A RAPID METHOD FOR THE DETERMINATION OF BACTERIAL GROWTH KINETICS[1]

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ABSTRACT

We have developed a rapid method for the determination of lag times and specific growth rates for bacteria. This method involves the use of a Bio-Tek model EL312 microtiter plate (MP) reader, controlled by KinetiCalc software, and uses a computer program that processes absorbance data from a 96-well MP. The program reads an ASCII file consisting of optical density readings generated by the MP reader, sorts the data into individual growth curves, and determines growth kinetics. No statistically significant difference ($P > 0.88$) was found between the MP method and standard batch methods. This method allows up to 95 growth curves to be carried out simultaneously. The MP method automates the difficult task of determining growth kinetics for bacterial strains under conditions that result in slow growth rates or long lag times. Using this method we determined the effect of NaCl on the growth kinetics of Listeria monocytogenes *and the effects of NaCl and temperature on the growth kinetics of* Leuconostoc mesenteroides.

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INTRODUCTION

Microbial growth in broth culture medium is characterized by a succession of phases: lag, log, stationary, and death phases. These phases are defined by changes in the growth rates of specific bacterial strains. Traditional methods for determining bacterial growth kinetics (Monod 1949; Pirt 1975), either in test tube or in continuous cultures, are laborious and time-consuming. A determination of kinetic parameters, however, is critically important for the evaluation of industrial cultures and compounds that affect bacterial growth.

Our laboratory has an ongoing interest in the development of starter cultures for vegetable fermentations. Determination of bacterial growth kinetics under varying media conditions is a useful method for the analysis of potential fermentation starter culture strains. In a previous report from our laboratory, McDonald *et al.* (1993) included an analysis of the growth rates and lag times of *Lactobacillus plantarum* cultures under various brine and temperature conditions as part of a general protocol to determine the ability of these strains to dominate cucumber fermentations. The inclusion in this study of growth conditions that are not favorable to rapid growth of the test strains required optical density readings to be monitored continuously for 24 h or more. The time necessary for a study of this type can be prohibitive.

Rapid and automated methods for monitoring bacterial growth have previously been developed. Thomas *et al.* (1985) developed a computer-controlled microtiter plate (MP) reader and determined the effect of ethanol and pH on the cell yield of various *Leuconostoc* and *Lactobacillus* strains used in the fermentation of wine. The MPs were incubated in polyethylene bags to prevent evaporation and read in the plate reader at 2, 5, and 9 days. This method demonstrates the utility of doing large numbers of bacterial fermentations simultaneously in a MP. Commercial products, such as the Bioscreen analyzing system (Labsystems Oy, Helsinki, Finland) that allow the determination of bacterial growth kinetics in multi-well MPs have recently become available, but at considerable expense. We have developed a relatively inexpensive method for determining bacterial growth kinetics that allows the use of a computer-controlled MP reader, common in many microbiology laboratories. The MP method could be easily adapted to work with a variety of MP readers.

MATERIALS AND METHODS

Bacterial Strains

Leuconostoc mesenteroides strain C33 (Stamer *et al.* 1971) was maintained in the U.S. Food Fermentation Laboratory culture collection (Raleigh, NC). *Listeria*

monocytogenes 4b (#F5069) was obtained from C. Donnelly (University of Vermont).

