tissue cultures for chemicals production. However, some oils and chemicals are made only in rather specialized organs. The use of organ cultures for chemicals production has been explored little. Such cultures would offer difficult challenges in bio-reactor design. Nonetheless, it may be quite valuable to consider such systems for products from such tissues.

ACKNOWLEDGMENT

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Production of Protein and Fatty Acids in the Anaerobic Fermentation of Molasses by *E. ruminantium*

KETAN I. MEHTA*, Anderson Clayton Foods, 3333 North Central Expressway, Richardson, TX 75080, and **C.D. CALLIHAN**, Department of Chemical Engineering, Louisiana State University, Baton Rouge, LA 70802

ABSTRACT

Production of protein and volatile fatty acids by anaerobic digestion of blackstrap molasses was investigated. This protein may have potential as a feed for animals (and, we hope, for humans) in the near future. Volatile fatty acids can be further fermented to produce methane. Fermentation of molasses by *Eubacterium ruminantium* was studied in a chemostat at a constant temperature of 37 C. This study focused on kinetics of growth of the pure culture. The maximum rate of protein production of about 0.326 g/l/hr was obtained when the pH and retention times were 6.2 and between 5 and 7 hrs, respectively. Average cell yield was 12.6% and carbohydrate conversion was 82 to 99%. Volatile fatty acids also were produced, with acetic acid and n-butyric acid being the predominant products. Two different kinetic models were used to fit the experimental data. The kinetic parameters obtained for the Monod model were: $\mu max = 0.207$ (1/hr); $k_s = 0.165$ g/l.

INTRODUCTION

A need exists for developing protein, preferably from materials not directly consumed by animals or humans (1). If a usable protein can be produced by fermentation of carbohydrate-rich agricultural wastes, it might be substituted for soybean meal. Benefits would be an economic solution to waste disposal problems; use of waste as substrate which has greater negative cost value; a feed supplement rich in crude protein for animal feed; a possible protein which can be purified for human consumption; the independence of protein production on agricultural land, and energy production in the form of methane.

In Germany during World War I bakers' yeast, *Saccharomyces cerevisiae,* was grown with molasses and ammonium salt for consumption as a protein supplement. Protein is produced by the aerobic cultivation of lactose fermenting yeast, *Saccharomyces fragilis* on cheese whey (2,3). The 'Symba' Process, using an *Endomycopsis* to hydrolyze starch in wastes followed by Torula yeast grown on sugar, was developed in Sweden many years ago (4). Callihan (5) et al. used *Cellulomonas uda* to ferment bagasse for protein production. Gautreaux (6) fermented sweet potato waste using primary sludge from a sewage treatment plant for producing protein and methane. An advanced commercial process for protein production from methanol has been developed by Imperial Chemical Industries, Ltd. They started up a 60,000 metric ton/year plant in 1981 at Billingham, England, and now are trying to adapt the technology to use sugar and other substrates (7). Besides ICI and Phillips Petroleum, a number of other organizations like Germany's Hoeclist and Japan's Mitsubishi are investigating SCP production for human consumption.

Most of the above processes use aerobic fermentation. Though the technology of aerobic fermentation is well developed, in this research anaerobic fermentation was used. This is because of the following proven advantages of anaerobic fermentation and the limitations of aerobic fermentation:

*To whom correspondence should be addressed.

- Energy requirements are high in aerobic systems compared to anaerobic processes.
- Due to the low rate of oxygen transfer, only limited organic loadings can be used in aerobic processes.
- Nutritional requirements (nitrogen and phosphorous) are low in anaerobic processes compared to aerobic processes.
- Anaerobic processes need comparatively lower capital investment.
- In anaerobic processes, it is easy to harvest bacterial cells by settling and decantation.
- Anaerobic processes produce methane.

