $y = \ln(N/N_0)$, $A = \ln(N_\infty/N_0)$, $e = 2.718$, t denotes the time in units of hours, μ denotes the specific growth rate in units of 1/h, and λ denotes the length of lag time in units of hours; N is the cell concentration at any time during the experiment in units of cells per milliliter; N_0 is the initial cell concentration (cells per milliliter); N_∞ is the stationary phase cell concentration (cells per milliliter).

Substrate Inhibition

L. mesenteroides was grown in media containing sucrose concentrations varying from 1 to 500 g/L and with an initial pH of 6.4 ± 0.15. The growth rates, lag times, and amount of each type of dextran produced were measured for each batch experiment. As can be seen in Figure 3, increasing the sucrose concentration significantly decreased the specific growth rate. A decreased growth rate accompanied an increase in the lag time for sucrose concentrations >75 g/L. Figure 4 shows that the lag time increased from 1 h when *L. mesenteroides* was grown in media containing 1 g/L sucrose to 12 h when grown in media containing 500 g/L sucrose.

The stationary phase cell density (Fig. 5) parallels the growth rate. The maximum growth rate occurred at an initial sucrose concentration of 75 g/L and the highest observed cell density increase occurred at an initial sucrose concentration of 125 g/L. While high sucrose concentrations were able to prolong the lag time and decrease the growth rate, Figure 5 shows that, within experimental error, the increase in cell density was the same using an initial sucrose concentration of either 15 g/L or 500 g/L.

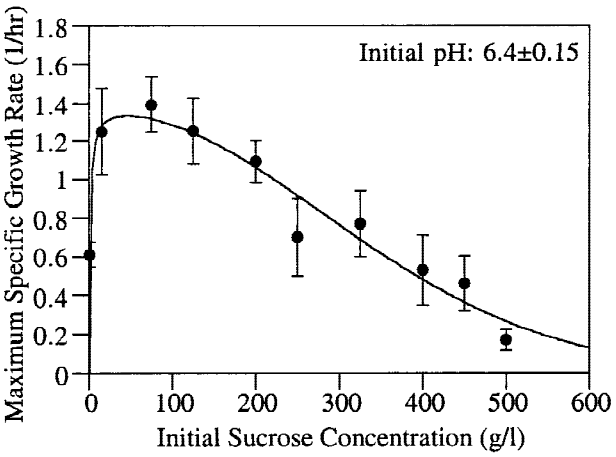

Figure 3. Sucrose inhibition of *L. mesenteroides* growth. The solid line represents the fit of the modified Edwards equation.

Dextran was assayed for both water-insoluble and water-soluble fractions because it is hypothesized that WID is more effective than WSD for the plugging of a porous matrix. When low initial sucrose concentrations were used (e.g., 15 to 75 g/L), the initially clear broth became noticeably opaque during the course of reaction and contained large aggregates of WID. At concentrations >200 g/L, the initially clear broth became slightly opaque, but visible WID aggregates were not present. The production of both WID and WSD was inhibited by increasing the initial sucrose concentration, as can be seen in Figure 6, and a decrease in dextransucrase activity was observed (Table I).