Culture Medium

Bacto lactobacilli MRS broth (MRS) was prepared according to the supplier's instructions (Difco Laboratories, Detroit, MI). Cabbage juice broth (CJB) was prepared using a modification of the method of Kyung and Fleming (1993). The cabbage was quartered, placed in an autoclave, and the temperature was raised to 121C and then immediately lowered, requiring a total of 10 min in the autoclave. The heated cabbage was then homogenized in a Waring Blendor and the juice extracted by pressing through cheesecloth. The juice was clarified by centrifugation at 40,000 × g (type 35 rotor, Beckman Instruments, Fullerton, CA) for 30 min at 5C and filtered through a sterile 0.22 μ filter (Costar Corp., Cambridge, MA). The addition of NaCl to CJB, as indicated below, was done prior to filtration. A glucose defined medium (GDM) shown to support the growth of *L. monocytogenes* was made according to the method of McFeeters and Chen (1986) with the modification of Premarante *et al.* (1991); D,L-thioctic acid (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 4.8 μM. The completed medium was aerated by bubbling air through ice cold medium for 2 h. The medium was then sterilized by filtration with a 0.2 μm Millipore filter (Millipore Corp., Bedford, MA), dispensed into sterile, screw cap, 15-ml glass test tubes, and held at 5C prior to inoculation. Dissolved oxygen was measured using a dissolved oxygen meter (YSI model 5739, Yellow Springs Instrument Co., Yellow Springs, OH).

Fermentations

L. monocytogenes 4b and *L. mesenteroides* strains were grown overnight for 15 h in GDM and CJB, respectively. The cell counts were determined using a Spiral Systems, model D2, spiral plater (Spiral Systems Inc., Cincinnati, OH). All optical density determinations were carried out by determining absorbance at 630 nm. The cells were washed and resuspended in growth medium and then diluted to inoculate broths in MP wells or screw capped, 15-ml test tubes for growth rate determination. A final volume of 12 ml was used for test tube fermentations and 200 μl for MP fermentations. For *L. monocytogenes* 4b, the cells were diluted with GDM with different NaCl concentrations (0–2.5%) in 0.25% increments to give a starting population of 1×10^4 CFU/ml for growth rate determinations. The growth curves were carried out in the MP reader (see below) using sterile, 96-well MPs (Costar type #3598). The MP fermentations were overlaid with 75 μl of sterile mineral oil, and the test tube fermentations were overlaid with 0.2 ml of sterile mineral oil. For the *Listeria* experiments, cells

were incubated immediately (upon dilution) in the MP reader, which was programmed to take optical density readings (630 nm) every hour for 36 h at 30C. For lag time determinations with the MP reader, the initial optical density of the cell suspension was 0.1 unit, corresponding to an inoculum of 3.6×18^8 CFU/ml (see below). The test tube fermentations were started at intervals by holding the inoculated broths at 5C (as indicated below) for no longer than 10 h. This procedure allowed fermentations with varying lag times to be initiated simultaneously. Optical density readings were taken hourly using a Pharmacia Novaspec II (Pharmacia Biotech Inc., Piscataway, NJ) upon incubation at 30C in a Lauda RC-20 circulating water bath (Brinkmann Instrument Co., Westbury, NY).

For the *L. mesenteroides* experiment, the MP was set up with CJB containing 7 different NaCl concentrations (0–6%) in 1% increments. The *L. mesenteroides* culture was diluted in MRS to give a starting population of 1×10^7 CFU/ml. The incubation period was 24 h, with optical density readings taken every hour. The range of temperatures used was 15–30C, in 2.5C increments, for 7 temperatures in all. All fermentations were carried out in duplicate.

Prior to each optical density reading, a programmed, 5-s vibrational shake of the MP was carried out, and a corresponding 5-s vortexing of the test tubes was also carried out. We did not observe any effect, due to an oil-water emulsion on the optical density readings from the mixing procedure either with the test tube or MP fermentations (data not shown). Optical density data for all rate determinations was limited to absorbance values above 0.1 and below 0.7 (at 630 nm), so as to remain within the range of the MP reader, which gave a linear response (data not shown) for absorbance vs. CFU/ml. The linear response was determined by comparison of the dry weight of the cells to the optical density readings. The R^2 value obtained for these data was 0.998.

Growth Kinetics and Computer Algorithm

The Regress program was written in C++ and compiled on a Borland Turbo C++ compiler (version 3.1 for Windows, Borland International, Inc., Scotts Valley, CA). Input data consisted of an ASCII file containing one column of optical density readings from an 8 (row) × 12 (column) MP, as produced by KinetiCalc software version 2.03 (Bio-Tek Instruments Inc., Winooski, VT). The KinetiCalc program controlled a Bio-Tek model EL312 MP reader with a temperature control heater unit (Bio-Tek). Incubation of the entire MP reader in an environment chamber allowed us to run the MP reader at constant temperatures above or below room temperature.