A comprehensive review of the fundamental microbiology and biochemistry involved in anaerobic fermentation has been presented by Torien and Hattinger (8). A fast growing bacteria, *Eubacterium ruminantium,* was used as an inoculum source for anaerobically fermenting blackstrap molasses during this research. Molasses consists mainly of sucrose, glucose and fructose. This makes it readily consumable by microorganisms without pretreatment. Figure 1 illustrates the mechanism of anaerobic fermentation of molasses (sugars). Bacteria was chosen for studying the kinetics of protein production because their generation time is 20-30 min, compared to 2-3 hr for yeast and 16 hr or more for algae and fungi. The acid-forming stage involves the acid forming bacteria which as a group obtain energy for growth by hydrolyzing sugars and fermenting the products to fatty acids and gaseous products like CO_2 and H_2. The methane forming bacteria obtain energy for growth by catabolizing the products of the first stage to CO_2 and CH_4.

The acidogenic and methanogenic microbial groups are believed to have significantly different environmental requirements, physiology, growth, metabolic characteristics and nutritional requirements. Because of this, culturing these organisms in isolated optimized environments should improve overall process efficiency as well as reaction rate, operational control and stability (9,10). Due to this, 2-stage anaerobic fermentation was chosen for the present research.

In a protein production process using this technology, bacterial cells containing protein and volatile fatty acids can be separated in the first stage of fermentation. The volatile fatty acids like acetic, propionic and butyric acids can be stored as a liquid in the acid pond and can be pumped into a methane pond where high rates of methane can be produced.

This research was focused mainly on the study of the kinetics of protein and fatty acids production in a chemostat. Limited research was conducted in the area of bacterial cell harvesting and studying the kinetics of methane production.

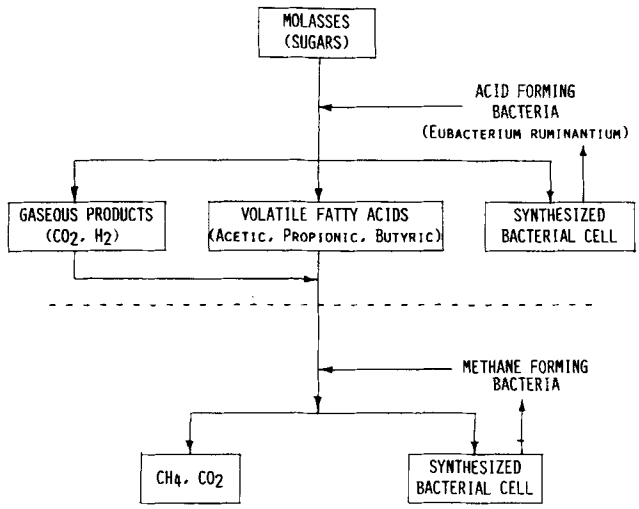

FIG. 1. Anaerobic digestion of molasses.

Two different mathematical models were created and compared for protein production using the multiresponse data obtained through the series of experiments in a chemostat. These models are shown in Table I (11). The importance of modelling was to predict the fermentor behavior; determine the optimum operation conditions, and design and scale-up the fermentor to commercial production unit.

MATERIALS AND METHODS

Inoculum: A fast growing culture, *Eubacterium ruminantium,* isolated from ruminant fluid obtained from a fistulated cow raised at the Louisiana State University dairy farm was used as an inoculum source. Culture was maintained during the entire study by transferring it into new test tubes filled with sterile substrate every 3 days. The transfer was performed under sterile conditions and the test tubes were kept in the New Brunswick controlled Environment Incubator Shaker at 150 rpm and 37 C.

