SINGLE-CELL PROTEIN FROM WASTE CELLULOSE

FINAL REPORT

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by

C. E. DUNLAP and C. D. CALLIHAN
Department of Chemical Engineering
Louisiana State University
Baton Rouge, Louisiana

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INTRODUCTION

The recycle, reuse, or reclamation of materials contained in solid or liquid wastes has now become an economic imperative due to the added impact of new legislation upon the already great demands of the environment. Cellulose, in its heterogeneity of forms, comprises the bulk of both the volume and weight of all municipal, industrial, and agricultural wastes. Already the fledgling refuse recycle industries are finding the fiber fraction of their segregated output hard to market and, indeed, hard to dispose of.

Four years ago our group at Louisiana State University (LSU) proposed to develop a process to convert waste cellulose to single-cell protein (SCP) by fermentation. We planned to give primary attention to agricultural waste fibers and to the optimization of the process for a critical economic and technical analysis.

This is the final report of our effort to define, operate, and evaluate the SCP-from-cellulose process. Several papers, articles, and reports have been generated during the course of the work and will be referred to in the body of this report.

This work has been generously supported over the past four years by grants from the Bureau of Solid Waste Management of EPA, the National Aeronautics and Space Administration and it's Mississippi Test Facility, the Rockefeller Foundation, the American and Florida Sugar Cane Leagues, and the LSU Research Foundation. We are deeply appreciative to these foundations and agencies for their interest.

SUBSTRATES

All materials which contain some cellulose as a component may be considered as possible substrates for the fermentation. Since this includes a great variety of materials, some parameters have been chosen to evaluate the technical and economic suitability of a cellulosic for use in the SCP fermentation. The availability of the material, the fraction of it that is cellulose, the cost, and the biodegradability, along with other factors such as toxicity and other possible uses, must be considered before deciding upon it as a suitable substrate.

AVAILABILITY

The availability of a cellulosic depends upon more than just the gross tonnage of the material produced. The distribution of the material, whether it is harvested and gathered as a by-product in an agricultural process, or collected as municipal refuse, or simply left where it is generated like grass or leaves, is of great economic importance in determining its availability. Many suitable substrates are very seasonal in that they may be generated in only one or two months of the year and, to be used for year-round SCP production, must be stored for the remainder of the year. Also, some very good substrates are produced in only small amounts, thus making it unprofitable to design an SCP system to accomodate them.

The largest volume of useable cellulose occurs as agricultural waste products. Some of these are already gathered and in sufficient amount to support the operation of an SCP plant. Sugarcane bagasse, rice hulls and straw, lumber and paper mill fibrous wastes, corn cobs, and bean vines are

examples of suitable agricultural wastes. However, most of these materials are seasonal--their generation occuring during the harvesting seasbn--and would have to be stored for the year-round supply of an SCP operation.

The cellulose gathered with municipal refuse is not seasonal; and since it represents from 50% to 55% of the dry weight of the refuse, is in sufficient amount to supply any number of SCP plants.

CELLULOSE CONTENT

All of the agricultural and municipal sources of cellulose are materials that contain a large fraction of noncellulosic components. Lignin and hemicelluloses in agricultural products, and plastics; ink; protein; lipids; and inorganic minerals in municipal wastes. The higher the cellulose content of the material, of course, the more efficient the fermentation will be. Table 1 lists the cellulose contents of several possible substrates [1].

TABLE 1. CELLULOSE CONTENT OF VARIOUS MATERIALS

Material	Cellulose, % dry, wt.
Filter paper	98+
Newsprint	85
Sugarcane Bagasse	51
Rice straw	34
Corn cobs	37
Prairie grass	37
Cotton linters	90
Cottonseed hulls	50
Paper, pulp - bagasse	96+

It can be seen that the SCP yield from a substrate like rice straw (34% cellulose) would be less than half as much as for a material like bagasse paper pulp (96+% cellulose). Thus, for a given production amount of SCP over twice the amount of rice hulls must be procured, transported, stored, and processed than paper pulp.

COST

Costs of the various materials suitable for use as SCP substrate are difficult to define on a general basis since the materials are usually wastes. If the material is a salable item like bagasse, definite sale prices are already fixed for it ($8 - $12 per ton dry weight); but they often vary widely with location, demand, available fuel substitute, etc. If the material has no fixed value, then the true cost will depend upon such factors as gathering or harvesting costs, transportation costs, storage and handling costs, replacement fuel value, and/or costs of production. Since these costs vary from country to country and with location within the country, it becomes necessary to carefully compute the true delivered cost for each material individually and reduce it to a basic value such as dollars per ton of dry cellulose. The example shown in Figure 1 illustrates how costs might be computed for excess bagasse in the United States. The same format might be used for any agricultural waste and for municipal refuse (although a negative value could be assigned in the case of refuse procurement).

BIODEGRADABILITY

Since the successful use of any cellulosic as a substrate for fermentation depends upon the ability of an organism(s) to break down and metabolize the cellulose fraction, the biodegradability of the material is of prime

Figure 1. Delivered Cost of Substrate

Material:	Sugarcane Bagasse
Cost of material at point of origin:	$3.00/ton
Percent moisture	50 percent
Percent cellulose on a dry basis:	57 percent
Gathering costs:	$0
Transportation costs:	$0.10/ton
Storage costs:	$1.35/ton
Replacement fuel costs:	$0.90/ton

Cost of material in dollars/ton dry cellulose:

$$\frac{3.00}{.50x.57} + \frac{0.10}{.50x.57} + \frac{1.35}{.50x.57} + \frac{0.90}{.50x.57} =$$

$10.53 + 0.35 + 4.74+3.16 = $18.78 per ton dry cellulose at SCP plant.

importance in the choice of a substrate. Since very few cellulosics can be degraded by microorganisms at economically feasible rates without some type of pretreatment, the response (in increased degradability) of the material to treatment must be determined. Table 2 gives a brief listing of the digestibilities of a few materials before and after a standard alkali pretreatment. Since the treatment of substrates has been covered in detail elsewhere [2-10] and will be discussed later in this report, no discussion will be given here except to stress the importance of this criteria in substrate evaluation.

OTHER FACTORS

Several other features of a cellulosic must be considered in its evaluation. Some agricultural cellulosics may have high pesticide and herbicide

TABLE 2. DIGESTIBILITIES OF TREATED AND UNTREATED MATERIALS

Material	Percent of Solids Digested[a]	
	Untreated Sample	Treated Sample
Filter paper	75	--
Bagasse pith	15-17	82
Bagasse, whole	7-10	80
Newsprint	40	79
Chipboard	5	81
Bagasse paper pulp[b]	--	90+
Rice hulls	15	64
Municipal refuse (Black-Clawson fiber fraction)	53	65

[a] Standard Substrate Evaluation Test

[b] Previously treated

residues; if these are persistant through the pretreatment, they will show up in the culture media with possible harmful consequences. Municipal refuse may contain high levels of heavy metals that could tend to limit culture growth and decrease productivities necessitating shut-down of the process and flushing of equipment.

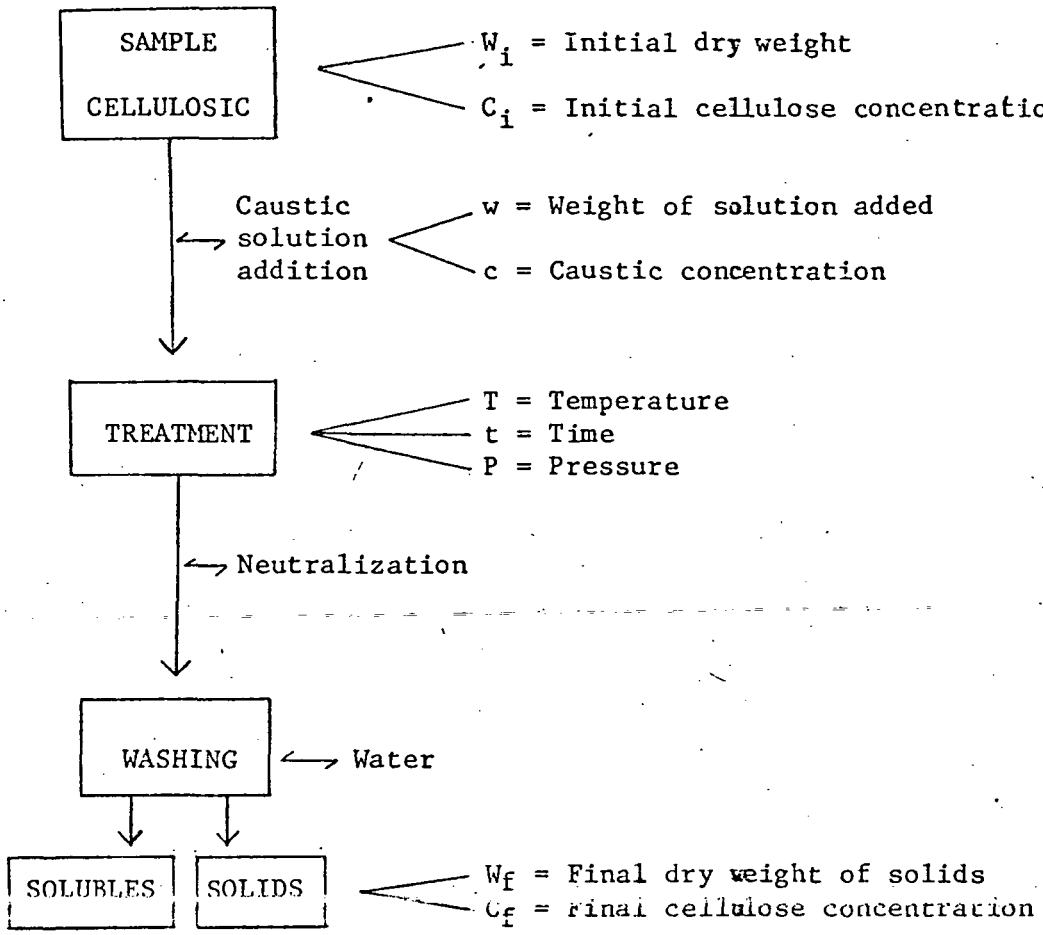
Other possible uses of the material should also be evaluated since it would be a disadvantage to be dependent upon the procurement of a material that either has, or will have, a high cost or high demand for a different use.

TREATMENT OF CELLULOSICS

Although a considerable amount of discussion has been previously presented concerning the theory of cellulosic pretreatment to increase the rate and extent of microbial digestion [2 to 10], it is important to review several aspects of the process. Native cellulosics such as grasses; straw; wood; etc. contain relatively large amounts of the heterocyclic polymer, lignin, which acts as a binder to maintain physical strength in the material as as a protective shield against microbial attack. Consequently, the native, untreated fibers are slow to digest and act as poor fermentation substrates.

To facilitate the digestion of these materials, it is necessary to disrupt the physical fine structure of the cellulose to allow more rapid and complete penetration of the material by water and the hydrolysing enzymes of the cellulase system. In our work, the application of sodium hydroxide in a liquid solution followed by heat and pressure serves to de-polymerize and disrupt the lignin physical structure while swelling the cellulose to increase water uptake and interstitial pore volume [11]. During the course of the treatment some of the lignin, hemi-celluloses, ash, and cellulose of the material is hydrolysed and solubilized. Also, some of the caustic apparently forms weak acid salts with some of the components of the material and cannot be titrated as free base at a neutral pH after the treatment.

The schematic of a general treatment process using a typical cellulosic, a caustic solution, and heat and pressure shows the various parameters used for the material balance around the process.



Evaluation Parameters:

$$Y_s = \frac{W_f}{W_i} = \text{Yield of solids through the treatment}$$

$$Y_c = \frac{Y_s C_f}{W_i C_i} = \text{Yield of cellulose through the treatment}$$

$$S/L = \frac{W_i}{w} = \text{Solids-to-liquid ratio used for treatment}$$

$$N = \frac{wc}{W_i} = \text{Caustic-to-solids ratio used for treatment}$$

and T, t, P, and N are always defined for a specific treatment.

EFFECTS OF TREATMENT ON SOLIDS

During the treatment process, some of the initially solid (insoluble) fractions of the cellulosic are solubilized by hydrolysis to soluble fragments. The hydrolytic action of the treatment process varies with the time, temperature, pressure, caustic concentration, and solids-to-liquid ratio. The degradation of a holocellulose in an alkaline environment has been well defined in the literature [11,12] and has shown rapid hydrolysis of the hemi-celluloses and lignin followed by the slower depolymerization of cellulose, and finally, degradation of the glucose ring structure. It follows then, that for a treatment of given severity, materials with higher hemicellulose and lignin fractions will lose respectively more of the initial solids than materials with lower hemicellulose and lignin levels. Values for yield of solids from the treatment for several different cellulosics subjected to treatment of identical severity, as expected, show that the samples with higher hemicellulose and lignin levels (bagasse pith, bagasse, rice hulls) lose more solids than the materials with higher cellulose fractions (the papers and chipboard).

Variation of the severity of the treatment by varying the time, temperature, pressure, caustic concentration, and solids-to-liquid ratio of the process also changes the amount of solids lost. Since our work has been primarily concerned with sugarcane bagasse, considerable definition of the yield of solids and yield of cellulose over the alkaline treatment has been obtained for this substrate.

Prior to any chemical treatment of bagasse, the solids were passed through a knife-cutter with a 1/8-in. screen. All treatment procedures were performed on this ground material. Yield tests for total insoluble solids and cellulose were run to define the effects of time of treatment (the time

that the material was subjected to the treatment temperature and pressure), temperature and pressure of treatment (these were not independent since saturated steam was used to generate both), caustic concentration, and the amount of caustic solution used per unit weight of dry solids.

The concentration of the caustic solution was varied in the range of from 1% to 8% by weight sodium hydroxide. Pressure ranged from 15 psig to 30 psig and was generated by saturated steam; therefore, the temperature ranged from 250°F to 275°F. The fraction of dry solids in the treatment slurry or sludge ranged from 0.05 to 0.50. Data from the treatment tests is shown in Table 3.

The solids yield varied directly with the solids-to-liquid ratio. Figure 2 shows a typical yield response in a 30-psi, 4-hour treatment with varying solids-to-liquid ratios and for different caustic concentrations.

We then attempted to define whether the amount of liquid per unit solids or the amount of caustic per unit solids (N) controlled the yield. Figure 3 shows that for a treatment at 30 psi, 4 hours, the yield of solids can be correlated very well with the amount of caustic used. The yield of cellulose is also shown to be correlated to the amount of caustic.

Figures 4 and 5 show solids yield (Y_s) as a function of amount of caustic per unit of solids (N) for different treatment pressures and times. The same Y_s and Y_c response to changes in N are seen for the different treatment severities. Figure 6 shows a compilation of the three Y_s versus N curves at different levels of treatment severity. Qualitatively, Y_s behaves as expected--becoming smaller as treatment severity is increased--but it is apparent that N is the controlling independent variable.

To determine the effects of the other two independent treatment variables--pressure (P) and time of treatment (t)--on solids yield, the yield was

TABLE 3. TREATMENT TESTS ON WHOLE, GROUND SUGARCANE BAGASSE

TREATMENT CONDITIONS									
Percent NaOH Conc.[a]	Solids to Liquid Ratio	Caustic to Solids Ratio	Time (Hrs.)	Pressure (psig)[b]	Solids Yield (Y_s)	Percent Cellulose	Cellulose Yield (Y_c)	Fraction Digested[c] (X_s)	(X_{os})[d]
0	0.1	--	Ambient	--	88.8	56.3	100	12.7	11.3
0	0.1	--	Ambient	--	89.0	57.0	100	7.0	6.2
1	0.1	.09	4	30	59.6	80.4	94	68.8	41.0
1	0.1	.09	4	30	54.5	72.4	77	75.6	41.0
1	0.1	.09	0.25	15	67.2	84.3	100+	62.1	41.7
1	0.1	.09	17	30	52.8	89.1	92	--	--
1	0.1	.09	0.25	30	67.6	--	--	56.8	38.4
1	0.1	.09	4	15	61.6	--	--	72.4	44.6
1	0.2	.04	4	30	71.2	69.5	97	56.5	40.2
1	0.3	.023	4	30	83.2	67.8	100+	22.0	18.3
1	0.4	.015	4	30	85.2	62.0	100+	20.3	17.3
4	0.1	.36	4	30	47.2	97.4	90	91.3	43.0
4	0.2	.16	4	30	56.0	90.3	99	82.6	46.2
4	0.3	.093	4	30	67.2	80.1	100+	70.0	47.0
4	0.4	.06	4	30	73.2	68.8	99	64.7	47.3

TABLE 3. TREATMENT TESTS ON WHOLE, GROUND SUGARCANE BAGASSE (Cont.)

TREATMENT CONDITIONS									
Percent NaOH Conc.[a]	Solids to Liquid Ratio	Caustic to Solids Ratio	Time (Hrs.)	Pressure (psig)[b]	Solids Yield (Y_s)	Percent Cellulose	Cellulose Yield (Y_c)	Fraction Digested[c] (X_s)	Fraction Digested[c] (X_{os})[d]
4	0.33	.081	0.25	15	72.0	75.2	100+	56.2	40.5
4	0.1	.36	0.25	15	49.6	96.8	94	80.3	39.8
4	0.33	.081	4	30	64.6	74.0	94	70.3	45.4
4	0.1	.36	4	30	47.0	95.5	88	95.1	44.7
4	0.33	.081	17	30	64.7	76.4	97	71.4	46.2
4	0.1	.36	17	30	47.6	95.0	89	96.6	46.0
4	0.1	.36	0.25	15	48.0	--	--	--	--
4	0.33	.081	4	30	65.0	--	--	--	--
4	0.33	.081	17	30	57.0	--	--	--	--
4	0.1	.36	0.25	30	51.6	--	--	79.3	40.9
4	0.1	.36	0.5	30	47.6	--	--	84.7	40.3
4	0.1	.36	1	30	44.8	--	--	89.1	40.0
4	0.1	.36	2	30	48.0	--	--	92.0	44.0
4	0.1	.36	3	30	47.2	--	--	88.2	41.6
4	0.1	.36	4	30	44.4	--	--	93.1	41.3

TABLE 3. TREATMENT TESTS ON WHOLE, GROUND SUGARCANE BAGASSE (Cont.)

TREATMENT CONDITIONS									
Percent NaOH Conc.[a]	Solids to Liquid Ratio	Caustic to Solids Ratio	Time (Hrs.)	Pressure (psig)[b]	Solids Yield (Y_s)	Percent Cellulose	Cellulose Yield (Y_c)	Fraction Digested[c] (X_s)	Fraction Digested[c] (X_{os})[d]
4	0.1	.36	0.25	15	53.6	--	--	75.5	40.5
4	0.1	.36	0.5	15	49.2	--	--	82.3	40.5
4	0.1	.36	1	15	49.2	--	--	84.6	41.6
4	0.1	.36	4	15	47.6	--	--	91.1	43.4
8	0.1	.72	4	30	36.8	100	72	94.6	34.8
8	0.3	.186	4	30	48.0	92.9	87	93.0	44.6

[a] Weight percent sodium hydroxide

[b] PSIG saturated steam

[c] Fraction of substrate digested in standard, substrate limited, 48-hr. test by mixed culture of *Cellulomonas* sp. and yeast Ycl3; corrected to a standard filter paper digestibility of 75%.

[d] Fraction of original substrate (before treatment) digested after a treatment process by procedure of [c].