To determine growth rates, individual growth curves were processed in sequential sets of n data values, where n was in the range of 3 to 10. This value may be determined by the user. In our experiment with $n = 3$, the data points

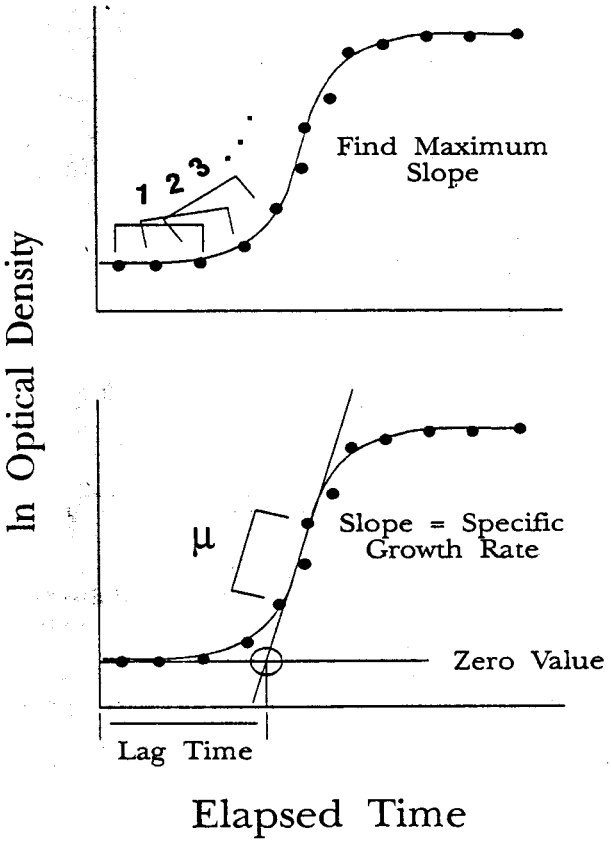

FIG. 1. METHOD FOR THE CALCULATION OF SPECIFIC GROWTH RATES AND LAG TIMES

The growth curves were processed as described in Materials and Methods.

(consisting of x-y pairs of elapsed time and the natural log of optical density) processed were 1 through 3, 2 through 4, then 3 through 5, etc. (see Fig. 1). The growth rate for each set of x-y data points was determined as the slope of the line generated by the data points used. The maximum slope obtained for all sets of data points in a growth curve was retained. This value represents the specific growth rate. In addition, the R^2 value for the regression line was also retained, so rates resulting from outlying points can be identified and discarded, as determined by a low R^2 value. Typically, R^2 values for our growth data were 0.980 or above; rate data with R^2 values below 0.900 were not considered to be accurate determinations of the growth rate. Each of the 96 growth curves were

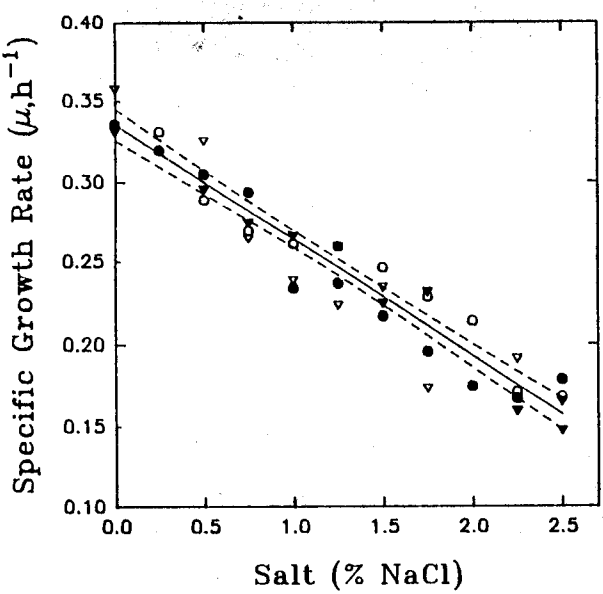

FIG. 2. COMPARISON OF THE TEST TUBE AND MP METHODS FOR GROWTH RATE DETERMINATION: THE EFFECT OF NaCl ON THE GROWTH OF *L. MONOCYTOGENES* F5069