Substrate: Crude blackstrap molasses was obtained from the Audubon Sugar Factory, LSU. This molasses was diluted with distilled water and then centrifuged at 2500 rpm

TABLE I

Kinetic Models with Steady-State Equation

Model No.	Kinetic Model	Steady-State Equation	Remarks
1.	$\frac{dX}{dt} = \frac{\mu_m SX}{(K_s + S)}$	$\bar{x} = Y(S_{in} - \bar{S})$	Monod Model
	$\frac{ds}{dt} = \frac{-\mu_m SX}{Y(K_x + S)}$	$\bar{S} = \frac{K_s D}{(\mu_m - D)}$	
2.	$\frac{dX}{dt} = \frac{\mu_m SX}{(K_x + S)} - K_d X$	$\bar{x} = \frac{YD(S_{in} - \bar{S})}{(D + K_d)}$	Monod Model with K_d
	$\frac{ds}{dt} = \frac{-\mu_m SX}{Y(K_x + S)}$	$\bar{S} = \frac{K_s(D + K_d)}{(\mu_m - D - K_d)}$	

TABLE II

Process Conditions and Experimental Program

Feed	2.85 wt % sugar solution of molasses: 1.920% sucrose 0.378% glucose 0.552% fructose + 1.5 g/l yeast extract + 3 g/l $(NH_4)_2$ HPO_4
Fermentor Volume	400 cc
Fermentor Temperature	37 C

pH Study	Retention Time Study
Kinetics studied At pH = 6.0, 6.2, 6.5, 6.7, 7.0 and Retention Time = 15.5 hr	Kinetics studied at retention time = 15.5, 13.5, 9.1, 6.5 and 5.0 hr and pH = 6.2

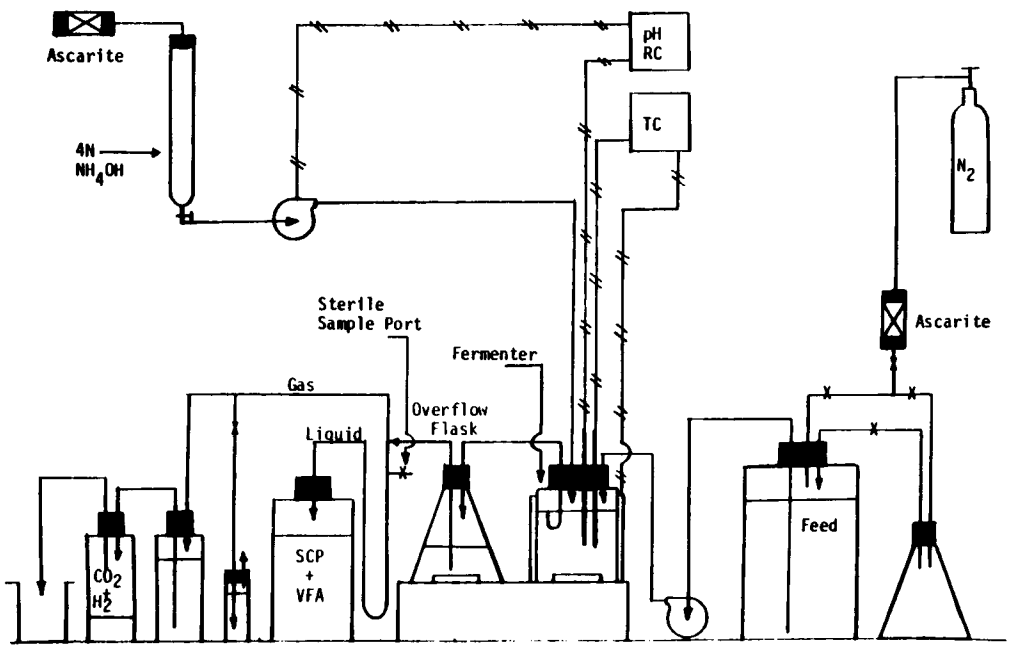

FIG. 2. Experimental set-up for kinetic study of cell protein and VFA.

for 15 min. Centrifuged molasses was further diluted with distilled water to bring the concentration of total sugar to the desired value. Water Associate's HPLC was used to find the wt % sugars in molasses. Sugar concentration of 2.85 wt % was used for the entire study. Table II shows detailed composition of substrate.