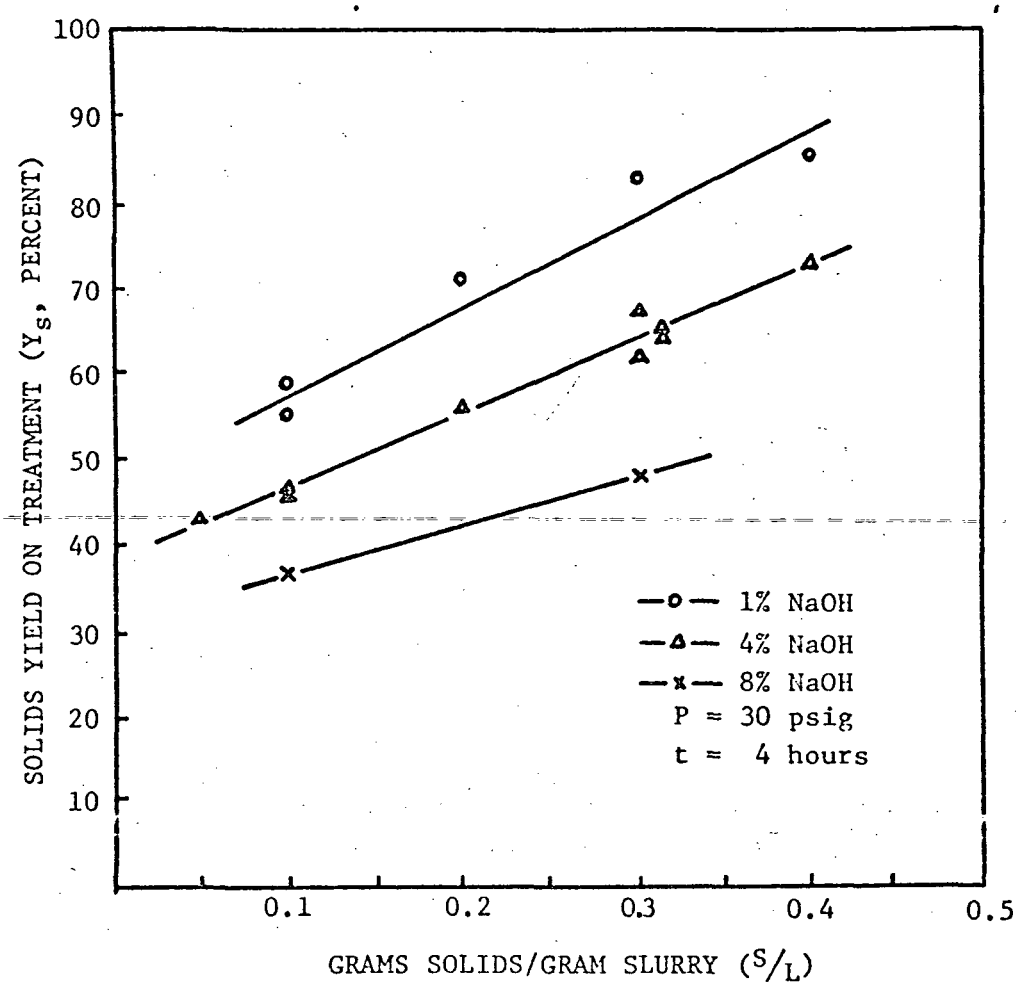


Figure 2. Effect of Treatment Variables on Yield of Solids

plotted versus time for two different pressures. Figure 7 shows that after about 30 minutes the yield is not effected by the time of treatment, and there was no difference between the yields of samples run at 15 and 30 psig. The caustic-to-solids ratio (N) was the same for all runs.

As stated previously, the kinetics of the alkaline hydrolysis of bagasse favors the preferential removal of lignin and hemicellulose. This should result in the effective concentration of the insoluble cellulose in

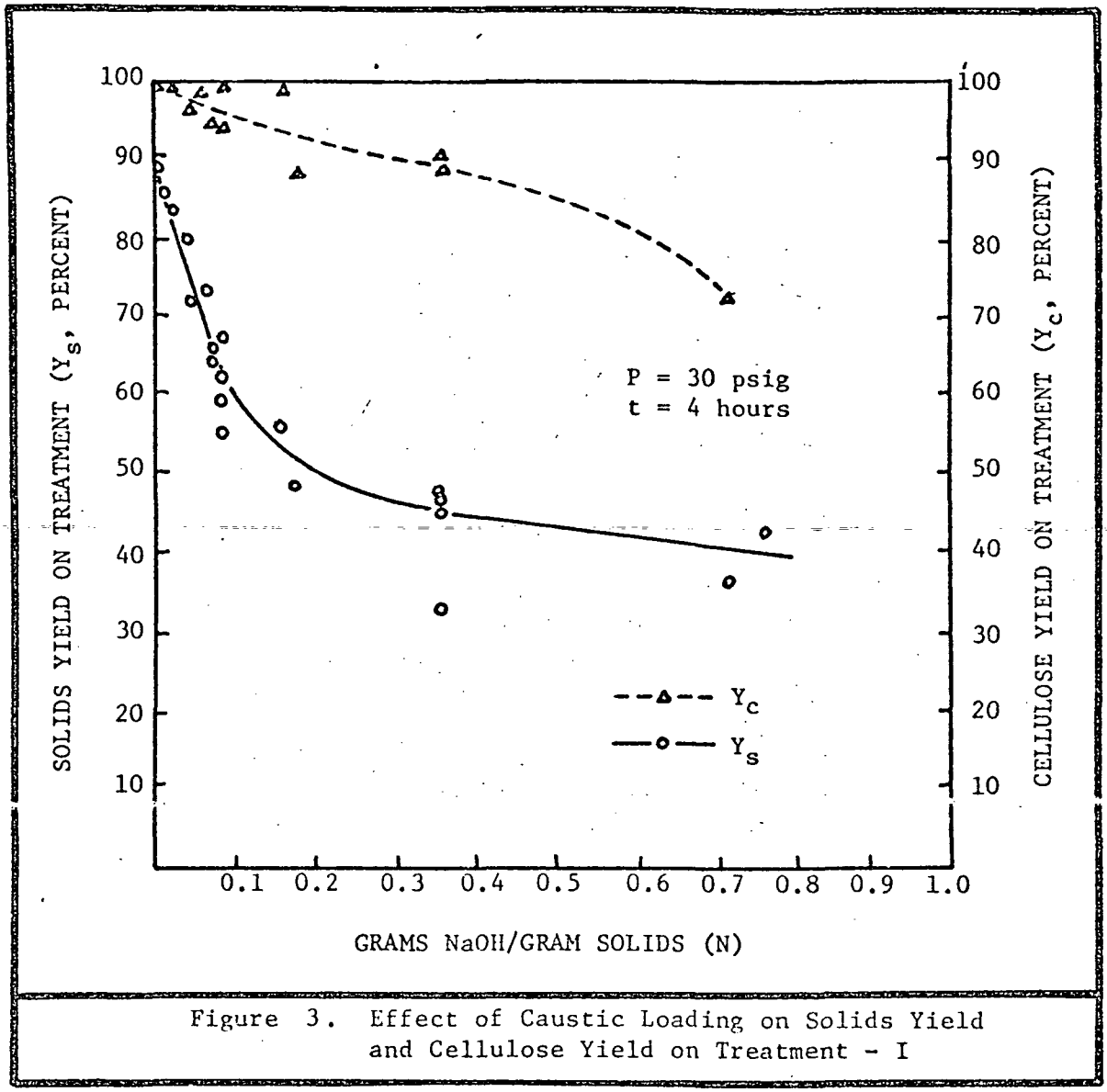


Figure 3. Effect of Caustic Loading on Solids Yield and Cellulose Yield on Treatment - I

the remaining solids. A plot of Y_s versus the percent cellulose in the solids remaining after treatment (Figure 8) shows that the fraction of cellulose in the remaining solids increases as Y_s decreases; but also, that progressively more of the original cellulose is lost. The magnitude of this loss over the range of treatment tested is evidently quite small.

Since the organisms metabolize cellulose, the fraction of the substrate that is cellulose will have an effect on the yield of cells on substrate.

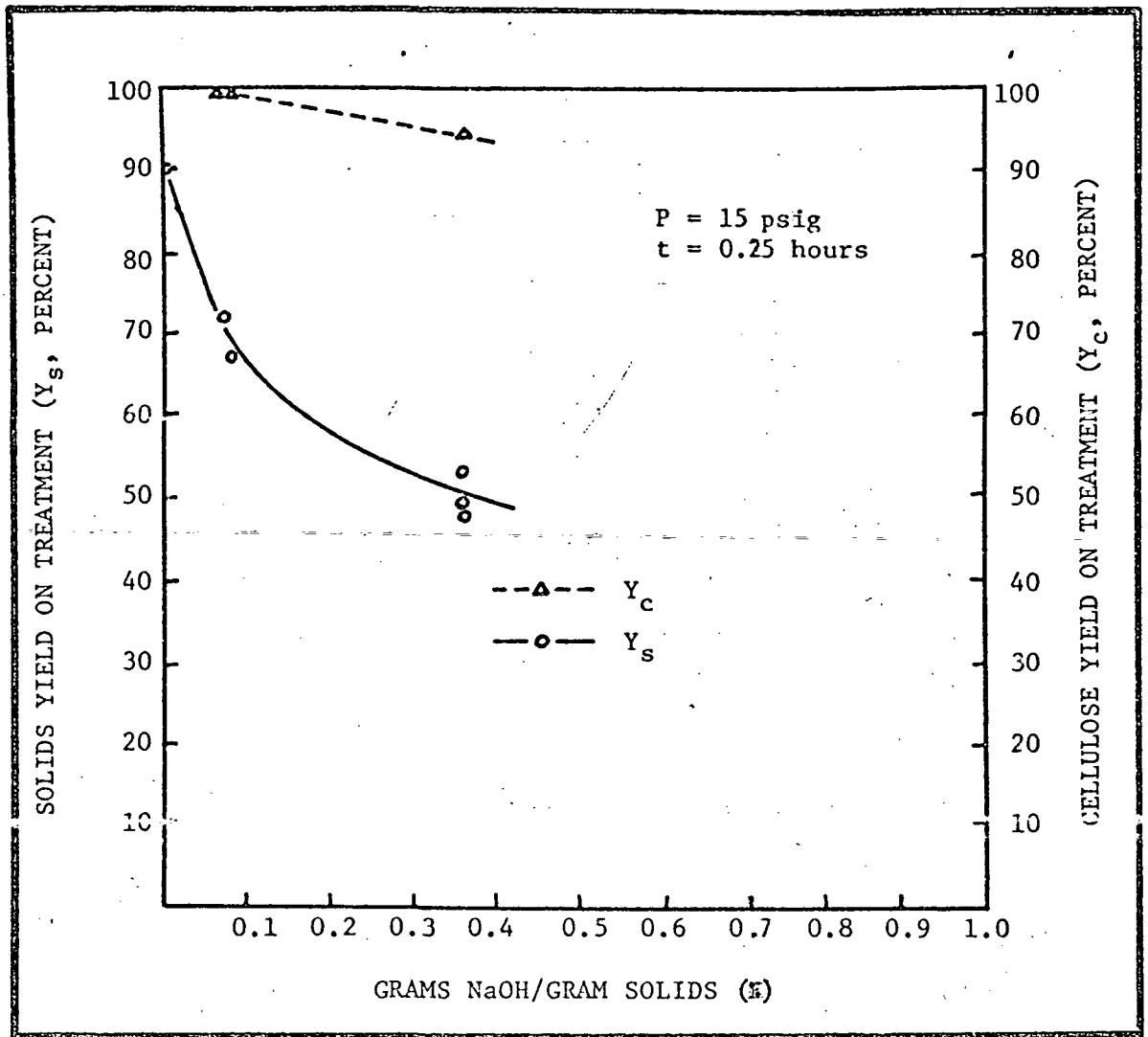


Figure 4. Effect of Caustic Loading on Solids Yield and Cellulose Yield on Treatment - II

The cell yield on pure cellulose is about 0.5 gm cells per grams of cellulose metabolized. If the treated bagasse has a very high cellulose content (95% - 100%), the cell yield on solids (Y) will be close to 0.50; on the other hand, as the fraction of cellulose in the treated solids decreases (with higher solids yields over the treatment process), the cell yield on solids should decrease. (i.e., at 75% cellulose in solids: $Y = (0.75)(0.50) = 0.375 \frac{\text{gm cells}}{\text{gm solids used}}$).

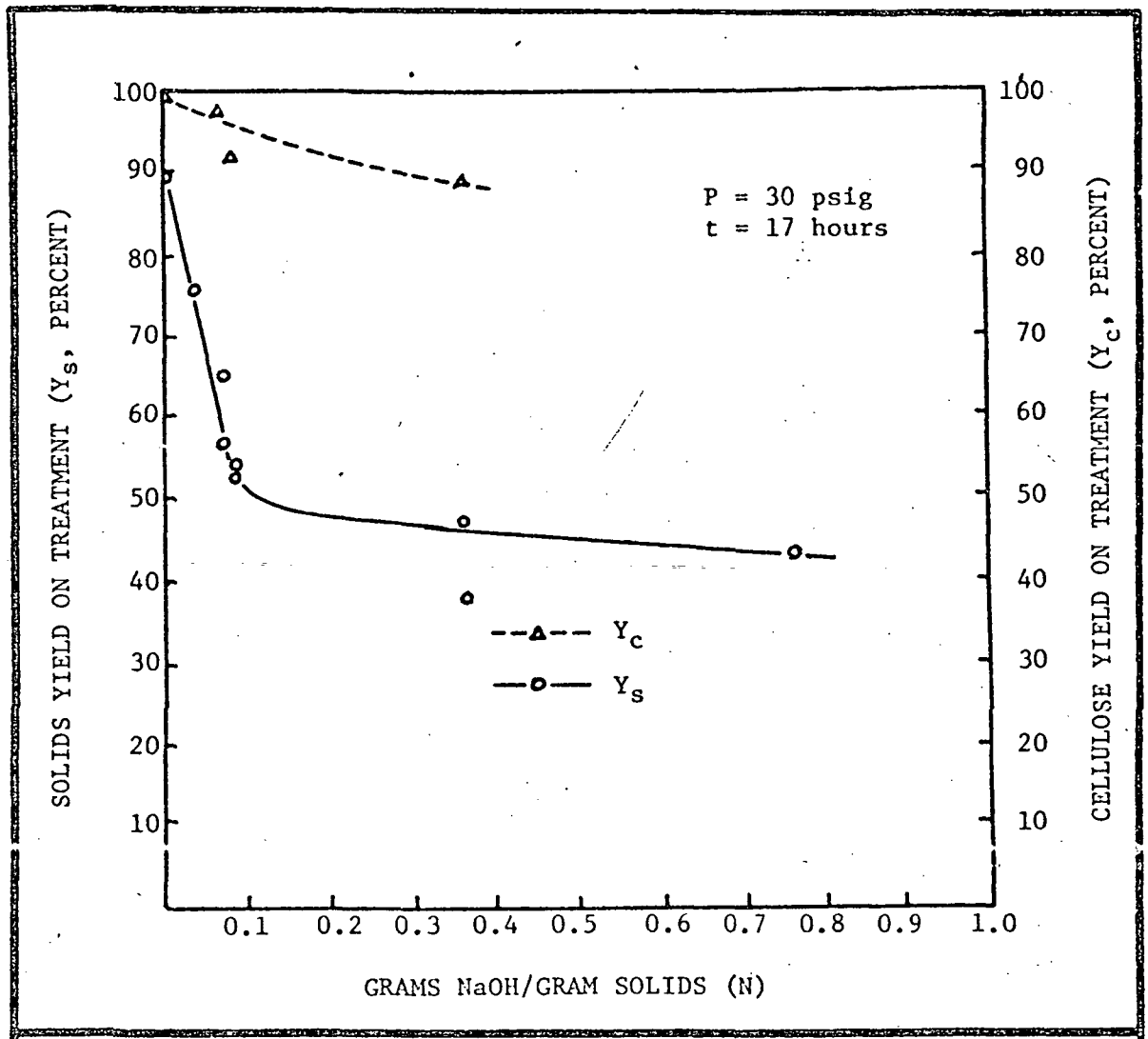


Figure 5. Effect of Caustic Loading on Solids Yield and Cellulose Yield on Treatment - III

CAUSTIC NEUTRALIZATION

After bagasse has been subjected to an alkali treatment, only a fraction of the sodium hydroxide that was initially added to the solids can be titrated with acid to neutrality. Different amounts of caustic were added to samples of bagasse, newsprint, and filter paper prior to a 30-psi saturated steam treatment for four hours. After the treatment, water was added to each sample and concentrated HCl used to titrate the sample back to pH 7.0 Figure 9

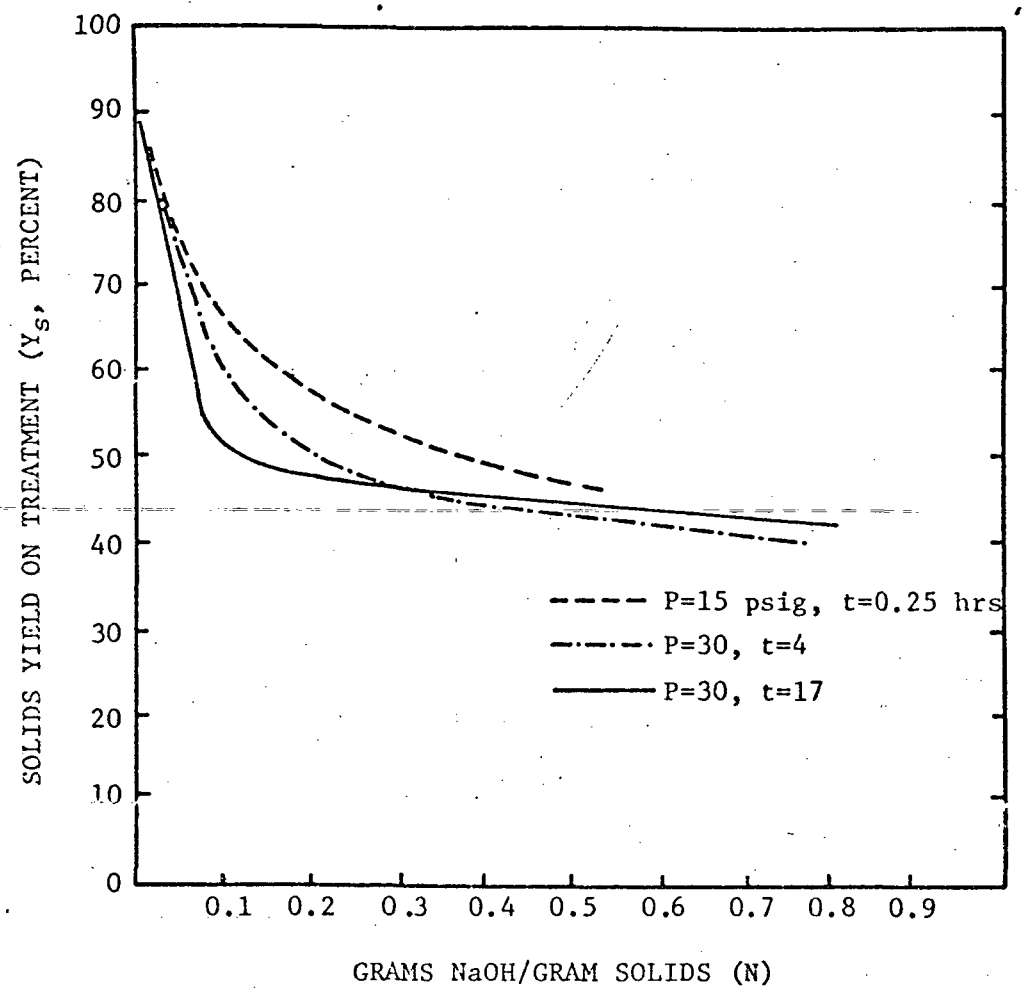


Figure 6. Comparison of N vs Y_S Curves for Different Treatment Severities

shows that when all three of the materials were completely neutralized, roughly 4% to 5% caustic based on the weight of dry solids was not titratable at 7.0 pH. This was expected for the bagasse since the hydrolysis of lignin and hemicelluloses would provide many weak acids for the production of sodium --weak acid salts that would be untitratable at pH 7.0, but could not be satisfactorily explained in this manner for the filter paper and newsprint. Since filter paper is almost pure cellulose and newsprint has a higher

