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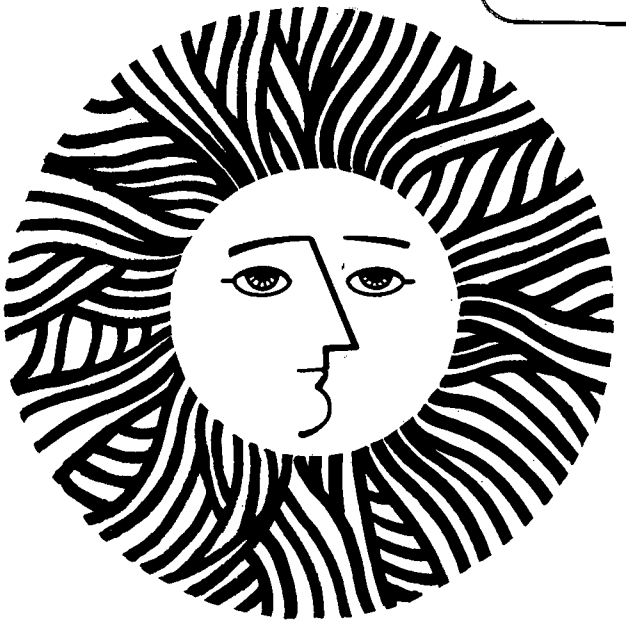
THE FERMENTATION OF XYLOSE BY BACILLUS MACERANS

Thomas A. Delfino*, Harvey W. Blanch,
and Charles R. Wilke
(*M.S. thesis)

September 1981

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Thomas A. Delfino*,
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*M.S. Thesis

September 1981

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This work was supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Division of Chemical Science Processes and Techniques Branch of the U.S. Department of Energy under Contract DE-AC03-76SF00098; and by the Assistant Secretary for Conservation and Solar Energy Research Institute under Contract DE-0-9058-1.

ABSTRACT

Plant stems, leaves, wood and most other sources of cellulose contain hemicelluloses. When cellulose from these sources is enzymatically hydrolyzed, hemicelluloses are also hydrolyzed by the action of xylanase. The hydrolysis of hemicelluloses yields pentoses, primarily xylose, and some hexoses.

Yeast are unable to ferment pentoses, therefore, schemes to produce ethanol from cellulose by fermentating glucose derived from cellulose will produce a waste stream containing the unfermented pentoses. Utilization of this pentose stream to produce ethanol is thus an attractive process option.

Bacillus macerans is capable of fermenting pentoses to ethanol. The ability of B. macerans to ferment pentoses to ethanol was examined. The organism was grown in a continuous flow stirred-tank fermenter. In continuous culture at steady state, ethanol and acetic acid were produced, whereas in batch culture and during transients in continuous culture, ethanol, acetic acid and acetone were produced. The organism was found to have a relatively high maximum specific growth rate. This maximum specific growth rate was observed to be higher than previously reported for B. macerans organisms.

It was found that B. macerans had difficulty attaining and maintaining a steady state when grown in continuous culture. A perturbation of the conditions in the fermenter, such as a step-change to a higher dilution rate or an injection of a small amount of xylose into the fermenter, would cause a growth stoppage. Several days were required for the fermenter to return to normal.

A possible explanation for this behavior is an interaction between the magnitude of the transmembrane pH gradient, the metabolic rate and protonated acetic acid inhibition. At a constant external pH, a decreasing metabolic rate would cause the pH gradient to decrease which would increase acetic acid inhibition which in turn would cause the metabolic rate to decline more. The effect of changing metabolic rates was amplified by the strong dependence of cell yield on metabolic rate. A small decrease in metabolic rate would produce a large decrease in specific growth rate because cell yield would decrease. A mathematical model of the growth and metabolism of B macerans was constructed to test the postulate that an interaction between metabolic rate, pH gradient and acetic acid inhibition was the explanation for the behavior observed. The model results confirm that the explanation is plausible.

Though B. macerans grows rapidly and produces ethanol from pentoses, its instability and transient behavior in continuous culture render it unsuitable for use in a commercial process for disposing of pentoses.

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1. INTRODUCTION

This work examines the feasibility of conversion of pentose sugars resulting from the biological hydrolysis of cellulose. Cellulose from most sources occurs together with the polysaccharide hemicellulose (1). Hemicellulose may contain any of a number of hexosan and pentosan, but primarily contains the pentosan xylan (2). Hemicelluloses may comprise as much as 20% of the dry weight of wood, of plant stems, of leaves, and other such cellulose sources (3).

In recent years much interest has been expressed in developing a fuel-ethanol industry based on renewable resources, such as whole trees or agricultural residues. Because yeast will not ferment most sugars derived from hemicelluloses, processes for producing ethanol from cellulose, using yeast to ferment hydrolyzed cellulose, will produce a waste stream containing hydrolyzed hemicellulose (4). The economic prospects for a fuel-ethanol from cellulose industry in this country are presently marginal, at best (5). For such an industry to develop, a method of disposal for hemicellulose sugars, which will not present an economic burden, will have to be developed. The disposal method will have to produce some product of more value than the cost of producing it from waste sugars. Because the disposal process would be located at a plant for producing ethanol by fermentation, converting the waste sugar to ethanol would seem an appropriate method of disposal that could pay for itself.

In this work, the ability of the bacterium, Bacillus macerans, to ferment hemicellulose sugars is assessed. Hemicellulose sugars were modeled by xylose alone. B. macerans exhibited unusual growth

characteristics which would make it unsuitable for use in a commercial waste disposal process. This unusual behavior was examined at the cellular level, and a model to explain the regulation of xylose metabolism was proposed.

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2. BACKGROUND

2.1. Comparison of Organisms Capable of Metabolizing Pentoses

Microorganisms can be found which will metabolize pentose sugars by one or both of two general metabolic routes--fermentation and respiration. The difference between these two is in the fate of the electrons removed from the organic molecules derived from the sugars. In fermentation these electrons are transferred to other organic molecules derived from the pentoses. In respiration they are transferred to inorganic molecules (1). Organisms metabolize sugars to obtain energy for growth. The yield of energy from respiration is many times greater than that obtain from fermentation, and therefore, the yields of cells and cellular metabolites is greater under respiration conditions. However, the metabolic products are CO_2, water and a reduced inorganic electron acceptor. In fermentation the metabolic products are small organic molecules, some of which have significant commercial value.

The most common inorganic electron acceptor is molecular oxygen. Respiration with molecular oxygen is called aerobic growth. In general, yeasts only grow aerobically on pentoses. Candida utilis, a torula yeast, is currently grown aerobically on waste pentoses produced as a by-product of paper pulp manufacture in a commercial operation (2). The dried yeast is sold as a food additive.

Some bacteria can grow aerobically on pentoses, particularly some of the members of the genus Bacillus. Some of these, including B. polymyxa, mentioned below, produce antibiotics during sporulation (1). Waste pentoses, therefore could replace the currently used substrate in

antibiotic production.

Some molds are also capable of fermenting pentoses. Some members of genus Fusarium show nearly stoichiometric yields of ethanol (3). However, Fusarium have two disadvantages for industrial application. Being filamentous organisms they have very low growth rates compared with bacteria or yeasts. They are also plant pathogens (4), and some produce carcinogenic compounds (16). Fusarium oxysporum, which has been studied extensively, has an ethanol yield on xylose of 0.41 gm/gm., similar to that of yeast fermenting glucose. However, the maximum growth rate is 0.04 hr^{-1} (5).

Bacteria are capable of fermenting pentoses to a wide variety of products. Some of the most common ones are ethanol, butanol, 2,3-butanediol, acetone, and acids such as lactic, formic, acetic propionic, butyric and succinic. Some members of the genus Clostridium are capable of producing ethanol in yields comparable to those of yeast. Other bacteria produce ethanol as a co-product with one or more other metabolites. An example is the mixed-acid fermentation, in which ethanol is produced with one or more of the acids (3). If 2,3-butanediol is produced in addition, this is known as a mixed acid plus, 2,3-butanediol fermentation. The acids do not have current commercial value, whereas the 2,3-butanediol is a precursor of 1,3-butadiene, a constituent of synthetic rubber, and so has considerable value (6).

Other product combinations exist. One that has great commercial significance was the butanol-acetone-ethanol fermentation carried out by Clostridium acetobutylicum. Between the World Wars this was the principle source of butanol. It was also an important source of the

vitamin riboflavin (1). As with torula yeast, the organism was grown on waste pentoses from paper pulp manufacture (7). Many members of the family Enterobacteriacae conduct a mixed-acid plus 2,3-butanediol fermentation, but yields of acids are high and yields of ethanol and 2,3-butanediol low. Bacillus polymyxa conducts the same fermentation, but the yields of ethanol and 2,3-butanediol are high, and only small amounts of acid are produced (8). Were it not for the cost of separation, this would likely be of commercial process.

Clostridium thermohydrosulfuricum shows great promise for a commercial fermentation producing ethanol from pentoses. Being an extreme thermophile--optimum temperature 65°C--it has a high maximum specific growth rate, 0.5 hr^{-1}. A recently isolated strain capable of giving high ethanol yields is currently under study (9).

Bacillus macerans conducts a modified mixed-acid fermentation. The principle products are ethanol, acetone and acetic acid (10). None of the other acids are produced in significant quantities. This product distribution has three advantages. Of the possible combinations where ethanol is coproduct with other metabolites, a combination with acetic acid or acetone provides the highest possible ethanol yield (1). Another advantage is that acetone could theoretically, entirely replace acetic acid as a coproduct--if the right conditions were found. The third advantage is the relative ease with which ethanol and acetone can be separated from a dilute aqueous solution. For these reasons B macerans was chosen as a candidate for possible commercial exploitation.

2.2. Bacillus Macerans--Previous Work

In the early part of this century several workers proposed

fermenting pentoses, derived by acid-hydrolysis of hemicelluloses to acetone and ethanol with B. macerans (11,12,13) Others at the time were using B. macerans to ferment hexoses to the same products (14). More recently, Rosenberg, et al. (10) and Rhodes and Riba (15) studied its ability to ferment pentose sugars.

Rosenberg (15) determined the optimum pH and temperature for growth to be 6.0 and 40°C, respectively. The best yields of acetone and ethanol were obtained at pH 5.5, but the growth rate was substantially lower than at pH 6.0 (see Fig. 2, 1). Northrop, Ashe and Senior (16) in 1920 found approximately the same optimum temperature, but not the same pH optimum. However, they used unagitated vessels and crude pH control techniques. Rosenberg (10) also observed that B. macerans made a considerable amount of slime. This too was observed by Northrop, Ashe and Morgan (14).

Rosenberg, Rhodes and Riba, unlike the early workers, were able to measure growth rates quantitatively. For batch growth at 40°C and pH 6.0, Rosenberg and Rhodes (10,15) found the maximum specific growth rate (μmax) to be 0.15 hr^{-1} with variations of ±10%. Riba (15) on the other hand, found μmax to be between 0.20 and 0.22 hr^{-1} (see Fig. 2,2). The maximum specific growth rates given above apply to initial xylose concentrations between 10 and 30 gm/L. At higher concentrations, μmax was smaller (see Fig. 2,2 and (10)).

Riba performed the experiments, from which Fig. 2,2 was prepared, as test tube cultures. pH control was provided by heavy buffering with $H_2PO_4^-$ - HPO_4^{-2} at pH 6.5. A shaker provided agitation to prevent the bacteria from settling to the bottom of the test tube. Cell density

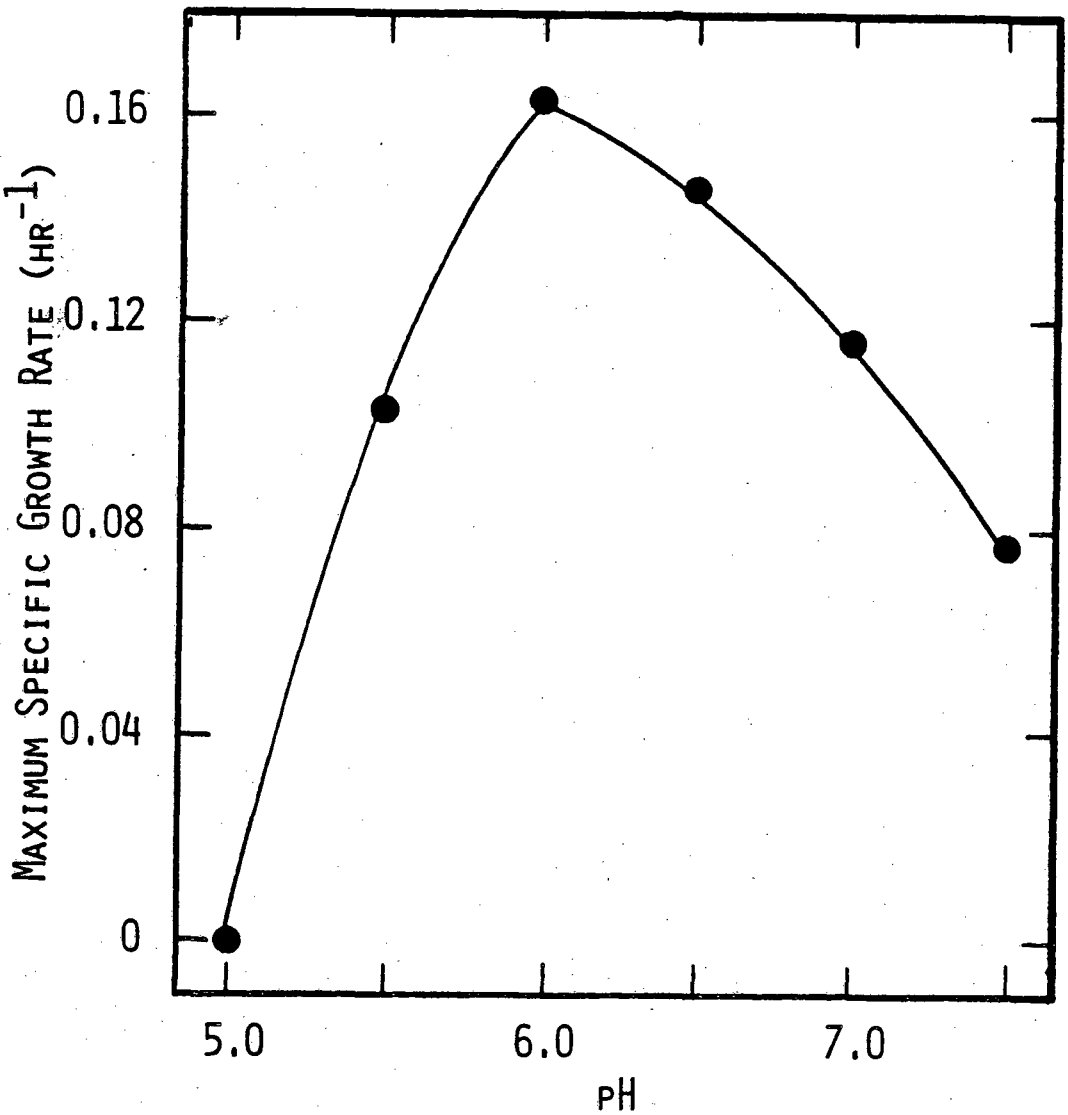
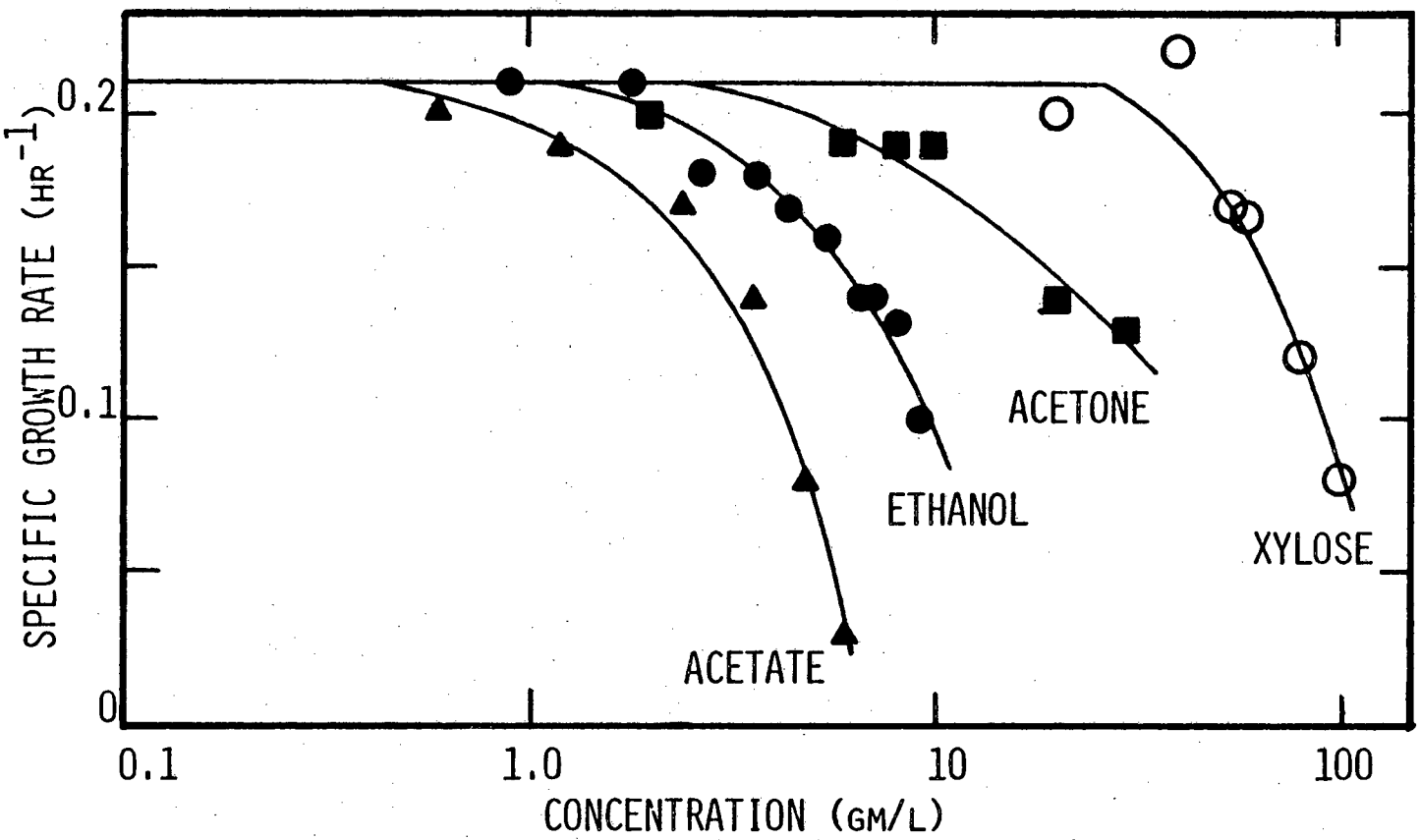


Figure 2.1 Effect of pH on maximum specific growth rate of Bacillus macerans. (Data from Rosenberg, S.L., Batter, T.R., Blanch, H.W. and Wilke, C.R., Hemicellulose Utilization for Ethanol Production, Lawrence Berkeley Laboratory Rept. 10880 (1980)

was measured by optical density, with the test tube serving as the cuvette. Inocula were drawn from the growing culture in the fermenter used in this work. Following inoculation, the gas space was purged with N_2 to remove air. The concentrations recorded in Fig. 2,2 were the initial concentrations. For the cultures with acetate, ethanol or acetone added, the initial xylose concentration was 5 gm/L. The growth rates reported were the highest measured for each fermentation.

The experiments of Rosenberg and Rhodes exhibited several interesting features. One of these was a long lag phase, of the order of two days or more. The inocula were prepared by growth anaerobically on xylose, and thus should have been well acclimated. Another feature was the inability of B. macerans to completely utilize the xylose. With an initial xylose concentration of 76 gm/liter this inability was extreme. Growth stopped after consumption of less than 20 grams per liter (see Table 2.1). However, after growth had stopped, metabolism, as indicated by an increase in product concentration, would continue (see Fig. 2,3). The most interesting feature, though, was the appearance of acetone in the fermentation broth. This occurred late in the fermentation, and marked the end of exponential growth. At the same time, the production rate of acetic acid, which had paralleled the production of ethanol up to that point, would drop substantially below that of ethanol. Since acetone production occurs at the expense of acetic acid and ATP production, these effects would be expected.

The appearance of acetone would occur when acetic acid concentration was between 1 and 2 gm/L. Riba's work (see Fig. 2,2) indicates the threshold for inhibition by acetic acid is approximately 0.5 gm/L.