Experimental Procedure: Protein (SCP) and VFA formation were studied in a chemostat using *E. ruminantium*. The experimental set up used for this study is shown in Figure 2. Table II shows the process conditions used during the entire study. The temperature was controlled at 37 C with a Cole Parmer 2157 temperature controller, thermocouple, voltage transformer and heating tape. An immersed pH probe with a Horizon 5650 pH monitor and controller maintained the desired pH by addition of 4N NH_4OH solution through a Masterflex pump.

Besides controlling pH, the NH_4OH also provided NH_4+ required for bacterial growth. Bryant (12) claimed that some of the rumen strains required amounts of NH_4+ approximately equal to or greater than the amount of cellular nitrogen produced during growth, regardless of the amount of amino acid and peptide nitrogen present in the medium, and did not require organic nitrogen compounds for growth. In the literature, NaOH generally is found to be a common neutralizer. But to provide NH_4+ needed and to avoid excess Na+ concentration, NH_4OH was selected as the neutralizer in the present study. Mixing in the chemostat was provided by a Curtin 19970-2 magnetic stirrer at setting 3. A Masterflex 7520-10 pump using a 7014-20 head and controller connected to a ratio controller was used for a very low flow to the chemostat. A sterile sample port was designed in the system. A 13-liter feed bottle was connected to a 2-liter conical flask using rubber tubings. Molasses having 2.85 wt % sugar solution with 1.5 g/l of yeast extract was added in the feed bottle while 3 g/l of $(NH_4)_2HPO_4$ was added in the conical flask prior to sterilization. This was to avoid carmelization at sterilization conditions of 230 F and 18 psi. The feed was sterilized at these conditions for 60 min. It was then cooled to room temperature and kept at that temperature for at least 12 hr. This cold feed was resterilized for at least 45 min. This procedure of double sterilization was adopted to kill all the

spore forming microorganisms. Feed which had been prepared by the above procedure and kept under nitrogen blanket remained sterile for days at room temperature without growth of unwanted organisms.

The whole system shown in Figure 1, besides controllers and pumps, was sterilized for 30 min prior to inoculation, which was done under sterile conditions in the presence of ultraviolet light.

During start-up the fermentor was operated for 16 retention times before a sample was drawn for analysis. After that, samples were withdrawn after every four retention times. Three samples were collected for each process variable. Average results are reported here with their coefficient of variation (C.V.).

The objective of this research was to optimize the rate of production of cell protein. Univariable optimization technique was used to search for optimum process conditions. As shown in Table II, kinetics of protein and VFA production at five different pH values and retention time of 15.5 hours was studied. The optimum pH for protein production was then selected and was used during retention time study.

Mathematical Modelling

For building the mechanistic mathematical models, three steps were taken: (i) Search for addquate model. Two different mathematical models most commonly used were chosen from the literature for this purpose. (ii) These Kinetic models with their steady state relationship were used for parameter estimation. These models are shown in Table I. Linear regression and iterative nonlinear optimization routine were used for parameter estimation. IBM 370-3033 was used for this work. The software created for this purpose is listed elsewhere (13). (iii) Graphic and statistical methodologies were used for comparison and discrimination of these two models.

Analytical Techniques

- *pH:* For pH measurement, a Corning Model 10 pH meter with temperature compensation was used.
- *Bacterial cell dry weight:* This was determined according to standard technique (14).
- *Protein:* Biuret protein was measured by the modified Biuret method of Herbert et al. (14).
- *VFA:* Individual VFA were determined using a Perkin Elmer Sigma 3B Gas Chromatograph. Detailed methods can be found elsewhere (13).
- *Carbohydrates:* A Water Associates HPLC was used for this analysis. Detailed method can be found elsewhere (13).
- *Staining of Culture:* Gram Stain. This was measured by Standard Hucker's gram stain method.
- *Gas Composition:* A Varian Aerograph Series 1800 Gas Chromatograph with a Varian Aerograph Model 20 recorder was used for this analysis. Detailed method can be found elsewhere (13).