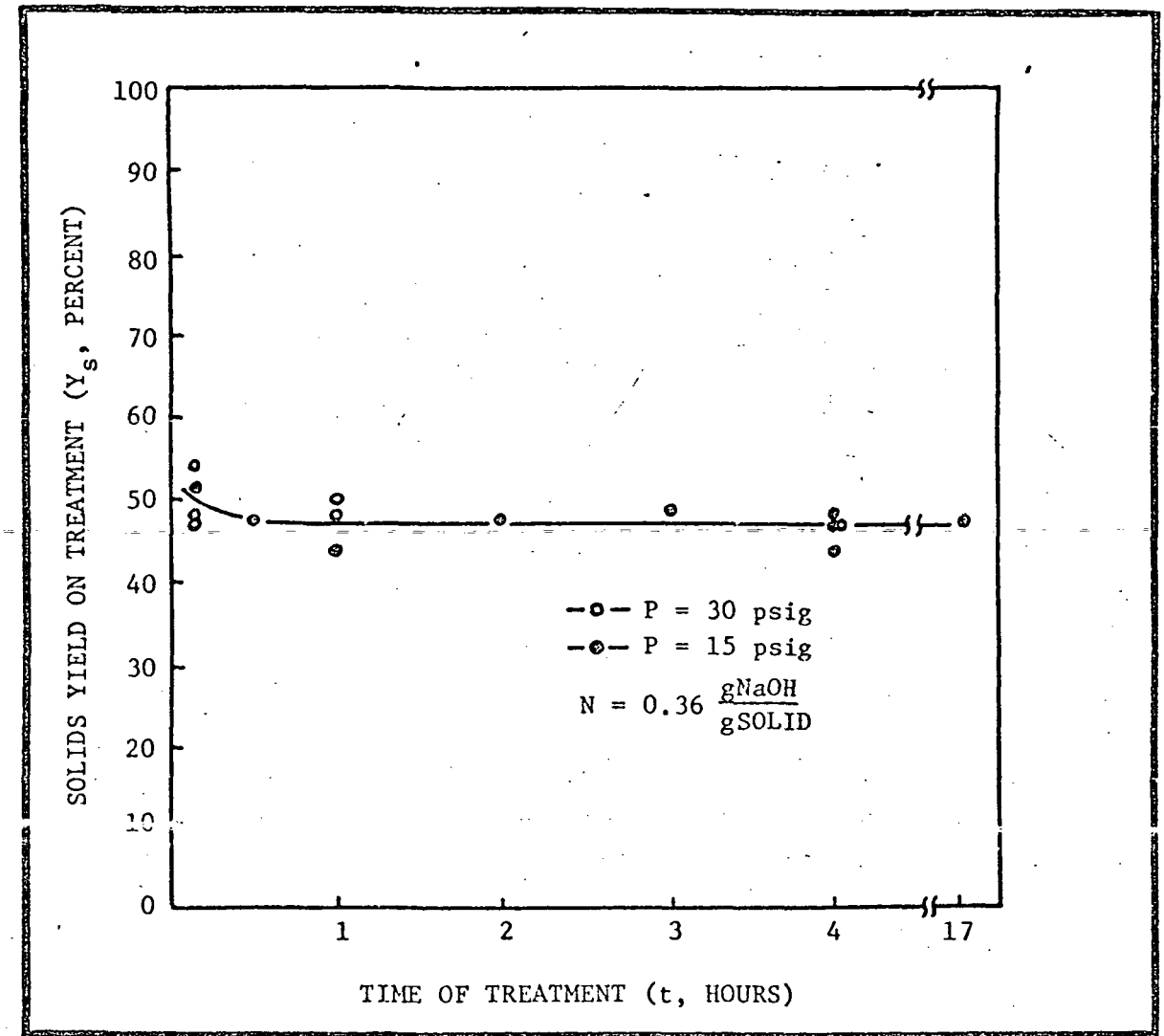


Figure 7. Effect of Treatment Time on Solids Yield

cellulose content than bagasse, it is probable that at the alkali concentrations and temperatures used for the treatment, some of the cellulose is hydrolysed to glucose which, under these conditions, degrades to dihydroxyacetone, glyceraldehyde, glycolic aldehyde, formaldehyde, and to various saccharinic acids. These weak acids certainly would contribute to the formation of sodium salts untitratable at a neutral pH. No attempt has been made to see if the amount of sodium hydroxide bound at pH 7.0 is effected by varying treatment severity or caustic concentration.

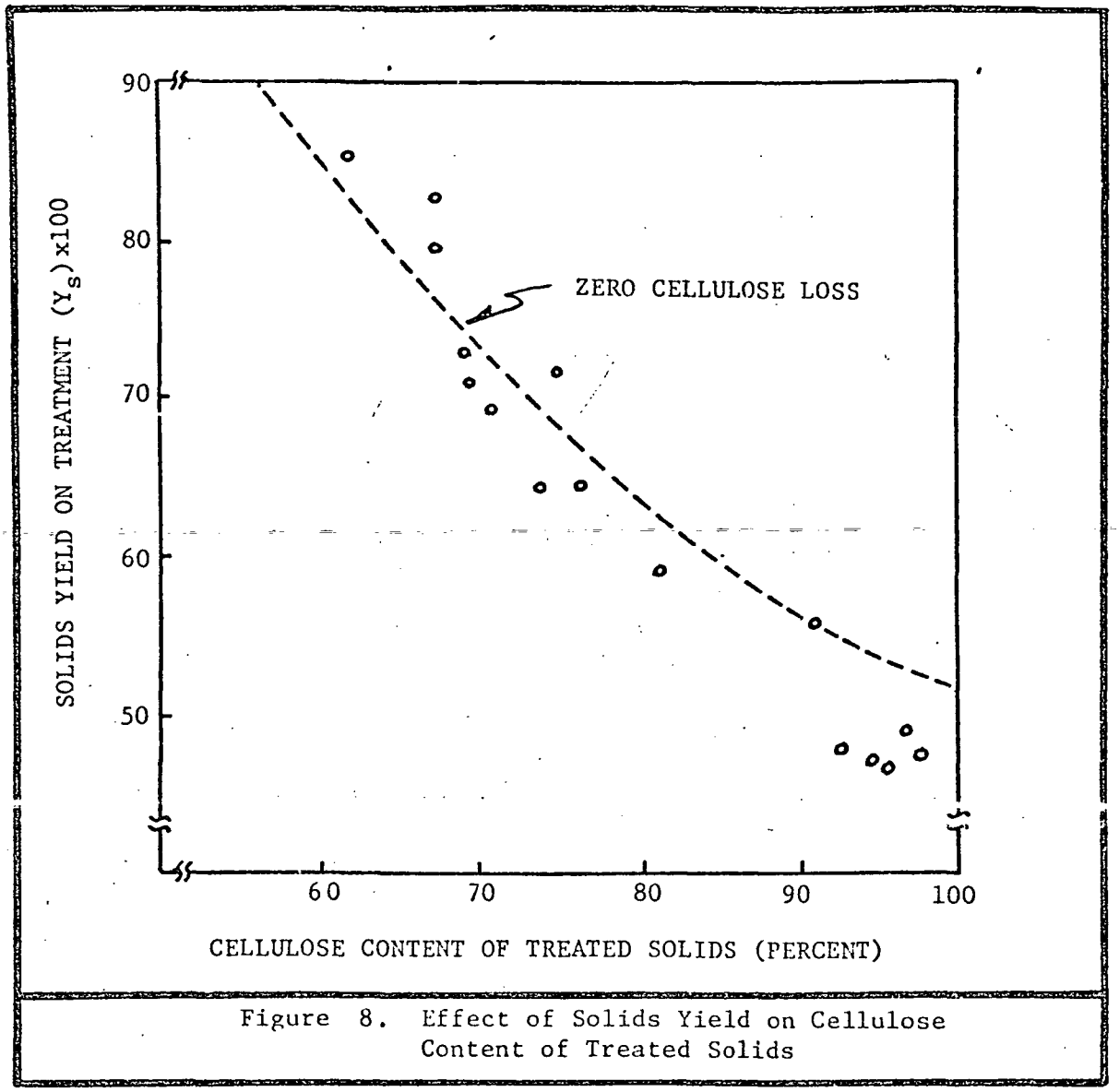


Figure 8. Effect of Solids Yield on Cellulose Content of Treated Solids

Titration of all samples was done automatically at 25°C. The time necessary to neutralize the treated bagasse was almost 24 hours, while filter paper and newsprint reached pH 7.0 within one hour.

EVALUATION OF THE DIGESTIBILITY

The purpose of the treatment step is to increase the biodegradability of the cellulose fraction of bagasse. The effects of the various treatment variables on the digestibility of the bagasse solids were defined in shake

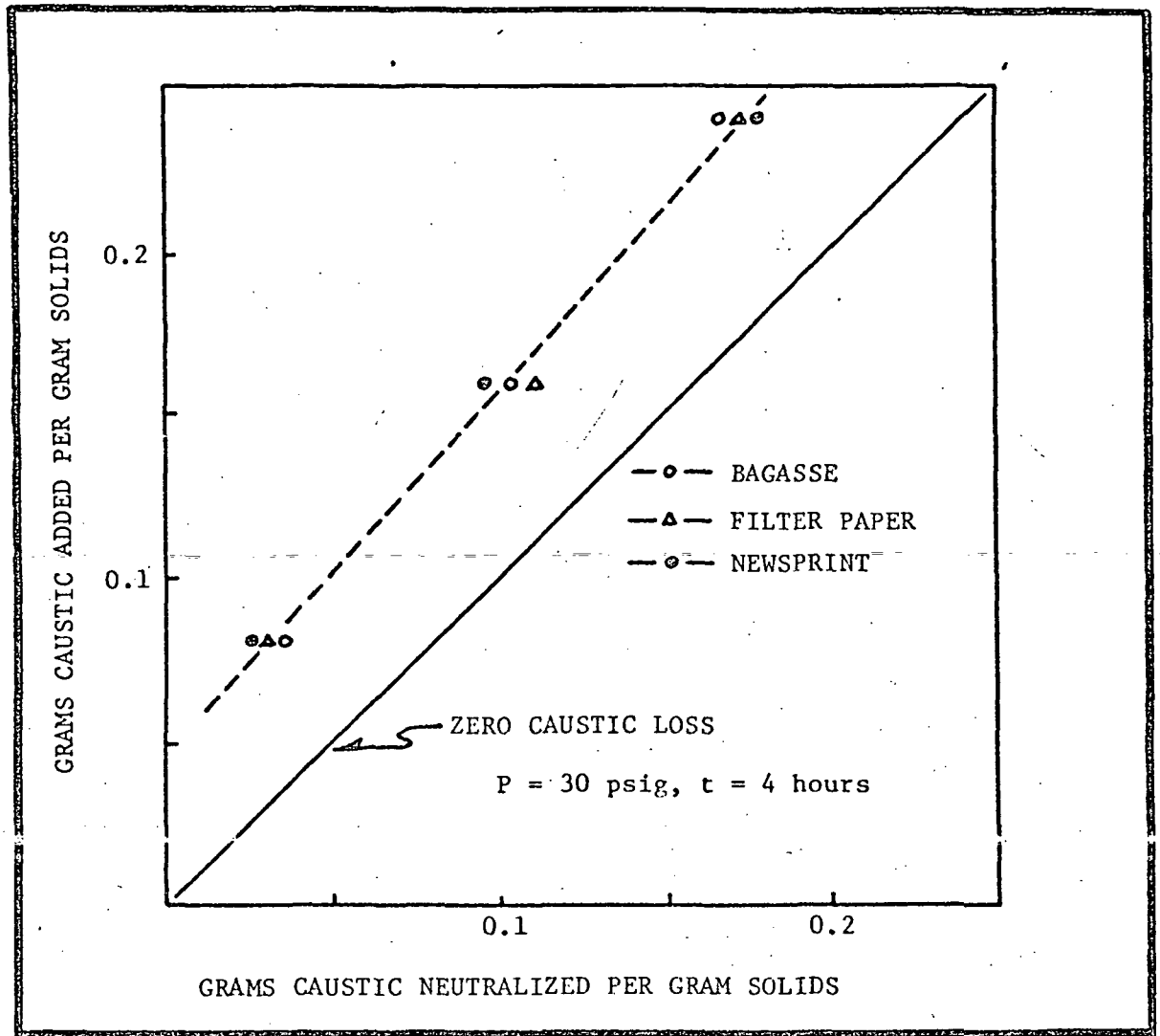


Figure 9. Loss of Caustic During Treatment

flask cultures using the standard Substrate Test Procedure given in the Appendix.

A substrate loading of 4.0 grams of solids per liter was chosen to assure substrate limitation within 48 hours, and to minimize effects of pH and media changes. The inoculum used was prepared according to the standard procedure given in the section on organisms and inoculum preparation. The amount of substrate lost after 48 hours was determined gravimetrically, and

the amount of digestion was reported as substrate consumed per unit of substrate initially added; i.e.,

$$X_s = \frac{S_o - S_f}{S_o}$$

where: S_o = Initial substrate concentration

S_f = Final substrate concentration (after 48 hours)

X_s = Fraction of substrate solubilized (consumed by the cells)

This data was reported in Table 3 with the data obtained for the treatment process. Also reported in that table is X_{os} where: $X_{os} = X_s Y_s$ and represents the fraction of the original substrate (prior to treatment) that was digested after a treatment process had been applied.

To correlate the values for digestibility of solids (X_s) with the three independent treatment variables P, N, and t, the general partial differential equation for the process was defined as:

$$dX_s = \frac{\partial X_s}{\partial P}_{t,N} dP + \frac{\partial X_s}{\partial t}_{P,N} dt + \frac{\partial X_s}{\partial N}_{t,P} dN$$

The functions of the partial derivatives were then presented graphically in Figures 10 (X_s versus P at constant t and N), 11 (X_s versus t at constant P and N), and 12 (X_s versus N at constant P and t).

Figure 10 shows that the digestibility is only a small function of the pressure (or temperature) of the treatment. It should be pointed out, however, that only two values of P were used; and these covered only a small range of relatively low pressure.

Digestibility is a rather strong function of the time of treatment from 15 minutes to about one to two hours, but a very weak function thereafter

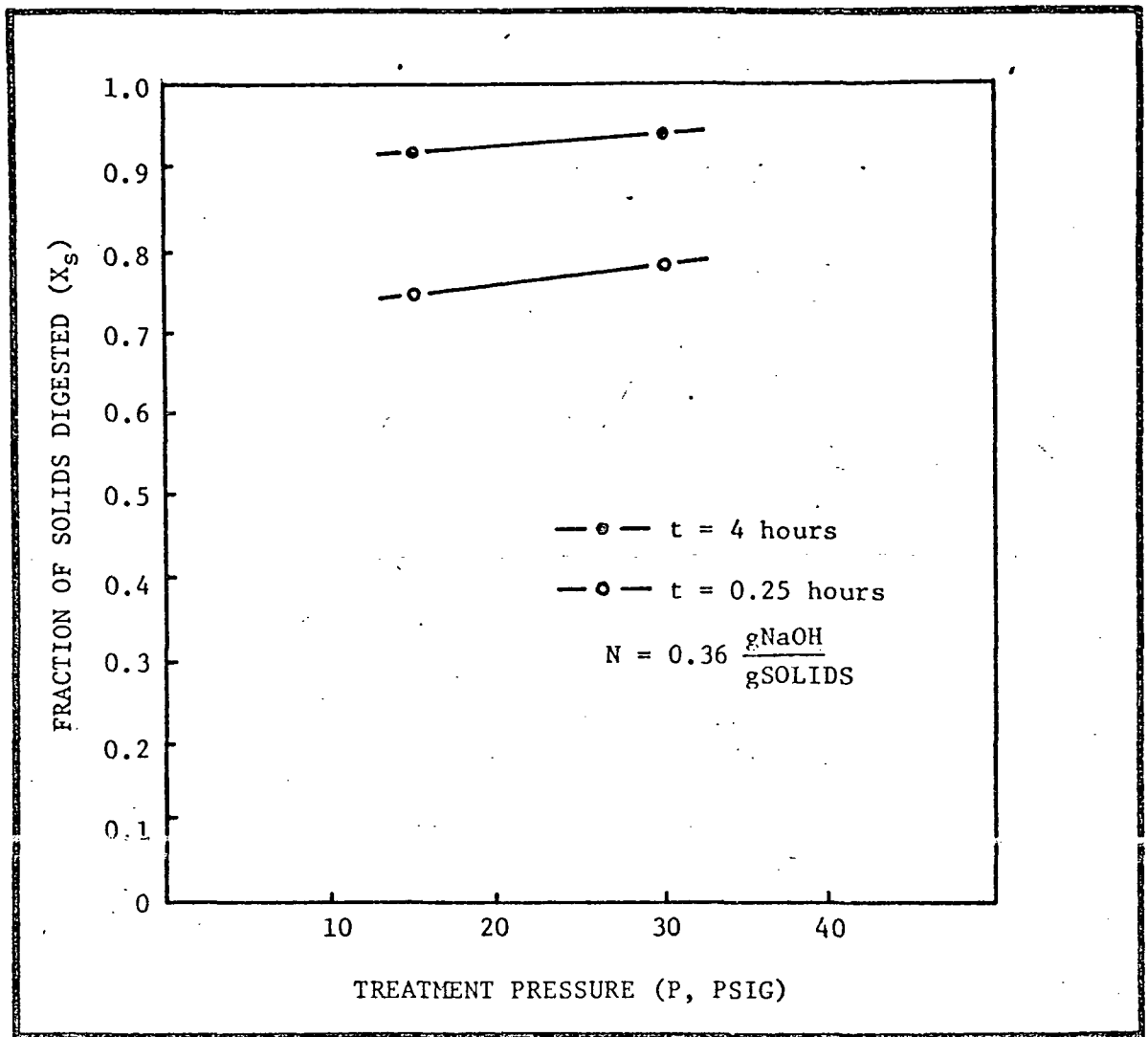


Figure 10. Effect of Treatment Pressure on Substrate Digestibility

(Figure 11). This plot suggests that any treatment should have a definite maximum time beyond which no further increase in digestibility occurs; but that up until that time is reached, digestibility is time dependent.