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Figure 2.2 Effect of acetate, ethanol, acetone and xylose on the specific growth rate of Bacillus macerans. (From Riba, J.P., Unpublished data.)

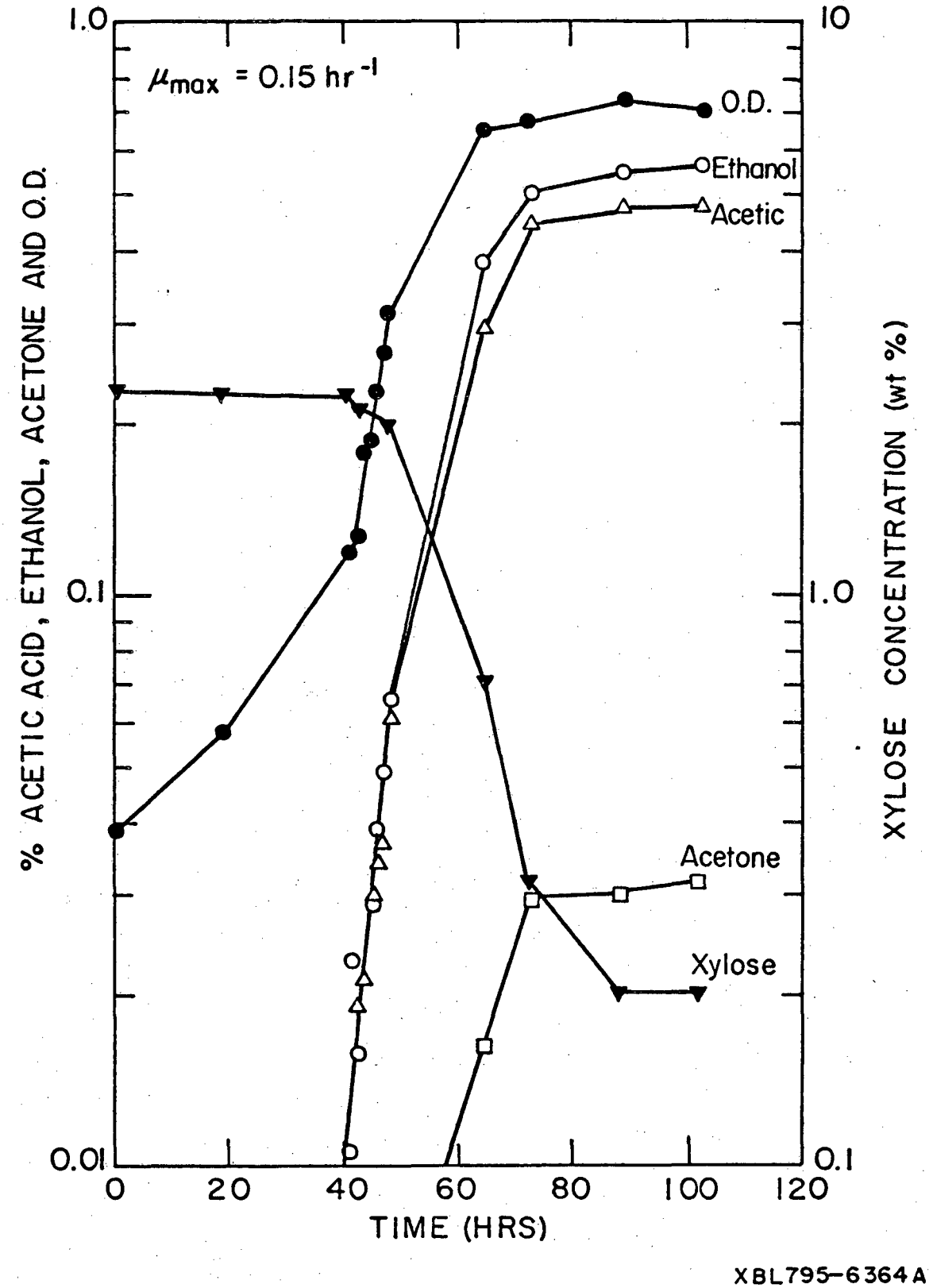


Figure 2.3 Batch growth of Bacillus macerans on xylose. (From Rosenberg, S.L., Batter, T.R., Blanch, H.W. and Wilke, C.R., Hemicellulose Utilization for Ethanol Production, Lawrence Berkeley Laboratory Report 10800 (1980), Berkeley, California

Acetic acid inhibition, therefore, is probably a factor in the triggering of acetone production.

From the foregoing work, it was decided to investigate further pentose metabolism of B. macerans in a continuous flow stirred tank fermenter at 40°C and pH 6.0. Continuous culture has two advantages over batch cultures. Because the substrate is low in such a culture, substrate inhibition would not be a problem. Since conditions in such a culture are like those late in a batch culture, the yield of acetone ought to be superior.

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3. XYLOSE METABOLISM AND MEMBRANE TRANSPORT

3.1. Xylose Metabolism

Bacillus macerans ferments xylose to ethanol, acetone, acetic acid, CO_2, and H_2. Only two previous reports, those of Speakman (1) and DeMey and Wilssens (2), shed any light on the mechanism. As a result, xylose metabolism in this organism must be inferred from other organisms.

The work of DeMey and Wilssens (2) demonstrates the existance of a pentose-phosphate pathway in B. macerans. Mortlock and Wood (3) have demonstrated in Aerobacter aerogenes the entrance to this pathway is through xylulose-5-phosphate. Xylose inside the cell is isomerized to xylulose then phosphorylated by ATP. Two fructose-6-phosphate molecules and one glycernaldehyde-3-phosphate molecule exit from the pathway for every three xylulose-5-phosphate molecules that enter (4). The molecules leaving the pentose-phosphate pathway enter the Embden-Meyerhof pathway which metabolizes them to pyruvate. DeMey and Wilssens also demonstrated the existence of the Embden-Meyerhof pathway in B. macerans.

Starting from xylose within the cell, the net generation of ATP is the same as for glucose on a per pyruvate basis. Each xylulose molecule is phosphorylated by an ATP. Each fructose-6-phosphate is also phosphorylated by an ATP. Each glyceraldehyde-3-phosphate picks up a second phosphate in the dehydration step in the Embden-Meyerhof pathway. Each diphosphoglycerate so formed is twice dephosphorylated to produce pyruvate. These dephosphorylations each produce an ATP from an ADP. Thus the overall balance for ATP production and xylose consumption is

5ADP + 5ATP + 5PI + 3 xylose = 10ATP + 5pyruvate + 10(H)

(The 10(H) represent the reducing equivalents residing on NADH molecules as a result of the dehydrogenation of glyceraldehyde-3-phosphate.) The net yield of ATP is 5/3 per xylose or one per pyruvate.

Pyruvate must be converted to materials which pass easily out of the cell. At the same time the reducing equivalents on NADH must be able to leave the cell. Under fermentation conditions some of the molecules derived from pyruvate accept these reducing equivalents and thereby carry them out of the cell.

In bacteria which produce ethanol, acetic acid and acetone such as B. macerans, these products are obtained via the intermediate acetyl CoA (5). Acetyl CoA is formed by cleaving the carboxyl group from pyruvate with the concurrent addition of co-enzyme A. The carboxyl group either appears as formic acid which is then split to H_2 and CO_2, or it appears as H_2 and CO_2 directly. No reducing equivalents are required to make acetic acid or acetone from acetyl CoA. Four are required to make ethanol (5). Two acetyl CoA's are required to make acetone (see Fig. 3.1). To balance reducing equivalents with pyruvate, the following stoichiometry must hold:

2 pyruvate + 4(H) = 1 ethanol + 1 acetic acid + $2CO_2$ + $2H_2$

or

2 pyruvate + 4(H) = 1 ethanol + 1/2 acetone + $2H_2$ + 2 1/2 CO_2

Given these stoichiometries the ethanol molarity must be equal to that of acetate plus twice that of acetone. Expressed as yields (gm/gm), ethanol should not exceed 0.26 gm ethanol per gm xylose consumed. The maximum acetate yield would be 0.33 gm/gm xylose.

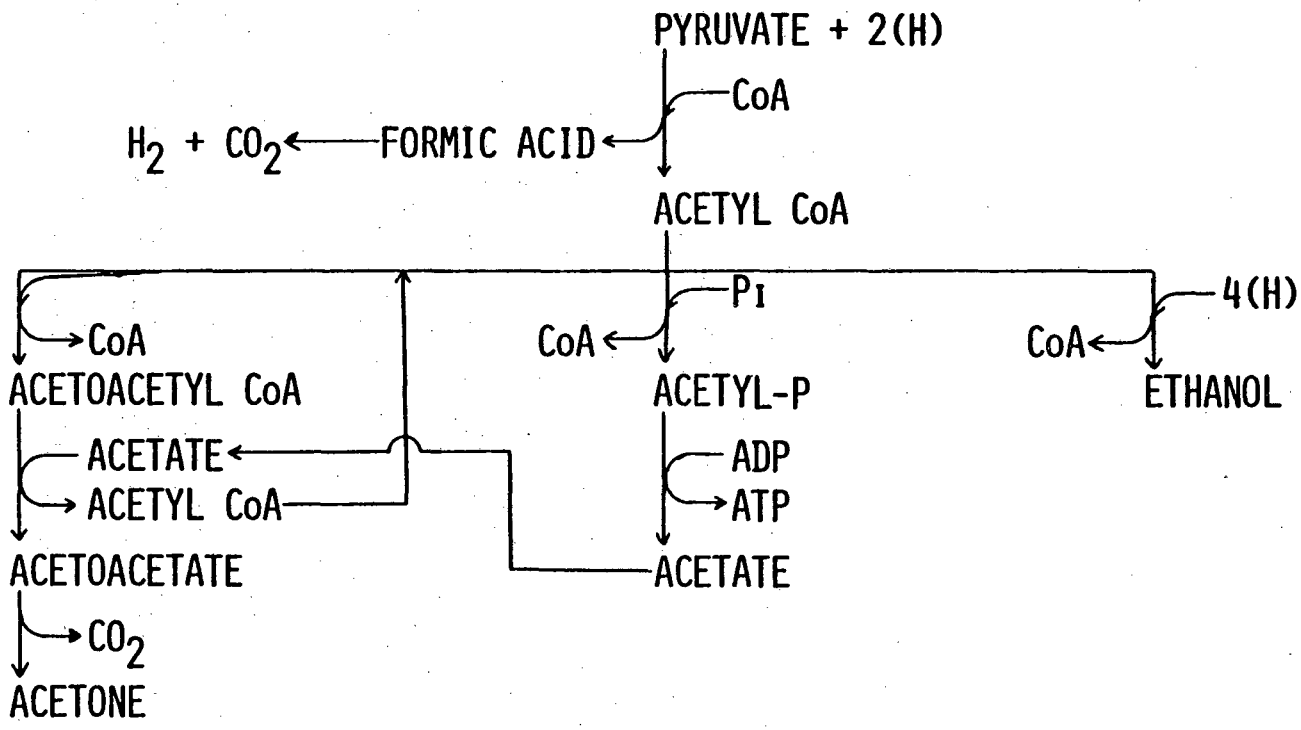


Figure 3.1. Pathways from pyruvate to acetone, acetate and ethanol.

Experimental results comply with the constraint on yields but not on stoichiometry (6,7). At a pH above 7.5, acetate yields are nearly equal to the theoretical, but ethanol is well below the theoretical and present at less than half the concentration of acetate. At a pH below 6, the opposite effect is found. Apparently this means the organism is capable of diverting product into slime formation.

The cell produces more energy, as ATP, when it produces acetic acid than when it produces acetone. The production of one molecule of ATP accompanies the production of each molecule of acetic acid or acetone. However, since two pyruvate are required to make one acetone, whereas only one is required for acetic acid, the yield of ATP per pyruvate is greater for acetic acid. No ATP is produced with ethanol (8). Overall the yield of ATP per molecule of pyruvate, assuming theoretical stoichiometry holds, would be 1.5 for ethanol and acetic acid production. For ethanol and acetone production it would be 1.25. This compares with 1.0 for a fermentation producing only ethanol or only lactic acid.

The ATP yields have been given on the basis of xylose already inside the cell. In the next section it will be seen that the true yields are lower due to a requirement for energy to enable xylose transport into the cell.

3.2. Membrane Transport

The cell membrane of bacteria is thought to be a continuous phospholipid bilayer studded with proteins (5). The phospholipid bilayer forms a barrier to penetration of the cell by non-lipophilic molecules. This means most sugars, amino acids and inorganic ions

(Na^+, K^+, H^+, PO_4^{-3}, SO_4^{-2}, etc.) cannot readily enter the cell, though they are essential to cell metabolism. On the other hand, the bilayer does prevent the escape of those materials from the inside of the cell. To provide for passage of these materials, some of the proteins in the membrane form channels. Some channels simply conduct solute molecules from the side with a higher concentration to the one with a lower concentration. This is known as facilitated diffusion. Others are coupled to an energy supply and so are able to pump solute molecules against a concentration gradient. This is referred to as active transport. These channel proteins resemble enzymes in their specificity and kinetics.

The currently accepted explanation for the mechanism of active transport involves the "Chemiosmotic Theory." (9, 10) The heart of this theory is that cells store energy in the form of a chemical potential gradient across the cell membrane. This gradient is due to a hydrogen ion gradient. The chemical potential is composed of a voltage gradient (negative inside) and a pH gradient (alkaline inside). In respirating cells this gradient is generated by hydrogen ion pumping by the electron transport chain (9, 10, 11). Similarly, in photosynthetic cells the operation of the photoreactive apparatus causes pumping of hydrogen ions (11, 12). In such cells the primary source of energy for phosporylating ATP is this chemical potential (9, 10, 13, 14). The reaction is performed by a transmembrane protein appropriately called ATPase. The phosphorylation takes place entirely within the cell, but two hydrogen ions must enter the cell through the ATPase for the reaction to occur.

In facultative anaerobes this ATPase is reversible (9, 10, 13, 14). When the cells are respiring the ATPase operates as described above. However, when the cells are fermenting the electron transport system no longer functions. The only means for building the chemical potential is pumping hydrogen ions by reversing the ATPase action. Hydrolysis of ATP results in the expulsion of two hydrogen ions.

The energy for active transport also derives from this chemical potential (9, 10, 14). The movement of one or more hydrogen ions through an active transport channel accompanies the movement of a solute molecule across the membrane. Because the movement of solute molecules is coupled to this gradient, solute molecules can move against an opposing gradient. Movement against a gradient as high as 10^6 (gm/L/gm/L) have been reported (15). This would require a chemical potential of 400 mV or more, which has not yet been observed (10). The sense of direction in energy transfer is not unidirectional. A high enough solute gradient will drive hydrogen ions against an opposing gradient (16). Xylose and most common, natural sugars, other than glucose, enter the cell by active transport (9). The stoichiometry for sugar movement is 1:1 (sugar, hydrogen ion) (17). Concentration ratios of 10^3 (inside over outside) are generally accepted as the maximum achievable (10) though ratios as high as 10^5 have been reported (18). To achieve a ratio of 10^3, a chemical potential of more than 180 mV is required. Details are available in Chapter 6.

In the first section of this chapter the yield of ATP was given as 1.5 or 1.25 per pyruvate, depending on product mix. When the energy required to transport xylose across the membrane is taken into account

($1H^+$ = 1/2 ATP per xylose transported) these numbers become 1.2 and 0.95, respectively. Thus membrane transport of xylose consumes 20% or more of the energy generated from the xylose.

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4. METHODS AND PROCEDURES

4.1. The Organism

Bacillus macerans (ATCC 8244) was obtained from the American Type Culture Collection. The culture was stored on aerobic slants of DIFCO Nutrient Agar, at 0-5°C.

To prepare an inoculum for the fermenter, organisms were transferred by loop to flasks of sterile DIFCO Nutrient Broth. The flasks were aerobically incubated, with shaking, at 40°C for two days. 5 ml of the grown culture were aseptically pipetted into the fermenter. Three days were allowed for the culture in the fermenter to grow-up before continuous operation commenced.

4.2. The Medium

The medium was that used by Rosenberg (1), with slight modification (see Table 4.1). The pH of the inlet medium was about 5.0, to prevent any growth of the organism into the medium inlet tube. By having the inlet medium at a pH of 5.0, more NaOH was required to maintain the fermenter pH at 6.0.

Medium was prepared in quantities of 19 liters. All ingredients were mixed, then filter sterilized through a 0.2 micron Nuclepore filter membrane. The sterile medium was stored in a 20 liter carboy which also served as a reservoir for the fermenter. All mineral ingredients were reagent grade. The xylose was Sigma grade II ($\sim$ 99% pure).

The medium was not optimized, but was designed so that xylose alone was growth limiting. The medium was designed to be sufficient for aerobic growth on an initial xylose concentration of 20 gm/L (2).

4.3. The Fermenter

The fermenter was a 1.8 liter glass, jacketed fermenter. The

Table 4.1

Medium Ingredients

Ingredients per Liter of Medium

6.0 gm KH_2PO_4

5.0 gm NH_4SO_4

1 mgm Biotin

1 mgm Thiamine hydrochloride

10 ml Trace mineral solution

Trace Mineral Solution (per liter)

24 gm EDTA tetrasodium

20 gm $MgSO_4$

0.8 gm $ZnSO_4$

2.0 gm $CaCl_2 \cdot 2H_2O$

2.0 gm $MgSO_4$

2.0 gm $FeSO_4$

working volume was 1.0 liters. A magnetic stirring bar provided agitation with a mixing time of approximately 10 seconds. Warm water at 41°C, from a thermostatic bath, circulated through the jacket maintaining the temperature at 40 ± 0.3°C. A Horizon model 5997 automatic pH controller, monitoring an Ingold pH probe, and operating a Sigmamotor AL4E Kineticlamp pump (small head) maintained the pH at 6.05 ± 0.05. The neutralizing agent was 4N NaOH. The storage reservoir was a 20 liter carboy, stoppered with rubber. The gas over the medium was air and the carboy was vented to the air. All hoses were rubber with the exception of two: a short section of silicone in the pump, and a short section of silicone in the sampling line. All rigid tubing was glass. All frequently changed connections were ground glass ball-and-socket joints, lubricated with silicone grease. The feed pump was a Sigmamotor AL4E Kineticlamp pump (large head). The hose in the pump was a 1 mm ID heavy wall silicone hose. With that hose and the particular unit used, the pumping rate at 10% of maximum speed was 30 ml/hr. The pumping rate was linear with percent of maximum speed up to 80%. The deviation from linearity was not more than 5%. The outlet from the fermenter was a tube extending down through the headplate to the 1 liter level. By pumping out at a rate approximately twice the inlet rate, the level could be maintained at 1 liter. The outlet went into a sterile polyethylene carboy, which was emptied and sterilized periodically. To control foaming, an antifoam agent, General Electric AF-70, was added periodically from a reservoir in the headplate. The antifoam was sterilized with the fermenter. For sparging N_2 into the fermenter, a gas dispersion tube, Pyrex 12 EC, extended down through the headplate to

within 4 cm of the bottom. The incoming N_2 passed through a water saturator, through a glass wool filter, and into the fermenter. The N_2 was bottled gas from Matheson, with a purity higher than 99.999% and less than 2 ppm O_2. The outlet gas passed out of the fermenter through a chilled condenser. When not connected to the CO_2 analyzer, the gas passed from there through a water trap exit to the atmosphere. The headplate was equipped with a plugged hole through which inocula could be added. A tube extending through the jacket, near the bottom of the fermenter, allowed the withdrawal of samples. A short section of silicone hose connected the tube to a specially adapted screwcap fitting 16 mm culture tubes. The tube and hose held approximately 3mm. A pinch clamp held the hose shut when not sampling. A sterile culture tube was kept screwed into the special cap at all times.

4.4. Sampling

Samples were drawn into sterile 16 x 125 culture tubes. Two samples were drawn at each sampling. The first, 5 to 10 ml, cleared the tube and hose of stagnant fluid. The second, 10 to 15 ml, was used for the analyses.

The sample was divided into two portions. One, 8 to 10 ml, was centrifuged at 500 g for 10 minutes. Tbe supernatant was poured into a clean culture tube, acidified with four drops of concentrated HCl, tightly capped, and stored in the freezer. The other portion was used for optical density measurements, then discarded.

4.5. Optical Density Measurement

Optical density was determined at 650 nm, using 10 quartz curvettes, in a Beckman model 1098 spectrophotometer. Distilled water

was used as a blank. For most samples, dilution was required to keep the absorbance below 0.4 units.