L. mesenteroides, a heterofermentative member of the lactobacillus family, produces lactic acid as it ferments glu-

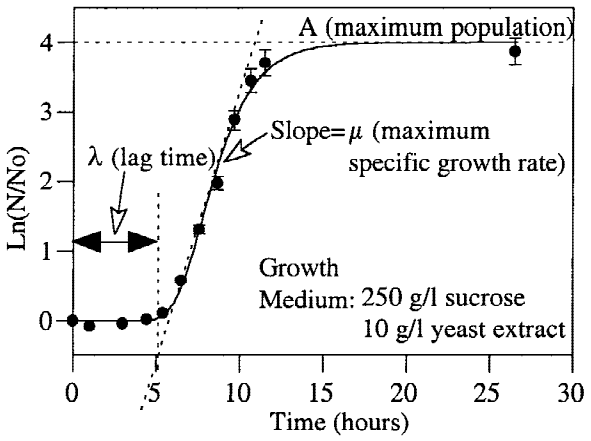

Figure 2. Typical batch growth curve of *L. mesenteroides.*

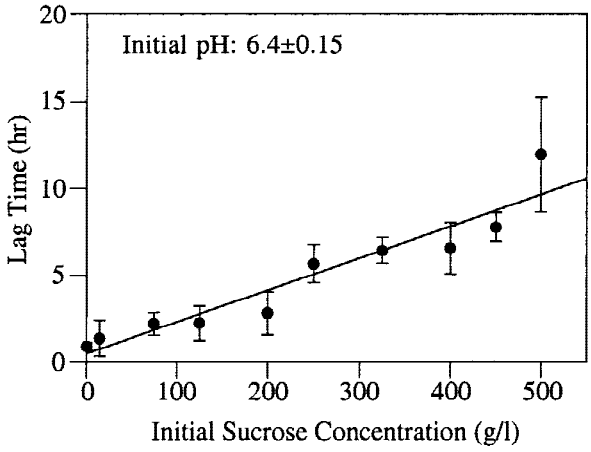

Figure 4. Sucrose delays onset of *L. mesenteroides* growth.

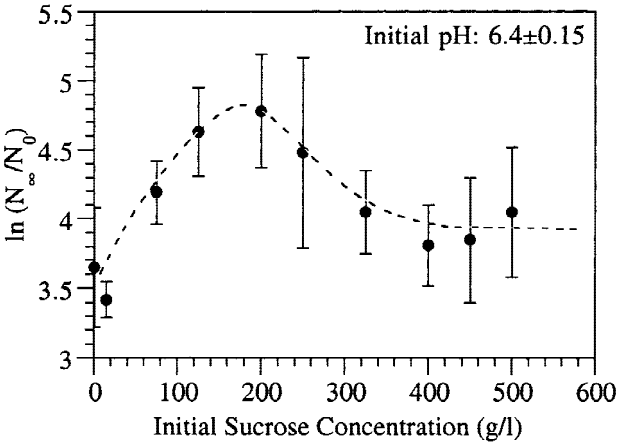

Figure 5. Cell density increase is a function of initial sucrose concentration.

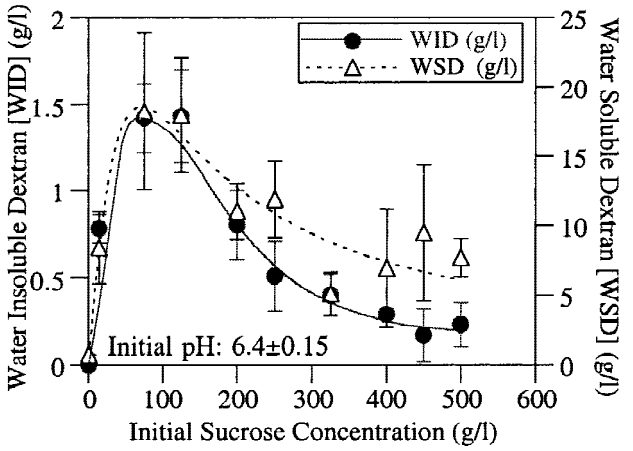

Figure 6. WSD and WID produced varies with initial sucrose concentration.

cose. While not able to increase in cell number during the lag phase, *L. mesenteroides* was able to ferment glucose with an accompanying drop in pH during the lag phase (data not shown). When the sucrose concentration was increased, the maximum specific growth rate decreased as did the rate of pH decrease. Acid production was coupled with the rate of cell metabolism and this rate was slowed by higher substrate concentrations. The rate of decrease in the broth pH during the exponential growth phase followed a similar trend as the specific growth rate (Fig. 7).

Hydroxide Ion Inhibition

Like other microorganisms, *L. mesenteroides* NRRL-B523 was able to grow in a wide range of pH values (Kim et al., 2000). The pH of the medium is typically maintained between 5.5 and 7 for maximum dextran yield (Lazic et al., 1993). To accentuate the effect of growth inhibition in a solution containing a sucrose concentration of 500 g/L, the solution pH was progressively increased until growth was completely inhibited. As the solution pH was increased, there was little change in *L. mesenteroides* growth characteristics until the solution pH reached 7.5 Above pH 7.5, the growth rate decreased to an average value of 0.1 1/h, and then dropped to 0 at a value of 8.2 (Fig. 8). It was determined that a pH value of 8.2 is the upper limit for *L. mesenteroides* growth because there was no measurable cell growth even after 300 h. Most importantly, Figure 9 shows that lag time was significantly increased (200 h) at pH 7.9 when compared with the 12-h lag time observed when *L. mesenteroides* was grown in solutions with an initial pH of 6.3.