Figure 12 shows that N is the strongest independent variable within the ranges tested. Digestibility increases with N until some limit is reached above which X_s remains constant. This "saturation point" for bagasse seemed to be about 0.3 gm NaOH per gram of solids. The shape of the curve shows a

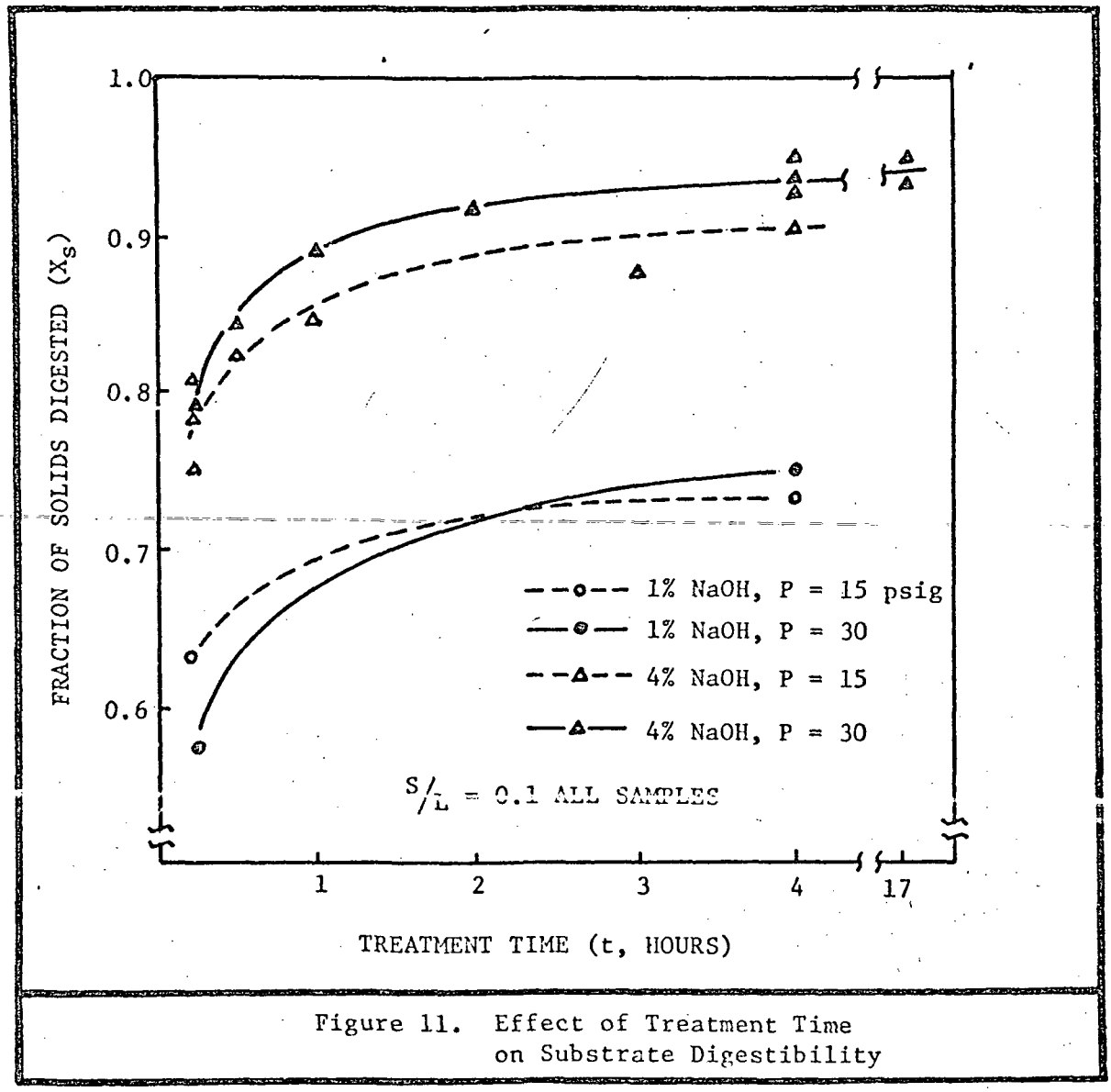


Figure 11. Effect of Treatment Time on Substrate Digestibility

very strong initial effect of NaOH level followed by an incrementally decreasing effect as N increases up to the "saturation point".

Due to the similarities in the effects of the independent variables P, N, and t on both Y_s and X_s, a plot of X_s versus Y_s was prepared (Figure 13). The graph shows an increase in X_s with decreasing yield of solids. The yield of solids from bagasse seemed to have a definite lower limit of from 45% to 50%. This value corresponds rather well with the value obtained for

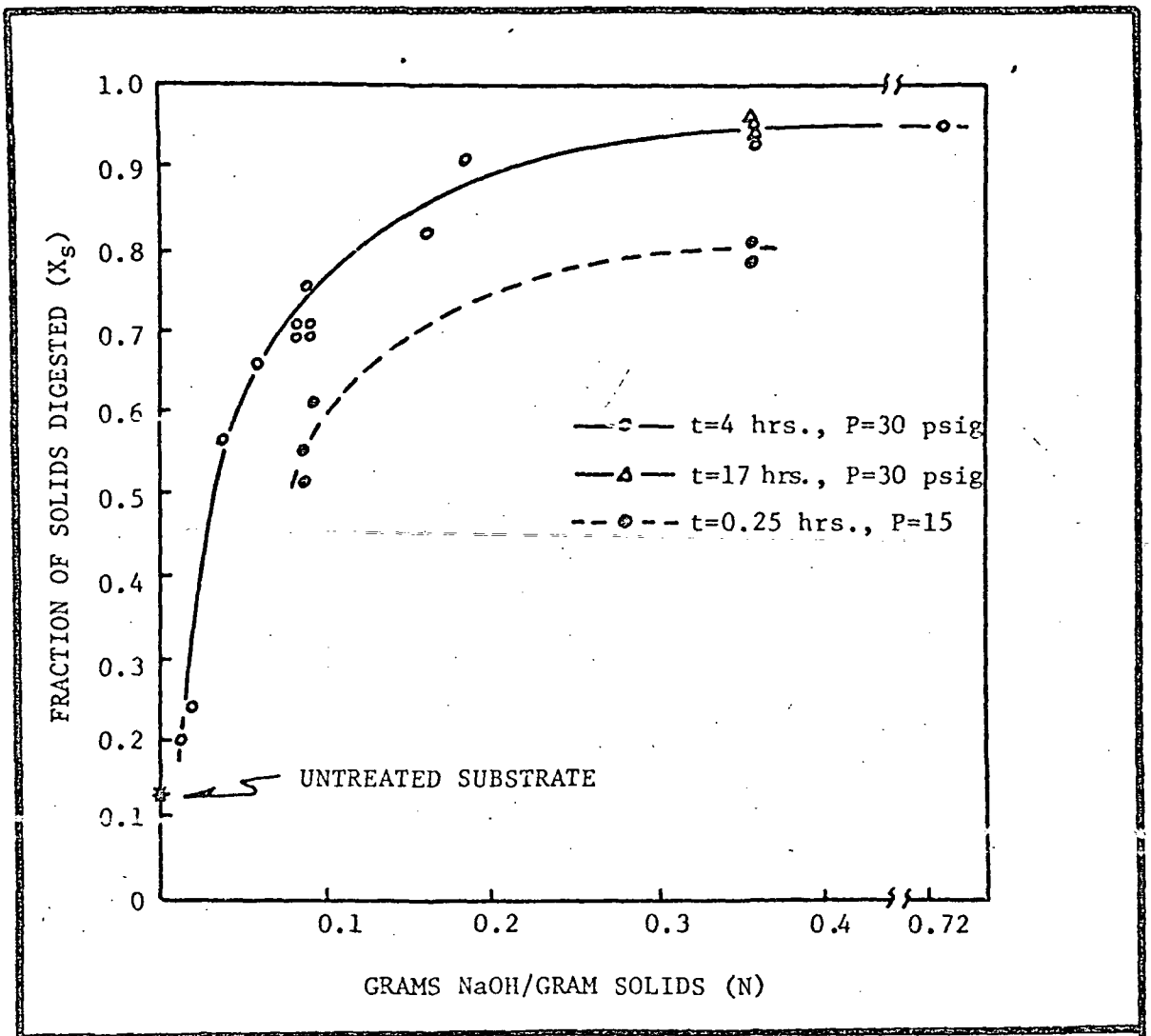


Figure 12. Effect of Caustic Loading on Substrate Digestibility

the percentage of crystalline cellulose present in a bagasse sample by x-ray diffraction studies--42.5% [1]. This lower limit probably reflects the alpha-cellulose content of the sample which is not solubilized by the treatment process. Cellulose concentration data from Figure 8 show the same relationship. The digestibility of this fraction continues to increase, however, as the treatment increases in severity and the cellulose continues to swell and free itself from lignin.

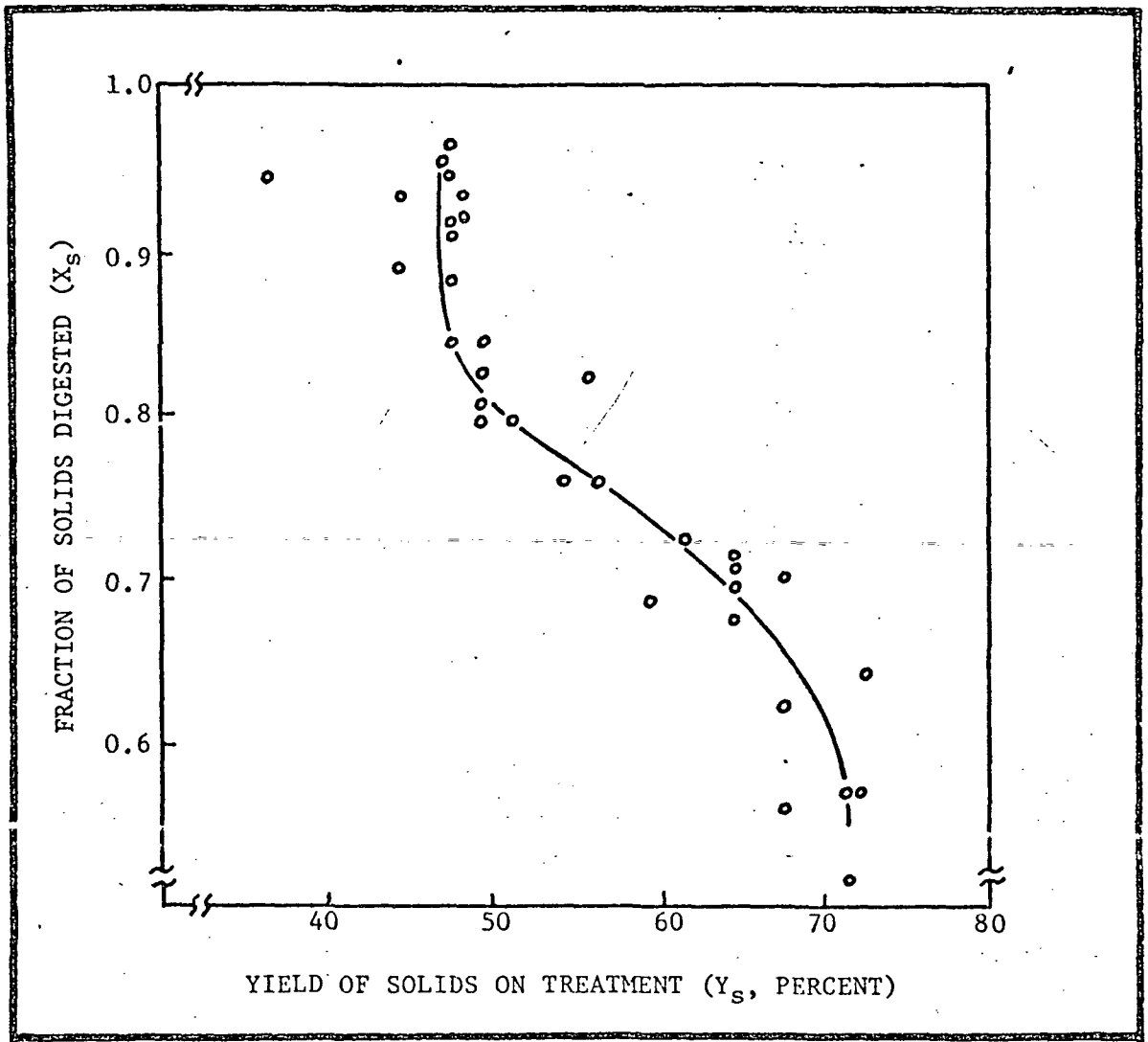


Figure 13. Correlation of Solids Yield and Fraction of Solids Digestible

An unexpected correlation of X_{os} (the fraction of original substrate prior to treatment that was digested after a treatment process had been applied) versus Y_s is seen in Figure 14. This plot shows that below a solids yield of about 75%, the severity of the treatment as measured by the yield of solids has very little effect on the amount of the substrate (measured prior to the treatment) that will be digestible. Also, between Y_s values of from 45% to 75%, the value of X_{os} remains in a relatively narrow band of 40% to

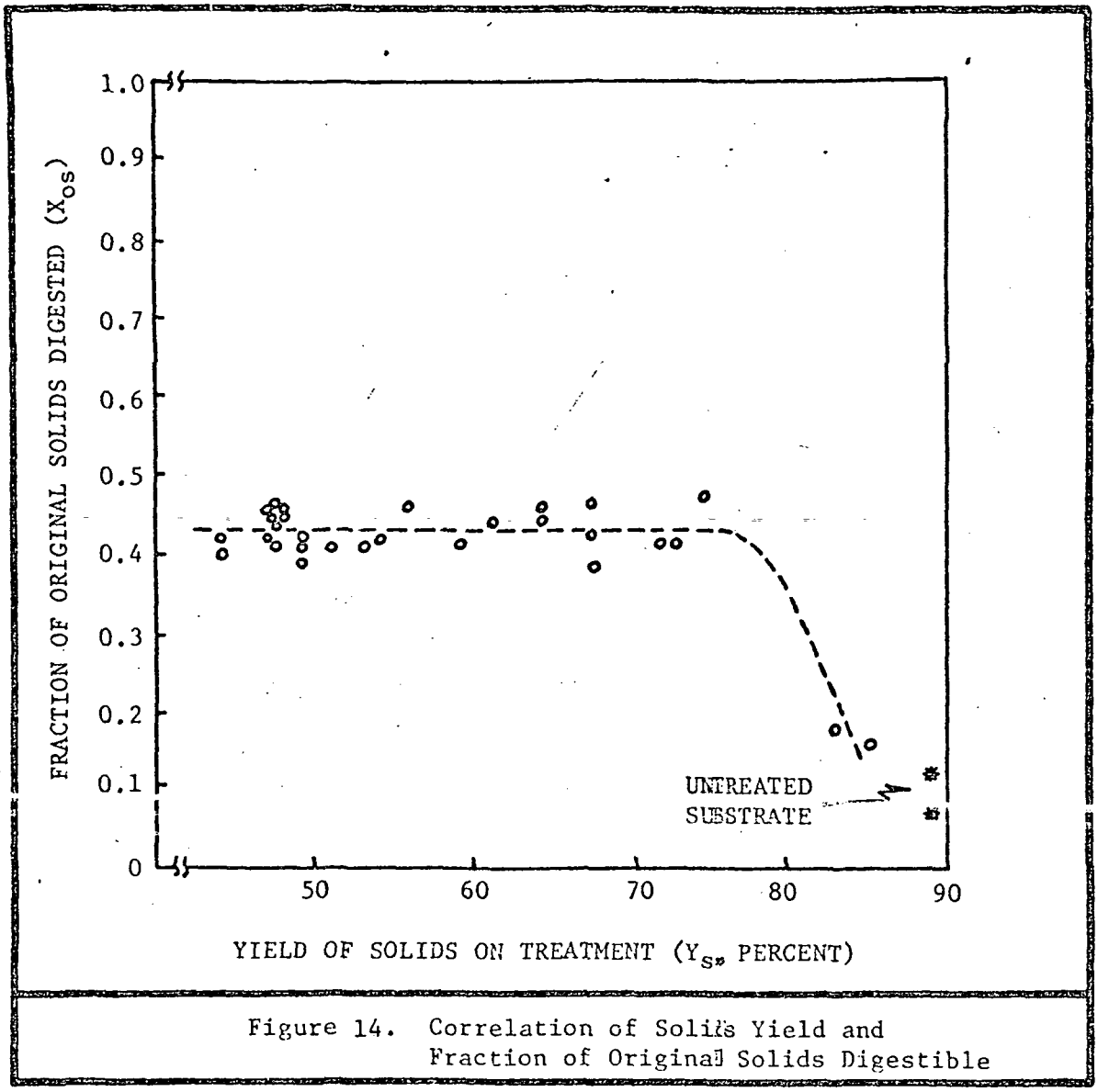


Figure 14. Correlation of Solids Yield and Fraction of Original Solids Digestible

45%. This indicates that after some minimum treatment, a relatively constant fraction of the original material is available for digestion and that this fraction remains about the same irrespective of the amount of other (undigestible) material that is removed from around it. Therefore, the severity of the treatment process, above a certain minimum, seems only to effect the relative amount of undigestible material that accompanies the digestible fraction through the fermentation.

It should be recognized that no measure of culture growth rate is made in the shake-flask tests and that this is an important parameter which may, indeed, be effected by treatment severity.

FEED CONDITIONING

After bagasse has been subjected to a treatment process, it is ready to be prepared for inclusion into the fermentation media. Sometimes the treated solids were washed prior to use, and sometimes they were simply neutralized and used without washing. The washing procedure included repeated slurrying with fresh water followed by dewatering by draining on a screen or by squeezing in a cloth bag. The washing resulted in removal of almost all of the soluble fraction and the residual caustic. Most of the dark-brown lignin was washed out, leaving a much brighter material than the original, untreated fiber. Most of the fermentation tests and kinetic studies were performed on this washed material.

If the material was mixed into the media with no washing, the media had to be neutralized with acid prior to use. The media retained a dark-brown color throughout the run, and residual sodium hydroxide had to be neutralized by acid addition throughout the first 10 to 18 hours of the run. The cell product obtained from a run using unwashed bagasse also had a brown coloration.

The composition of the material that is solubilized in the treatment process has not been quantitatively determined; however, the initial holocellulose fractions of lignin (7% - 15%) and hemicellulose (20% - 30%) are preferentially removed from the material, and in the alkaline environment are probably depolymerized and degraded to some extent. Very little free glucose can be found in the soluble fraction, but a positive test for

carbohydrates can be obtained by a phenol-sulfuric acid procedure. Apparently about 15 % of the soluble materials can be degraded and metabolized by the cells.

STERILIZATION

When the substrate fiber is mixed with the nutrient media, the entire media is subjected to a steam sterilization at 15 psig for one hour (in the 500-liter vessel) or at 30 psig for one hour (in the 7- and 14-liter vessels).

When continuous operation of the unit is desired, two different methods of sterilization and feeding are used in the 14-liter and 500-liter fermenters. For the 14-liter vessel, a carboy with a liquid solution of all nutrients except the solid substrate is autoclaved for one hour at 30 psig. This liquid media is then pumped into the vessel continuously at the desired dilution rate. The solid substrate is divided into predetermined portions in separate beakers which are covered with foil and autoclaved. The solids are then fed manually into the culture at specific time intervals (thereby establishing an effective S_o value).

The liquid media and solid substrate for the 500-liter vessel are slurried in a pre-mix tank where the feed stream is heated. A metering pump then pumps a specific amount of material per unit time through a sterilizing heat exchanger and a cooling heat exchanger prior to entering the fermenter.

ORGANISMS AND INOCULUM PREPARATION

The classification and testing of cellulomonas sp. bacterium and alcaligenes faecalis bacterium have been discussed in previous papers by Han and Srinivasan [4,9]. Both are non-spore forming, short rod-shaped, gram-negative bacteria. Cellulomonas sp. (ATCC #21399) gives a positive test for C_x cellulase activity, and is the bacterium responsible for cellulose depolymerization. Alcaligenes faecalis (ATCC #21400) gives a positive test for beta-glucosidase and is responsible for the depolymerization of short-chain oligosaccharides. Another organism used at times to replace alcaligenes faecalis in the culture is a beta-glucosidase-positive yeast obtained from an LSU culture collection. This yeast is referred to as Yc13.

Mixed cultures of cellulomonas sp. and alcaligenes faecalis or cellulomonas and Yc13 were used for all fermentations. Methods used for storing and carrying cultures are given in the Appendix.

Inocula for all runs were prepared by standard techniques and used only if they met standard criteria. The standard inoculum preparation methods for all cultures are given in the Appendix.

NUTRIENTS

Nutrients and growth factors for all cultures were supplied by inorganic salts and yeast extract. Extensive studies of various nutrients and nutrient levels have been previously reported [3,9,10].

Media of different composition were used for shake-flask and fermentor cultures because of the greater pH buffering capacity needed in the flasks. The flask basal media contained 6.0 g/l $(NH_4)_2SO_4$, 1.0 g/l NaCl, 4.45 g/l K_2HPO_4, 3.40 g/l KH_2PO_4, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2$, 1.0 g/l yeast extract (Difco), and 1.0 ml/l trace minerals solution. The media was formulated to buffer at a pH of about 6.8.

The trace minerals solution was composed of 16.7 g/l $FeCl_3 \cdot 6H_2O$, 0.18 g/l $ZnSO_4 \cdot 7H_2O$, 0.16 g/l $CuSO_4 \cdot 5H_2O$, 0.18 g/l $CoCl_3 \cdot 6H_2O$, and 20.1 g/l EDTA (ethylenedinitrilotetraacetic acid) dissolved in water.

Media used for pH-controlled runs in the fermentors was identical to the flask media except levels of the two phosphate buffers was 0.4 g/l K_2HPO_4, and 0.4 g/l KH_2PO_4. About 0.1 ml/l to 0.3 ml/l of polyglycol P-2000 (Dow Chemical Co.) was used in all media as antifoam.