To convert optical density measurements into cell density (dry weight) a graph of absorbance versus dry weight was prepared. An aliquot was drawn from the fermenter and centrifuged. The supernatant was discarded, and the cells resuspended in distilled water. The cells were centrifuged and resuspended in fresh distilled water. From the resuspended cells an aliquot was drawn. This was dried at 105°C to determine dry weight. The remainder was diluted to 10 different concentrations and the absorbances determined. The resulting graph was linear up to 0.3 gm dry wt/liter with a slope of 0.447 gm dry wt/liter/absorbance unit.

4:6. Sugar Assay

Sugar was assayed by the DNS (dinitrosalicylic acid) method. 0.5 ml of the supernatant or diluted supernatant, was placed in a test tube. To this 1.5 ml of DNS reagent (2) was added. The result-mix was vortexed, capped and boiled for 5 minutes. 10 ml of distilled water was added and the test tube shaken. The absorbance was measured on a Bausch and Lomb model 400 automatic spectrophotometer at 600 nm with distilled water as a blank. Absorbance was linear up to 2.5 gm/liter. Assays were done in batches of 8 to 18 samples. With each batch five standards were also run. All samples and standards were done in triplicate.

4:7 Ethanol, Acetone and Acetate Assay

Volatile products were measured by gas chromatography. An Aerograph model 1520 GC connected to a Varian CDC111 digital integrator

was used for nearly all the samples. The column was a 60 x 1/4 inch OD stainless steel tube filled with Porapak Q or Chromasorb 101. The column temperature was 170°C, and detection was by flame ionization. With a helium flow rate of about 50 ml/min, the emergence times for ethanol, acetone and acetate were 0.9, 2.3 and 4.5 minutes after the air peak, respectively. Acetate tended to tail, particularly for the first few injections of the day. To compensate for this tailing a half-dozen injections were made prior to each series of assays.

With each series of assays a standard containing all three compounds was also injected. The standards were done in quadriplicate, and the samples in triplicate. An average of the standard injections was compared with an average of the sample injections to determine the sample concentration. 3 microliters of standard or acidified supernatant (see section 4:4) were used per injection.

4:8. Carbon Dioxide Analysis

Carbon dioxide production was measured in two ways. CO_2 content in the outlet gas was measured with a Hartman and Braun model URAS2T infrared CO_2 analyzer. Its range of operation was 0 - 3% CO_2. Standardization was made with the bottled N_2 mentioned in section 4:3 above, and with 2.04% CO_2 standard gas from Matheson. Flow through the analyzer was about 250 cc/min. A refrigerated condenser at 4°C located in-line just before the analyzer kept the water vapor content low and constant.

CO_2 production, integrated over time, was obtained by bubbling the outlet gas through 1N NaOH, then titrating to pH 8.5 to determine CO_2 absorbed. The two methods were not used simultaneously.

4.9. Residual Carbon Assay

Residual carbon, i.e. carbon in non-volatile compounds, in the supernatant was analyzed. Samples were prepared by freeze-drying 5 ml aliquots of acidified supernatant (see section 4.4). The dried samples were weighed, then stored with dessicant. The results were reported as percent carbon in the dried sample.

4.10. Lactic and Formic Acids Assay

The initial samples showed negligibly small quantities of either acid so routine analyses were not performed.

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5. RESULTS AND DISCUSSION

5:1. Steady-State Continuous Culture of B. macerans

Rosenberg, et al. (1) and others (2,3) noted that acetone appeared near the end of a batch culture. Because the conditions in a steady-state culture in a CFSTF are similar to those near the end of a batch culture--low substrate, high product concentrations--it was postulated that acetone yield would be higher in a CFSTF. In addition neither Rhodes (4) nor Rosenberg, et al. (1) were able to account for all the carbon in the xylose consumed, i.e. some of the carbon was appearing as a product not identified by them.

Table 5.1 presents material balances for a batch culture and several steady-state continuous cultures at dilution rates of 0.03 and 0.06 hr^{-1}. In general, for a dilution rate of 0.03 hr^{-1}, the cell yield was lower, and acetate and ethanol yields somewhat higher than at a dilution rate of 0.06 hr^{-1}. Acetone yields in continuous cultures at steady-state were very low or zero. High acetone yields were only observed during transient growth.

The maximum theoretical yields for acetate and ethanol are 0.333 and 0.256 gm product/gm xylose consumed, respectively (see section 3:1). The observed yields were about 0.25 and 0.20 gm/gm, respectively. The shortfall in acetate and ethanol yields appears to be compensated by the some unknown, water-soluble, non-volatile organic compounds(s), labeled "Residual Carbon" in Table 5.1. This unknown compound(s) was found when samples of fermenter broth were analyzed by the residual carbon assay (see chapter 4). Early investigators (5) using another

Table 5.1

Material Balances

Dilution Rate (hr^{-1})	Inlet Xyl. Conc. (gm/L)	Cells* Yield (gm/gm)	Cells* %C** (%)	Ethanol Yield (gm/gm)	Ethanol %C (%)	Acetone Yield (gm/gm)	Acetone %C (%)	Acetate Yield (gm/gm)	Acetate %C (%)	CO_2 Yield (gm/gm)	CO_2 %C (%)	Residual Carbon*** (%)	Carbon Recovery (%)
0.03	11.2	0.026	3.2	0.194	25.2	0	0	0.228	22.8	0.47	32	5.2	88
0.03	11.4	0.022	2.8	0.201	26.0	0	0	0.230	23.0	0.44	30	6.2	88
0.03	11.4	0.023	2.9	0.228	29.7	0	0	0.272	27.2	0.53	36	4.8	101
0.03	11.2	0.028	3.5	0.198	25.8	0	0	0.225	22.5	0.46	31	5.1	88
0.03	11.6	0.023	2.9	0.216	28.1	0	0	0.297	29.7	0.47	32	4.8	98
0.06	11.6	0.031	3.9	0.194	25.2	0.001	0.1	0.259	25.9	0.41	28	5.3	88
0.06	10.2	0.033	4.2	0.198	25.8	0.010+	1.6	0.251	25.1	NM++	(33)++	7.0	97
Batch	10.7	0.061	7.6	0.215	27.9	0.012	1.9	0.183	18.3	0.47	32	10.2	98

*Cells assumed to be 50% carbon.

**%C is the yield of product expressed as the percent of the carbon in the xylose consumed appearing as product

***The "Residual Carbon" is the carbon contained in unknown non-volatile, water soluble organic compounds produced by B. macerans.

+The high acetone yield indicates the culture may not have been at steady-state.

++CO_2 was not measured, 33% is an estimate.

strain of B. macerans postulated that the organism made lactic acid, a compound which would be measured by the residual carbon assay were it present in the broth. However, several assays for lactic acid in the fermentation broth of the strain used here showed no lactic acid production. The CO_2 yield data confirm that little or no lactic acid is formed. If it is assumed that all the CO_2 is formed in the reaction

$$\text{Pyruvate} + \text{CoA} = \text{acetyl CoA} + CO_2 + H_2 \qquad (5:1)$$

and acetyl CoA is an intermediate in the formation of all products, i.e., cells, slime, ethanol, etc, then the percentage of carbon from xylose consumed appearing as carbon in CO_2 would be 33.3%. Within the experimental error of the measuring techniques and the accuracy of the assumptions made, this is the percentage found experimentally (6). Because lactic acid is formed directly from pyruvate the yield of CO_2 would have to be lower if lactic acid were made. If it were assumed that 33.3% of the carbon from xylose consumed appeared as carbon in CO_2, the recovery of carbon would be better than 90%.

Batch culture results show the same yields as those reported by Rosenberg, et al.(1) The ethanol and acetone yields of about 0.20 and 0.01 gm/gm, respectively, were the same as those reported by Rosenberg et al. Rosenberg, et al. did not report an acetate yield for fermentations with an initial xylose concentration of 10 gm/liter, but did report one for an initial concentration of 20 gm/liter. With an initial xylose concentration of 20 gm/liter the ethanol yield was about 0.02 gm/gm higher than the acetate yield. If the same difference existed for fermentations starting with 10 gm/liter of xylose, the acetate yield would be about 0.18 g/gm, the same as was observed here.

Compared to batch culture results, steady-state continuous culture results were very different. Instead of the acetate yield being lower and the acetone yield being higher, the opposite was found. In the batch culture results of Rosenberg, et al. and Rhodes, the acetate yield was was consistently lower than the ethanol yield. For continuous culture steady-state yields at dilution rates of 0.03 and 0.06 hr^{-1} the acetate yields were consistently higher than the ethanol yields. Cell yields were about 0.024 and 0.032 gm/gm, for dilution rates of 0.03 and 0.06 hr^{-1}, respectively, compared to 0.06 gm/gm for batch cultures. In all, for steady-state continuous cultures, the ethanol yields were about the same as for batch culture, while the acetate yield increased and the acetone, cell and residual carbon yields declined.

The number of experiments in which steady-state was attained was very limited because a steady-state was difficult to attain. The data shown in Table 5.1 may not represent true steady-states. Tables 5.2a,b illustrate the lack of constancy at what was deemed to be steady-state. The variations seem to show trends, but the variations are only slightly larger than the variance in the measuring procedures, making determination difficult.

A more extreme example of the difficulty in establishing steady-state is seen in Table 5.3. This shows qualitative of the condition of the culture during the eight days following start-up of continuous operation. The culture started as a batch until fully grown, at which time continuous operation began. The irregular cycling from normal cell density to very low cell density indicates that steady-state is unstable. From these observations it was decided that an investigation

Table 5.2a

Steady State at Dilution Rate 0.03 hr^{-1}

Inlet Xylose Concentration was 11.6 gm/L.

Time (hr)	Cell Yield (gm/gm)	Ethanol Yield (gm/gm)	Acetone Yield (gm/gm)	Acetate Yield (gm/gm)
0	0.025	0.209	0	0.252
2.25	0.024	0.214	0	0.247
17.0	0.023	0.216	0	0.284
18.5	0.024	0.214	0	0.266
22.0	0.023	0.216	0	0.289
24.5	0.023	0.216	0	0.297
30.0	0.023	0.206	0	0.267
40.75	0.024	0.184	0	0.273
42.75	0.024	0.197	0	0.265
Average	0.024* ±0.001	0.208 ±0.011	0	0.271 ±0.017

During the entire period xylose concentration was below the detectable limit -0.029 gm/L.

Table 5.2b

Steady-State at Dilution Rate 0.06 hr^{-1}
Inlet Xylose Concentration was 11.6 gm/L.

Time (hr)	Cell Yield (gm/gm)	Ethanol Yield (gm/gm)	Acetone Yield (gm/gm)	Acetate Yield (gm/gm)
0	0.031	0.184	0.001	0.266
2.25	0.031	0.194	0.001	0.259
6.5	0.031	0.197	0	0.245
8.0	0.032	0.208	0	0.268
24.25	0.031	0.214	0	0.261
28.5	0.031	0.201	0.001	0.241
30.5	0.029	0.224	0	0.241
48.0	0.031	0.207	0	0.234
Average	0.031 ±0.001	0.240 ±0.012	-	0.252 ±0.013

During the entire period xylose concentration was below the detectable limit -0.02 gm/L.

Table 5.3

Observations of Irregular Cycling

of *B. macerans* at a dilution rate of 0.06 hr^{-1}

Day	Conditions	
1	Normal Cell Density	continuous operation started with culture at normal density.
2	Very Low Cell Density	
3	Normal Cell Density	
4	Very Low Cell Density	
5	Very Low Cell Density	
6	Normal Cell Density	
7	Morning-Very Low/ Evening-Normal	
8	Normal Cell Density	
9	Normal Cell Density	

of transient behavior was necessary to understand the behavior of the organism under steady-state growth.

5:2. Transient Behavior

The unusual transient behavior of B macerans was first noticed during early attempts to establish steady-state conditions. It was noted that following some changes in dilution rate the culture would almost wash-out, but would recover rapidly. The realization that something was very unusual about B. macerans arose following the experiment shown in Fig. 5.1.

The experiment was designed to measure inhibition by acetate. For this purpose acetate was added to the inlet medium. The dilution rate, which had been held at 0.06 hr^{-1} for four days, was increased to 0.18 hr^{-1} as a step-change. Because the dilution rate was greater than the maximum specific growth rate reported by Rosenberg, et al., 0.15 hr^{-1}, wash-out was expected to take place. It was expected that once the xylose concentration rose, the specific growth rate would rise to the maximum possible with the concentration of acetate present. In spite of a forty-fold increase in xylose and a 60% decrease in acetate concentrations, the specific growth rate remained constant at 0.06 hr^{-1}. Five hours after the change in dilution rate, acetone appeared. At the sixth hour its concentration rose to a peak of 1.2 gm/L, then decreased to 0.1 gm/L by the eighth hour. When continuous operation was halted at the eighth hour, the cell density was 0.11 gm/L. The next day the cell density had only increased to 0.12 gm/L. The second day it was 0.14 gm/L. The third day it was still only 0.17 gm/L. On the fourth day cell density had increased to 0.40 gm/L and the xylose

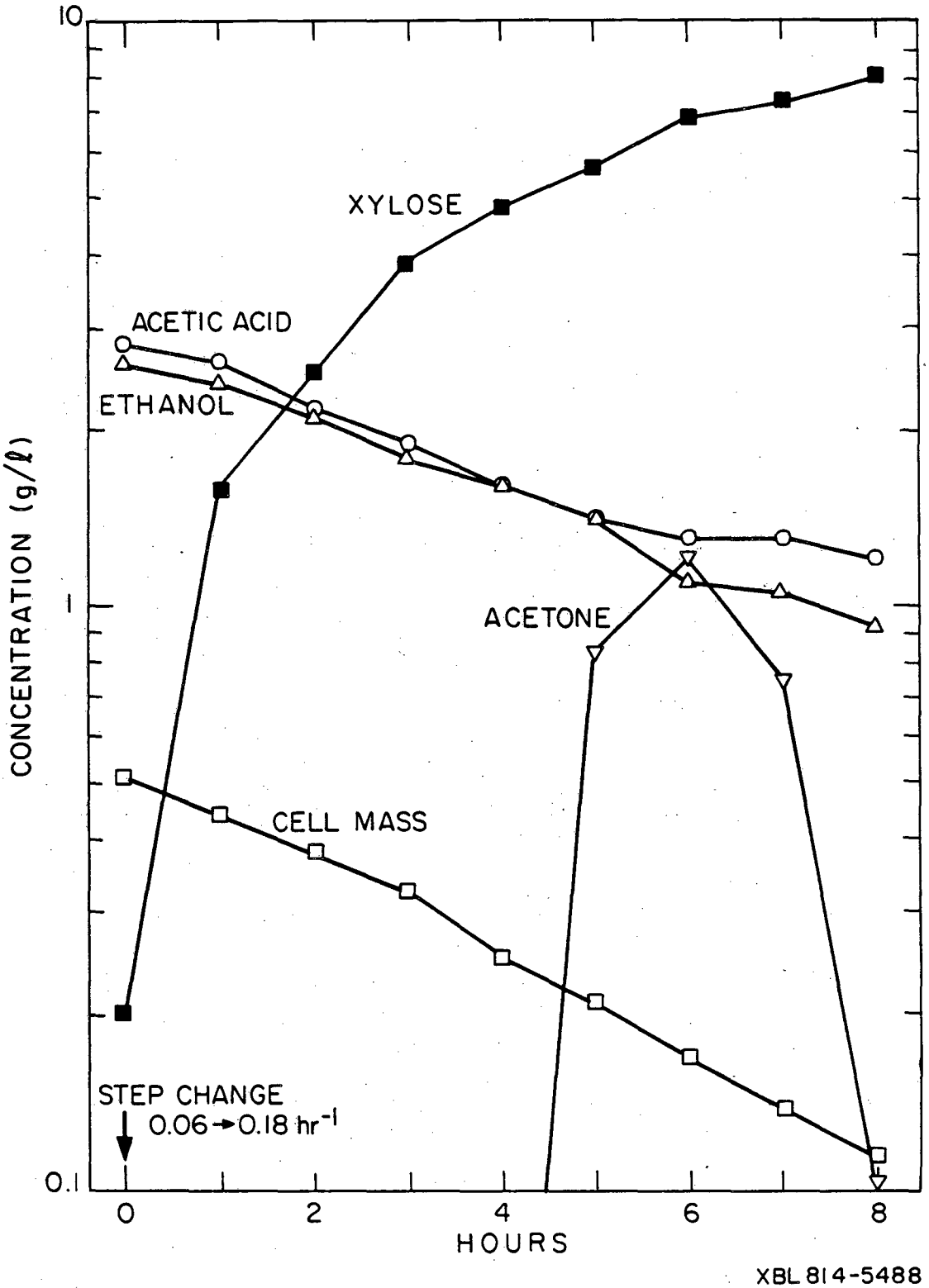
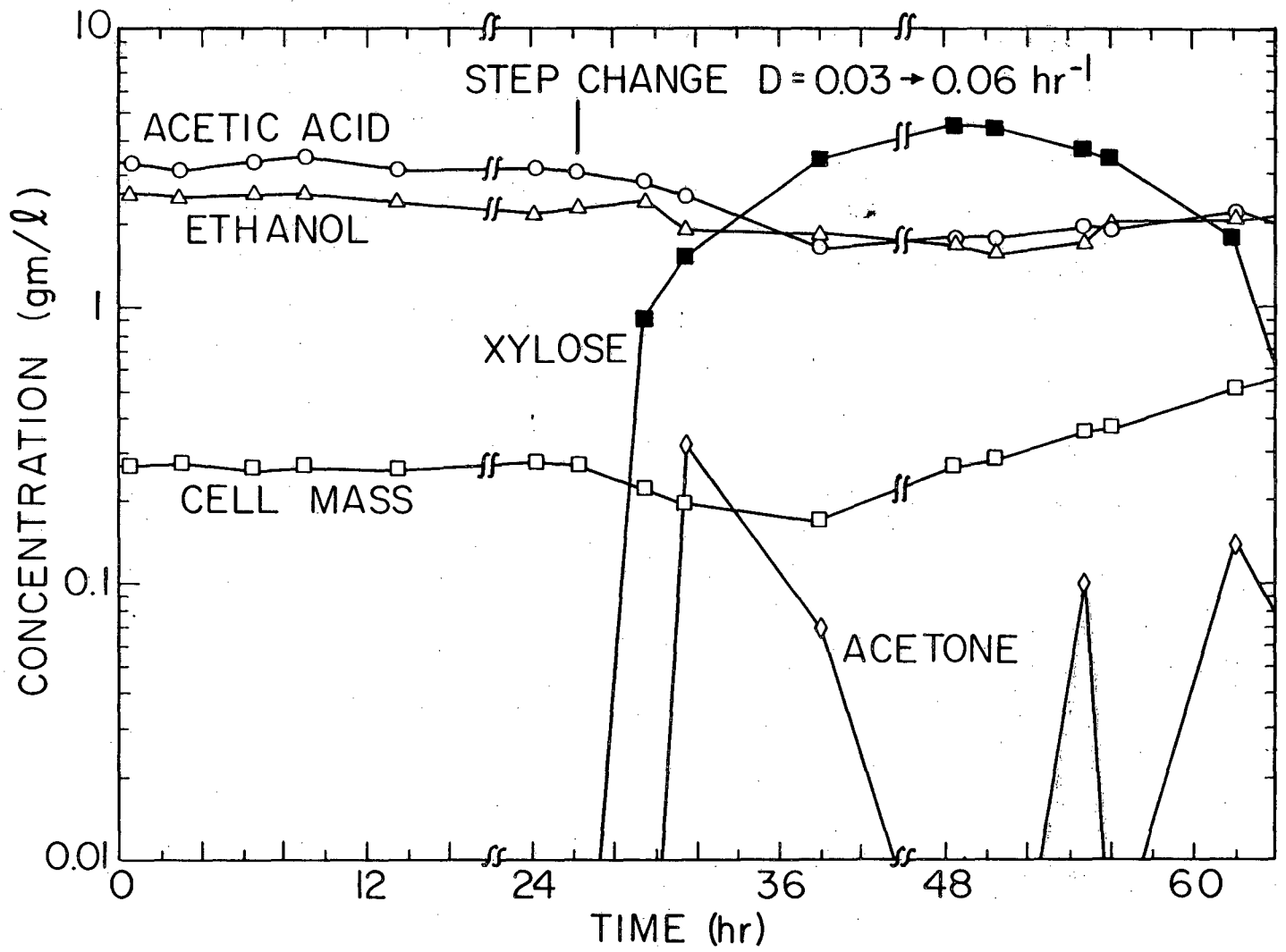


Figure 5.1 Conditions in a culture of B. macerans following a step-change in dilution rate from 0.06 to 0.18 hr^{-1}. Inlet xylose concentration was 11.4 gm/L, and inlet acetate concentration was 0.66 gm/L.

was fully consumed. This long delay in regrowing resembled the long lag phase observed by Rosenberg, et al. (1).