RESULTS AND DISCUSSION

pH Study: Initially the investigation was performed over a pH range from 6.0 to 7.0 at a retention time of 15.5 hr. Terminal pH of 5.5 was obtained. Bryant (15) found similar results. The performance was evaluated on the basis of rates of production of protein and other microbial products. The results of these analyses are given in Table III. Table IV shows statistical analyses of experimental data points. It can be seen that maximum rate of protein production was obtained at a pH of 6.2. It also can be seen that pH did not appear to have any effect on the % consumption and the rate of consumption of sugar. High concentration of acetic and butyric acid and low concentration of propionic acid were observed. This agrees with the observation of many other investigators who worked with *Eubacterium* in the past. Results of an overall carbon balance calculation are given in Table V. The following assumptions were used in this calculation: (i) Dry cell mass contained 48% carbon (16). (ii) The carbon in the gas produced was computed assuming the gas was pure CO_2 (11). (iii) Equilibrium between the liquid phase and the gas phase was assumed for calculating the carbon present in the liquid phase (HCO_3^- ion, dissolved carbon dioxide). Using the definition of equilibrium constant (K) and relating CO_2 (aqueous) to the partial pressure of CO_2 in the gas phase (pCO_2) by Henry's constant (H), the following relationship could be derived:

TABLE III

Effect of pH on Molasses Fermentation

pH	6.0	6.2	6.5	6.7	7.0
Rate of Production (g/l hr)					
Cells	0.245	0.255	0.236	0.223	0.184
Protein	0.152	0.158	0.146	0.138	0.114
Acetic acid	0.385	0.417	0.475	0.625	0.823
Propionic acid	0.121	0.047	0.064	0.140	0.039
n-Butyric acid	0.440	0.501	0.312	0.146	0.192
Yield Coefficient (g product/g sugar consumed)					
Cell	0.135	0.139	0.130	0.124	0.104
Protein	0.084	0.086	0.081	0.077	0.064
Acetic acid	0.211	0.228	0.261	0.349	0.465
Propionic acid	0.067	0.026	0.035	0.078	0.022
n-Butyric acid	0.242	0.273	0.171	0.082	0.109
Sugar Consumption (%)	99.1	99.7	98.9	97.3	96.2
Rate of Sugar Consumption (g/l hr)	1.82	1.83	1.82	1.82	1.77
Gas Produced (l/l hr)	0.242	0.225	0.175	0.094	0.069
Ammonium hydroxide consumption (ml/l hr)	2.188	2.750	4.300	4.925	5.500

TABLE IV

Statistical Analyses[1] of Experimental Data Points

pH		6.0	6.2	6.5	6.7	7.0
Bacterial cells (g/l)	Mean	3.80	3.95	3.65	3.45	2.85
	C.V.(%)	2.77	1.66	2.39	3.11	3.86
Total sugar (mg/l)	Mean	270	77	310	760	1090
	C.V.(%)	5.24	5.51	4.56	4.84	2.47
Gas produced (l/l hr)	Mean	0.242	0.225	0.175	0.094	0.069
	C.V.(%)	1.76	5.09	6.55	3.01	5.47
Ammonium hydroxide	Mean	2.188	2.750	4.300	4.925	5.500
(ml/l hr)	C.V.(%)	2.86	9.00	0.60	1.01	4.55
Retention Time (hrs)		15.5	13.5	9.1	6.15	5.0
Bacterial cells (g/l)	Mean	3.95	3.86	3.78	3.23	2.50
	C.V.(%)	1.66	9.89	2.85	2.98	7.37
Total sugars (mg/l)	Mean	77	87	220	460	5050
	C.V.(%)	5.51	6.50	2.57	3.69	2.52
Gas produced (l/l hr)	Mean	0.225	0.254	0.821	1.067	0.250
	C.V.(%)	5.09	8.84	0.52	0.43	1.82
Ammonium hydroxide	Mean	2.75	5.21	9.69	8.33	10.45
(ml/l hr)	C.V.(%)	9.00	2.37	2.74	23.78	2.37

[1]Coefficient of variation (C.V.) = (S.D./Mean) x 100.