CONTROL OF pH

As fermentation of the cellulose substrate progressed, the pH of the culture media became increasingly acid; and if allowed to continue to fall, would finally reach a pH of 5.0 or below and all cell growth would cease.

The buffer system used in shake-flask media could maintain a pH above 6.2 - 6.4 (the lowest effective pH for cell growth) only if the substrate loading was 4.0 g/l or below. If the substrate loading in the flask was

higher than 4.0 g/l, the culture would become pH limited before becoming substrate limited. (Thus, the 4.0 g/l substrate loading for the standard substrate test procedure).

The pH was controlled in the fermenters at 6.6 by the addition of base on demand. Anhydrous ammonia gas was used in the 500-liter vessel, and 10 N NaOH used in the 14-liter fermenters. Experimental data from several runs, Table 5, showed that the culture was using about 0.3 gm NaOH per gram of dry cells produced; or about 0.15 gm NaOH per gram cellulose metabolized. This unusually high base usage rate could not be attributed to formation of by-product acids because the cell yield on substrate (Y) was close to the 40% to 50% theoretical maximum. Rhodes and Fletcher [13] had stated that the use of a nitrogen source like ammonium sulfate could cause excess acidity through the preferential use of the inorganic ammonium, thus freeing the negatively charged sulfate into the media. A theoretical material balance assuming that this was indeed true and that the cells contained 10% nitrogen by weight gave a NaOH demand of 0.283 gm NaOH per gm cells. The difference between this value and the actual 0.3 gm NaOH per gram cells could now be attributed to by-product acid production by the cells. Rhodes and Fletcher suggested that urea would be a suitable "neutral" nitrogen source [13]. Prior testing of urea with cultures of cellulomonas and alcaligenes or Yc13 had shown that urea as such was not used as a nitrogen source, but that if the urea were included in the basal media prior to steam sterilization, the resulting media after sterilization could be used successfully. (Transamination between urea and the other inorganic salts and cellulose could have provided ammonia in other than urea forms).

Subsequent fermentation runs showed that the use of urea as a nitrogen source caused a base demand of only 0.1 gm NaOH per gram cells and supported

cell growth at approximately the same rate and to the same extent as ammonium sulfate when both were added to provide the same level of nitrogen in the media. Figure 15 shows the cell growth and caustic demand versus time for cultures using ammonium sulfate and urea as nitrogen sources. The three-fold difference in base demand can be seen.

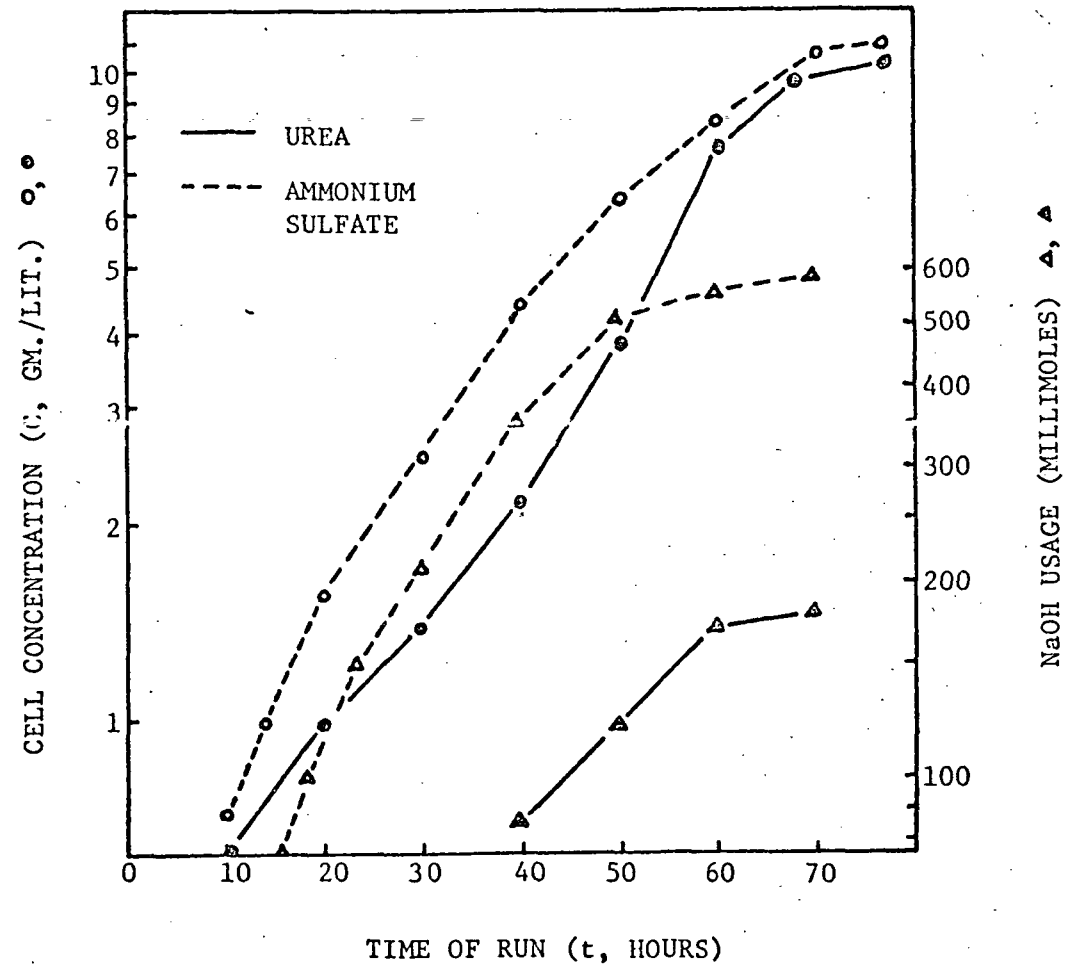


Figure 15. Base Demand with Ammonium Sulfate and Urea as Nitrogen Sources

BATCH FERMENTATION

Cultures of cellulomonas and alcaligenes or cellulomonas and Ycl3 were grown in 7-, 14-, and 500-liter fermenters under batch culture conditions on various cellulosics. All further discussion on these cultures will be limited to those with bagasse as a substrate unless stated otherwise. The nutrient media used was the same as given in the section on nutrients for fermenters.

The major purpose of the batch cultures was to provide basic data on cell growth conditions, fermentation kinetics, and overall culture dynamics. It was planned that the kinetic data obtained from the batch cultures could be used to effectively define cell-substrate interaction under controlled conditions; and to predict, or at least define more completely, the kinetic behavior of the culture under continuous fermentation conditions. (Continuous fermentation is the continuous feeding of media and substrate to the culture at equilibrium conditions and the continuous removal of culture from the fermenter.)

TECHNIQUE AND CONDITIONS

Standard techniques were used to prepare, sterilize, inoculate, operate, sample, and analyze all batch runs. These were the same for 7-, 14-, and 500-liter vessels except where size limitations existed (as in the sterilization procedures).

The whole media containing nutrient salts, growth factors, antifoam, and cellulose substrate was mixed in the fermenter. Acid was added if necessary to adjust the pH to between 6 - 7. The fermenter was then subjected to sterilization by heating to 275°F at 30 psig for one hour (for the 7- and

14-liter vessels) or to 250°F for one hour (for the 500-liter vessel). The contents were then cooled to the operating temperature and brought to a pH of 6.6 by the addition of acid or base.

All cultures were maintained at a temperature of 33°C ± 2° throughout the run. The pH was maintained at 6.6 by the addition of acid or base on demand. Cultures were sampled by the withdrawal of a homogeneous sample of the whole culture media. These samples were analyzed for cell concentration and undigested solids concentration by standard techniques given in the Appendix. At the time each sample was taken, values were recorded for time elapsed since inoculation, aeration, agitation, internal fermentor pressure, pH, temperature, and amount of acid or base added.

Aeration was maintained at about 0.2 to 0.3 volumes of air per volume of culture per minute in all runs. Agitation was maintained at 300 rpm for the dual-turbine agitators in the 7- and 14-liter (New Brunswick Scientific Co.) fermenters.

The type of culture being used and the type of treatment given the cellulosic substrate were also recorded for each run.

EVALUATION OF BATCH CULTURES

The cell concentration, C (grams dry weight cells per liter of culture), amount of caustic used to maintain pH at 6.6 (millimoles), and amount of solids per liter that had been solubilized since the batch had been started, $S_o - S$ (S_o = grams of solids per liter of culture at start of the run; S = grams of solids per liter at time t), were plotted on semi-logarithmic graph paper versus time t (hours) for all runs. The resulting plot as shown in Figure 16 represents the basic kinetic data obtained from a batch run. All further analysis of the run could then be taken from this plot.

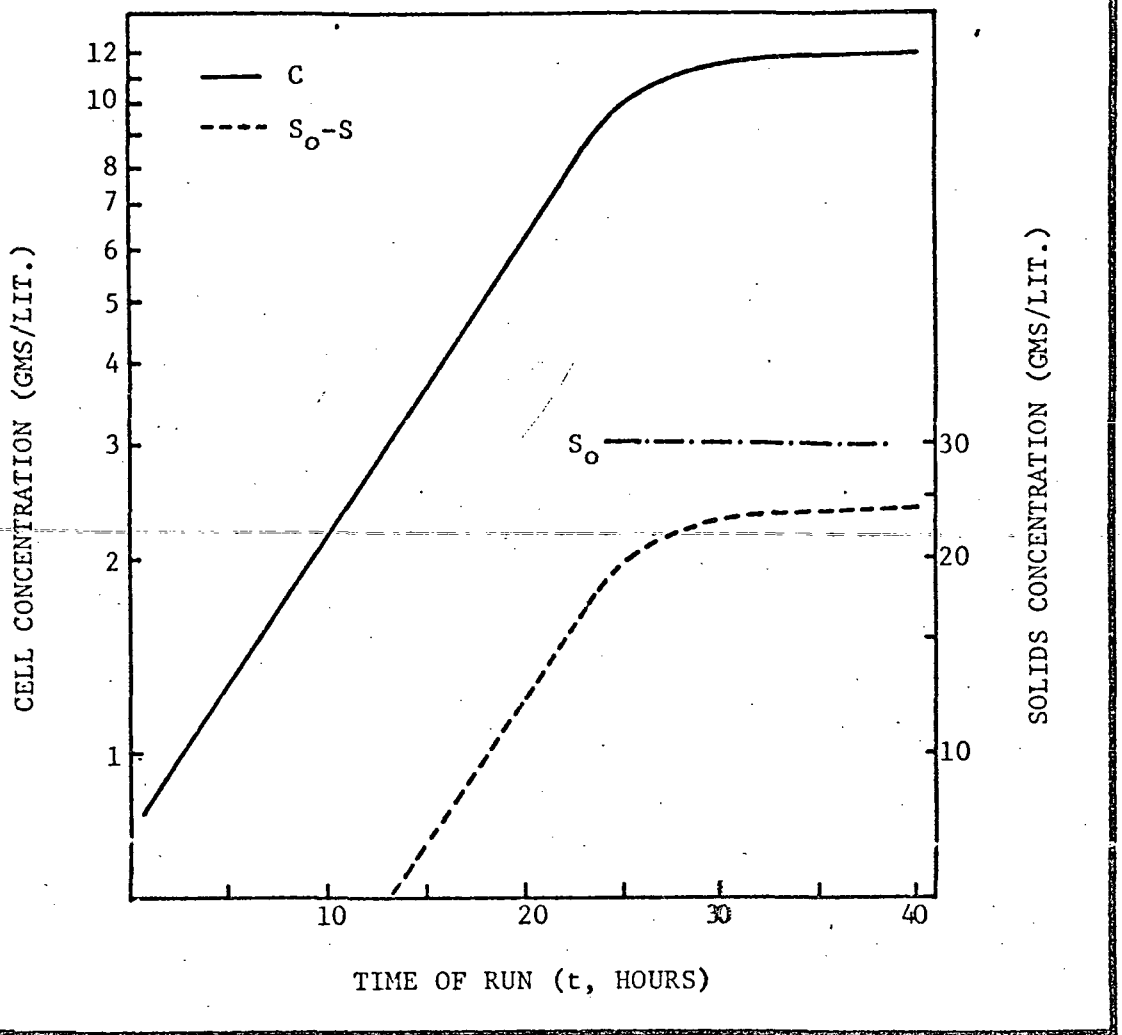


Figure 16. Theoretical Batch Run
Basic Kinetic Data

The slope of the S_o-S versus t curve, $\left(\frac{dS}{dt}\right)$, indicates the activity of the culture on the substrate. If $\frac{dS}{dt}$ is divided by C, the resulting $\frac{1}{C}\left(\frac{dS}{dt}\right)$ defines the specific activity of the culture per unit of cell mass at any time during the batch.

The basic equation for the growth of cells in a culture is

$$\frac{dC}{dt} = \mu C \qquad [1]$$

where μ is the growth rate constant of a first-order autocatalytic reaction with units of reciprocal hours (hrs.$^{-1}$). If we then define the cell yield on substrate as

$$Y = \frac{dC}{dS} \qquad [2]$$

where the yield, Y, is the grams of dry cells formed per gram of substrate metabolized, we can combine [1] and [2] to get:

$$\frac{\mu}{Y} = \frac{1}{C}\frac{dS}{dt} \qquad [3]$$

since:

$$\frac{dC}{dt} = Y\frac{dS}{dt} \qquad [4]$$

Now, it is apparent from Figure 16 that both $\left(\frac{dC}{dt}\right)$ and $\left(\frac{dS}{dt}\right)$ change with the state of the culture and that it is difficult to assign a typical logarithmic growth rate to the overall curve. If, then, we calculate values for the fraction of substrate utilized, X, for all values of $\frac{1}{C}\frac{dS}{dt}$ (hence μ/Y) as shown in Table 4, and plot μ/Y versus X [where $X = \frac{S_o - S}{S_o}$], Figure 17 results.

Figure 17 gives a relationship between μ and X which indicates that the cell growth rate is some function of the extent of substrate utilization. Two points of this curve are of major interest: Point A (the μ/Y intercept at X = 0) indicates the maximum growth rate of the culture and will be termed μ_{max}/Y; Point B (the X intercept) indicates the maximum extent of substrate digestion and will be termed X_{max}.

In cultures obeying Monod-type kinetics--almost all cultures where cells are grown on a soluble substrate under only substrate limitations--the μ/Y

TABLE 4. CALCULATION OF SPECIFIC ACTIVITY FROM BATCH RUN DATA

Time (t) (Hours)	$\frac{dS}{dt}$	C (gms/lit.)	$\frac{S_o-S}{S_o} = X$	$\frac{1}{C}\frac{dS}{dt} = \frac{\mu}{Y}$
18	0.59	3.0	0.20	0.196
20	0.74	3.66	0.24	0.202
22	1.0	4.5	0.30	0.222
24	1.15	5.58	0.37	0.206
26	1.43	6.9	0.45	0.207
28	1.64	8.55	0.56	0.191
30	1.15	10.05	0.67	0.114
33	0.58	11.25	0.75	0.051
36	0.23	11.7	0.78	0.019
40	0	12.0	0.80	0

versus X curve would be a straight line beginning at $\mu_{max}/_Y$ and extending at that same value to almost X = 1.0. In other words, the growth rate μ is a function of the fraction of substrate used only when very low substrate concentrations are reached (the saturation point, or K_s value).

Hence, Figure 17 indicates that the growth of our cultures on solid cellulose particles departs from classical Monod kinetics in that μ is a function of X. This is certainly not unexpected since enzyme penetration and product diffusion must obviously play a major role in the kinetics of degradation of the solid cellulose particles.

Substrate Limitation

The evaluation of a treated cellulosic substrate in shake-flask cultures was carried out with low initial substrate loadings to insure that the

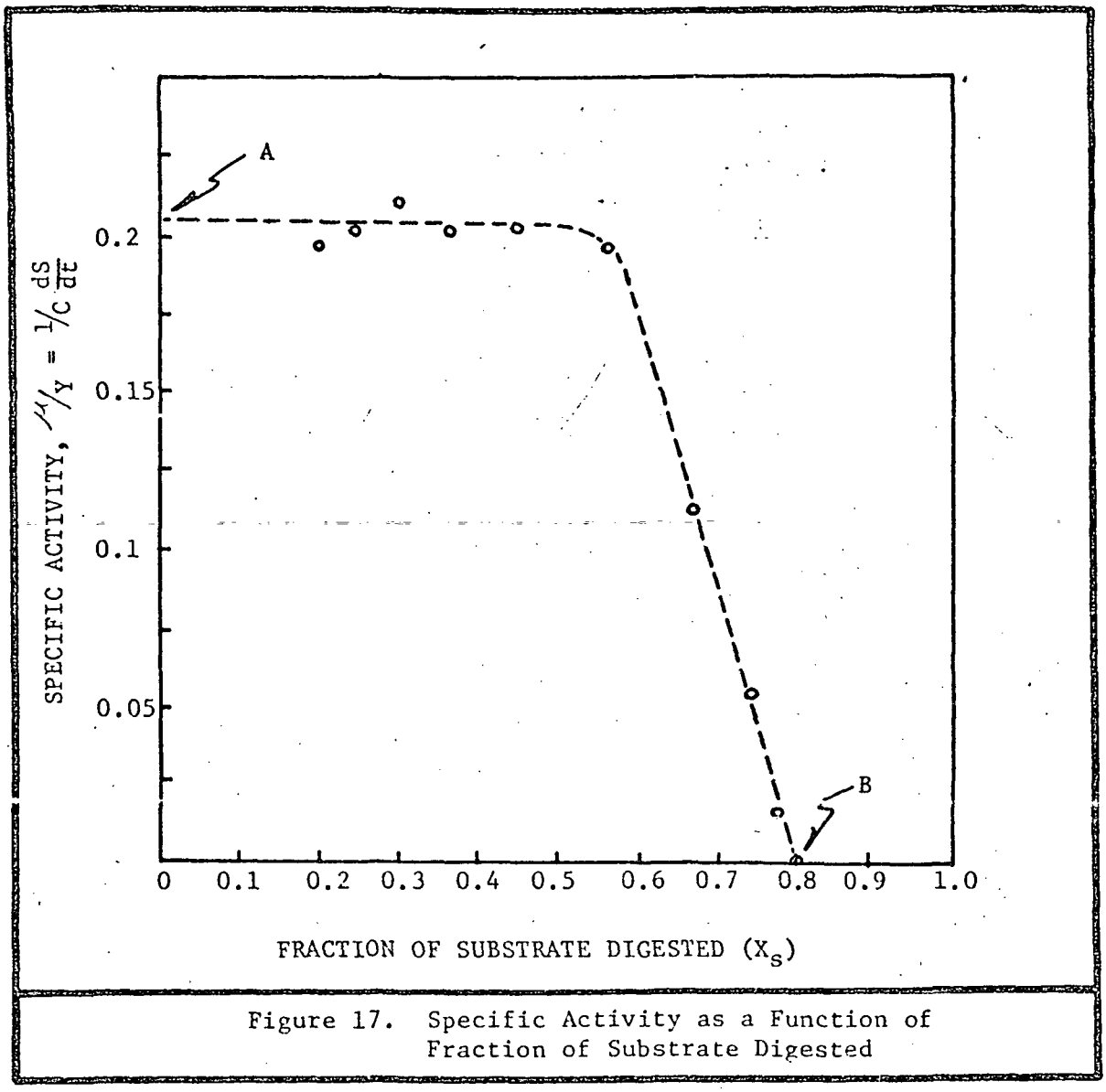


Figure 17. Specific Activity as a Function of Fraction of Substrate Digested

culture would be substrate limited. The maximum cell density that could be achieved in the flask cultures was 2.0 grams/liter of dry cell mass. In agitated and aerated fermenters, it was desirable to increase the cell density to economically feasible operating levels of over 10 grams/liter. This additional biomass production placed higher requirements on control of pH, temperature, nutrient supply, and aeration for proper culture growth. The growth limiting effects of several of these variables were determined in order to assure substrate limitation in those cultures with higher cell concentrations.

pH Limitation

As discussed previously, the pH of a growing culture tends to drop as more and more acidic products are formed. In several batch cultures the pH was allowed to fall without control. Since the fermenter media was essentially unbuffered, the pH dropped to about 5.0 after only two or three grams/liter of cell growth. As the pH dropped below 6.0 the growth rate slowed, and finally stopped altogether at a pH of 5.0 to 5.5. In several instances, the pH was readjusted to 6.8 or 7.0. In some cases the culture would resume growth after a lag time of arbitrary length, and other times the culture would not respond at all.