The specific growth rate data were plotted along with the regression line (solid line) for the combined data. The broken lines represent the 95% confidence limits for the means of the Y values at given X values. The open and filled circles represent the rates determined from the duplicate MP wells. The open and filled triangles represent the data obtained from duplicate test tube fermentations.

similarly processed. Determination of specific growth rates for the test tube fermentations used the same method described above, except the points used for the rate calculations were determined by inspection.

Lag time was calculated using the equation of the regression line that was used to determine the growth rate for a given growth curve. The initial optical density reading for the growth curve was used for lag determinations. By entering this value (Ln of optical density or "zero value") in the regression equation, elapsed time was calculated and represents the lag time (Fig. 1). To ensure the initial optical density value was not an outlying point, conditions governing the acceptance/rejection of this point were built into the algorithm. The criterion for acceptance/rejection was dependent upon the slope of the first three O.D. readings taken of the growth curve.

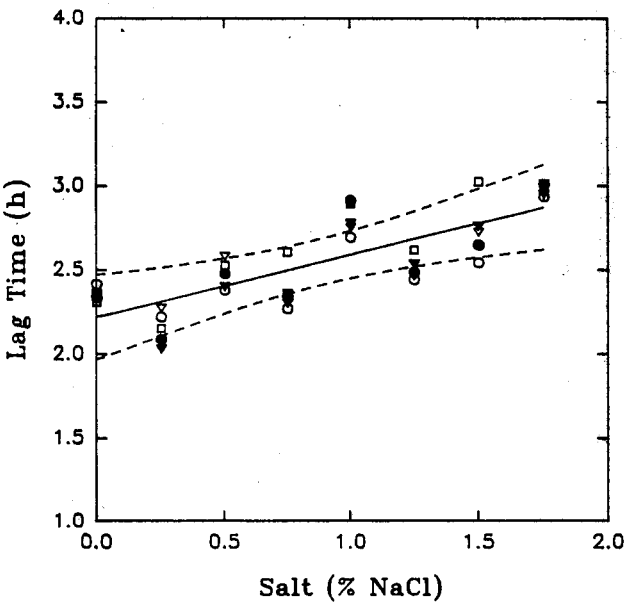

FIG. 3. THE EFFECT OF NaCl ON LAG TIME FOR THE GROWTH OF *L. MONOCYTOGENES* F5069

The solid line represents the predicted values as determined by linear regression. The broken line represents the 95% confidence limits for the means of the Y values at given X values. The symbols represent the results from 5 growth curves carried out with various salt concentrations in the MP wells. Lag values were calculated as descibed in Materials and Methods.

Statistical Analysis

A randomized complete block design was used for the statistical comparison of the two methods. The General Linear Models Procedure (GLM) of PC-SAS (SAS Institute, Cary, NC) was used to compute the analysis of variance and the response surface.

RESULTS

Specific growth rates for *L. monocytogenes* F5069 were calculated from data generated by the MP and test tube methods. There were no statistically significant differences between the two methods ($P > 0.88$). The specific growth rates