These observations led to experiments to examine how B. macerans would react to a step change to a higher dilution rate. Figure 5.2 is a typical example of the reaction to such a change. The dilution rate had been held constant for four and one-half days at 0.03 hr^{-1}. Conditions in the fermenter were reasonably steady at the time of the change. Immediately after the step-change to 0.06 hr^{-1}, the cell density began to decline. Early the next morning cell density started to recover. The cell density continued to increase until the xylose concentration went to zero. Cell density then declined to the new steady-state level.

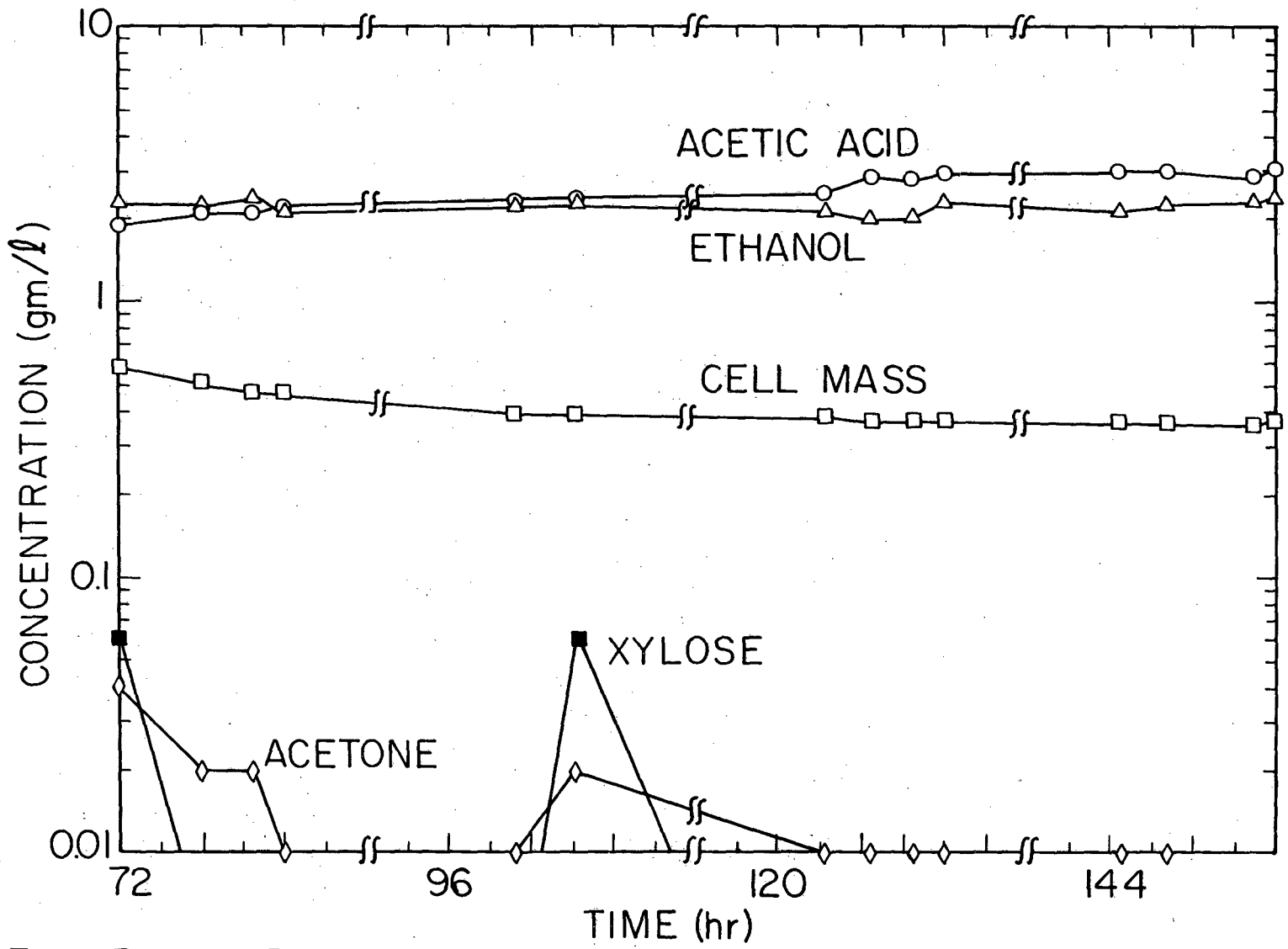
When the Hartman and Braun continuous CO_2 analyzer (see Chapter 4) was acquired it became possible to monitor continuously the metabolic rate of the organism. The first experiment in which continuous CO_2 monitoring was used was the one shown in Figure 5.2. Fifteen to twenty minutes after the step-change the rate of CO_2 production began declining, reaching a low point, about half the original rate, an hour later (see Figures 5, 3a, b). From there it increased again, slowly at first, then faster as the cell density recovered. It reached a peak at about the time the growth rate was a maximum, then declined to the new steady-state level. From the result it was reasoned that the condition triggering the sudden decline in metabolism must have been a sudden increase in xylose concentration. When an impulse of xylose was injected into the fermenter so that the xylose concentration rose to 0.5 gm/L, the same kind of transient was observed. It was also possible to produce the same transient with an impulse of glyceraldehyde or



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Figure 5.2 Conditions in a culture of B. macerans before and after a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 11.6 gm/L.



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Figure 5.2 continued

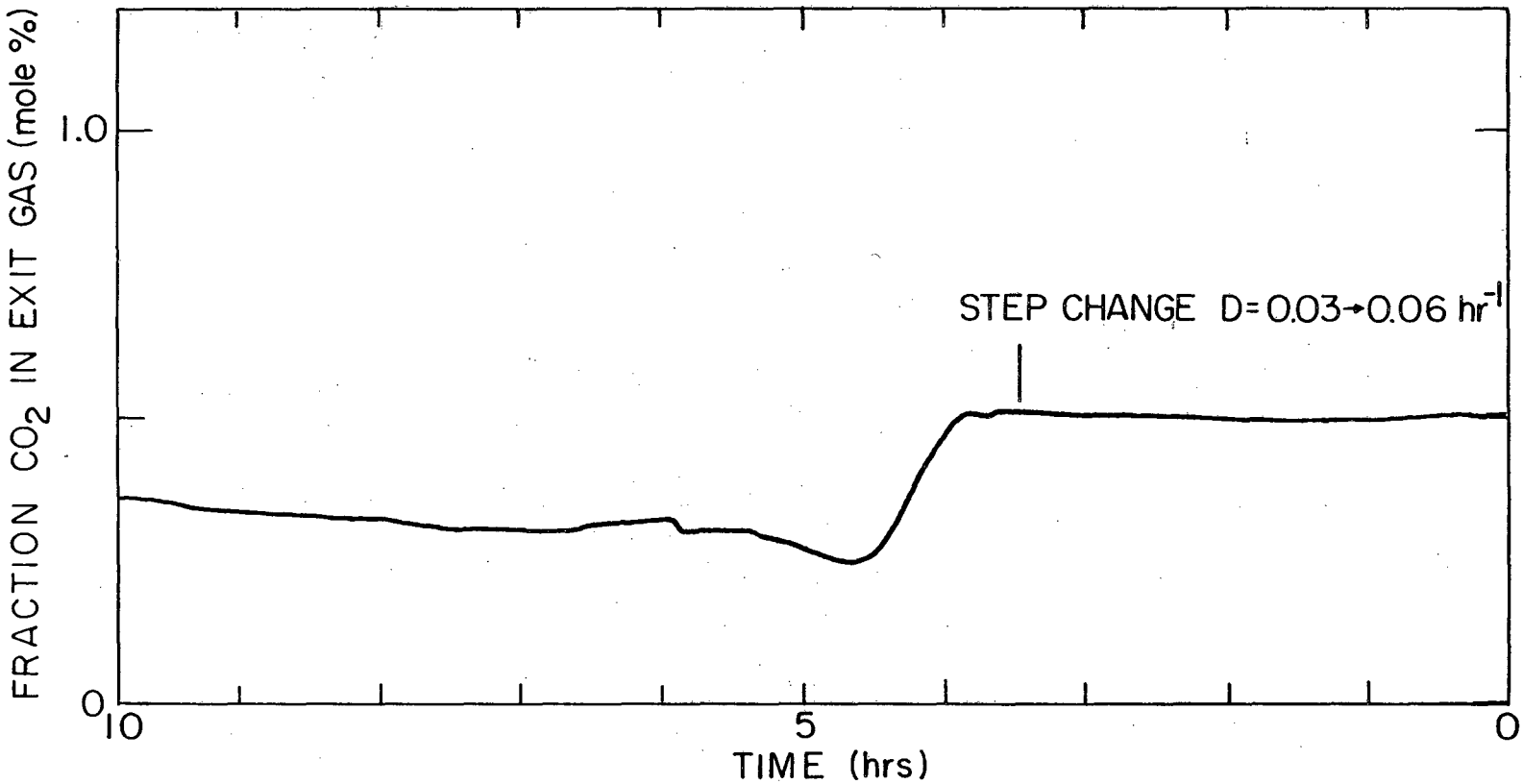


Figure 5.3a Strip chart trace of the fraction of CO_2 in exit gas from the culture in Figure 5.2 at the time of the step-change.

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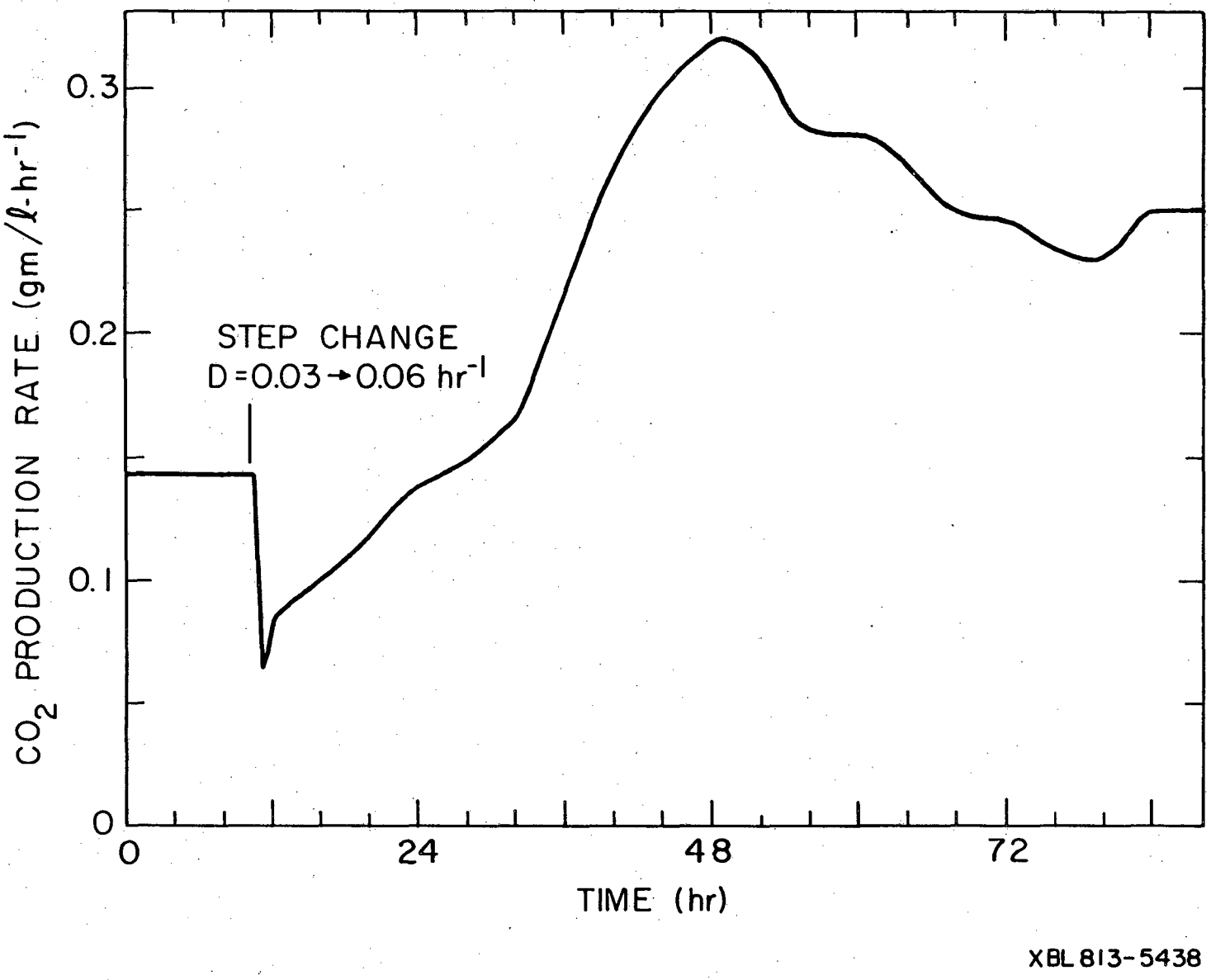


Figure 5.3b CO_2 production rate at the culture in Figure 5.2.

sedoheptulose. An impulse of erythrose or fructose produced a slight increase in metabolic rate. These sugars were chosen because their phosphate derivatives are intermediates in the pentose-phosphate pathway.

During the first five hours following the step-change in Figure 5.2, the specific growth rate was zero. For six other such transients the specific growth rate ranged from 0.06 to -0.02 hr^{-1} (see Table 5.4). A negative specific growth rate could be due to cell shrinkage or cell lysis. The length of the declining - and the rate of specific growth during recovery are also tabulated in Table 5.4. The length of the declining phase ranged from about 15 hours to about 90 hours. The specific growth rate during recovery ranged from 0.10 to 0.26hr^{-1}.

A specific growth rate of 0.26 hr^{-1} is 70% higher than the maximum specific growth rate observed by Rosenberg, et al. (1). An important difference between that and this, besides the mode of operation, was in sparging to remove CO_2 and H_2. Rosenberg, et al. only used N_2 to sweep air out of the fermenter headspace before starting a fermentation. In this work all experiments were performed with continuous sparging of N_2. That this sparging is necessary is illustrated by Table 5.5. Without sparging, at a dilution rate of 0.03 hr^{-1}, B. macerans could only consume 7.1 gm/L out of 22.5 gm/L of xylose available in the inlet medium, whereas with sparging at rates of 0.05 and 0.10 vessel volumes of gas per minute, the organism could completely consume all available xylose. The fermenter used by Rosenberg, et al. had a headspace of 0.8L. The fermentation of only two or three grams of xylose would produce enough gas to displace the nitrogen. By the time the growth rate had increased to about 0.15 hr^{-1}, two or three grams of xylose would have been consumed. Thus, 0.15 hr^{-1} is the maximum specific growth rate under CO_2/H_2

Table 5.4

Specific Growth Rates During Declining and Recovery Phases and Length of Declining Phases

Change	Specific Growth Rate During Decline (hr^{-1})	Specific Growth Rate During Recovery (hr^{-1})	Length of Declining Phase (hrs)
0.03-0.06	0.01	0.25	25
0.03-0.06	-0.02[b]	0.14	20
0.03-0.06	-0.02[b]	> 0.10[9]	15
0.03-0.06	0.00	0.11	20
0.03-0.09	< 0.04[a]	0.26	40
0.03-0.09	-0.02	0.26	40
Impulse	-0.02[b]	> 0.10[a]	25
Impulse	-0.01[b]	0.12	90
0.06-0.18	0.06	MM	110

[a]Samples were not taken during the critical part of the phase so specific growth rates must be estimated

[b]A negative growth rate could be due to cell lysis or cell shrinkage. Cell density was measured by optical density, which is sensitive to cell shrinkage.

Table 5.5

Effect of Sparging on Growth of B. macerans at 0.03 hr^{-1} dilution rate

Sparging Rate (Vessel Volume/Min)	Cell Mass (gm/L)	Inlet/Fermenter Substrate Conc. (gm/L)(gm/L)
0	0.29	22.5/15.4
0.05	0.62	20.6/0*
0.10	0.64	22.5/0*

*means below the detectable limit - 0.02 gm/L.

inhibition.

Another factor involved in specific growth rate is cell yield. At a specific growth rate of 0.03 hr^{-1}, the yield was about 0.025, with some yields as low as 0.022 gm/gm. When the specific growth rate was 0.26 hr^{-1} the cell yield exceeded 0.10 gm/gm. If the consumption of xylose is assumed to be controlled by a single rate limiting reaction, then the consumption rate can be expressed as

$$\text{Xylose} \xrightarrow{R} \text{Cells} + \text{Products} \qquad (5.2)$$

The rate of xylose consumption can be related to the yield and the growth rate by

$$R = \mu/Y \times \text{Cell Mass} \qquad (5.3)$$

where μ is the specific growth rate, and Y is the cell yield. Calculating xylose consumption rate for a specific growth rate of 0.03 hr^{-1} and cell yield of 0.022 gm/gm, the specific rate is 1.4 gm xyl/gm cell-hr, whereas the rate at a specific growth rate of 0.26 hr^{-1} and cell yield of 0.10 gm/gm, the rate is 2.6 gm xyl/gm cell-hr. Hence a doubling of metabolic rate produces a nine-fold increase in specific growth rate. In the transient in Figure 5.2 the metabolic rate, i.e. rate of CO_2 production, fell to half what it has been before the change. If a halving of metabolic rate produces a nine-fold decline in specific growth rate then the specific growth rate would be expected to be 0.003 hr^{-1}, a value indistinguishable from zero. Zero was the value found.

Judging by the work of Riba (8) (see section 2.2) inhibition by acetone or xylose would not be a factor in the transient behavior of

B. macerans. The thresholds of inhibition for these compounds are 2.5 40 gm/L, respectively - far above the concentrations present. Ethanol inhibition may have played a role, but only a very insignificant one. The maximum concentrations of ethanol in any of these transients was just over 2 gm/L, the threshold was between 1 and 2 gm/L. Acetate, on the other hand, may have played a large role, but pH was a key factor. Riba found the threshold of inhibition to be at an acetate concentration of approximately 0.5 gm/L. Nearly all acetate concentrations were above this threshold concentration. During the periods when the specific growth rate exceeded 0.20 hr^{-1} the acetate concentration was above the threshold. The explanation for this apparent discrepency is that the relevant concentration is not total acetate, but only the concentration of the acid form, acetic acid. This has been found to be true with other organisms (7). Though the pH outside the cell was controlled at pH 6.05 ± 0.05, the pH inside the cell could vary depending upon the pH gradient the cell was capable of maintaining (see section 2:B). Because the inside of the cell is alkaline with respect to the outside, the greater the pH gradient, the higher the interior pH, and thus the lower the acetic acid concentration - for a given total acetate concentration. Thus, for a cell whose metabolic rate is decreasing, which therefore can only maintain a progressively smaller pH gradient, inhibition by acetate becomes progressively greater, even if total acetate hardly changes. Thus, when a small perturbation causes either the metabolism to slow or the interior pH to fall, a cascade can be triggered which results in a large disruption in metabolism.

At steady-state the rate at which the cell exports hydrogen ions

just matches the rate at which the cell imports them. If the external xylose concentration rises slightly, the rate of import rises as the rate of xylose transport increases (see section 2.2). The rate of export does not rise instantaneously, so the interior pH falls. If the acetate concentration is in the right range, the decline in interior pH increases the acetic acid inhibition, slowing the metabolic rate, which causes the pH to decline further, which increases the acetic acid inhibition, this cycle is repeated until a large disruption in metabolism has occurred. With the sensitivity of the specific growth rate of B. macerans to small changes in metabolic rate, even a small disruption will produce the growth stoppage observed in the transients.

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6. COMPUTER SIMULATION OF TRANSIENT BEHAVIOR

6.1. The Model

In section 5.2 it was proposed that the reason B. macerans exhibited unusual transient behavior was because of an interaction between the magnitude of the transmembrane pH gradient, the acetic acid concentration and the metabolic rate. To determine the plausibility of this proposal, a mathematical model was constructed. The model represents B. macerans fermenting xylose in a continuous-flow stirred-tank fermenter (CFSTF).

The fermenter was modeled as a CFSTF, from which the cells consumed xylose at a rate RT and excreted acetate at a rate such that the acetate concentrations inside and outside the cell were equal. The cells were assumed to occupy no significant volume in the fermenter. This assumption is reasonably accurate as the volumetric cell concentration was less than 1%.

The metabolism of the cell was represented by two principle reaction steps. In the first step, xylose inside the cell was transformed into an intermediate metabolite at the rate RAA. Concomitant with this step was an ATP to ADP reaction, representing the phosphorylation steps in the pentose-phosphate pathway. The second step, with a reaction rate RA, represents the conversion of intermediate metabolites to fermentation products. The only product of interest was acetate, so it was the only one modeled. The other products were assumed to have had no effect on cellular behavior. The yield of acetate was assumed to be constant under all conditions. Concomitant with the second reaction step was the production of ATP and ADP.