In those batches where unwashed, treated bagasse was used as substrate (the soluble fractions generated during treatment were left in the material), the pH would first rise to 7.5 or 8.0, and in some cases subsequently fall as cell growth on cellulose started; in others, it would simply remain at 8.0 or above if no cellulose degradation began.

The pH set-point usually used was 6.6, and it was working practice to maintain pH of the culture at any stage of growth between 6.6 and 7.0. No data was obtained for the quantitative effects of pH on growth rate or cell yield on substrate.

Temperature Limitation

The temperature of all cultures was maintained at 33°C $\pm$ 2°. Although no studies were undertaken to quantify the effect of temperature on culture dynamics, data from selected runs has shown a decrease in growth rate below 30°C or above 40°C, and cessation of growth above 45°C. The culture, therefore, seem to behave as true mesophiles with a growth optimum between 32°C and 36°C.

Nutrient Limitation

The inorganic nutrient composition of the fermenter media was designed to make nitrogen the first limiting nutrient. Ammonium sulfate at 6.0 grams/liter provides the only nitrogen source (other than the small amount provided by the yeast extract amino acids) at 1.27 grams nitrogen per liter of media. Assuming the cells contain 10 percent nitrogen, the nitrogen provided in the initial media should become limiting at about 12 grams/liter of cell mass. Runs in which sufficient substrate was fed to assure growth higher than 12 grams/liter cell mass, and in which no additional nitrogen was added, have shown a cessation of cell growth at about the 11 or 12 grams/liter level.

Aeration Limitation

Air is fed to the 7- and 14-liter New Brunswick fermenters and to the 500-liter pilot plant vessel at about 0.3 volumes of air per volume of culture per minute. Agitator speeds on the 7- and 14-liter vessels are 300 rpm, and about 200 to 220 rpm for the 500-liter vessel. Cell yield on oxygen for these aerobic cultures should be about 1.0 gm oxygen per gram cell mass formed. Oxygen demand is therefore dependent on cell mass concentration and on growth rate. No data have been obtained on oxygen transfer rates in any of these vessels; however, cultures have been grown to productivity values of 1.4 gms. of cells per liter per hour in batch cultures. This would create a theoretical oxygen demand of 1.4 gms. O_2/liter·hour or about 45 m moles O_2/liter·hour, which is well within the capabilities of the fermenter units being used. Since all batch and continuous culture productivities have been under the 1.4 maximum that was attained, it was assumed that the oxygen transfer rate was non-limiting.

Increasing S_o

A definitive test of substrate limitation at high substrate loadings was carried out by operating three 7-liter fermenters under identical conditions except for the level of the initial substrate concentration. It was felt that if the three vessels exhibited the same μ/Y versus X curves and had the same X_{max} values, then batches with S_o values within the range of the test could be assured of meaningful X_{max} values in a pH-controlled batch run.

Initial S_o values of 9.3, 16.7, and 23.3 grams of dry solids/liter were used in the three vessels. Figure 18 shows the basic C versus t data, and

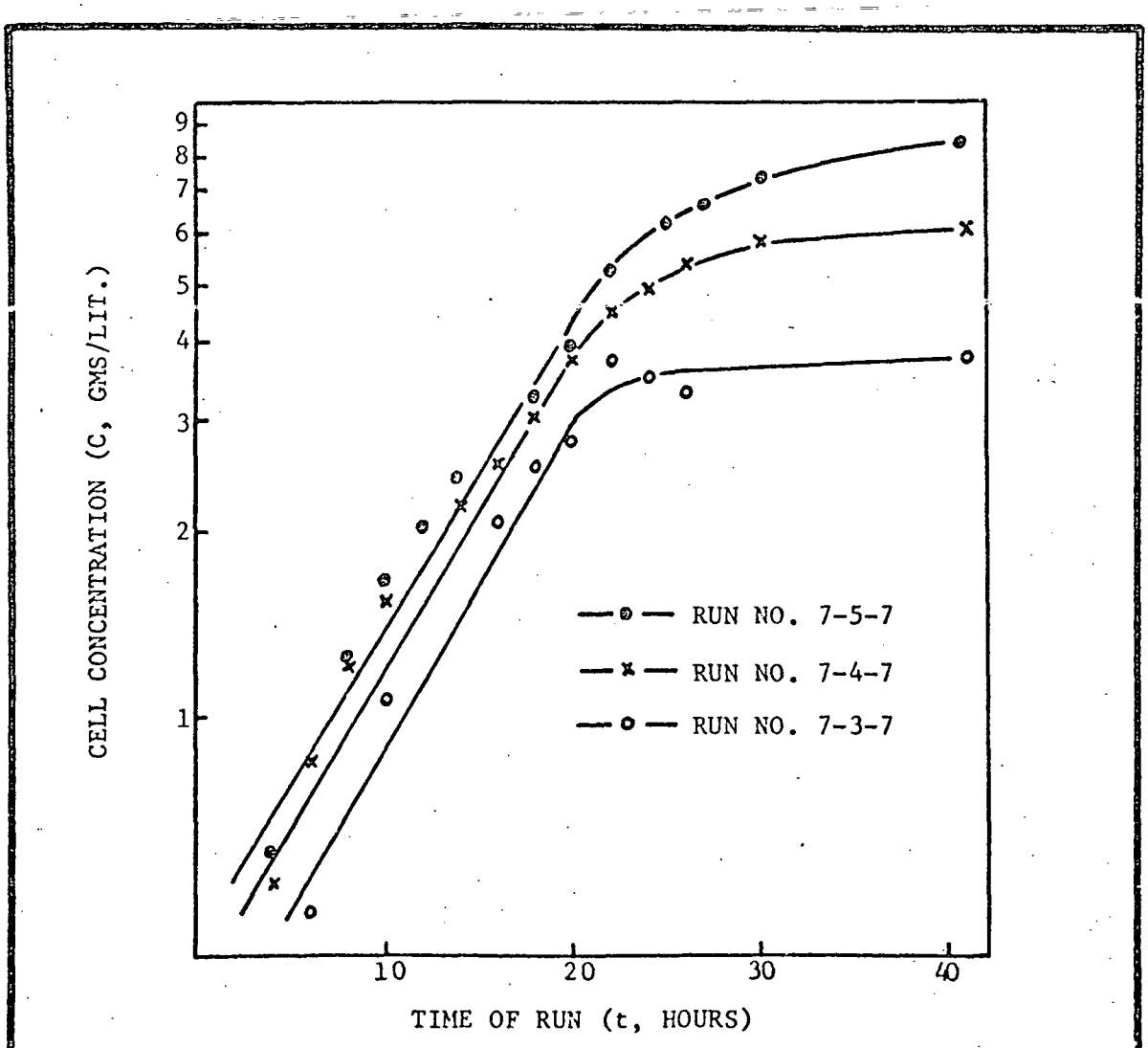


Figure 18. Cell Growth Rate Data for Different S_o Values

Figure 19 shows the S_o-S versus t plot. Data from both plots were used to calculate values for μ/Y and X for the three runs. The μ/Y versus X curves

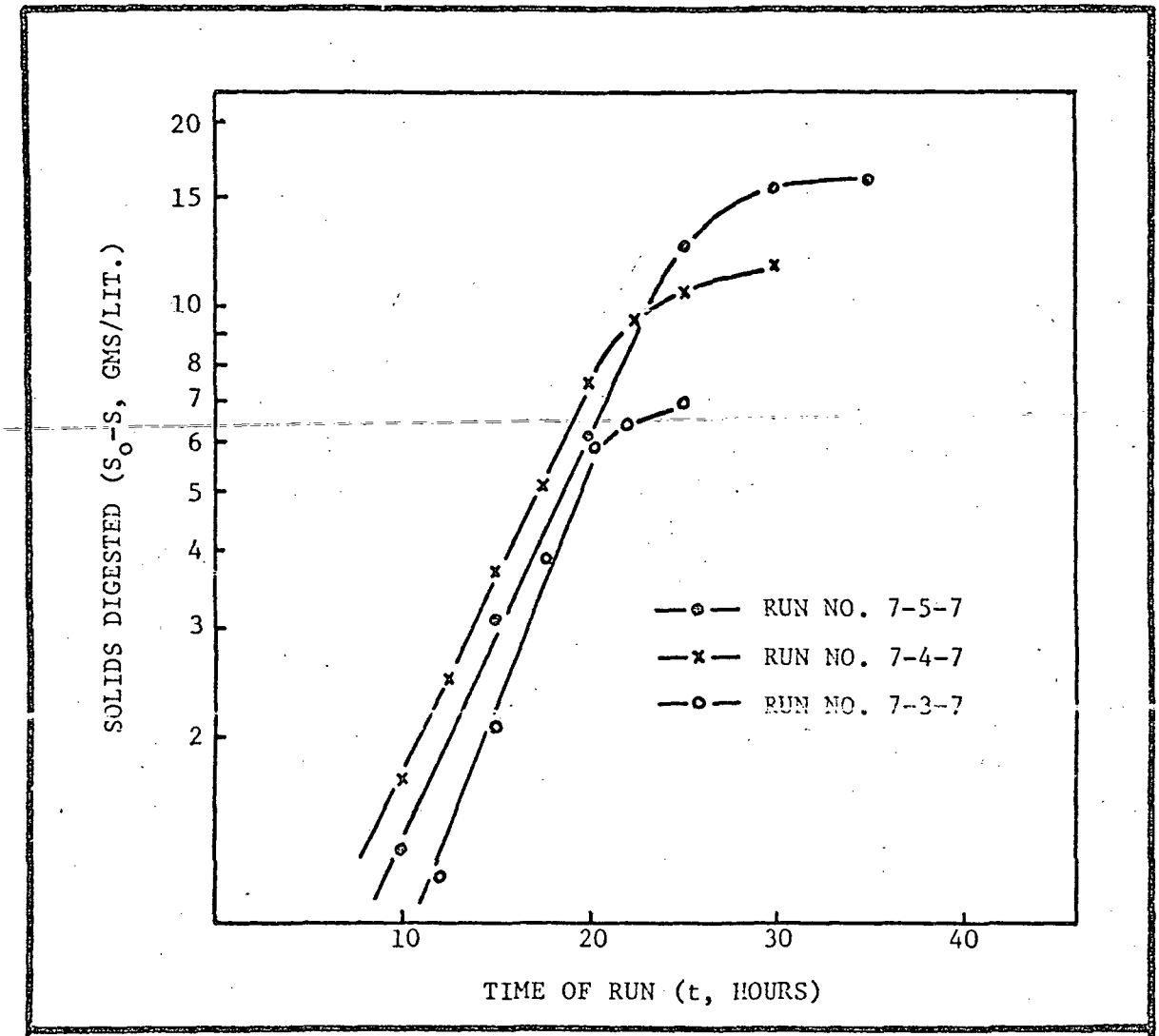


Figure 19. Solids Digestion Rate Data for Different S_o Values

for the three runs are shown in Figure 20. It can be seen from these curves that all three μ_{max}/Y and X_{max} values are within ± 3 percent of each other and that the $\mu = f[X]$ equation could be the same for the three cultures.

The conditions of the substrate treatment for this experiment were: N = 0.0812 gm NaOH/gm solids, t = 17 hrs., and P = 30 psig. The X_s value for

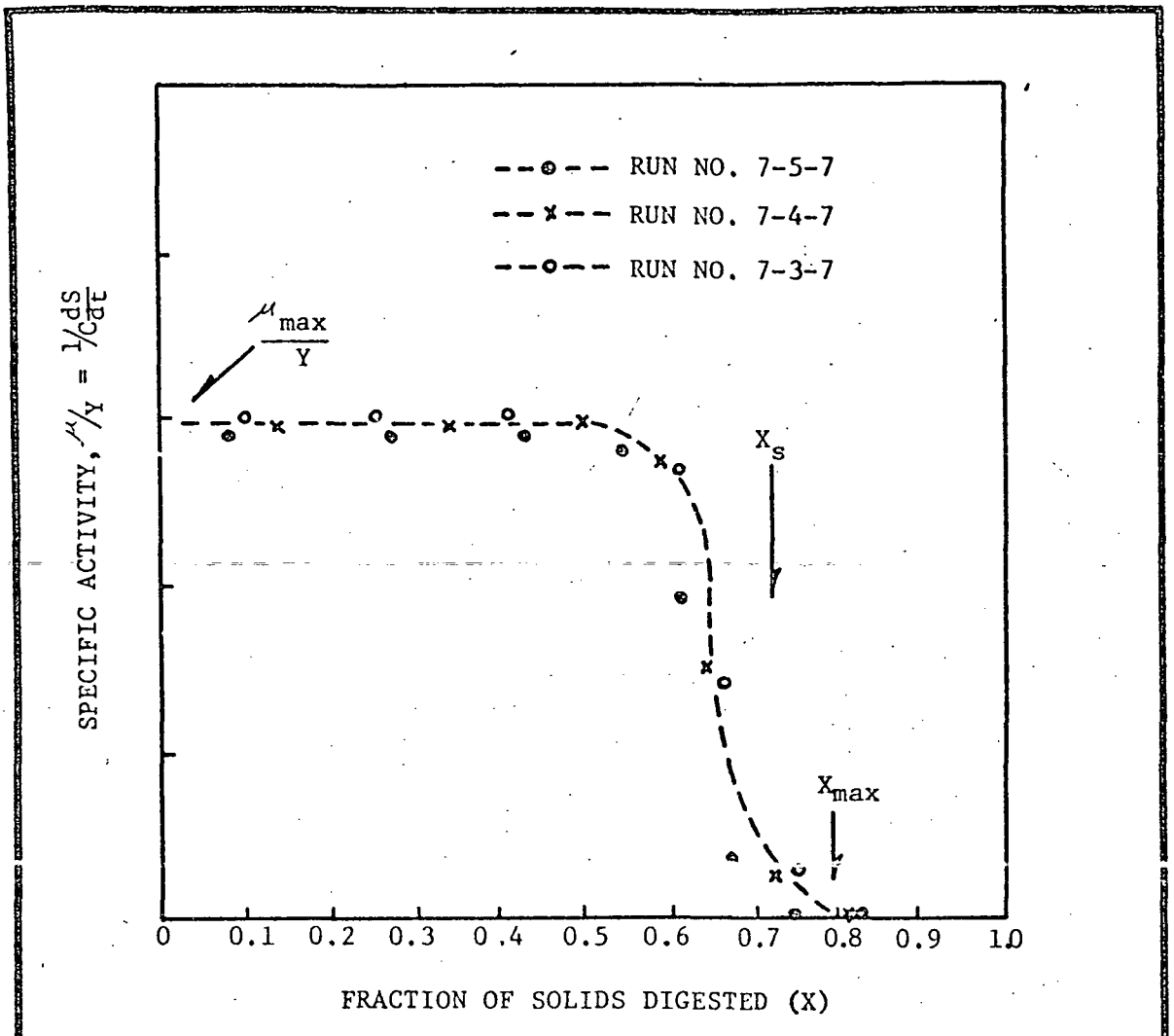


Figure 20. Specific Activity versus Fraction of Solids Digested for Different S_o Values

fraction of substrate degraded in the standard substrate test (from Figure 5) is about 0.72. The range of X_{max} values obtained in the three fermenters was from 0.75 to 0.82 which compares favorably with the predicted value obtained in the shake-flask test.

PERFORMANCE OF BATCH CULTURES

Data from all representative batch culture runs (cultures which were not grossly contaminated and which were within the test design) were tabulated to

present the substrate and treatment data in correlation with the kinetics and material balances of the resulting runs. Table 5 gives substrate, substrate treatment conditions, and types of organisms used correlated with the fraction of the substrate digested in the Standard Substrate Test (X_s). Values for initial substrate loading (S_o), specific culture activity ($\mu max/Y$), final cell concentration (C_{Final}), cell yield on substrate (Y), maximum fraction of substrate utilized (X_{max}), caustic use rate ($\frac{dNaOH}{dC}$), and productivity calculated from the batch data (P_C) are presented in Table 6 for the substrates characterized in Table 5.

The specific culture activity ($\mu max/Y$ = grams of substrate digested per liter per hour per gram of dry cell mass) in the mixed cultures of cellulomonas and Ycl3 yeast ranges from about 0.15 to 0.32 grams substrate per lit·hr. per gram cells. To make these values more meaningful, consider that an average cell yield on substrate is 0.45; the unit productivity of the culture, then, is from 0.075 to 0.16 grams of cells generated per lit·hr. per gram cells. If, for example, a continuous culture was being operated at an equilibrium (C_{eq}) cell density of 10 grams/liter dry cell mass, the productivity of the cultures would be from 0.75 to 1.6 grams/liter per hour. The $\mu max/Y$ values for mixed cultures of cellulomonas and alcaligenes fall into the same general range as for cellulomonas and yeast cultures.

The values for cell yield on substrate (Y = grams cells produced per gram substrate solubilized) range from about 0.40 to 0.55. Figure 21 shows the distribution of yield values obtained from 43 batch runs. It is obvious from the plot that cell yield is distributed around 0.50 with more occurring between 0.40 and 0.50 than above 0.50 or below 0.40. This is in accord with the known cellulose concentrations of the substrates and with values for classic cell growth on carbohydrates.

TABLE 5. BATCH CULTURE DATA - SUBSTRATES AND ORGANISMS

RUN NO.	SUBSTRATE	N[a]	t[b]	P[c]	ORGANISMS	X_s[d]
14-2-78	Bagasse, treated, washed	.2	4	30	Cellulomonas + Yc13	0.88
14-1-78	" "	.2	4	30	"	0.88
14-2-77	" "	.36	4	30	"	0.92
14-1-77[e]	" "	.36	4	30	"	0.92
14-2-76	Paper pulp	--	--	--	"	0.90+
14-1-76	Paper pulp	--	--	--	"	0.90+
14-2-74	Bagasse, treated, washed	.081	17	30	"	0.55
14-1-74	Bagasse, treated, aged 1 year	.36	1	ATM. 160F	"	0.90
14-2-73	Bagasse, treated, unwashed	.081	17	30	"	0.70
14-2-72	Bagasse, treated, washed	.081	17	30	"	0.70
14-2-71	" "	.081	17	30	"	0.70
14-2-70[e]	" "	.081	17	30	"	0.70
7-5-8	Paper pulp	--	--	--	"	0.90+
7-4-8	Bagasse, treated, washed	.081	17	30	"	0.70
7-3-8	Bagasse, treated, unwashed	.081	17	30	"	0.70
7-5-7	Bagasse, treated, washed	.081	17	30	"	0.70
7-4-7	" "	.081	17	30	"	0.70
7-3-7	" "	.081	17	30	"	0.70
7-5-5	" "	.081	17	30	"	0.70
7-4-5	" "	.081	17	30	"	0.70

TABLE 5. BATCH CULTURE DATA - SUBSTRATES AND ORGANISMS (Cont.)