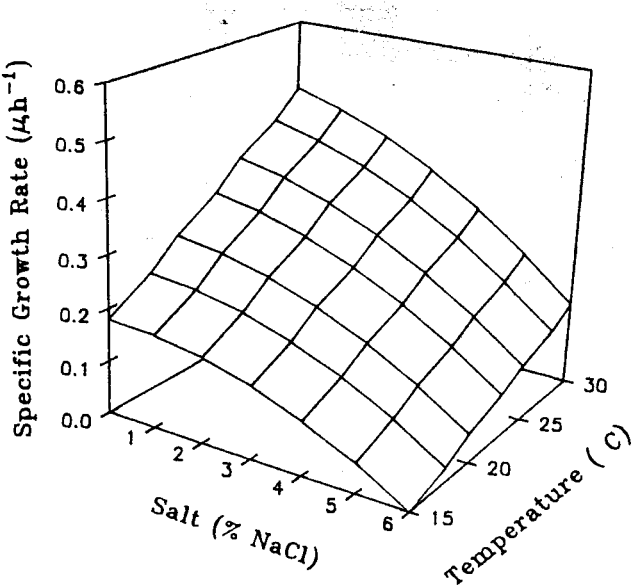

FIG. 4. THE EFFECTS OF TEMPERATURE AND NaCl ON THE GROWTH KINETICS OF *L. MESENTEROIDES*

Response surface graph showing the surface determined from the predicted values for specific growth rates. The predicted values were determined as described in Materials and Methods.

for both methods were regressed against salt concentration, the R^2 values for the MP and tube methods were 0.969 and 0.916, respectively, indicating a slightly better fit for the MP method. Pooling the data from the two methods produced an R^2 value of 0.938 (Fig. 2). The effect of salt concentration on lag time, as determined by the MP method, is shown in Fig. 3, with an R^2 vlaue of 0.708. Lag time data were not available for the test tube method due to the sequential delay of starting times. The starting times for the test tube method were staggered to minimize operator time on the spectrophotometer, and this illustrates one advantage of the MP method.

An *L. mesenteroides* strain (C33) was used for bacterial growth rate determinations by the MP method. A response surface graph showing predicted values was produced from the following equation, as generated by SAS software using the GLM procedure:

$$\mu = -0.1119 + 0.0194t + 0.02262s - 0.005022s^2 - 0.00150ts$$

where μ is the calculated specific growth rate, t is temperature in °C, and s is

the salt concentration in percent. With this equation, the coefficient of determination for the specific growth rate data was found to be 0.979.

DISCUSSION

We have developed a rapid method for the determination of bacterial growth kinetics. Because of the large amount of data generated by 96 simultaneous fermentations, an automated method was needed for the analysis of growth kinetics. A computer algorithm was developed that sequentially processed sets of optical density readings from the growth curve data. Curve fitting methods for growth rate determinations were rejected because bacterial growth in the presence of some inhibitory substances may not always result in sigmoidal growth curves.

For the growth of *L. monocytogenes*, aerated medium was used to allow continued aerobic growth in the MP and test tube systems covered with oil. The dissolved oxygen in GDM was monitored and determined to be adequate to support aerobic growth through logarithmic phase, as seen by comparable growth rates and metabolic end products when cells are grown in shake culture (Romick and Fleming 1993). Medium overlay with mineral oil was essential for accurate MP readings by preventing evaporation loss during incubation that would decrease the optical path length. The utility of the MP method was demonstrated by the response surface data shown in Fig. 4. Seven 24-h MP fermentations were needed to generate the data for calculating the response surface equations. This amounted to a total of 2,352 optical density readings. Generation of this amount of data by standard test tube methods would clearly be impractical. The utility of the MP method was further demonstrated by the ability to generate lag time data for bacterial strains. The MP method may also be adapted for measuring the concentration of biological compounds by correlating concentration with the observed effects on growth kinetics or the maximum optical density achieved by an indicator bacterial strain. An example of this is the determination of vitamin concentration by microbiological assay (Snell 1950).

The MP method offers a simple, rapid, and efficient means for determining bacterial growth kinetics. The method requires an automated MP reader with a controlled temperature MP chamber.

The Regress program reported herein could be easily adapted to a variety of ASCII data formats, but is currently available to read ASCII data from a Bio-Tek MP reader controlled with KinetiCalc software, and can be obtained by anonymous ftp to ftp.ncsu.edu. The program and documented source code has been placed in the /pub/ncsu/fbreid directory.

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