The ATP produced was accumulated in a pool. ATP was expended in

three ways; for phosphorylation, for hydrogen ion pumping (see section 3.2), and for replication and maintenance of cells. The rate at which it was used for phosphorylation was governed by a fixed relationship with RAA. The rate at which ATP was used for hydrogen ion pumping was termed RB. The magnitude of RB was a function of the size of the ATP pool and the magnitude of the transmembrane pH gradient. The rate at which ATP was used for cell replication and maintenance, RC, was proportional to the size of the ATP pool. Cell replication was assumed to be unaffected by the magnitude of RA, i.e. the rate at which the materials for replication was produced.

The re-entry of hydrogen ions into the cells occurred by two mechanisms. One route was the transport of xylose, where the transport was accompanied by the import of hydrogen ions (see section 3.2). The other route represented all other means of entry for hydrogen ions, including leaks, ion transport, and flagella rotation (1). This route was assumed to be a function of the proton motive force (see section 3.2).

RT, the rate of xylose transport into the cell, was given the expression,

$$RT = RTP \left(\frac{E(1)}{E(1)-QS}\right) [2DH-\log 10\ (E(3)/E(1))] \frac{QI}{QI+E(5)} \qquad (6.1)$$

see Table 6.1 for the definitions of symbols). The first two terms, RTP and $\frac{E(1)}{E(1)+QS}$ are the maximum reaction velocity and the effect of substrate concentration on reaction velocity, respectively. The last term $\frac{QI}{QI+E(5)}$, is a term for noncompetitive inhibition by the intermediate metabolite. The regulation of substrate transport is claimed to be accomplished by feedback inhibition from an intermediate metabolite

Table 6.1

Definitions of Symbols

Symbol	Definition
AA, AAM*	Acetic acid concentration.
COP	The magnitude of the proton motive force below which ATP cannot be made by the transmembrane ATP ase.
D	Dilution rate.
DF, DFP	The hydrogen ion "leakage" rate and its maximum rate, respectively. "Leakage" represents all other ways by which hydrogen ions enter the cell besides concurrently with xylose transport.
DH, DHM*	Transmembrane pH gradient.
DLT	Integration step size.
EPP	Cell maintenance ATP requirement.
H, HM*	Internal hydrogen ion concentration.
MU, MUM*	Specific growth rate.
QA, QB	Ka's for acid groups on the enzyme involved in the reaction represented by Equation 6.3.
QAA	Ka for acetic acid.
QC	A proportionality constant in the expression for RC.
QFA, QRA	First-order forward and reverse reaction rate constants in the expression for RAA.
QI, QII	Inhibition constants in theexpressions for RT and RA, respectively.
QP	A Michaelis-Menten-like constant in the rate expression for DF.
QPH	A proportionality constant relating the net flux of hydrogen ions into the cell to the change in internal pH.
Y	ATP requirement for cell replication.

Table 6.1 continued

Symbol	Definition
RA, RAM*	The rate of conversion of intermediate metabolite to fermentation products.
RAP	The maximum reaction velocity constant in the expression for RA.
RAA, RAAM*	The rate of conversion of xylose inside cell to intermediate metabolite.
RB	The rate at which ATP is expended to pump hydrogen ions out of the cell.
RC, RCM*	The rate at which ATP is expended for cell replication and maintenance.
RT, RTM*	The rate of xylose transport into the cell.
RTP	The maximum reaction velocity constant in the expression for RT.
SO	The inlet xylose concentration.
Y	Acetic acid yield.
YXS, YXSM*	Cell yield.
Z	A proportionality constant in the rate expression for RB.
E(1-7)*	The seven variables modeled.
E(1)	External xylose
E(2)	Cell density
E(3)	Internal xylose concentration.
E(4)	Internal pH
E(5)	Intermediate metabolite concentration.
E(6)	ATP concentration
E(7)	External acetate concentration
EM(1-7)*	
F(1-7)	The values of the seven differential equations.

*When used in calculating constants and initial conditions no postscript indicates the value is for steady-state growth at a dilution rate of 0.03 hr^{-1} and M postscript indicates the value is for steady-state growth at a dilution rate of 0.26 hr^{-1}.

derived from the regulated substrate (2). The remaining term is an expression for the electrochemical potential available for driving xylose transport. DH represents the magnitude of the pH gradient. The gradient is multiplied by two to represent the total proton motive force. It was assumed that the pH gradient accounted for half the proton motive force under all conditions. $Log_{10}(E(3)/E(1))$ represents the chemical potential of the xylose gradient across the cell membrane.

The rate of conversion of xylose to intermediate metabolite was given the form

$$RAA = QFA \times E(3) - QRA \times E(5) \qquad (6.2)$$

The simple first-order equilibrium kinetics were chosen for simplicity of calculation. The rate of the ATP consuming reaction concomitant with RAA was fixed at 5/3 moles ATP per mole of xylose converted.

The expression for the rate of conversion of intermediate metabolite to products was

$$RA = RAP \left(\frac{E(5)}{E(5)+QSI}\right)\left(\frac{QII}{QII+AA}\right)\left(\frac{QA}{QA+H}\right)\left(\frac{H}{H+QB}\right) \qquad (6.3)$$

Once again the first two terms are the maximum reaction velocity and the effect of substrate concentration on reaction velocity, respectively. The third term represents noncompetitive inhibition by acetic acid. The acetic acid concentration was calculated by multiplying the total acetate concentration by the fraction of acetate in the protonated form. Thus

$$AA = E(7)\left(\frac{H}{H+QAA}\right) \qquad (6.4)$$

The last two terms represent pH effects on the enzyme involved in the

the reaction represented by Equation 6.3. The first is for inactivation of the enzyme by protonation of the active form. The second is for inactivation by deprotonation of the active form. pQA and pQB were selected to 6.0 and 9.0, respectively, based on the shape of the curve in Figure 2.1.

The rate of production of ATP was assumed to be 10/3 moles ATP/mole xylose equivalent consumed in the reaction represented by the Equation 6.3. This assumption ignores the ATP produced when acetate is produced from acetyl CoA (see section 3.1). Calculations with ratio of 12.5/3 mole/mole, the rate achieved with the maximum theoretical acetate yield, showed only a higher hydrogen ion leakage rate, DF, and a higher cell maintenance requirement.

RB and RC, the rates at which ATP was expended for hydrogen ion pumping, and cell maintenance and replication, respectively, were assumed to be proportional to the ATP concentration. Thus,

$$RB = Z \times E(6)\ [COP - E(4)] \tag{6.5}$$

and

$$RC = QC \times E(6) \tag{6.6}$$

Z and QC being proportionality constants. The term, (COP-E(4)), is a term representing the magnitude of the proton motive force. COP represents the magnitude of the proton motive force below which ATP cannot be produced by the transmembrane ATPase/hydrogen ion pump (see section 3.2). Since an organism performing a fermentation maintains the proton motive force by expending ATP (3), the organism would not be expected to maintain the force above COP. COP has been observed to be about 187 mV in Streptococcus lactis (4). 187 mV translates to 1.6 pH units

using the assumption of pH being half the potential. For simplicity of calculation, in this model the external pH was included in COP so that COP had the value 7.6.

The form of the rate expression for DF was chosen to be

$$DF = DFP \frac{DH}{DH + QP} \tag{6.7}$$

This form was chosen because it was assumed that when the proton motive force (expressed as the pH gradient) is in its normal range, > 1.0 pH units, DP is fairly insensitive to changes in gradient - only increasing when the gradient increases because the leaks become larger. When the gradient is small, DF is sensitive to changes in gradient because the organism ceases some of its activities, e.g. flagella rotation, as the gradient becomes smaller.

The model used seven differential equations to simulate the internal and external conditions of the organism. The variables thus modeled were the external xylose concentration, the cell density, the internal xylose concentration, the internal pH, the internal concentration of intermediate metabolite, the internal ATP concentration, and the external acetate concentration. The rate of change of external xylose concentration was expressed as

$$\frac{d\,E(1)}{dt} = F(1) = D[SO - E(1)] - RT \times E(2) \tag{6.8}$$

D(SO-E(1)) is the difference between the amount of xylose flowing into the fermenter in the continuous feed and the amount leaving by the continuous overflow. RT x E(2) is the amount of xylose transported into the cells.

The rate of change in the cell density was represented by

$$\frac{dE(2)}{dt} = F(2) \times [QY(RC-EPP) - D] \tag{6.9}$$

E(2) x QY(RC-EPP) represents the rate at which cells are replicated (RCC-EPP) is the amount of ATP available for new cell production. E(2) x D is the rate at which cells are removed from the fermenter in the continuous overflow.

The rates of change in internal xylose and intermediate metabolite concentrations were expressed as

$$\frac{dE(3)}{dt} = F(3) = RT - RAA \tag{6.10}$$

and

$$\frac{dE(4)}{dt} = F(5) = RAA - RA \tag{6.11}$$

respectively. These are the differences between the rates at which they appear and the rates at which they are consumed.

The equation for the rate of change in internal pH was

$$\frac{dE(5)}{dt} = F(4) = QPH\ [2RB - (RT + DF)] \tag{6.12}$$

2RB is the rate at which hydrogen ions are pumped out with the expenditure of ATP (see section 3:2). RT + DF is the rate at which hydrogen ions re-enter the cell. QPH is a proportionality constant relating net hydrogen ion flux to rate of change in internal pH. Assuming the net flux is proportional to the rate of change in pH means the cell is assumed to be buffered at infinitely many pK's over the range of pH's of interest, but only a limited amount of buffering is available at each pK.

The rate at which the size of the pool of ATP changes was expressed as

$$\frac{dE(6)}{dt} = F(6) = \frac{10}{3} RA - (RB + RC + \frac{5}{3} RAA) \quad (6.13)$$

which is the rate which ATP is made less the rates at which it is expended.

The rate of change in external acetate concentration was expressed as

$$\frac{dE(7)}{dt} \quad F(7) = Y \times RA \times E(2) - D \times E(7) \quad (6.14)$$

This is the rate at which it is produced less the rate at which it is removed in the continuous overflow. It was assumed that the yield of acetate was a constant 0.25 gm/gm, under all conditions. To set the 21 constants and 7 initial conditions so that specific growth rates of 0.03 and 0.26 hr^{-1} was achievable, conditions observed at these two growth rates were used. It was assumed that steady-state could be attained at these two growth rates. With this assumption the reaction rates, RT, RAA, and RA, were set equal to each other and given by

$$RA = MU/Y \quad (6.15)$$

The yields were assumed to be 0.10 and 0.022 gm dry wt./gm xylose consumed for the specific growth rates 0.26 and 0.03 hr^{-1}, respectively. The cell yields and specific growth rates were assumed to be determined by observation and therefore not subject to arbitrary adjustment. Other constants assumed to be determined by observation were QAA, QA, QB, COP and Y. For E(1), EM(1) and SO the range of possible values was limited by observation. E(1) was observed to be less than 0.02 gm/L, EM(1) was observed to be about 8.0 gm/L. The inlet xylose concentration, SO, in the experiments on transient behavior had been between 10 and 13 gm/L. It was found that by arbitrarily choosing values for QP, QI, QII, QPH and QFA as well as E(1), E(4), EM(1), EM(3), EM(4), Em(5), and EM(6), the

values for QC, Z, DFP, EPP, QY, E(2), E(3), E(5), E(6), E(7) could be calculated.

The first four values to be calculated were for E(6), Z, QC and DFP. Four simultaneous equations involving these for values could be written. They were

$$\frac{5}{3} RA = E(6)[Z(COP - E(4)) + QC] \tag{6.16}$$

$$2Z \times E(6)[COP - E(4)] = RT + DFP \frac{DH}{DH + QP} \tag{6.17}$$

$$\frac{5}{3} RAM = EM(6)\ [Z(COP - EM(4) + QC \tag{6.18}$$

$$2Z \times EM(6)[COP - EM(4)] = RTM + \frac{DHM}{DHM + QP} \tag{6.19}$$

Equations 6.16 and 6.18 are ATP balances, and equations 6.17 and 6.19 are hydrogen ion flux balances. These equations were rearranged to

$$E(6) = 1/2\left[RT + DFP \frac{DH}{DH + QP}\right] /[Z(COP-E(4))] \tag{6.20}$$

$$Z = \left(\frac{5}{3} \frac{RA}{E(6)} - QC\right)/[COP-E(4)] \tag{6.21}$$

$$QC = \frac{5}{3} \frac{RAM}{EM(6)} - Z(COP - EM(4)] \tag{6.22}$$

$$DFP = [2Z \times EM(6) \times (COP - EM(4)) - RTM \frac{QD + DHM}{DHM} \tag{6.23}$$

An iterative method was used to solve these equations.

With values for E(6) and QC, RC and RCM could be calculated. Because steady-states were assumed.

$$MUM = QY(RCM - EPP) \tag{6.24}$$

and

$$MU = QY(RC - EPP) \tag{6.25}$$

By rearranging, EPP and QY could be calculated by

$$EPP = \frac{RCM \times MU - RC \times MUM}{MUM - MU} \tag{6.26}$$

and

$$QY = \frac{MUM}{RCM + EPP} \tag{6.27}$$

To proceed further required the calculation of E(2), E(7), Em(7) and QSI. These were calculated from

$$E(2) = SO - E(1)\ YXS \tag{6.28}$$

$$E(7) = (SO - E(1))\ Y \tag{6.29}$$

$$EM(7) = (SO - EM(1))Y \tag{6.30}$$

$$QSI = \frac{EM(5)}{0.95} - EM(5) \tag{6.31}$$

QSI was chosen such that $\frac{EM(5)}{EM(5) - QSI} = 0.95$, i.e., RA was 95% of the maximum possible rate under the given conditions of pH and acetic acid concentration.

By using the rearranged expression for RAM,

$$RPA = RAM \left(\frac{HM + QB}{HM}\right)\left(\frac{HM + QA}{QA}\right)\left(\frac{QSI + EM(5)}{EM(5)}\right)\left(\frac{QII + AA}{QII}\right) \tag{6.32}$$

RAP was calculated. Using RA and RAP, EI(5) was calculated by

$$E(5) = RA \times QSI/\left[RAP \left(\frac{QA}{QA + H}\right) \left(\frac{H}{QB + H}\right)\left(\frac{QII}{QII + AA}\right) - RA\right] \tag{6.33}$$

To calculate QRA, the expression

$$QRA = [QFA \times EM(3) - RAM]/EM(5) \tag{6.34}$$

was used. E(3) was then calculated with

$$E(3) = \{RAA + (QRA \times E1(5))]/QFA \tag{6.35}$$

The final two constants to be evaluated were ATP and QS. The two simultaneous equations

$$RT = RTP \left(\frac{E(1)}{E(1) + QS}\right)\left(\frac{QS}{E(5) + QI}\right)[2OH - Log\ 10(EI(3)/EI(1))] \tag{6.36}$$

$$RTM = RTP \left(\frac{EM(1)}{EM(1) + QS}\right)\left(\frac{QI}{EM(5) + QI}\right)[2DHM - Log10(EM(3)/EM(1))] \tag{6.37}$$

were combined and rearranged to yield

$$QS = \frac{E(1) \times TERMA - EM(1) \times TERMB}{TERMB - TERMA} \tag{6.38}$$

where TERMA and TERMB are given by

$$TERMA = RT[2DHM - Log10(EM(3)/EM(1))]\ EM(1)[E(5)+QS] \tag{6.39}$$

and

$$TERMB = RTM[2DH - Log10(E(3)/E(1))]\ E(1)[EM(5)+QI] \tag{6.40}$$

respectively, and

$$RTP = RT\ \frac{E(1) + QS}{E(1)}\ \frac{E(5) + QI}{QI}/[2DH - Log10(E(3)/E(1))] \tag{6.41}$$

6.2. Results of the Simulation

The model was used to simulate the transient behavior induced by a step-change in dilution rate from 0.03 to 0.6 hr^{-1}. The first step in simulating a transient was to select and evaluate the constants and initial conditions by the methods described in section 6.1. A set of arbitrarily chosen constants and conditions, which produce the desired results, was found by trial and error. The initial conditions simulated the steady-state conditions at a dilution rate of 0.03 hr^{-1}, so changing the dilution rate to 0.06 hr^{-1}, after the initial conditions has been evaluated, simulated the step-changes performed in the experiments.

The results of a simulation are shown in Figures 6.1a and b and c. These figures show the changes in xylose concentration, cell density and total metabolic rate, i.e., the product of the specific metabolic rate and cell density, respectively. The total metabolic rate could be monitored as the rate of CO_2 production, assuming the yield of CO_2 was a constant. For comparison, Figures 6.2 a, b and c show the changes in xylose concentration, cell density and rate of CO_2 production measured experimentally. Up to the point where the xylose concentration

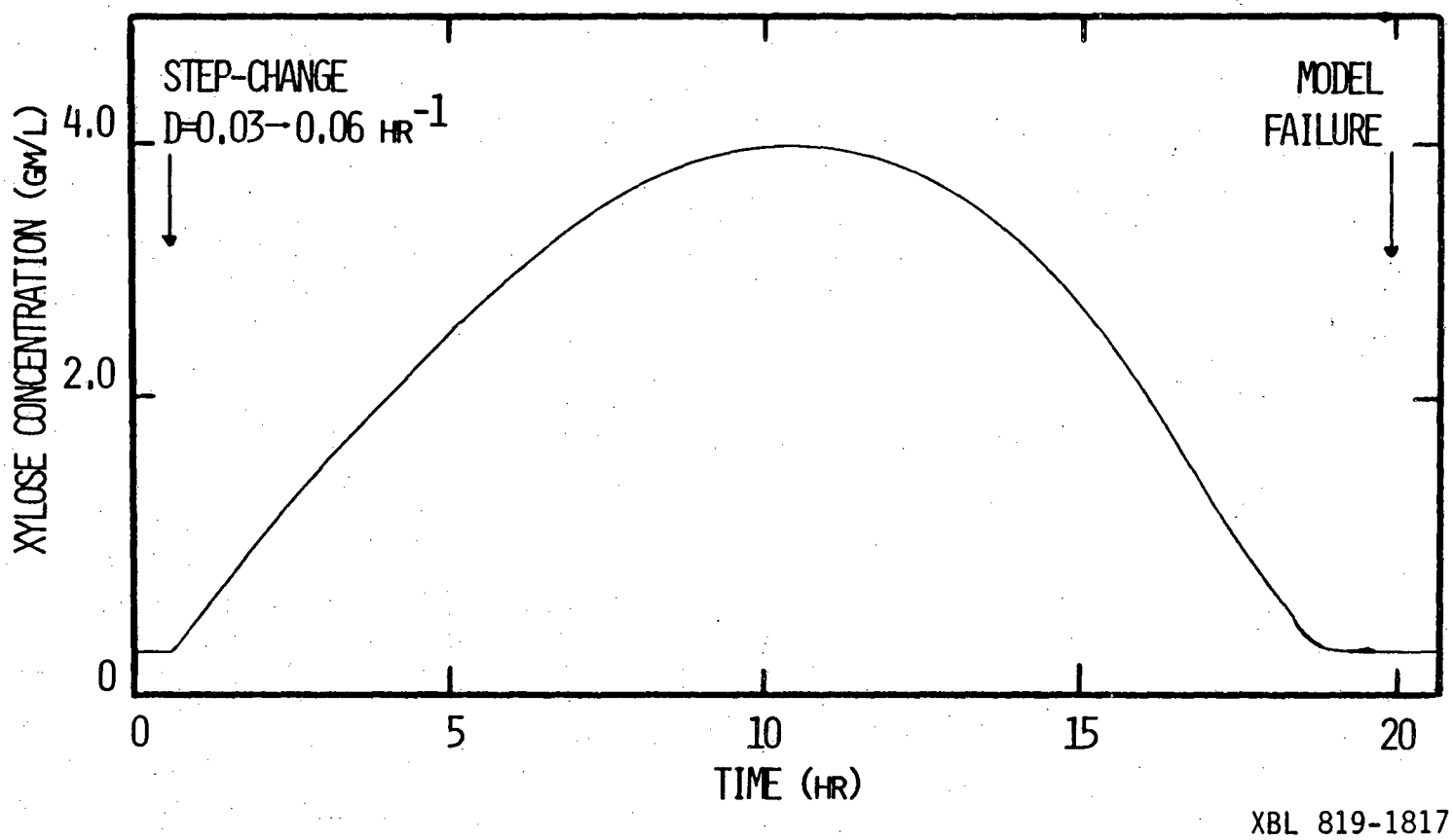
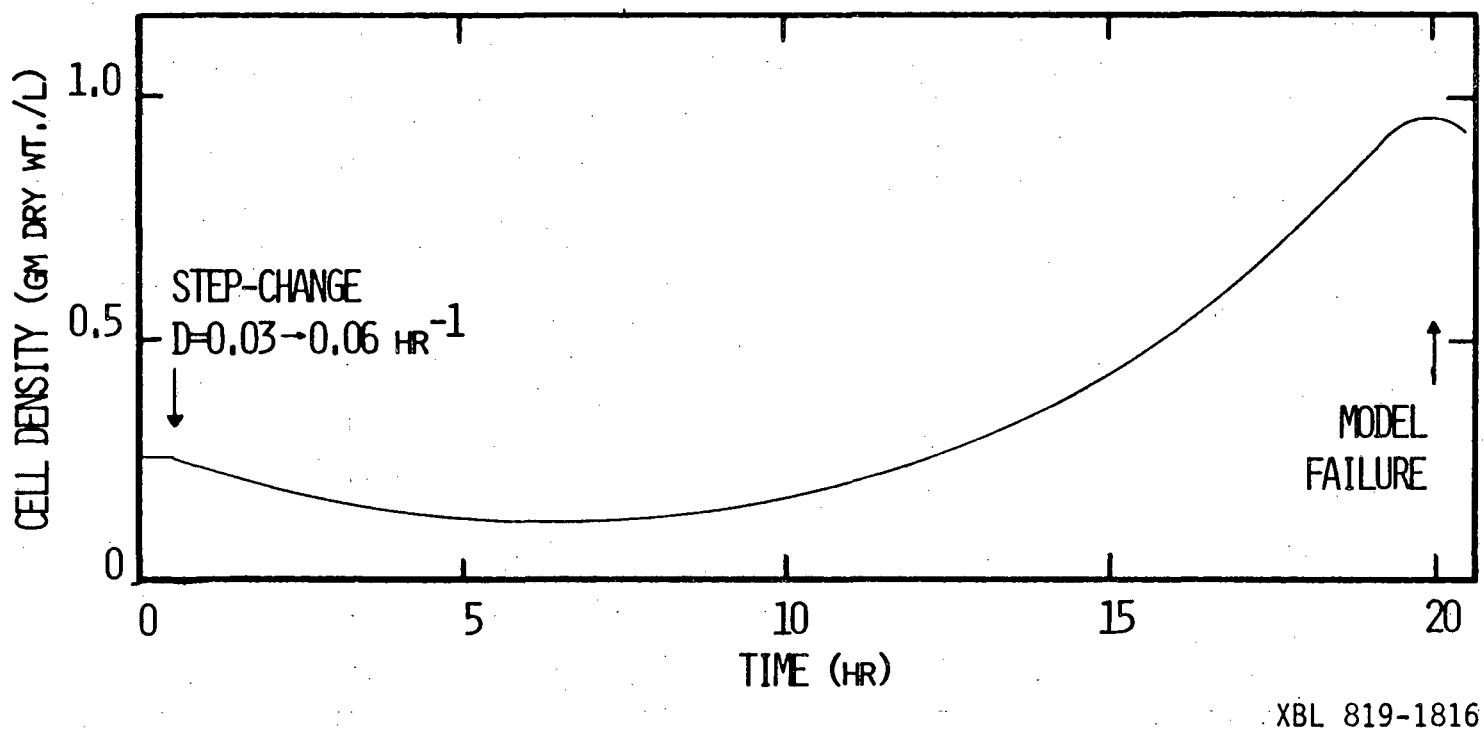