While the lag-phase hydrogen ion concentration increased slightly during the lag period of the substrate inhibition experiments, there was an exponential increase in the hydrogen ion concentration during the exaggerated lag phase observed during the hydroxide ion inhibition experiments. The observed rate of decrease in solution pH during the lag phase varied linearly from a minimum of 0.015 1/h at an initial pH value of 8.1 to a maximum of 0.077 1/h at an initial pH value of 7.1. Interestingly, for all hydroxide ion inhibition experiments, the bacteria did not enter the exponential growth phase until the solution pH dropped to a value of 6.79 ± 0.06.

The growth of *L. mesenteroides* in alkaline solutions had, within experimental error, no effect upon the production of water-insoluble exopolymers and showed an increase in the amount of low-molecular-weight exopolymers produced (Fig. 10).

Sodium Benzoate Inhibition

Sodium benzoate is a common weak acid food preservative used to prevent the growth of yeast and bacteria. Initially, a 0.1% w/v (weight/volume) concentration of sodium benzoate was added to a solution containing 15 g/L sucrose and no growth inhibition was observed. To accentuate the inhibition of media containing 500 g/L sucrose, solutions containing 0.1%, 0.2%, 0.4%, and 0.6% w/v sodium benzoate were made. Viable plate counts (data not shown) confirmed that *L. mesenteroides* was unable to grow in solutions containing a benzoate concentration of >0.6%. As the benzoate concentration was increased, the lag time remained approximately constant, the growth rates and the final cell densities decreased, and the final pH increased. Table II summarizes these results.

Sodium Dodecylsulfate Inhibition

Another substance that can inhibit the growth of *L. mesenteroides*, sodium dodecylsulfate (SDS), also known as lau-

Table I. Maximum dextransucrase activity observed throughout the course of a batch reaction.

Initial sucrose concentration (g/L)	Maximum dextransucrase activity (units/mL)
125	4.5 ± 0.3
500	2.5 ± 0.2

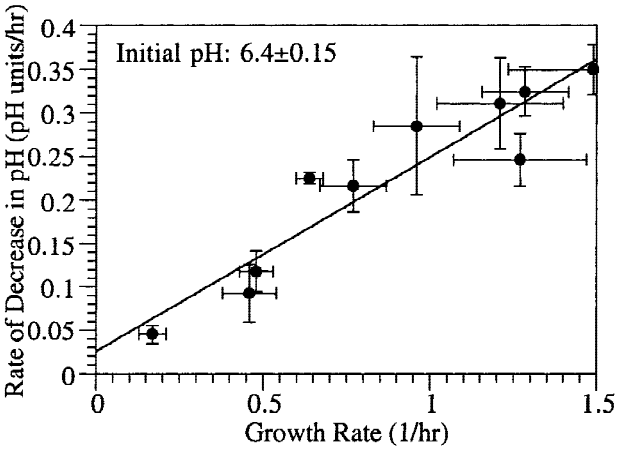

Figure 7. Rate of decrease in pH varies linearly with growth rate.

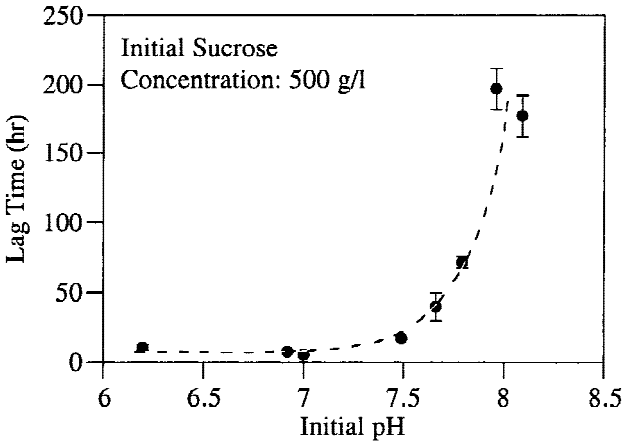

Figure 9. Effect of alkaline solution pH values on *L. mesenteroides* lag time.

ryl sulfate, was added to solutions containing 15 g/L sucrose. SDS was chosen as a growth inhibitor because of its documented success in reducing the effectiveness of sucrose cleavage by dextransucrase (Kobayashi et al., 1985). For the conditions used in the growth experiments, the SDS critical micelle concentration (CMC), was approximately 0.8 m*M* (Hiemenz and Rajagopalan, 1997). *L. mesenteroides* was grown in solutions containing SDS in concentrations above and below the CMC. A sharp change in growth characteristics was found above an SDS concentration of 0.050 m*M*. The lag time at 0.050 m*M* was approximately 1 h, whereas the lag for 0.070 m*M* was near 200 h, as shown in Table III.