TABLE V

Carbon Balance in SCP and VFA Production

Feed concentration : 28.25 g sugar/l; (0.735 g carbon/l of feed - hr)
Temperature : 37 C.
Retention time : 15.5 hrs
Yeast extract : 1.5 g/l; (0.046 g carbon/l of feed - hr)[1]

pH	6.0	6.2	6.5	6.7	7.0
	(g/l - hr)				
Acetic acid carbon	0.154	0.167	0.190	0.250	0.329
Propionic acid carbon	0.059	0.023	0.031	0.068	0.019
n-Butyric acid carbon	0.240	0.273	0.170	0.080	0.105
Cell carbon	0.118	0.122	0.113	0.107	0.088
Gas carbon	0.118	0.109	0.085	0.046	0.034
Dissolved carbon	0.030	0.035	0.050	0.069	0.118
Unconverted carbon	0.007	0.002	0.008	0.020	0.028
Total carbon	0.726	0.731	0.647	0.640	0.721
Recovery (%)	92.96	93.60	82.84	81.95	92.32

[1]It was assumed that 48% of the yeast extract is carbon.

$$(C)\ (mole/l)\ in\ liquid\ phase = (pCO_2/H)\ (1 + K/10^{-pH})$$

Where $H = 39.25\ atm/(mole/l)$

$K = 4.96 \times 10^{-7}\ 1/(mole/l)$

Bryant (15) studied *E. ruminantium* using glucose agar and reported CO_2, acetic acid, butyric acid, lactic acid and formic acid as the major microbial products. No methane, hydrogen, ammonia, propionic acid, succinic acid or ethanol was detected. Nghiem (17) reported traces of hydrogen during fermentation of glucose using ruminant fluid. Thauer et al. (18) have pointed out that the production of butyric acid consumed hydrogen. Thus, a high concentration of butyric acid in the present study may support the assumption that only traces of hydrogen were present in the gas. Infrequent gas analysis showed only traces of ammonia and hydrogen. Recovery of total carbon computed on the basis of cell mass, VFA and gaseous and dissolved carbon dioxide amounted to 82-94%. The discrepancy in the carbon recovery could be because of formic and lactic acid which were not accounted for in the calculations.

Retention Time Study: After the results of the pH study were evaluated, a growth rate study at the optimum pH of 6.2 was conducted. Results of this study are given in Table VI. Table IV shows statistical analysis of experimental data points. The maximum rate of protein production of about 0.326 g/l hr was obtained at a retention time of 6.15 hr. At this condition 62% of dry cell mass was evaluated to be cell protein. Cell washout occurred at a retention time of about 4.8 hr. As before, the overall carbon material balance calculations were performed and the results are reported in Table VII. Recovery of total carbon was 84-93%. At all the process conditions studied, VFA accounted for the maximum percentage of the carbon. This shows that *E. ruminantium* is very desirable for VFA production.

Mathematical Modelling: The arithmetic mean of the five cell yields obtained through the retention time study and given in Table VI was used as the yield coefficient Y in all the calculations. The arithmetic mean of 0.126 was obtained. To evaluate the parameters K_s and μ_m in the Monod model, a Lineweaver-Burk plot (19) was used with linear regression. This plot gave a correlation of 0.99, a slope of 0.80 and an intercept of 4.84. The calculated values of K_s and μm using these results are given in Figure 3. The values of the cell mass concentration and the sugar concentration