Figure 6.1a Computer simulation of xylose concentration during a transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 12.0 gm/L.



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Figure 6.1b Computer simulation of cell density during a transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 12.0 gm/L.

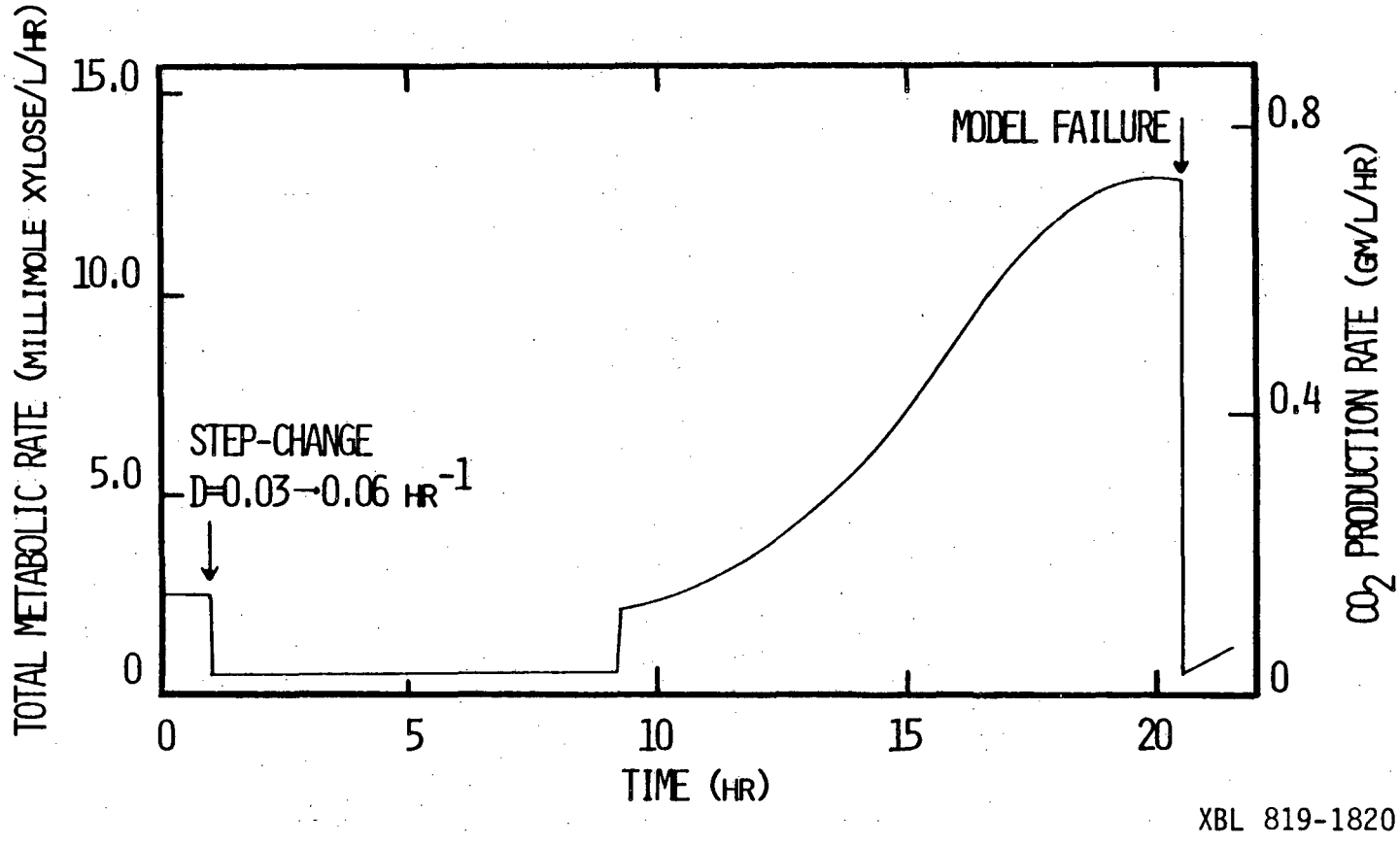


Figure 6.1c Computer simulation of the total metabolic rate change transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 12.0 gm/L.

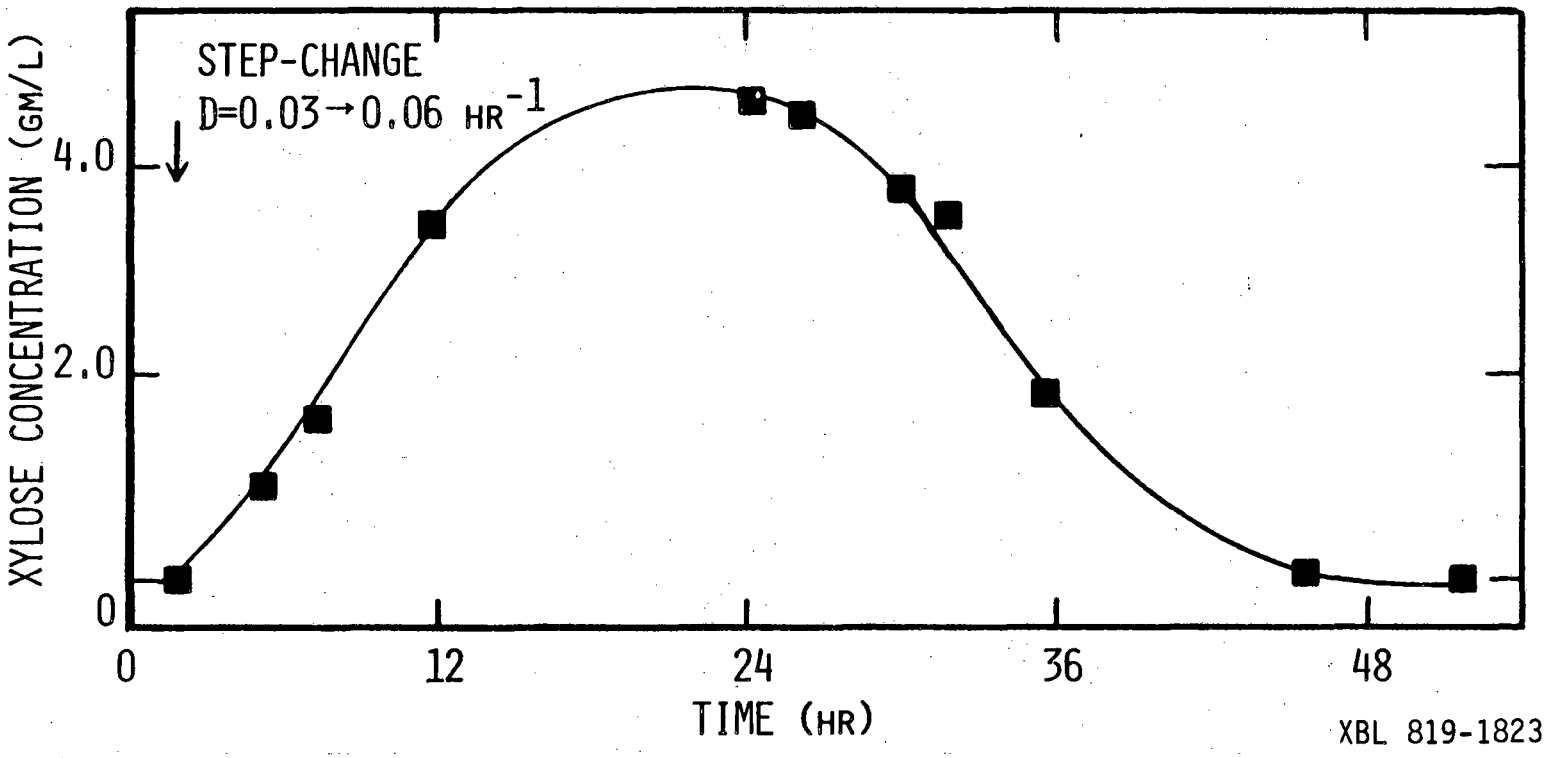


Figure 6.2a Xylose concentration during a transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 11.6 gm/L.

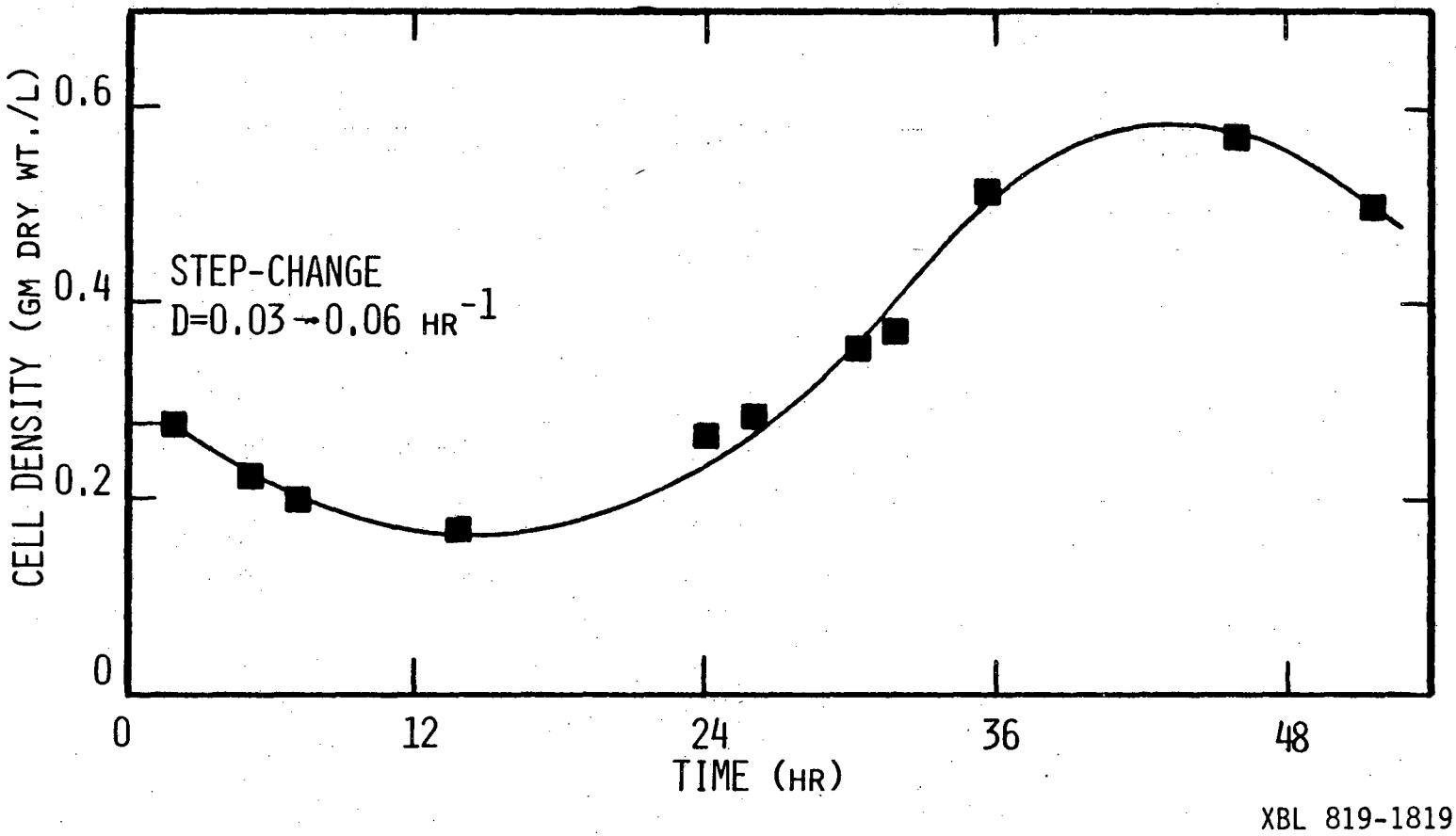


Figure 6.2b Cell density during a transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 11.6 gm/L.

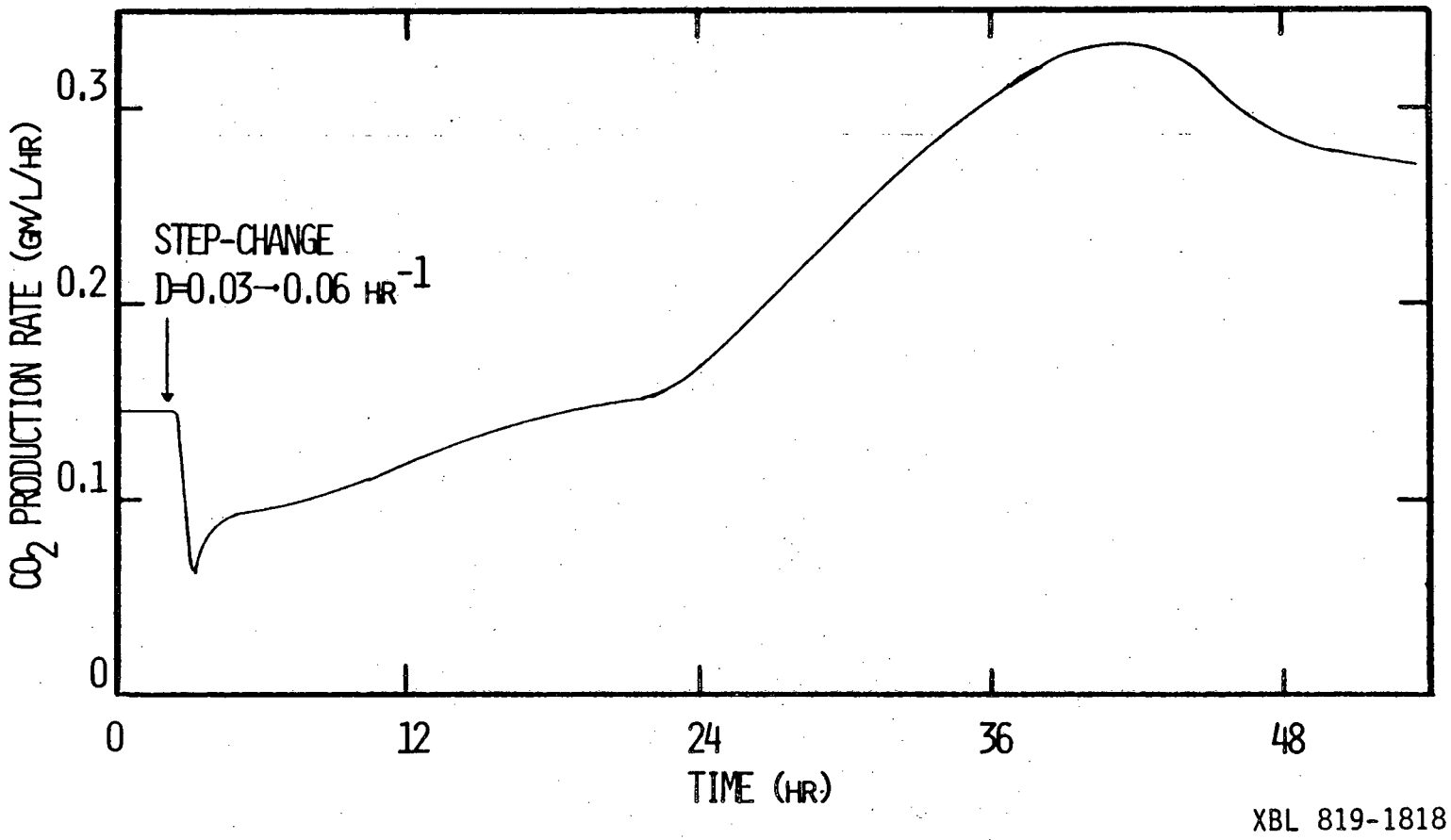


Figure 6.2c CO_2 production rate during a transient induced by a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 11.6 gm/L.

fell to zero, the simulation transient followed the observed transient quite well with the exception that the time span of the simulated transient was shorter. In both transients xylose concentration was virtually zero at the beginning, increased after the step-change, reached a maximum of approximately 4 gm/L, then decreased to zero. The simulated transient behavior of cell density and total metabolic rate was more exaggerated than was observed experimentally, The decline in simulated total metabolic rate was much more rapid, after the step-change, than was observed experimentally. Whereas the observed CO_2 production rate did not begin to decrease for fifteen to twenty minutes after the step-change, the simulated total metabolic rate decreased immediately. After reaching a minimum, the simulated total metabolic rate increased slowly for approximately 8 hours, but then made a sudden increase to a much higher rate and increased rapidly thereafter. Except for the sudden increase, the observed CO_2 production rate exhibited similar behavior, but the maximum was not as high. The simulated cell density also exhibited a higher maximum than was observed experimentally, though it had the same decline and recovery the experimental transient cell density had. After the xylose concentration fell to zero, the model failed to follow the experimental transient. The failure was caused by the metabolic rate falling rapidly to nearly zero.