DISCUSSION

Substrate Inhibition

Excess sucrose acted on growth and dextran production in two ways: (1) by interfering with the catalytic activity of dextransucrase, the enzyme that *L. mesenteroides* secretes in the presence of sucrose for the production of dextran and cleavage of sucrose; (2) by creating a strong osmotic gradient across the bacterial cell membrane. It is hypothesized that the first effect dominated at relatively low sucrose concentrations, and that the second effect explains the lower-than-predicted growth rates in solutions containing >350 g/L sucrose.

The inhibition of dextransucrase may explain the decrease in growth rate with increasing initial sucrose concentration. When sucrose is present in concentrations that are not inhibitory, dextransucrase binds sucrose, forms a covalent glucosyl intermediate by releasing fructose, and then transfers the glucosyl intermediate to the growing dextran chain covalently attached to another site. The reaction is as follows:

$$\text{Sucrose} + G_n \xrightarrow{\text{Dextransucrase}} \text{Fructose} + G_{n+1}$$

where G_n is the growing dextran polymer.

In addition, glucose can be released either by dextran chain growth termination or by an invertase-like activity of dextransucrase (Lappan and Fogler, 1994). Tanriseven and Robyt (1993) suggested that dextransucrase's active sites are independent of a third acceptor site that binds glucosyl

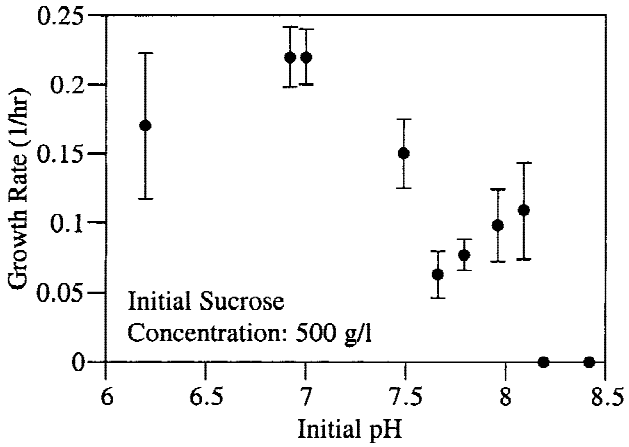

Figure 8. Effect of alkaline solution pH values on *L. mesenteroides* growth.

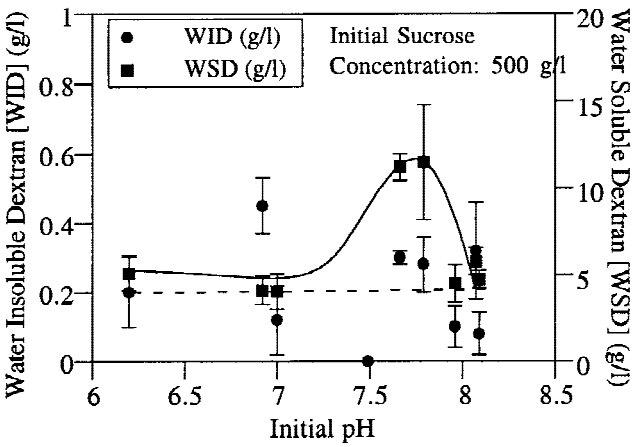

Figure 10. Effect of alkaline solution pH values on *L. mesenteroides* production of exopolymers.

Table II. Effect of sodium benzoate on growth in 500-g/L sucrose solutions.