TABLE VI

Effect of Retention Time on Molasses Fermentation

Retention Time (hrs)	15.5	13.5	9.1	6.15	5.0
Rate of Production (g/l hr)					
Cells	0.255	0.290	0.415	0.526	0.500
Protein	0.158	0.180	0.257	0.326	0.310
Acetic acid	0.417	0.778	0.440	0.738	1.080
Propionic acid	0.047	0.087	0.066	0.259	0.640
n-Butyric acid	0.501	0.319	0.444	0.774	1.114
Yield coefficients (g Product/g sugars consumed)					
Cells	0.139	0.136	0.134	0.115	0.107
Protein	0.086	0.084	0.083	0.071	0.066
Acetic acid	0.228	0.370	0.141	0.162	0.230
Propionic acid	0.026	0.041	0.021	0.057	0.136
n-Butyric acid	0.273	0.152	0.143	0.170	0.238
Conversion (%)					
Total sugar	99.7	99.69	98.95	98.39	82.28
Rate of Consumption (g/l hr)					
Total sugar	1.830	2.105	3.108	4.559	4.690
Gas Produced (l/l hr)	0.225	0.254	0.821	1.067	0.250
Ammonium hydroxide Consumed (ml/l hr)	2.750	5.20	9.688	8.325	10.450

TABLE VII

Carbon Balance in SCP and VFA Production

pH : 6.2
Temperature : 37 C.

Retention Time (hrs)	15.5	13.5	9.1	6.15	5.0
	(g/l/hr)				
Feed carbon	0.781	0.897	1.332	1.971	2.424
Acetic acid carbon	0.167	0.311	0.176	0.295	0.432
Propionic acid carbon	0.023	0.042	0.032	0.126	0.311
n-Butyric acid carbon	0.273	0.174	0.242	0.422	0.608
Cell carbon	0.122	0.137	0.200	0.252	0.240
Gas carbon	0.109	0.123	0.399	0.518	0.121
Dissolved carbon	0.035	0.040	0.060	0.089	0.109
Unconverted carbon	0.002	0.003	0.010	0.031	0.404
Total Carbon	0.731	0.830	1.119	1.733	2.225
Recovery (%)	93.60	92.53	84.01	87.92	91.79

as given in Table VI were used for these calculations. To estimate the parameter values in Monod with K_d, pattern search with the least square criteria was used. Given the value of Y, the value of K_d was estimated using the steady state relationship of the cell mass concentration X as given in Table I. To estimate the rest of the parameters, iterative search technique with the following objective function was used:

$$\text{Minimize } I = \sum_{i=1}^{5} (\bar{s}_{exp} - \bar{s}_{pred})_i^2$$

The parameter values for this model are given in Figure 4. Figures 3 and 4 give graphic comparison of experimental data with mathematical models. Variance for both the models are shown in Table VIII. It can be seen through graphic and variance analysis that the experimental data was best fitted using the Monod model. The Monod model gave an optimum cell protein production of 0.366 g/l hr at a retention time and pH of 5.3 hr and 6.2, respectively. Also the model showed that the cell washout occurred at a retention time of 4.8 hr. This also was found experimentally as mentioned before.

Amino Acid Composition

Table IX shows the amino acid profile of cell protein obtained at the optimum conditions. The amino acid profile of soybean cake is shown for comparison (20). It can be seen that an appreciable amount of methionine was present in the cell protein. A project proposal is being considered for conducting a study of feeding this cell protein to living shrimp and other animals at Louisiana State University.

This technology for producing cell protein and VFA from molasses can be applied to any indigenous carbohydrate waste with required modifications (e.g., hydrolysis of polysaccharides). Commercial-scale production of such protein appears to be promising in those regions of the world where carbohydrate waste is available and conventional animal feedstuff proteins, such as soybean meal and fish meal, are in short supply.