The rapid increases and decreases in total metabolic rate resulted from the model being too sensitive to the interaction between pH gradient, metabolic rate and acetic acid inhibition. The sensitivity is in part due to the simplifications of the model. Only two reaction steps were used to model the metabolic pathway, which actually contains more than 10 reaction steps. More reaction steps would provide the system with

additional inertia. Cell function was treated as a uniform, continuous process. No account was taken of the individuality of the cells, nor the existence of growth cycles in cell reproduction. Finally, no account was taken of changes in metabolic function which occur as metabolic rate changes. For example, the acetate yield was assumed to be a constant 0.25 gm/gm, however, experimentally the yield was substantially lower at high metabolic rates, and slightly higher at low metabolic rates (see section 5.1). If the reduction in acetate yield at high metabolic rates had been used in the model, the acetate concentration could have been much lower at the time the model failed to simulate experimental data, thereby reducing the increase in acetic acid inhibition as the internal pH dropped as the metabolic rate dropped.

The high maximums in simulated cell density and total metabolic rate were due to a high specific metabolic rate. In the model, as in the organism, the cell yield was quite sensitive to specific metabolic rate (see section 5.2). Because the specific metabolic rate was higher, the cell yield was higher. thus the maximum cell density was higher. Because the total metabolic rate is the product of cell density and specific metabolic rate, it too was higher.

In spite of the defects in it, the model confirms the plausibility of the postulate that an interaction occurs among the magnitude of the transmembrane pH gradient, the metabolic rate and acetic acid inhibition. That a model could be constructed which is more sensitive to such an interaction, indicates the possibility of the interaction taking place in the organism.

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7. CONCLUSION

This work examined the ability of B. macerans to convert pentose sugars to ethanol. It was found that B. macerans produces ethanol from xylose with a yield of 0.20 gm/gm when growing at steady-state with a dilution rate of 0.03 or 0.06 hr^{-1}. It also produces acetic acid with a yield of approximately 0.25 gm/gm under the same conditions. B. macerans also produces acetone, and could, theoretically, entirely replace its acetic acid production with acetone production. Batch culture results suggested that continuous culture at low dilution rates would enhance acetone production, however this was not true. Steady-state growth at dilution rates of 0.03 and 0.06 hr^{-1} produced immeasurably low yields of acetone. It was also found that approximately 33% of the carbon in the xylose consumed appeared as CO_2 and 5 to 10% appeared as an unidentified, non-volatile, water soluble organic compound.

The maximum specific growth rate, measured during batch culture, was reported to be 0.15 hr^{-1}. It was found that B. macerans has a maximum specific growth rate of at least 0.26 hr^{-1}. Because the batch culture experiments were done without N_2 sparging, carbon dioxide inhibition appears to be the reason a lower maximum specific growth rate was measured. The effect of carbon dioxide inhibition on the metabolic rate need not have been great to produce the large difference in maximum specific growth rates. The cell yield was quite sensitive to metabolic rate, amplifying small differences in metabolic rate into large differences in specific growth rate. Metabolic rates of 1.4 and 2.6 gm xylose/gm cell (dry wt.)/hr produced cell yields of 0.022 and 0.10 gm (dry wt.)/gm, respectively, and specific growth rates of 0.03 and 0.26 hr^{-1}, respectively.

A steady-state continuous culture was difficult to attain or maintain with B. macerans, and when perturbed B. macerans exhibited unusual transient behavior. It was found that when growing in continuous culture, a step-change to a higher dilution rate produced a cessation of growth. Using CO_2 production as an indicator of metabolic rate, it was found metabolic rate began to decrease within twenty minutes of the step-change. The same behavior could be induced by injecting a small amount of xylose, glyceraldehyde or sedoheptulose directly into the fermenter. The cell density would continue to decline for as long as 90 hours after the induction of a transient. Following the decline, cell density would rapidly recover, reaching a maximum, then declining towards steady-state within a day.

It was postulated that the cause of this behavior is an interaction between metabolic rate, the magnitude of the transmembrane pH gradient and acetic acid inhibition. A high metabolic rate permits a large pH gradient to be maintained which keeps acetic acid inhibition low, but a decreasing metabolic rate causes the pH gradient to decrease which increases acetic acid inhibition causing the metabolic rate to decrease more. The effect of this interaction is amplified by the decrease in cell yield as metabolic rate decreases. A computer model was constructed to confirm the plausibility of this postulate. The model was even more sensitive to the interaction than the organism. The postulate was, therefore, confirmed as a possible explanation of the behavior of B. macerans.

The instability and transient behavior renders B. macerans unacceptable for use in a commercial process to convert pentose sugars to

ethanol. In one experiment, over a period of a week, a continuous culture of B. macerans, at constant dilution rate, made three cycles of high cell density to low cell density back to high cell density. Such behavior could not be suitable for a commercial process.

APPENDIX A TRANSIENTS

Presented here are the results of six transients. Figures A,1 and A,2 are from transients induced by step-changes in dilution rate from 0.03 to 0.06 hr^{-1}. In both transients the length of the declining phase was less than 24 hours.

Figures A,3 and A,4 are from transients induced by step-changes in dilution rate from 0.03 to 0.09 hr^{-1}. In both transients the length of the declining phase was less than two days. Figures A,5a and A,6a are from transients induced by injecting xylose into cultures growing at steady-state at a dilution rate of 0.03 hr^{-1}. In Figure A,5a the declining phase lasted about 20 hours, whereas the declining phase in Figure A,6a lasted 90 hours. Accompanying Figures A,5a and A,6a are figures of the production rates of CO_2 during these transients. In both cases the production rates fell precipitously within 15 minutes of the injection of xylose, and rose to peak values when cell mass was a maximum. In all six transients ethanol and acetate showed less overall variation than cell mass, although changes between individual samples were often greater for ethanol or acetate. The larger amplitude in cell mass variations can be attributed to the sensitivity of cell yield, and thus specific grwoth rate, to metabolic rate.

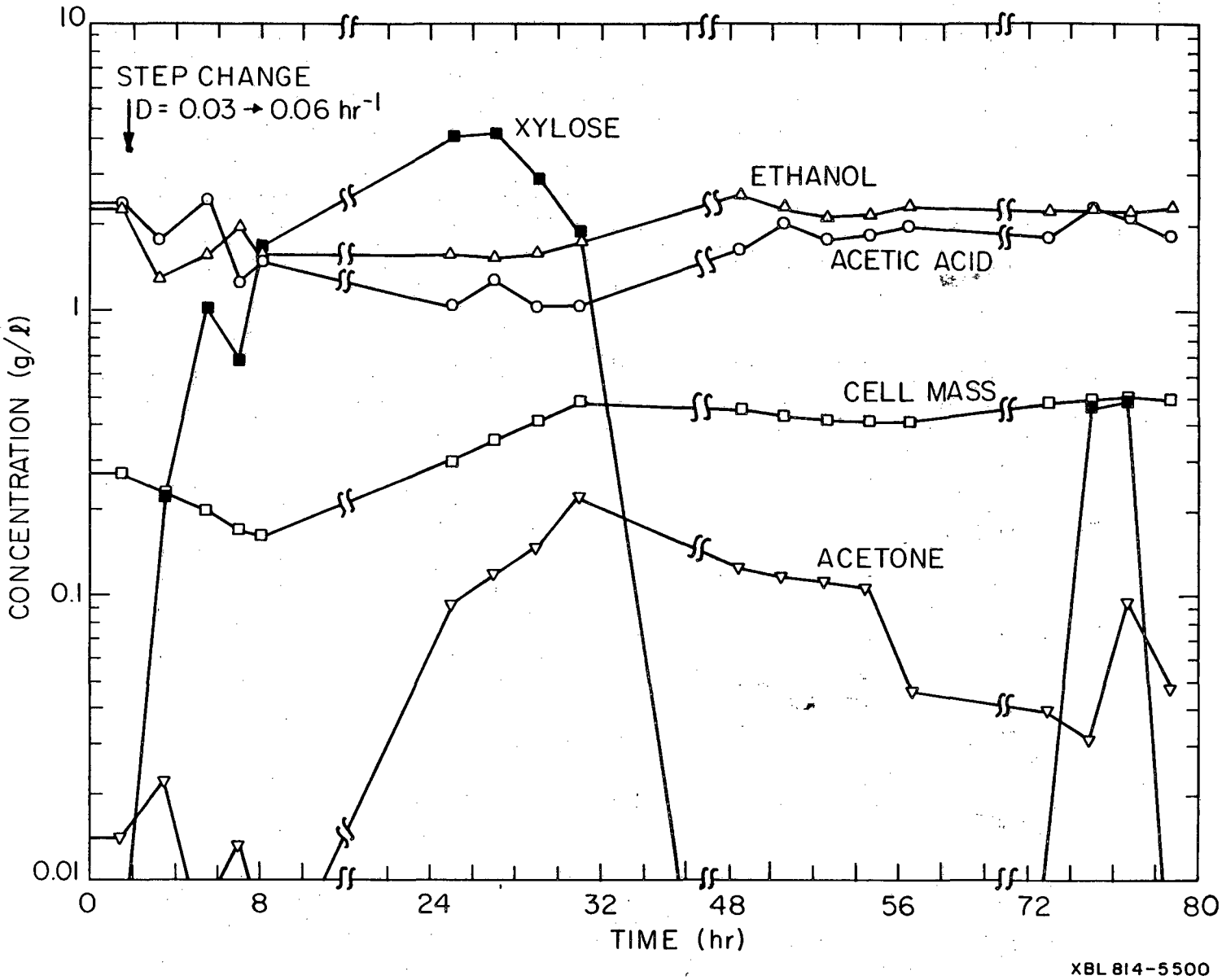


Figure A.1 Conditions in a culture of B. macerans following a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 11.2 gm/L.

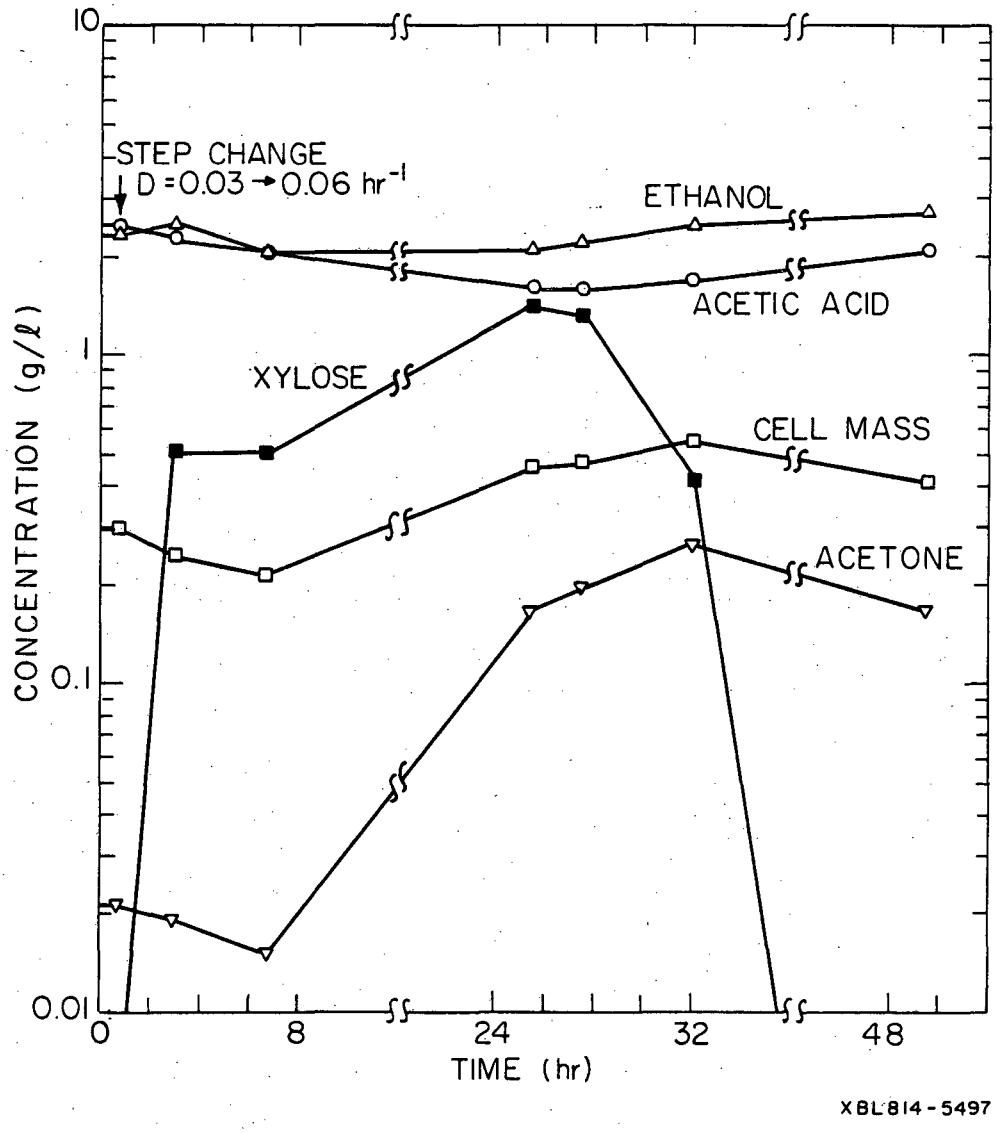


Figure A.2 Conditions in a culture of B. macerans following a step-change in dilution rate from 0.03 to 0.06 hr^{-1}. Inlet xylose concentration was 10.9 gm/L.

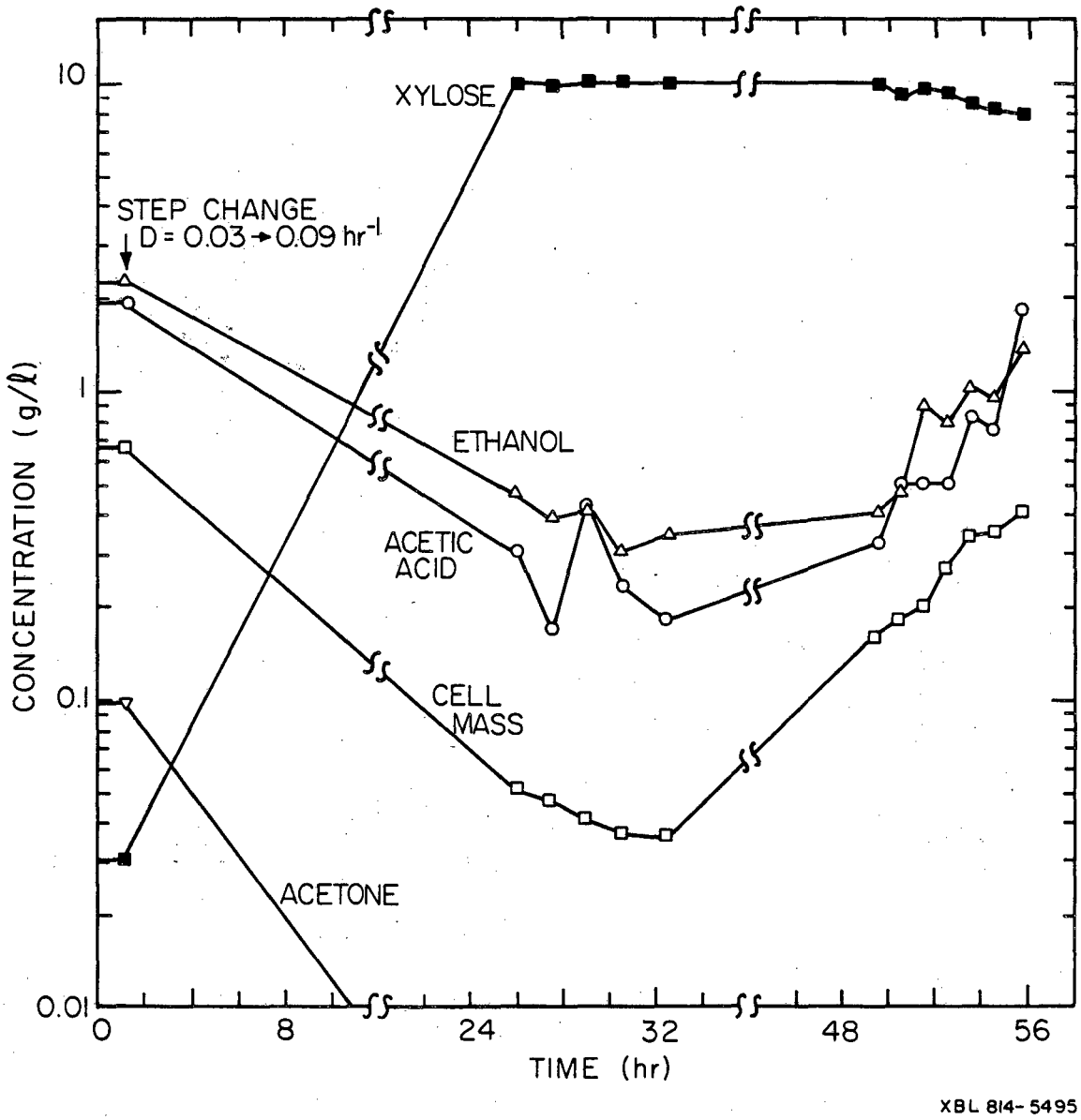


Figure A.3 Conditions in a culture of B. macerans following a step-change in dilution rate from 0.03 to 0.09 hr^{-1}. Inlet xylose concentration was 12.0 gm/L.

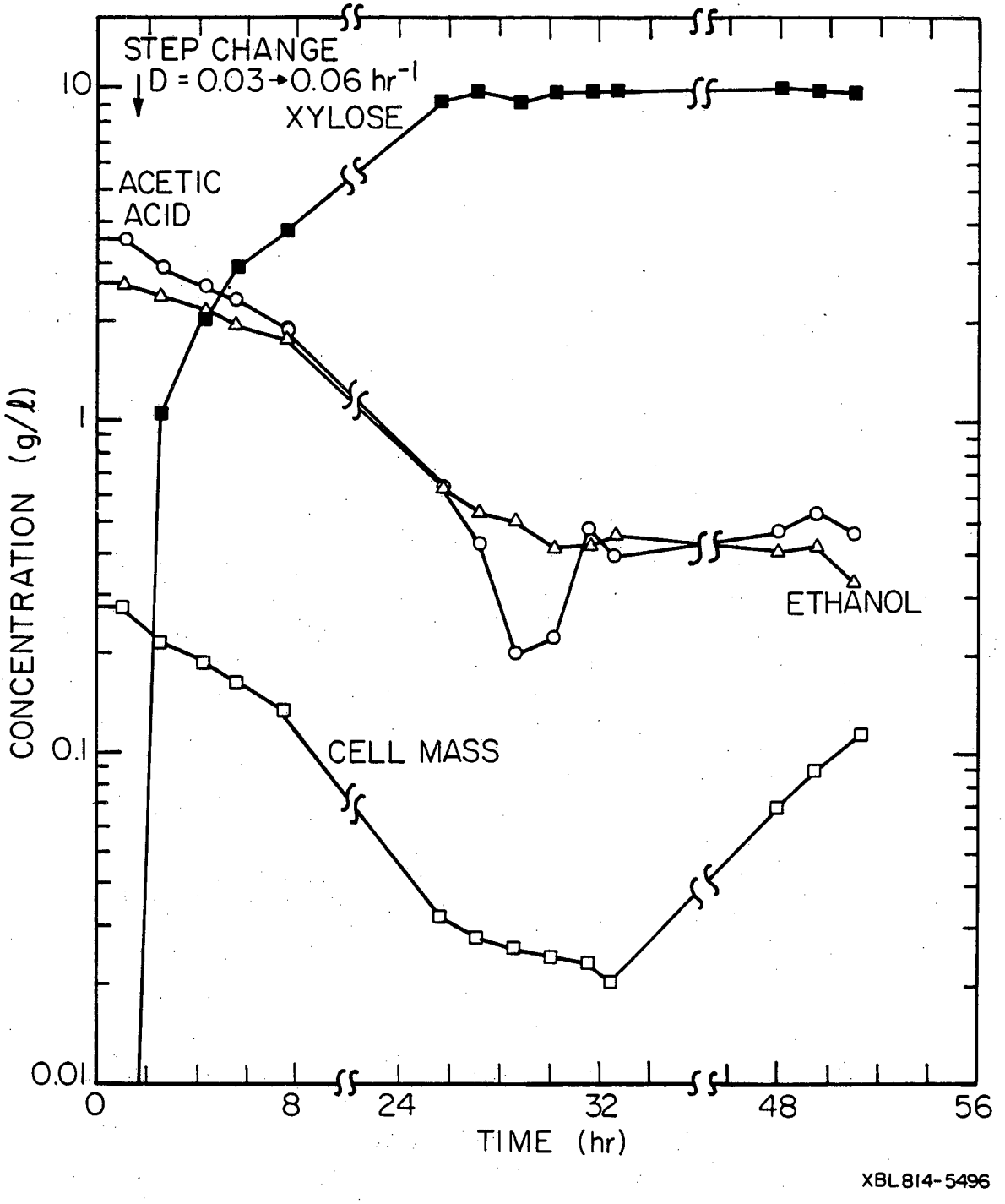


Figure A.4 Conditions in a culture of B. macerans following a step-change in dilution rate from 0.03 to 0.09 hr^{-1}. Inlet xylose concentration was 12.0 gm/L.