RUN NO.	SUBSTRATE	N[a]	t[b]	P[c]	ORGANISMS	X_s[d]
7-3-5	Bagasse, treated, washed	.081	17	30	Cellulomonas + Ycl3	0.70
7-3-6	" "	.081	17	30	Cellulomonas	--
7-5-6	" "	.081	17	30	Cellulomonas + Alcaligenes	0.70
7-4-6	" "	.081	17	30	"	0.70
14-1-65	" "	.36	4	30	"	0.94
14-1-64	Bagasse, treated, washed	.081	17	30	"	0.70
14-1-52[e]	Bagasse, treated, washed, aged 1 yr.	.36	1	ATM. 160F	"	0.90
14-2-50	Bagasse, treated, washed	.081	0.25	15	"	0.55
14-2-47	Lactose	--	--	--	Alcaligenes	--
14-1-46	Lactose	--	--	--	"	(Uncontrolled treatment)
14-2-45	Bagasse, treated, unwashed, aged 6 mos.	.36	1	ATM. 160F	Cellulomonas + Alcaligenes	
14-2-44	" "	.36	1	ATM. 160F	"	"
14-2-35	Bagasse, treated, washed, aged 6 mos.	.36	1	ATM. 160F	"	"
14-2-34	Bagasse, treated, unwashed, aged 6 mos.	.36	1	ATM. 160F	"	"
14-2-33	Bagasse, treated, dried, aged 6 mos.	.36	1	ATM. 160F	"	"
14-1-33	Bagasse, treated, unwashed, aged 6 mos.	.36	1	ATM. 160F	"	"

TABLE 5. BATCH CULTURE DATA - SUBSTRATES AND ORGANISMS (Cont.)

RUN NO.	SUBSTRATE	N[a]	t[b]	P[c]	ORGANISMS	X_s[d]
14-1-31	Bagasse, treated, unwashed, aged 6 mos.	.36	1	ATM. 160F	Cellulomonas	(Uncontrolled treatment)
14-1-30	" "	.36	1	"	"	"
14-1-27	" "	.36	1	"	"	"
14-1-21[e]	Bagasse, treated, washed, aged 6 mos.	.36	1	"	"	"
14-1-19	Bagasse, treated, unwashed, aged 6 mos.	.36	1	"	"	"
14-2-18	" "	.36	1	"	"	"
14-1-14	" "	.36	1	"	"	"
14-1-11	" "	.36	1	"	"	"
535-43	" "	.36	1	"	"	"
535-39	Bagasse, treated, washed, aged 6 mos.	.36	1	"	"	"
535-38	Bagasse, treated, unwashed, aged 6 mos.	.36	1	"	"	"
535-26	" "	.36	1	"	"	"
535-19[e]	" "	.36	1	"	"	"

[a] N = Grams NaOH/gm. cellulosic solids used in treatment.

[b] t = Time (hours) of treatment.

[c] P = Pressure in psig saturated steam pressure (or temp. if atmospheric pressure).

[d] X_s = Fraction of substrate digestible in Standard Substrate Test. (Aged bagasse was not tested due to lack of control of the aging process.)

[e] These runs were used to start continuous runs.

TABLE 6. BATCH CULTURE DATA - OPERATIONAL RESULTS

RUN NO.	S_o	$\mu_{max/Y}$	C_{FINAL}	Y	X_{max}	$\frac{dNaOH}{dC}$	P_c [b]
14-2-78	24.2	0.38	5.78	0.40	0.58	0.32	0.61
14-1-78	24.6	0.32	6.23	0.47	0.53	0.33	0.76
14-2-77	19.5	0.32	5.6	0.47	0.61	0.29	0.38
14-1-77[a]	20.0	0.26	4.7+	0.54	0.48+	0.30	0.64
14-2-76	20.0	0.11	10.5	0.52	0.90	0.29	0.36
14-1-76	23.2	0.12	10.6	0.49	0.87	0.09 (Urea)	0.42
14-2-74	29.5	0.19	2.8	0.40	0.24	0.21	--
14-1-74	21.5	0.19	3.4	0.46	0.34	0.29	--
14-2-73	30.0	0.22	7.8	0.49	0.45	--	--
14-2-72	26.0	0.17	6.0	0.50	0.52	0.32	0.42
14-2-71	33.0	0.17	9.0	0.53	0.50	0.30	0.72
14-2-70[a]	20.7	0.22	--	0.50	0.43+	--	0.53
7-5-8	21.5	0.14	8.5	0.51	0.72	0.30	0.55
7-4-8	27.5	0.28	9.0	0.50	0.79	0.31	0.95
7-3-8	35.5	0.20	13.0	0.50	0.78	--	0.91
7-5-7	23.5	0.24	8.4	0.48	0.75	0.24	0.84
7-4-7	16.6	0.23	5.6	0.42	0.82	0.35	0.58
7-3-7	9.3	0.23	3.6	0.47	0.82	0.29	0.46
7-5-5	16.1	0.15	5.3	0.43	0.72	0.32	--
7-4-5	25.0	0.15	8.2	0.44	0.70	0.33	--
7-3-5	26.4	0.16	9.2	0.49	0.68	0.32	--
7-3-6	23.9	0.20	7.3	0.32	0.85	0.32	0.55
7-5-6	21.9	0.33	7.3	0.45	0.72	0.35	0.50

TABLE 6. BATCH CULTURE DATA - OPERATIONAL RESULTS (Cont.)

RUN NO.	S_o	$\mu_{max}/_Y$	C_{FINAL}	Y	X_{max}	$\frac{dNaOH}{dC}$	P_c[b]
7-4-6	22.0	0.20	7.5	0.45	0.72	0.36	0.57
14-1-65	24.5	0.25	7.0	0.52	0.55	0.30	0.65
14-1-64	30.6	0.14	14.8	0.56	0.81	0.30	0.99
14-1-52[a]	20.0	0.27	12.0+	0.50	--	0.34	0.83
14-2-50	21.0	0.13	4.4	0.34	0.62	0.26	--
14-1-47	--	--	11.2	--	--	0.30	--
14-1-46	--	0.76	20.1	0.35	--	--	5.3
14-2-45	18.5	0.17	3.8	0.46	0.45	--	--
14-2-44	20.9	0.34	2.4	0.34	0.34	0.15	--
14-2-35	15.5	0.22	8.5	0.62	0.79	0.25	1.19
14-2-34	16.2	0.18	3.0	0.41	0.45	--	--
14-2-33	22.3	0.22	3.1	0.39	0.36	--	--
14-1-33	13.8	0.25	3.0	0.34	0.64	--	--
14-1-31	16.4	0.10	6.1	0.50	0.74	--	0.32
14-1-30	19.3	0.20	6.8	0.41	0.71	0.24	0.55
14-1-27	14.6	0.15	3.9	0.50	0.57	--	0.23
14-1-21[a]	27.0	0.21	5.0+	0.42	0.54+	--	0.61
14-1-19[a]	28.5	0.29	6.0+	0.48	0.59+	--	0.83
14-2-18	30.6	0.35	5.8+	0.22	0.83	--	0.27
14-1-14	26.8	0.33	10.2	0.49	0.78	--	1.44
14-1-11	8.0	0.43	2.0+	0.44	0.50+	--	0.38
535-43	13.0	0.19	4.9	0.54	0.62	--	0.50
535-39	29.0	0.14	5.1	0.33	0.53	--	--

TABLE 6. BATCH CULTURE DATA - OPERATIONAL RESULTS (Cont.)

RUN NO.	S_o	$\mu_{max}/_Y$	C_{FINAL}	Y	X_{max}	$\frac{dNaOH}{dC}$	P_c [b]
535-38	21.0	0.28	5.8	0.41	0.91	--	0.71
535-26	13.7	0.11	4.7	0.53	0.78	--	0.37
535-19[a]	10.0	0.23	4.7^+	0.50	--	--	0.60

[a] These runs were later made continuous, X_{max} values; therefore, are not representative of true X_s values.

[b] Values for productivity calculated from batch data are the maximum $C\mu$ values for the run.

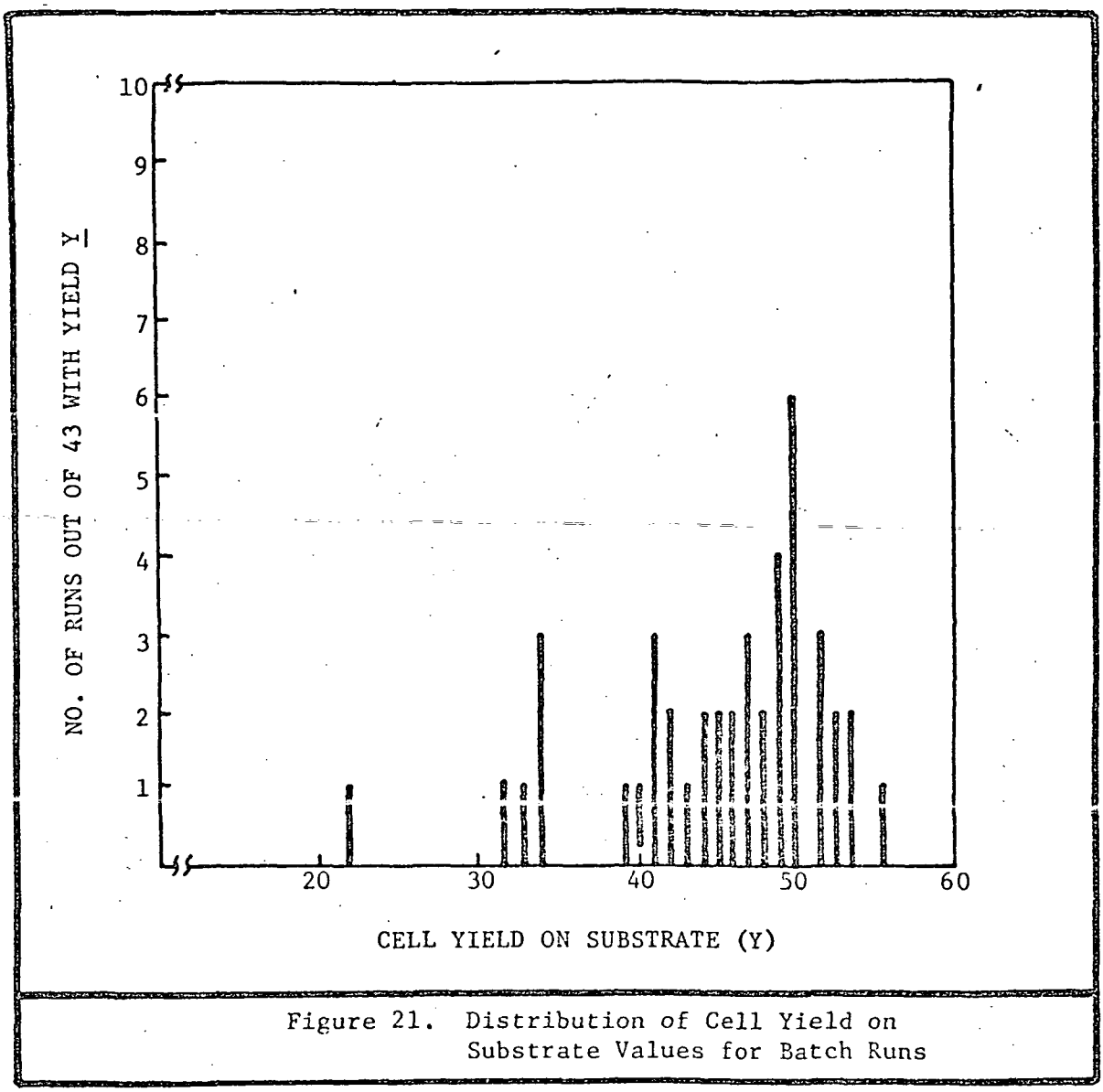


Figure 21. Distribution of Cell Yield on Substrate Values for Batch Runs

A comparison of fraction of substrate digested in Standard Substrate Tests (X_s) versus the actual fraction digested in the more concentrated fermenter cultures (X_{max}) in Figure 22 shows a clustering of points around the theoretical line with a considerable number of points falling below the line (cultures in which X_{max} was less than the X_s predicted by the tests). No single explanation could be found for this anomaly. In actuality, the arbitrary problems of foaming, unsuitable inocula, inefficient substrate

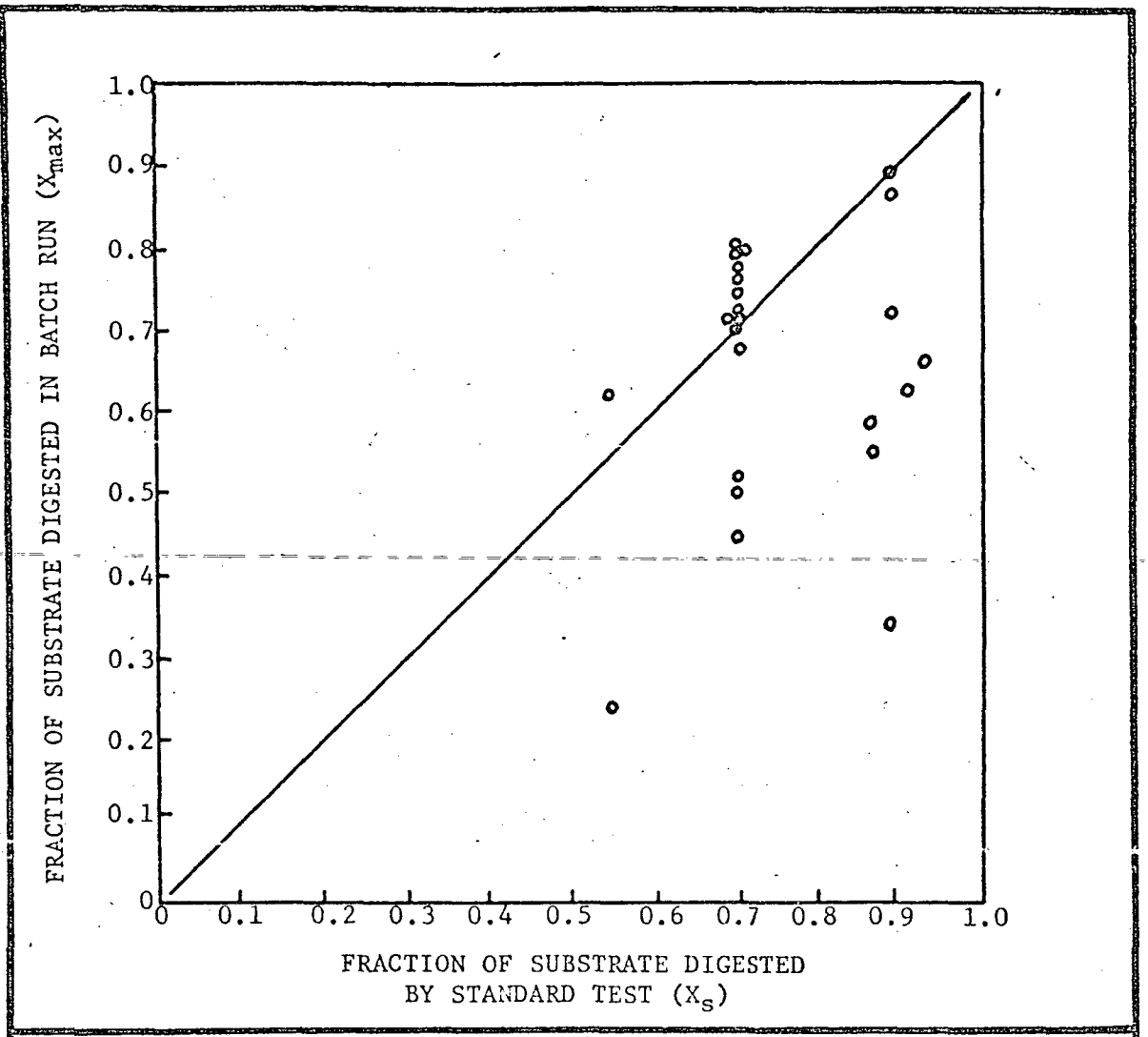


Figure 22. Comparison of X_S and X_{max} Values

treatment, and low levels of culture contamination probably account for the sub-optimal activity the lower points indicate.

The amount of sodium hydroxide necessary to maintain a pH of 6.6 is expressed as $\frac{dNaOH}{dc}$ (grams NaOH per gram of cells produced) and, as previously discussed, is about 0.3 when ammonium sulfate is used as the nitrogen source, and 0.1 when urea is used.

The values for productivity calculated for the batch cultures (P_c = grams of cells/lit·hr) are for the maximum that the culture attained. These

values should be indicative of the productivity of a continuous culture with the same growth rate and the same cell concentration.

Culture Flocking

Both cellulomonas and Yc13 yeast cultures and cellulomonas and alcaligenes cultures had an apparent tendancy to arbitrarily form microflocs during batch culture runs; however, cultures did not form flocs with an identifiable change in any monitored variable, and some cultures did not form flocs at all. On the other hand, some cultures which had flocked would sometimes spontaneously unfloc. No additive was found that would prevent flocking, but extensive observation permitted some definition of conditions where flocking tended to occur:

1. Cultures entering the stationary or idiophase of growth formed flocs more often than young cultures, possibly due to the release of some intracellular protein or other surfactant.
2. Cultures experiencing a rapid pH change of over 1.0 pH point were susceptible to floc formation.
3. Floc formation was more apparent in cultures that had relatively high initial soluble carbohydrate in the media (unwashed substrate).
4. Cellulomonas and alcaligenes tended to floc more than cellulomonas and yeast.
5. Cultures tended to form floc after an abrupt change in the ionic strength of the media (addition of supplementary nutrient salts).

When cultures did form flocs, the measurement of optical density was impossible; but culture activity could still be monitored by base usage rate for pH control.

A theory exists which proposes that cellulomonas is itself strongly absorbed onto the cellulose substrate and that the cellulase enzyme(s) is

primarily cell-wall bound, thereby serving as a point of attachment of the cell to the substrate. The shape of the μ/Y versus X curves seem to suggest some effect of surface area on growth rate, but culture optical density seems to correlate cell density quite well with chemically measured protein in unflocked cultures of over two or three grams/liter cell concentration.

CONTINUOUS FERMENTATION

Cultures of cellulomonas and alcaligenes faecalis, and cellulomonas and Ycl3 yeast were operated as continuous fermentations with continuous (or regularly spaced) additions of media and substrate and removal of culture to maintain a constant volume. All continuous cultures were operated with treated sugarcane bagasse as substrate.

TECHNIQUE AND CONDITIONS

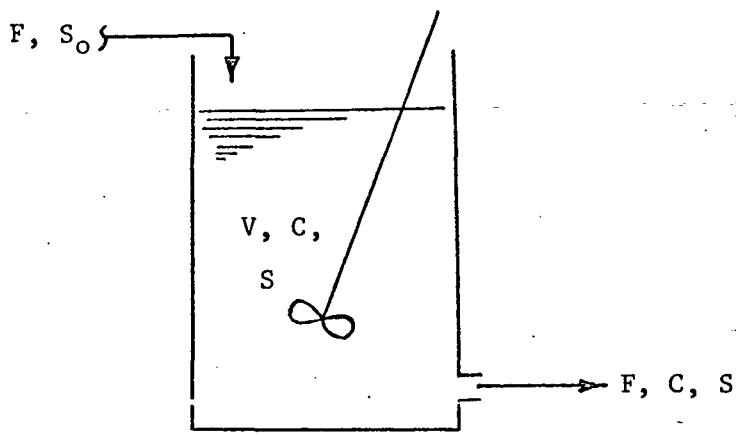
Continuous cultures were started by initiation of feed and culture removal at a specific rate from batch cultures still in the logarithmic phase of cell growth. In some cases the solid substrate was fed with the liquid media as a slurry of constant solids content. An easier, although less aseptic, method of feeding that came to be used with the 14-liter vessels was the constant feeding of pre-sterilized liquid media and manual addition of solids at predetermined intervals. Whole culture was withdrawn just prior to each solids addition at a rate to maintain a constant volume. This technique served to "smooth out" the cell concentration curve since no slugs of liquid media were added to the culture.

All liquid media and solid substrates were autoclaved prior to use, but the technique of manual solids addition certainly did not insure aseptic operating conditions. However, no continuous culture with reasonably high cell density showed any adverse effects of gross contamination.

As in most batch cultures, the temperature of continuous runs was maintained at 33°C $\pm$ 2 C, the pH at 6.6, aeration at from 0.2 to 0.3 VVM, and the agitation (in the 7- and 14-liter New Brunswick vessels) at 300 rpm. Liquid media was of the same composition as that used in the batch runs.