Sodium benzoate concentration % (w/v)	Growth rate (1/h)	Lag time (h)	Stationary phase cell concentration (#/mL)	Stationary phase pH
0.0	0.17 ± 0.04	13.2 ± 5.0	$4.5 \times 10^8 \pm 1 \times 10^8$	4.2 ± 0.1
0.1	0.095 ± 0.02	13.1 ± 2.2	$2.4 \times 10^8 \pm 0.7 \times 10^8$	4.8 ± 0.2
0.2	0.090 ± 0.03	12.2 ± 2.1	$1.4 \times 10^8 \pm 1.1 \times 10^8$	5.2 ± 0.1
0.4	0.078 ± 0.01	13.1 ± 2.4	$2.5 \times 10^8 \pm 0.8 \times 10^8$	5.2 ± 0.2
0.6	0.026 ± 0.01	12.5 ± 1.5	$1.6 \times 10^8 \pm 0.9 \times 10^8$	5.7 ± 0.1
0.7	0	∞	Not applicable	Not applicable

intermediates but does not bind sucrose or the growing dextran molecule. They further proposed that excess sucrose binds to a distinct low-affinity sucrose-binding site, allosterically inhibits the formation of dextran by altering the conformation of one of the two high-affinity sucrose-binding sites, and prevents the elongation of dextran. Under these conditions, the reaction is as follows:

$$\text{Sucrose} \xrightarrow{\text{Dextransucrase}} \text{Fructose} + \text{Glucose}$$

Tanriseven and Robyt (1993) stated that the formation of glucosyl intermediates is not affected by excess sucrose. However, Martinez-Espindola and Lopez-Munguia (1985) observed that excess sucrose, up to a concentration of 400 g/L, inhibited *L. mesenteroides* NRRL B-512 dextransucrase. They were also able to describe changes in the fructose production rate using a substrate-inhibition kinetics model.

The presence of high concentrations of WSD observed in this study verifies that sucrose interferes with the growing dextran polymer. The bacterial growth rates observed in this study confirm that fructose (and glucose) is produced at reduced rates in the presence of high sucrose concentrations. Furthermore, the observed decrease in dextransucrase activity above a sucrose concentration of 125 g/L validates this hypothesis. Sucrose inhibition began above a sucrose concentration of 100 g/L, as can be seen in Figures 6 and 7.

The decrease in water activity (a_w) due to an increase in sucrose concentration may also explain the reduction in growth rate. A correlation between a_w and growth rate has been observed when other species of bacteria are grown in solutions containing high concentrations of salts or sugars (Troller and Christian, 1978). While a decrease in a_w may describe the observed reduction in growth rate of *L. mesenteroides*, the change in a_w provides no mechanistic description of growth inhibition, and it cannot explain the increase in growth rate at low sucrose concentrations.

Longer lag times may have been caused by bacterial osmoregulation strategies initiated to compensate for high sucrose concentrations. Although sucrose is able to pass through the cell wall, sucrose is unable to pass through the bacterial cell membrane, and bacteria rapidly lose water when they are exposed to a medium containing a high sucrose concentration (Yoneyama et al., 1986). When sucrose concentrations are sufficiently high, bacteria accumulate inorganic and/or organic low-molecular-weight compounds, known as compatible solutes, within the cytoplasm to compensate for the loss of water (Brown, 1976; Csonka and Hanson, 1991). The reallocation of cellular resources for the import of compatible solutes may have delayed the initiation of exponential growth for *L. mesenteroides.*

To model the growth rate of *L. mesenteroides* in a medium containing excess sucrose, an inhibition kinetics model was used as a first approximation. This model assumes unsegregated and unstructured growth kinetics (Bailey and Ollis, 1986). Excess sucrose interfered with dextransucrase by binding to the enzyme–substrate complex and hindered the cleavage of sucrose into glucose and fructose; this mechanism is known as noncompetitive inhibition. Noncompetitive inhibition has been used successfully by Hill and Robinson (1975) to describe substrate inhibition of *Pseudomonas putida* by phenol. Eq. (2) is derived after assuming quasi-steady-state conditions. Eq. (2) relates the specific growth rate, μ, to the maximum growth rate, μ_{max}, the Monod constant, K_m, and the inhibition constant, K_i:

$$\mu = \frac{\mu_{max} S}{K_m + S + S^2/K_i} \tag{2}$$

where S is the sucrose concentration (g/L).

While Eq. (2) describes the experimental data well at low initial sucrose concentrations, it predicts higher growth rates than were observed at sucrose concentrations >350 g/L. Above a sucrose concentration of 350 g/L, it is believed that the model does not predict the observed results because of the use of ATP for osmoregulation. Because the growth of *L. mesenteroides* is hypothesized to be slowed by a combination of effects, empirical growth inhibition models fit the data more closely than did a noncompetitive inhibition model. Model parameters are tabulated in Table IV. The modified Edwards equation is plotted with the experimental data in Figure 3.