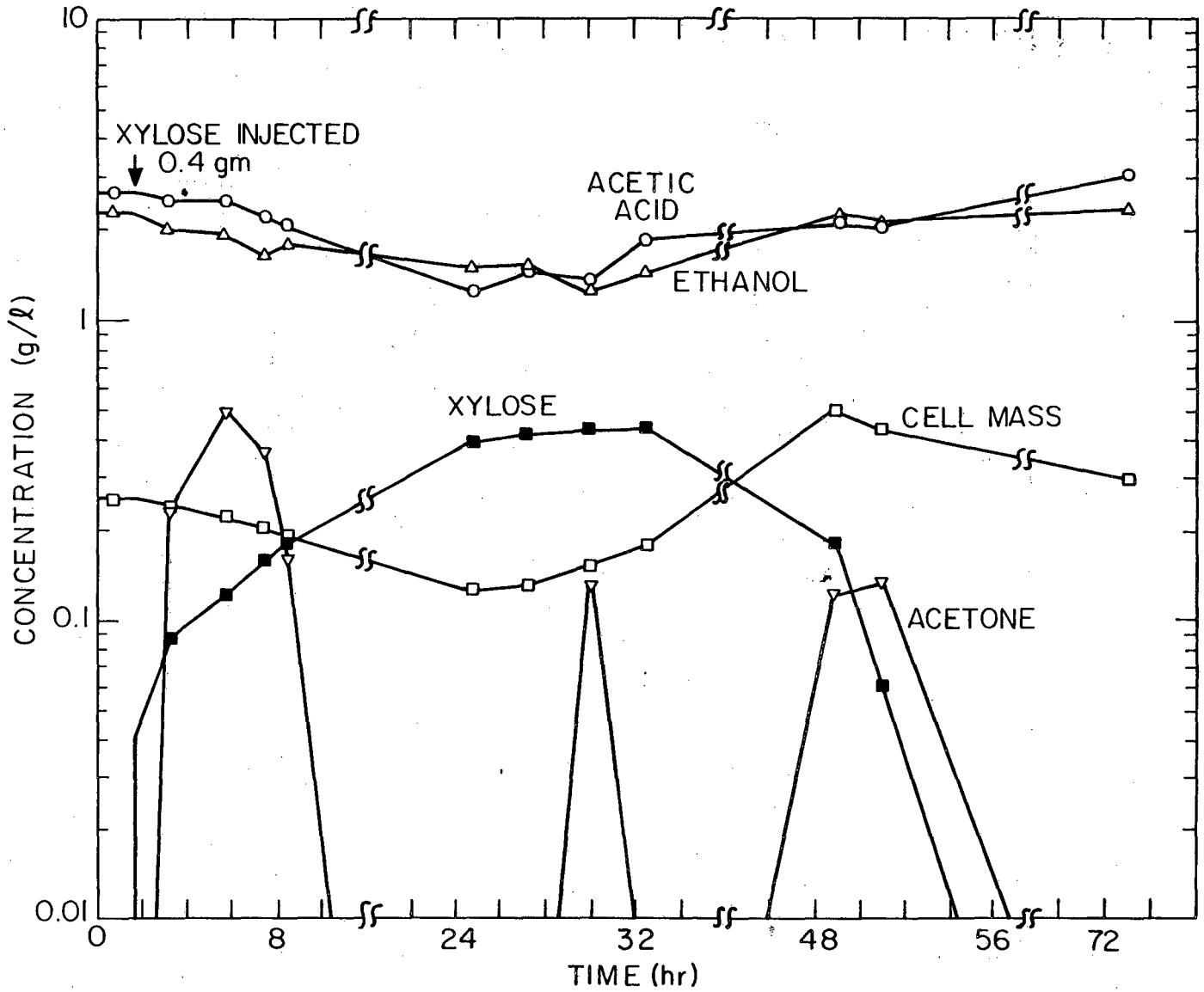


Figure A.5a Conditions in a culture of B. macerans following an impulse of 0.4 gm of xylose. Inlet xylose concentration was 11.4 gm/L.

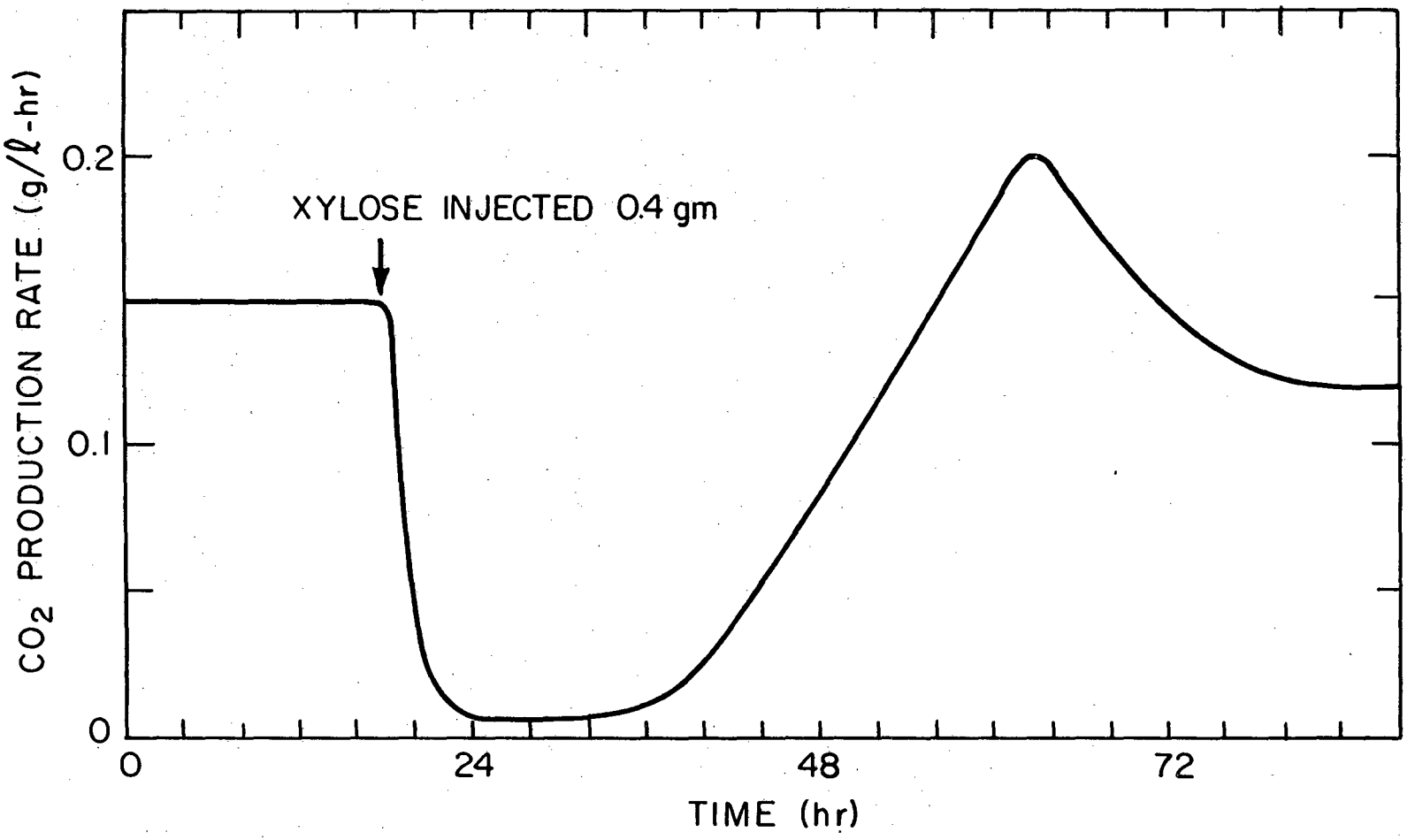
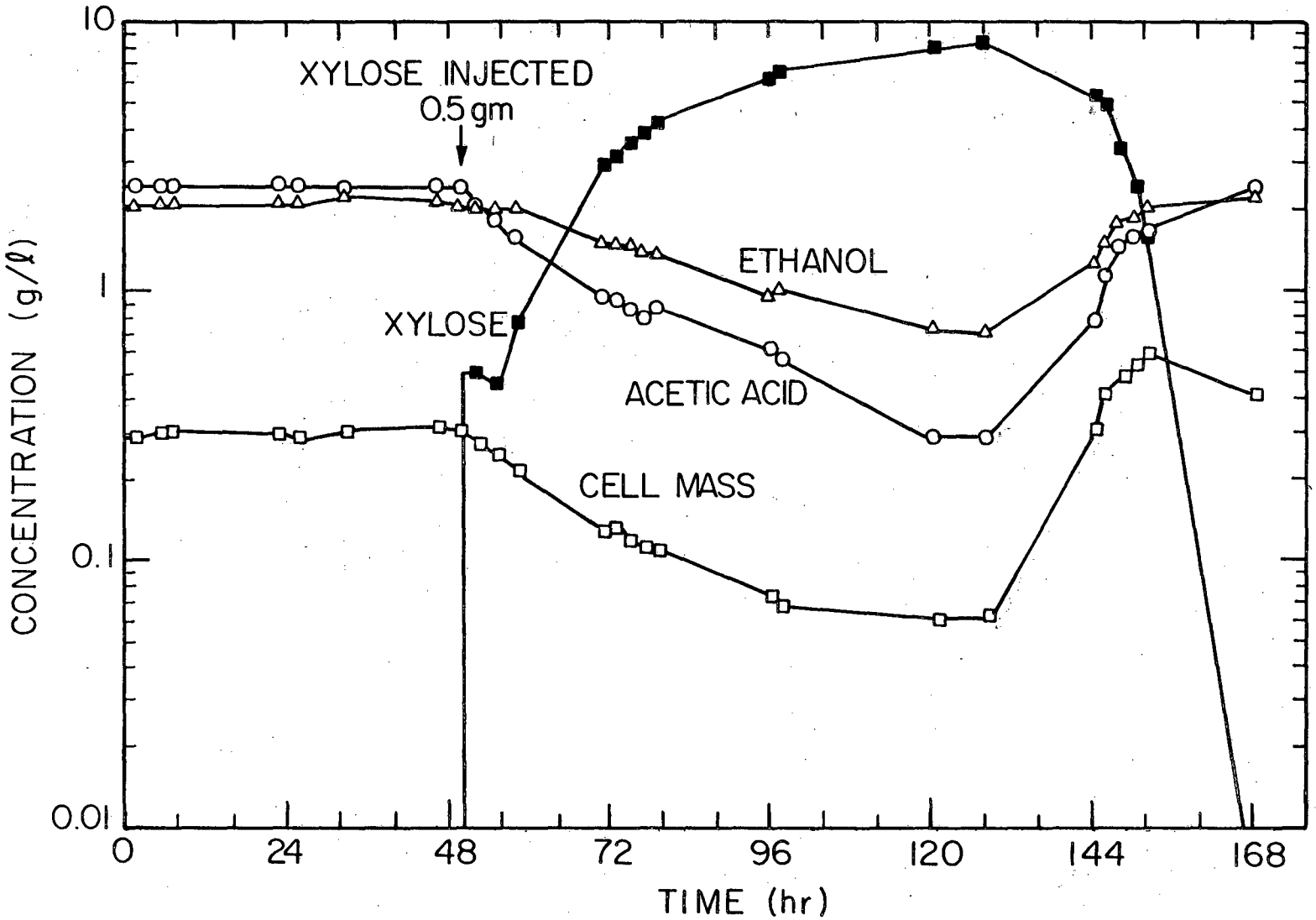


Figure A.5b CO_2 production rate of the culture in Figure A.5a.

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Figure A.6a Conditions in a culture of B. macerans following an impulse of 0.5 gm of xylose. Inlet xylose concentration was 11.7 gm/L.

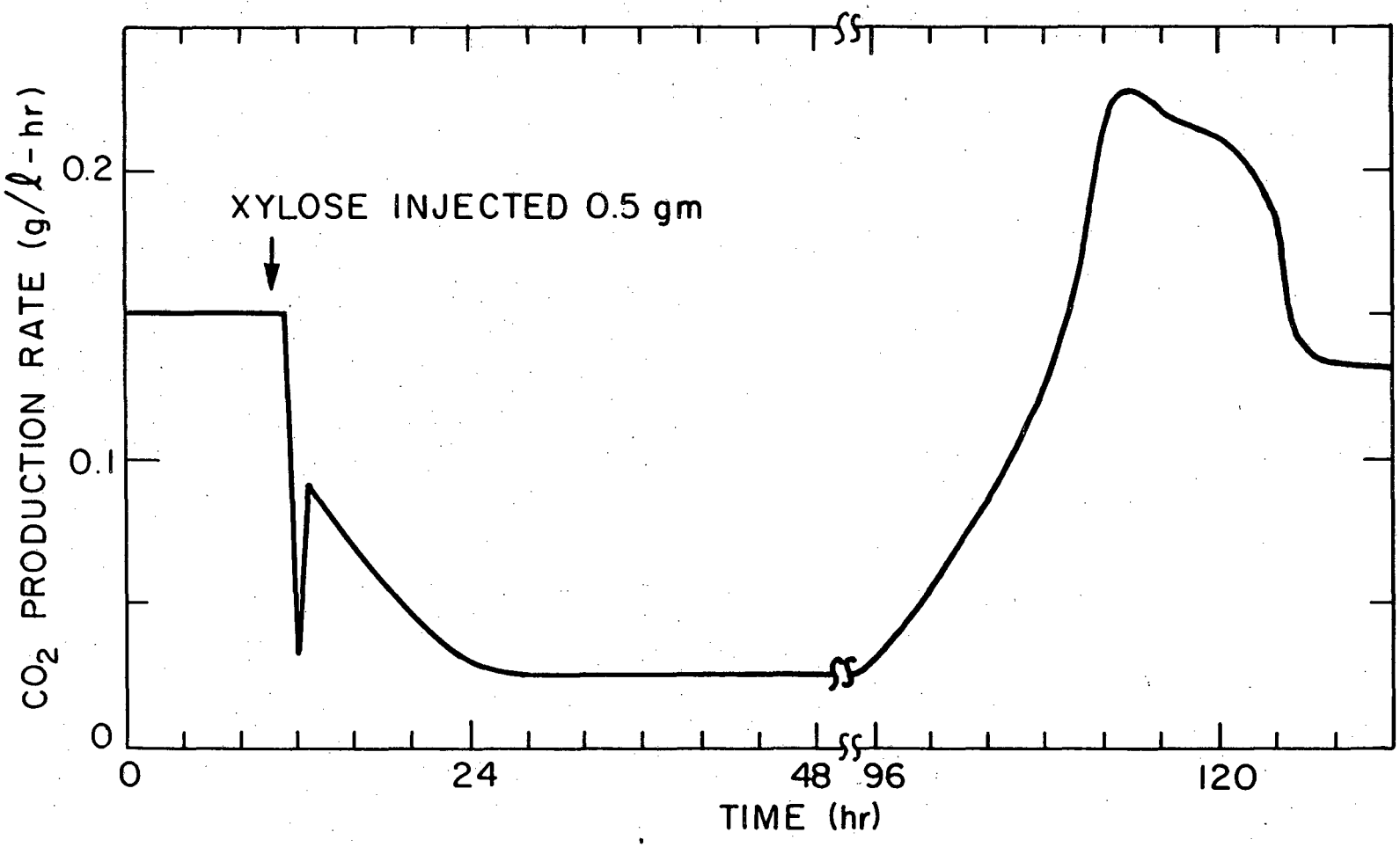


Figure A.6b CO_2 production rate for the culture in Figure A.6a.

APPENDIX B

THE PROGRAM FOR THE COMPUTER SIMULATION OF TRANSIENT BEHAVIOR IN B. MACERANS

The program was written in Fortran for use on the CDC 6400 computer at the University of California, Berkeley. The program used the equations and methods described in Section 6:1 to evaluate constants and initial conditions. The integration of the differential equations were done with a fourth-order Runge-Kutta method from McCracken and Dorn(1). The program, BMAC, evaluated initial conditions and constants, changed the dilution rate and printed out the results of the investigation. It called a subroutine, BUCUO, to perform the Runge-Kutta integration. BUCUO, in turn, called another subroutine, PODU, to evaluate the seven differential equations.

The program printed the results as a graph. Four variables were displayed in the graph: external xylose concentration, cell mass, internal pH and total metabolic rate - the product of cell mass and reaction rate RA. After every 250 steps of the integration, the program would print a new line of characters representing the four variables. Because the integration step size was 0.001 hours, the interval between printed lines was 0.025 hours.

The dimensions used in the program were millimoles, grams wet wt of cells, liters and hours. Concentration terms, with one exception, had units of millimoles per liter (see Table B.1). The external xylose and acetate concentrations were millimoles per liter of fermenter, whereas internal xylose and intermediate metabolite concentrations were millimoles per liter of cell. Intermediate metabolite was treated as

Table B.1

Units for Symbols Used in the Program

Symbol	Units
AA, AAM	millimole acetic acid/L
COP	pH units
D	$hour^{-1}$
DF, DFP	millimole hydrogen ion/gm wet wt. cell/hr
DH, DHM	pH units
DLT	hour
EPP	millimole ATP/gm wet wt. cell/hour
H, HM	mole/L hydrogen ions
MU, MUM	$hour^{-1}$
QAA, QA, QB	mole/L hydrogen ion
QC	$hour^{-1}$
QFA, QRA	$hour^{-1}$
QI, QII	millimole/L
QP	pH units
QPH	pH units/millimole hydrogen ions
QS, QSI	millimole/L
QY	gm wet wt. cell/millimole ATP
RA, RAM, RAP, RT, RTM, RTP, RAA, RAAM	millimole xylose (or equivalent xylose)/ gm wet wt. cell/hour
RB, RC, RCM	millimole ATP/gm wet wt. cell/hour
SO	millimole/L
Y	millimole acetate/millimole xylose
YXS, YXSM	gm wet wt. cell/millimole xylose

Table B.1 continued

Symbol	Units
Z	$hour^{-1}$ pH units
E(1), EM(1), A(1)	millimole xylose/L (fermenter)
E(2), EM(2), A(2)	gm wet wt. cell/L (fermenter)
E(3), EM(3), A(3)	millimole xylose/L (cell volume)
E(4), EM(4), A(4)	pH units
E(5), EM(5), A(5)	millimole equivalent xylose/L (cell volume)
E(6), EM(6), A(6)	millimole ATP/gm wet wt. cell
E(7), EM(7), A(7)	millimole acetate/L (fermenter or cell volume)

equivalent xylose. That is one millimole of xylose consumed in RAA appeared as one millimole of intermediate metabolite. ATP concentration was expressed as millimoles per gram wet wt. of cells. The rate expression had units of millimoles per hour per gram wet wt. of cells. Because concentration and rates had different concentration units, a reconciliation factor had to be added to expressions for F(3) and F(5). The cells were assumed to have a specific gravity of 1, so the reconciliation factor relating reaction rates to rates of change in concentration was 1000 grams wet wt. of cells per liter of cells. Therefore, equations 6:10 and 6:11 became

$$\frac{dE(3)}{dt} = F(3) = 1000(RT - RAA) \qquad (B.1)$$

and

$$\frac{dE(5)}{dt} = F(5) = 1000\ (RAA - RA) \qquad (B.2)$$

Whole cells were assumed to be 90% water, thus cell yields of 0.022 gm/gm and 0.10 gm/gm became 0.033 and 0.15 gm wet wt. cells/millimole xylose consumed, respectively. The yield of acetate, 0.25 gm/gm, was 0.625 when expressed in millimoles acetate per millimole xylose consumed. The yield and consumption factors for ATP remained 10/3 and 5/3, respectively.

The symbols used in the program, which follows, were the same as those used in this Appendix and in Chapter 6. The equations in PODU used A(N) in place of E(N). A(N) represented intermediate values of E(N) generated in BUCUO. E(N) were values of the variables at the beginning and end of each integration step.

References

1. McCracken, D. and W. Dorn, Numberical Methods and Fortran Programming with Applications in Engineering and Science, John Wiley and Sons, New York 1964.

Acknowledgment

This work was supported by the Director, Office for Energy Research, Office of Basic Energy Sciences, Division of Chemical Science Processes and Techniques Branch of the U.S. Department of Energy under contract DE-AC03-76SF00098, and by the Assistant Secretary for Conservation and Solar Energy Research Institute under contract DR-0-9058-1.

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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