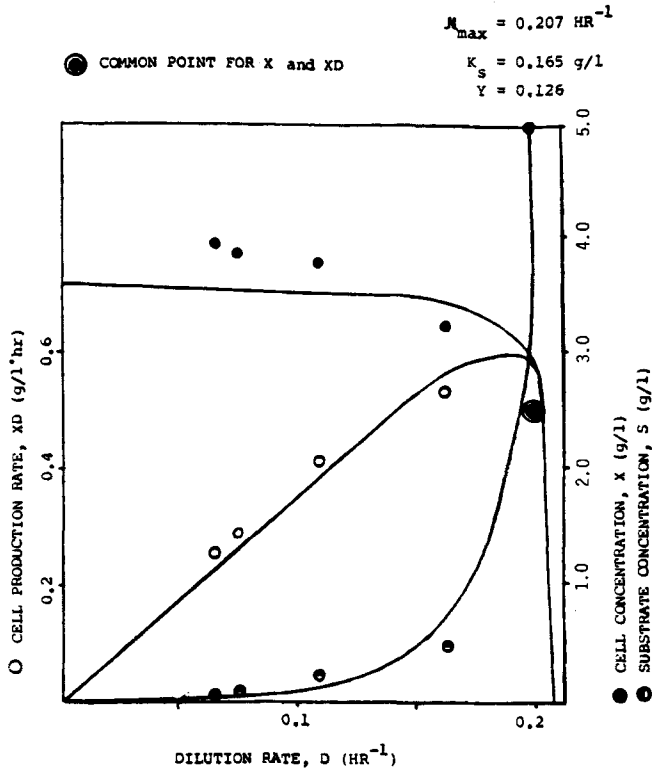

FIG. 3. Monod Model.

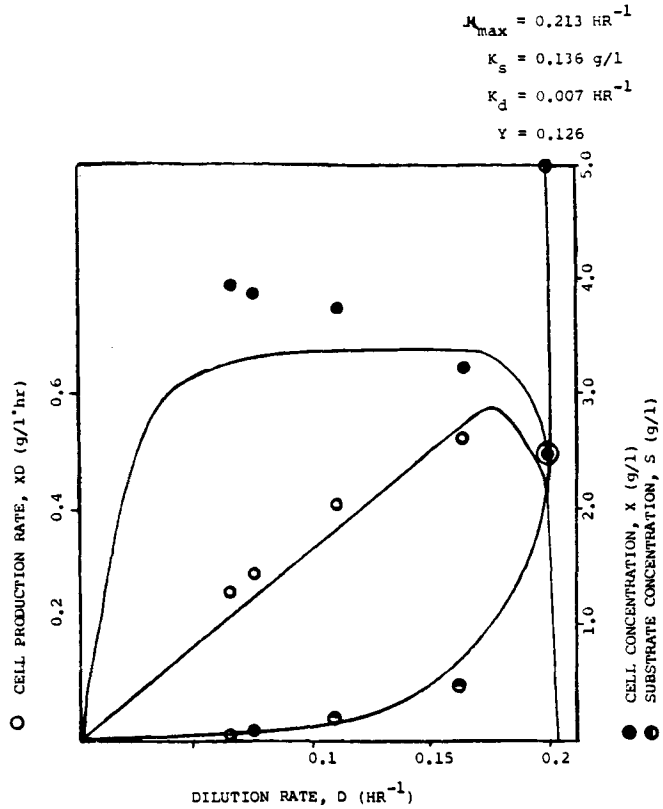

FIG. 4. Monod Model with death contribution.

TABLE VIII

Statistical Analysis for the Evaluation of Mathematical Models:

Model No.	Number of Parameters	Residual Sum of Squares T	Degree of Freedom D	Variance V^2
1.	3	0.72	2	0.36
2.	4	1.37	1	1.37

TABLE IX

Amino Acid Composition

Amino acids	Cell Protein	Soybean Cake
	mg a.a./g Nitrogen	
Isoleucine	157	302
Leucine	299	489
Lysine	279	380
Methionine	83.26	89
Cystine	–	104
Phenylalanine	–	313
Tyrosine	–	237
Threonine	181	267
Tryptophan	–	–
Valine	304	327
Arginine	42	443
Histidine	55	159
Alanine	466	284
Aspartic acid	707	719
Glutamic acid	661	1,157
Glycine	164	278
Proline	65	351
Serine	165	347

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