Table III. Effect of SDS on growth in 15-g/L sucrose solutions.

SDS concentration (m*M*)	Lag time (h)	Growth rate (1/h)
0.10	∞	0
0.075	∞	0
0.070	190 ± 8	0.01 ± 0.001
0.060	95 ± 13	0.009 ± 0.001
0.050	1.0 ± 0.2	0.20 ± 0.03
0.010	1.0 ± 0.15	0.70 ± 0.05

Table IV. Fitted parameters for *L. mesenteroides* sucrose inhibition.

Model	χ^2	μ_{max}	K_m	K_i
$\mu = \frac{\mu_{max} S}{K_m + S + S^2/K_i}$ (noncompetitive inhibition)	0.273	1.78 1/h	2.40 g/L	199 g/L
$\mu = \frac{\mu_{max} \cdot S \cdot \exp(-S/K_i)}{K_m + S}$ (Edwards, 1970)	0.147	1.70 1/h	2.16 g/L	343 g/L
$\mu = \frac{\mu_{max} \cdot S \cdot \exp(-(S/K_i)^2)}{K_m + S}$ (Modified Edwards)	0.080	1.39 1/h	1.29 g/L	389 g/L

Hydroxide Ion Inhibition

Bacterial metabolism depends on hundreds of enzymes to catalyze the reactions required for maintenance and reproduction. The amino acids that are the building blocks of enzymes contain ionizable groups that lose activity in the presence of high concentrations of hydrogen or hydroxide ions (Bailey and Ollis, 1986). The unfavorable ionization of key enzymes slows or completely stops bacterial growth. Intracellular enzymes are protected from extremes in pH by the cell membrane's active transport of undesirable ions out of the cytosol, but extracellular enzymes, such as dextransucrase, are immediately ionized by the ions present in solution.

Although dextransucrase was completely inactivated at pH 8 or higher, its activity may be restored by lowering the solution pH, and Otts and Day (1988) found that 7 h were required for the complete inactivation of dextransucrase at pH 8 in a cell-free system. When *L. mesenteroides* was grown in media with an initial pH of 8.1, the solution pH decreased exponentially at a rate of 0.015 1/h during the initial 100 h (lag phase) of the growth experiment; this rate was sufficient to prevent permanent inactivation of dextransucrase. It has also been suggested by Otts and Day (1988) that, at pH values >8.0, sucrose phosphorylase, an intracellular enzyme, may be responsible for the decomposition of sucrose into utilizable glucose-1-phosphate and fructose (Vandamme et al., 1987). Above a pH value of 8.2, either sucrose phosphorylase activity was insufficient to maintain cell metabolism, or dextransucrase decomposed more quickly than the rate at which lactic acid lowered the medium pH. As the initial pH was increased, it is believed that the more severe conditions increased the rate at which dextran polymerization was terminated, and the result was an increase in the concentration of WSD.

Sodium Benzoate Inhibition

Although benzoic acid is the active form of the preservative, the sodium salt is commonly used because of its high solubility in aqueous solutions (Reddish, 1957). Microbial growth is effectively prevented by concentrations of sodium benzoate of <0.3% w/v when the medium has a pH value ranging from 2.5 to 4.0 (Chichester and Tanner, 1972). While growth inhibition has been observed in a less acidic medium, an order of magnitude or higher concentration of sodium benzoate may be required as the pH of the medium is increased (Cruess and Richert, 1929).

In an acidic medium, benzoic acid readily diffuses through the cell membrane. Once in the cytoplasm, benzoic acid deprotonates and causes a drop in the internal cell pH. According to Krebs et al. (1983), a low cytosolic pH inhibits the activity of key metabolic enzymes such as phosphofructokinase. Warth (1991) suggested that the internal pH reduction is too small to account for growth inhibition caused by enzyme inactivation. Warth concluded that reduced growth rates were caused by the use of ATP for proton pumping (proton pumps in the membrane maintain the cytosolic pH near neutrality) at the expense of normal metabolic pathways.

While sodium benzoate has proven most effective in acidic conditions, for this study *L. mesenteroides* was grown in media with an initial pH of 6.1 to 6.3 to achieve maximum cell densities. As lactic acid accumulated in the extracellular environment sodium benzoate became a more effective growth inhibitor, an effect confirmed by a decreased final cell density and increased final solution pH.

SDS Inhibition

Unlike the gradual growth inhibition caused by excess sucrose, SDS suppressed the growth of *L. mesenteroides* at a threshold concentration. SDS interfered with growth because it was able to inhibit the activity of enzymes and because it may have disrupted the cell membrane. For short time periods, SDS may inhibit the activity of an enzyme, but given enough time, the enzyme will be completely inactivated by the binding of SDS to active sites (Vincenzini et al., 1985). Higher concentrations of SDS were able to more fully alter the charge of the membrane and disrupt the normal functioning of membrane-bound receptors and structural proteins (Helenius and Simons, 1975; Seufert, 1973).

When *L. mesenteroides* was grown in media containing <0.060 m*M* SDS there was a reduction in growth rate, but no change in the lag time. Above this concentration, a small increase in the cell population was observed after lag times of 300 to 400 h. This result indicates that there was a critical concentration of SDS that denatured enzymes and/or the membrane structural proteins critical for growth.

CONCLUSIONS

Batch growth experiments have demonstrated that the lag time and growth rate of *L. mesenteroides* NRRL-B523 can be controlled by substrate inhibition and by the addition of inhibitors. A combination of substrate inhibition and a high initial medium pH was the most successful technique for delaying the growth of *L. mesenteroides* while allowing the

bacteria to reach a stationary population concentration approximately equal to the population observed during uninhibited growth. Unlike growth in media containing <100 g/L sucrose, growth at a high substrate concentration significantly reduced the amount of WID and increased the amount of WSD. While the specific inhibitor regimes used in this study may not improve bacterial profile modification for all oil reservoirs, the current results provide a framework for further inhibition research that may improve the depth penetration of exopolymer-producing bacteria.

The authors would like to acknowledge the financial support of the University of Michigan Cellular Biotechnology Training Program, National Institutes of Health grant #TGM GM08353. This work was also supported by the members of the University of Michigan Industrial Affiliates in Flow and Reaction in Porous Media: Baker Petrolite, Chevron, Conoco, Dowell Shlumberger, and Halliburton Services, PDVSA-Intevep, and Phillips Petroleum.

References

Bailey J, Ollis D. 1986. Biochemical engineering fundamentals. New York: McGraw-Hill. 984 p.

Brown AD. 1976. Microbial water stress. Bacteriol Rev 40:803–846.

Chichester DF, Tanner RW. 1972. Antimicrobial food additives. In: Furia TE, editor. Handbook of food additives. Cleveland, OH: CRC Press. p 115–184.

Cruess WV, Richert PH. 1929. Effect of hydrogen ion concentration on the toxicity of sodium benzoate to microorganisms. J Bacteriol 17: 363–371.

Csonka LN, Hanson AD. 1991. Prokaryotic osmoregulation: Genetics and physiology. Ann Rev Microbiol 45:569–606.

Cusack F, Lappin-Scott HM, Costerton JW. 1987. Bacteria can plug waterflood injection wells. Oil Gas J 85:59–64.

Edwards VH. 1970. The influence of high substrate concentrations on microbial kinetics. Biotechnol Bioeng 12:679–712.

Helenius A, Simons K. 1975. Solubilization of membranes by detergents. Biochim Biophys Acta 415:29–79.

Hiemenz PC, Rajagopalan R. 1997. Principles of colloid and surface chemistry. New York: Marcel Dekker. 650 p.

Hill GA, Robinson CW. 1975. Substrate inhibition kinetics: phenol degradation by *Pseudomonas putida.* Biotechnol Bioeng 17:1599–1615.

Holló J, László E. 1971. The synthesis of dextran, I. Period Polytech Chem Eng 15:35–42.

Jeanes A, Haynes WC, Wilham CA, Rankin JC, Melvin EH, Austin MJ, Cluskey JE, Fisher BE, Tsuchiya HM, Rist CE. 1954. Characterization and classification of dextrans from ninety-six strains of bacteria. J Amer Chem Soc 76:5041–5052.

Jeanes A. 1965. Preparation of dextrans from growing *Leuconostoc* cultures. In: Whistler RL, Wolfrom ML, editors. Methods in carbohydrate chemistry, New York: Academic Press. p 118–126.

Jenneman GE, Knapp RM, McInerney MJ, Menzie DE, Revus DE. 1984. Experimental studies of in-situ microbial enhanced oil recovery. Soc Pet Eng J 24:33–37.

Jenneman GE, McInerney MJ, Knapp RM. 1985. Microbial penetration through nutrient-saturated berea sandstone. Applied and Environ Microbiol 50:383–391.

Kim DS, Fogler HS. 1999. The effects on cell morphology and culturability of *Leuconostoc mesenteroides* during starvation. Appl Microbiol Technol 52:839–844.

Kim DS, Thomas S, Fogler HS. 2000. Effects of pH and trace minerals on long-term starvation of *Leuconostoc mesenteroides.* Appl Env Microbiol 66:976–981.

Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F. 1983. Studies on the antifungal action of benzoate. Biochem J 214:657–663.

Kobayashi M, Yokoyama I, Matsuda K. 1985. Effectors differently modulating the dextransucrase activity of *Leuconostoc mesenteroides.* Agric Biol Chem 49:3189–3195.

Lappan RE, Fogler HS. 1992. Effect of bacterial polysaccharide production in formation damage. SPE Prod Eng 7:167–171.

Lappan RE, Fogler HS. 1994. *Leuconostoc mesenteroides* growth kinetics with application to bacterial profile modification. Biotechnol Bioeng 43:865–873.

Lazic ML, Veljkovic VB, Vucetic JI, Vrvic MM. 1993. Effect of pH and aeration on dextran production by *Leuconostoc mesenteroides.* Enzyme Microbiol Technol 15:334–338.

Martinez-Espindola JP, Lopez-Munguia CA. 1985. On the kinetics of dextransucrase and dextran synthesis in batch reactors. Biotechnol Lett 7:483–486.

Otts DR, Day DF. 1988. Dextransucrase secretion in *Leuconostoc mesenteroides* depends on the presence of a transmembrane proton gradient. J Bacteriol 170:5006–5011.

Press B, Flannery BP, Teukolsky SA, Vetterling WT. 1992. Numerical recipes in C. Cambridge, UK: Cambridge University Press. 994 p.

Reddish GF, editor. 1957. Antiseptics, disinfectants, fungicides and sterilization, 2nd ed. Philadelphia: Lea & Febiger. 975 p.

Sarkar AK, Georgiou G, Sharma MM. 1994. Transport of bacteria in porous media: I. An experimental investigation. Biotechnol Bioeng 44:489–497.

Seufert WD. 1973. The interaction of anionic detergents with lipid bilayer membranes. Biophysik 10:281–292.

Tanriseven A, Robyt JF. 1993. Interpretation of dextransucrase inhibition at high sucrose concentrations. Carbohyd Res 245:97–104.

Taylor SW, Jaffé PR. 1990. Biofilm growth and the related changes in the physical properties of a porous medium: 1. Experimental investigation. Wat Res Res 26:2153–2159.

Troller JA, Christian JHB. 1978. Water activity and food. New York: Academic Press. 235 p.

Vandamme EJ, Van Loo J, DeLaporte A. 1987. Dynamics and regulation of sucrose phosphorylase formation in *Leuconostoc mesenteroides* fermentations. Biotechnol Bioeng 19:8–15.

Vandevivere P, Baveye P. 1992. Saturated hydraulic conductivity reduction caused by aerobic bacteria in sand columns. Soil Sci Soc Am J 56:1–13.

Vincenzini MT, Favilli F, Stio M, Vanni P, Treves C. 1985. Detergents as selective inhibitors and inactivators of enzymes. Physiol Chem Phys Med Nucl Magn Reson 17:279–295.

Warth AD. 1991. Mechanism of action of benzoic acid on *Zygosaccharmoyces bailii:* Effects on glycolytic metabolite levels, energy production, and intracellular pH. Appl Env Microbiol 57:3410–3414.

Yoneyama H, Akastuka A, Nakae T. 1986. The outer membrane of *Pseudomonas aeruginosa* is a barrier against the penetration of disaccharides. Biochem Biophys Res Commun 134:106–112.

Zwietering MH, Jongenburger I, Rombouts FM, van'T Riet K. 1990. Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875–1881.