EVALUATION OF CONTINUOUS CULTURES

Continuous cultures with treated sugarcane bagasse were evaluated as substrate limited cultures in steady-state equilibrium in a continuous-flow backmix reactor. The schematic drawing of the continuous fermentation process shown below gives the variables and operating parameters that define the system.



where: F = feed rate, (lit./hr.)

S_o = substrate concentration in feed, (gms./lit.)

S = substrate concentration in fermenter, (gms./lit.)

C = cell concentration, (gms./lit.)

V = volume of cultures, (lit.)

D = F/V = dilution rate, ($hrs.^{-1}$)

and, μ_{max} = maximum cell growth rate constant, ($hrs.^{-1}$)

μ = cell growth rate constant, ($hrs.^{-1}$)

Y = cell yield on substrate, (gms. cells produced/gms. substrate used)

$E = \frac{S_o - S}{S_o}$ = reactor efficiency, (dimensionless).

Using the system and culture growth parameters, a general material balance may be written around the fermenter for cells and for substrate.

Material balance on cells:

$$\begin{matrix}\text{cells in}\\ \text{by flow}\end{matrix} + \begin{matrix}\text{cells in}\\ \text{by growth}\end{matrix} + \begin{matrix}\text{cells out}\\ \text{by flow}\end{matrix} - \begin{matrix}\text{cells out}\\ \text{by death}\end{matrix} = \begin{matrix}\text{accumulation}\\ \text{of cells}\end{matrix}$$

or: $$FC_o + \frac{dC}{dt}\Big|_{GROWTH} - FC - \frac{dC}{dt}\Big|_{DEATH} = V\frac{dC}{dt}$$

which may be simplified with the assumption that $C_o = 0$ and $\frac{dC}{dt}\Big|_{DEATH} = 0$, to:

$$-FC + \frac{dC}{dt}\Big|_{GROWTH} = V\frac{dC}{dt}$$

and since $D = \frac{F}{V}$ and $\frac{dC}{dt}\Big|_{GROWTH} = \mu C$; the material balance on cells becomes;

$$\boxed{C(\mu - D) = \frac{dC}{dt}}$$

The overall material balance on substrate may be written similarly to obtain:

$$\boxed{D(S_o - S) - \frac{\mu C}{Y} = \frac{dS}{dt}}$$

Now, with the addition of an expression for the growth rate (μ) as a function of the substrate concentration;

$$\boxed{\mu = f[S]}$$

the three equations may be solved simultaneously to give solutions for the equilibrium cell and substrate concentrations given values for D, μ, and S_o.

It is in the calculation of μ that the cellulose fermentation departs from classic Monod kinetics. From evaluation of the kinetic data of batch

runs μ has been shown to be some function of X, the fraction of substrate digested. In a continuous flow, backmix fermenter at steady-state conditions using a nondistributed substrate such as bagasse (the substrate is concentrated into discreet bits), the substrate particles have a definite age distribution pattern. This also occurs with molecules of soluble substrate, but is artifactual since μ is not a function of age or X in those reactions. However, with μ being a strong function of X (Figure 17) the age distribution of the substrate will definitely effect the values of C and S in the outlet stream.

Values of μ and X from batch data have been correlated with reasonable accuracy by the expression:

$$\mu = \mu_{max}[1 - (\frac{X}{X_{max}})^N]$$

where the value for N is about 6.0 for most cultures. This expression now gives us the necessary growth rate function to solve simultaneously with the material balances to give values for C, S, and E. No attempt has been made to obtain the mathmatical solutions to these equations at this time.

Experimental data have been obtained from several continuous runs; and the critical values of fermenter efficiency, equilibrium cell density, productivity, and equilibrium substrate concentration have been tabulated with the system parameters of S_o and D.

Table 7 shows that a continuous cell productivity of 0.91 grams/liter·hr. is the maximum that has been obtained; with 25 percent of the substrate digested in a single pass. In run 14-1-77 where 52% of the substrate was digested in a single pass, the yield was decreased (apparently) to 34%, thus giving a productivity of 0.47 grams/lit·hr.

A point that is evident from experimental data and qualitative consideration of the $\mu = f[X]$ relationship is that the single-pass efficiency value

TABLE 7. CONTINUOUS CULTURE DATA

RUN NO.	S_o (gm/lit)	D ($hrs.^{-1}$)	C (gm/lit)	S (gm/lit)	P (gm/lit·hr.)	P_c [a] (gm/lit·hr.)	$E=\frac{S_o-S}{S_o}$	Y
535-19	--	0.130	3.3	--	0.43	0.38	--	--
14-1-51	--	0.0572	7.2	23.2	0.412	--	--	--
14-1-52	24	0.096	9.4	18.0	0.91	0.83	0.25	0.59
14-2-70	--	0.09	3.0	17.0	0.27	0.53	--	--
14-1-77	25	0.105	4.4	12.0	0.47	0.64	0.52	0.34

[a] Productivity calculated from the batch run from which continuous run was started.

(E) for a culture operating at a reasonable productivity (above 0.7 gm./lit·hr.) will be much lower than that expected of a culture with Monod kinetics and $\mu \neq f[X]$. From consideration of the shape of the μ vs X plot, it would be reasonable to assume that a culture may be operated in continuous flow with a value of E = 0.7 or 0.75 X_{max}, since the $\mu = f[X]$ curve is almost flat to that point. The value for 0.7 X_s for the substrate of run 14-1-77 is 0.63 which is not too much in error from the 0.52 experimental value.

The yield value of 0.34 of run 14-1-77 is quite low, but may be due to the rapid absorption of cells onto the new solid substrate thus removing them from optical density measurements. (That phenomenon is apparent when new media is inoculated with cells.)

Data for continuous fermentation is relatively difficult to obtain with the major drawback being the maintenance of a homogeneous and constant feed. We could obtain meaningful S_o values only by the manual feeding of the solids separate from the liquid media. This presented an obvious problem of maintaining aseptic conditions that was never solved. Data for continuous fermentation of cellulose in the literature is unavailable.

RECYCLE

In an industrial SCP production operation it becomes important to recycle as much of the liquid media as possible to preserve nutrient salts and to avoid the dumping and subsequent treatment of the large water volumes involved. To test the recyclability of used liquid media a test was devised wherein spent liquid media from a batch culture was centrifuged to remove all cells and solids, recharged with nutrient salts and substrate, sterilized, and reinoculated with cells. The rate and extent of cell growth was monitored, and the procedure was repeated. Cell growth was within ± 5% for all three

runs. From this data it would follow that at least 66% of the liquid media from a run could be recycled with a good possibility that even more could. Some of the spent liquid must be removed from the process, however, to prevent accumulation of spent salts such as sodium sulfate (if ammonium sulfate is used as a nitrogen source) and by-product acid salts.

PRODUCT HARVESTING

Whole culture exiting the fermenter contains undigested cellulose fibers and cells. To effectively separate the cellulose and cells, the cells must be free and unflocked upon leaving the vessel. If it is desired to harvest cellulose and cells together, the cells may or may not be flocked.

FIBER REMOVAL

Undigested fiber may be removed from the culture by screen filtration or by low-speed de-sludging centrifugation. Although screening is probably the most economical means of fiber removal, centrifugation has an advantage in removing a sludge or mud-like sediment that is washed or freed from the bagasse during fermentation and carried with the cells through a screening. The combination of a rotating-drum screen filter with a 40-mesh stainless steel cloth for bulk fiber removal followed by a small de-sludging centrifuge for cell-cream polishing would provide the cleanest SCP product at a modest operating cost.

CELL CONCENTRATION

Several methods of cell concentration have been tested on the 500-liter pilot unit. Among those were direct cell centrifugation in a high-speed solid bowl centrifuge, flocculation followed by precipitation of the floc, flocculation followed by dissolved air flotation, and flocculation followed by centrifugation. Of the methods tested, the flocculation followed by centrifugation seemed to be the most efficient and least costly.

Cells could be flocculated most efficiently by the addition of high molecular weight polycationic polyelectrolytes such as Nalco N-610 and 71D09

added in dilute solution. Low-to-medium weight polycationic materials like Dow PEI 1000, Nalco 107, 603, 607, and 634 worked well also, but produced floc of smaller size. Polyanionic and nonionic materials formed floc very poorly.

After floc were formed, the cells could be easily cleared from the culture by low-speed centrifugation in a de-sludging centrifuge. The resulting cell sludge contained about 14% to 16% dry weight solids. This sludge could then be diluted with water for a washing step and then spun back to 16+% solids. This washed cell cream could then be spray dried or drum dried to form a cream to cream-yellow colored SCP product. The analysis of the protein and amino acid content of this material has been determined and discussed at some length in previous reports [8,10,14].

CO-HARVESTING OF CELLS AND FIBER

If it was desirable to harvest cells and fiber at one time; i.e., for a ruminant animal feed, the screening and de-sludging fiber-separation steps could be bypassed. The cells would simply be flocculated in the presence of the undigested fiber, and the resulting fiber/floc mass could be removed from the media by a de-sludging centrifuge. Usually the nutrient salts remaining with the sludge would be beneficial to the feed use and could be left in the dried product; therefore, the washing could be bypassed and the material dried directly on a drum dryer or in a rotary kiln.

ENZYME PRODUCTS

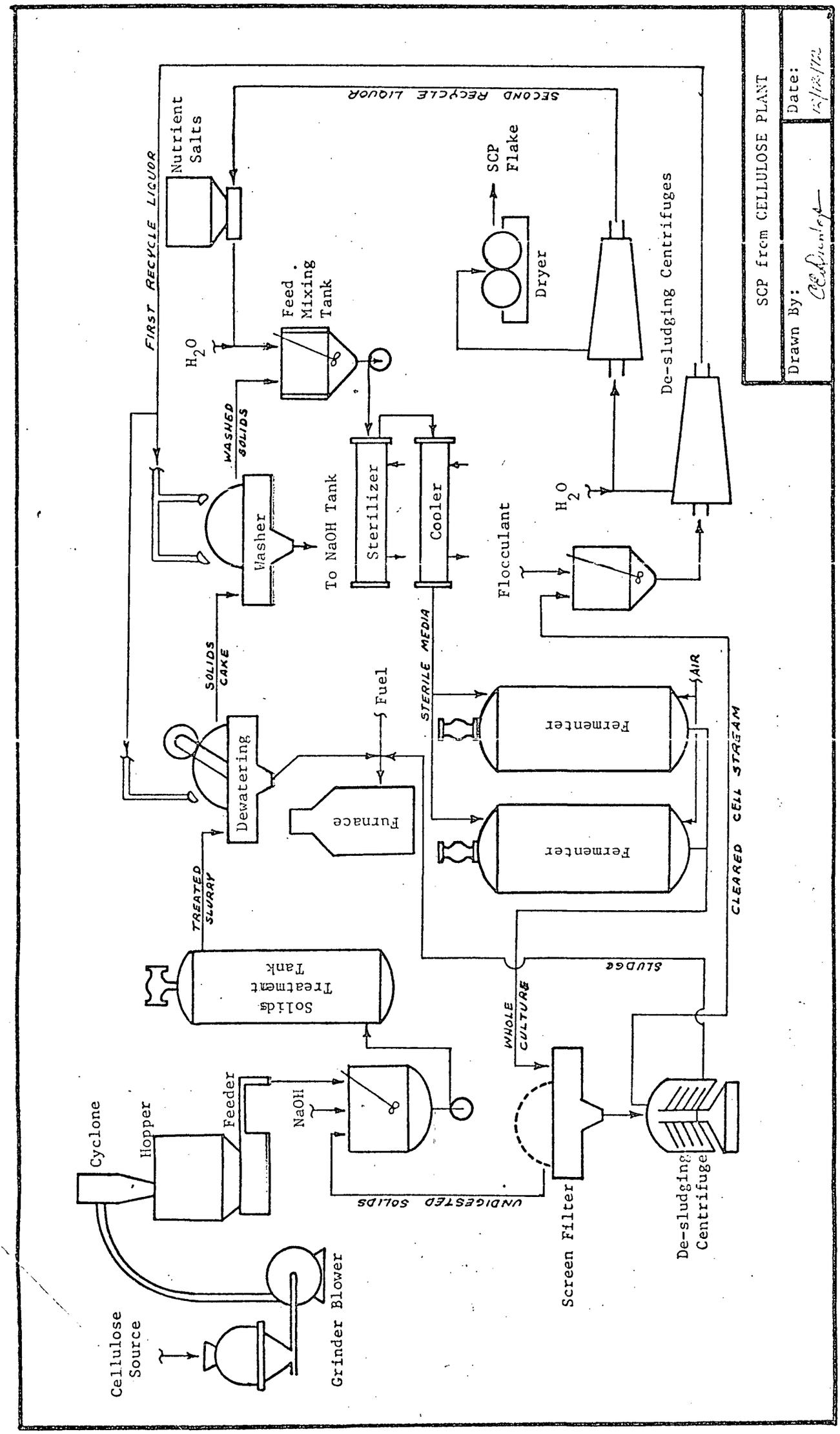
No large-scale enzyme isolation has been carried out at the pilot facility, but laboratory results have shown that a high-activity C_x cellulase can be harvested from the culture media [15].

PLANT FLOW SHEETS

The most promising methods of feed treatment, fermentation, and cell harvesting were chosen for a plant to produce whole-cell SCP in dry flake form from a waste cellulose source. The flow sheet that follows shows the overall pattern that we envision the integrated process to have.

It should be recognized that the data presented in this report provides a sufficient basis for the construction of overall material and energy balances. These overall balances could be programmed for a computer; and the overall effects of a change in such variables as growth rate, dilution rate, fermenter efficiency, etc. could quickly and easily be obtained.

A production plant may well desire to market SCP in forms other than dried, whole cells. An SCP-protein isolate is one product possibility, animal feed rations are another. In those cases, the final dryer shown on the flow sheet presented here would be replaced with protein extraction and purification equipment, or ration blending and pelleting machines. The basic process, in any case, would be about the same.


SCP from CELLULOSE PLANT
Drawn By:
Date:
Nutrient Salts
FIRST RECYCLE LIQUOR
SECOND RECYCLE LIQUOR
SCP Flake
Dryer
De-sludging Centrifuges
H_2O
Feed Mixing Tank
WASHED SOLIDS
Washer
To NaOH Tank
Sterilizer
Cooler
Flocculant
H_2O
SOLIDS CAKE
Fuel
STERILE MEDIA
Fermenter
AIR
Dewatering
Furnace
Fermenter
CLEARED CELL STREAM
TREATED SLURRY
Solids Treatment Tank
SLUDGE
WHOLE CULTURE
Cyclone
Hopper
Feeder
NaOH
UNDIGESTED SOLIDS
Screen Filter
De-sludging Centrifuge
Cellulose Source
Grinder Blower

LITERATURE REFERENCES

1. Dunlap, C. E., "Proteins from Waste Cellulose by Chemical-Microbial Processing", Ph.D. Dissertation, Louisiana State University, 1969.

2. Callihan, C. D., "How Engineers are Putting Microbes to Work", Chemical Engineering, 77(20):161, 1970.

3. Han, Y. C., C. E. Dunlap, and C. D. Callihan, "Single-Cell Protein from Cellulosic Wastes", Food Tech., 25:32-35, 1971.

4. Srinivasan, V. R., and Y. W. Han, "Utilization of Bagasse, Cellulases and Their Applications", Advances in Chemistry Series, 95:447, American Chemical Society, Washington, D.C.

5. Callihan, C. D., and C. E. Dunlap, "Single-Cell Proteins from Cellulose Wastes", Document of U.S. Environmental Protection Agency, 96 pages, 1971.

6. Dunlap, C. E., "Single-Cell Protein from Waste Cellulose", Presented at the 64th Annual Meeting of the American Institute of Chemical Engineers, San Francisco, California, Nov. 28-Dec. 2, 1971.

7. Han, Y. W., "Studies of the Bacterial Enzymes Involved in the Degradation of Cellulose", Ph.D. Dissertation, Louisiana State University, 1969.

8. Callihan, C. D., and C. E. Dunlap, "The Economics of Protein Production from Cellulosic Wastes", Compost Science, 10(1-2), 1969.

9. Han, Y. W., and V. R. Srinivasan, "Isolation and Characterization of a Cellulose Utilizing Bacterium", Appl. Microbiol., 16:1140-1145, Aug. 1968.

10. Callihan, C. D., and C. E. Dunlap, "Pilot Production of Single-Cell Proteins from Cellulosic Wastes", Report No. SW-24C, U.S. Environmental Protection Agency, Sup't. of Doc. Stock No. 5502-0027, 1971.

11. Warmicker, J. O., et al, A Review of the Literature on the Effect of Caustic Soda and Other Swelling Agents on the Fine Structure of Cotton, Shirley Inst. Pamphlet No. 93, Shirley Inst., Didsbury, Manchester, :55, December 1966.

12. Stamm, A. J., and E. E. Harris, Chemical Processing of Wood, Chemical Publ. Co., New York, :300, 1953.

13. Rhodes, A., and D. L. Fletcher, Principles of Industrial Microbiology, Pergamon Press, London, :72, 1966.

14. Yang, S. P., and W. F. McKnight, "Nutritional Studies of Single-Cell Protein", In Single-Cell Protein from Cellulosic Wastes", Final report, (2), Baton Rouge, Louisiana State University, :527-549, 1970.

15. Fleanor, M., and V. R. Srinivasan, "Studies on the Isolation, Purification, and Properties of a Cellulase from Cellulomonas sp. ATCC #21399", Presented to S.W. Regional ACS Meeting, Baton Rouge, Louisiana, December 6, 1972.

APPENDIX

A. MAINTENANCE OF ORGANISMS

   1. *Cellulomonas*

   2. *Alcaligenes faecalis*

   3. Ycl3 yeast

B. STANDARD INOCULUM PREPARATION

C. CULTURE SAMPLING AND ANALYTICAL TECHNIQUES

D. STANDARD SUBSTRATE TEST PROCEDURE

A. MAINTENANCE OF ORGANISMS

1. Cellulomonas

Colonies are routinely carried on slants of brain-heart infusion agar at 34°C and are transferred weekly. A healthy culture of cellulomonas can be stored in a refrigerator for about three months. The colonies are smooth and have a characteristic yellow color. The most reliable key to the identification of cellulomonas is its ability to digest filter paper. Should a culture fail to cause filter paper degradation within five days, the slant from which the culture was taken should be discarded. Only active, reliable cultures should be continued. Microscopically, cellulomonas is recognizable as a gram-negative, non-motile rod.

Brain-Heart Infusion Agar Slants -

37 gms brain-heart infusion

18 gms nutrient agar

1 liter D.I. water

Mix ingredients and put in autoclave for 10 min. at 15 psi and 250°F in order to melt the agar. Pour the melted agar into screw-top test tubes using only enough agar to form a slant 3/4 the length of the tube when the tube is placed on a slight incline. Screw the caps on the test tubes loosely, autoclave for 15 min. at 15 psi and 250°F. Remove the tubes from the autoclave, tighten the caps, and immediately place them on an incline to form a slant in the tube not less than 1/2-in. from the mouth of the tube. Allow an hour or so for the slants to solidify, then place them in a rack in the cold room.

Filter Paper Tubes -

Use 10-ml flask media, containing 1 g/l yeast extract and a strip of filter paper, in a screw-top test tube. Autoclave for 15 min. at 250°F and 15 psi, and store in cold room. Filter paper tubes should be inoculated directly from the slant and incubated in the incubator shaker at 33°C. In three to five days there should be evidence of filter paper degradation (positive cellulase reaction).

2. Alcaligenes faecalis

Colonies are routinely carried on slants of cellobiose agar at 34°C and should be transferred weekly. The parent cultures should be grown in the absence of yeast extract or other growth-enhancing factors; therefore, it may take four to five days after inoculation for colonies to appear on slants. Cultures of alcaligenes cannot reliably be stored for long periods of time in cold. The colonies are smooth, shiny, and almost transparent in appearance. Microscopically, alcaligenes is recognizable as a gram-negative, motile rod. Flask cultures of alcaligenes are grown on 0.6% lactose media, and flocculation occurs within 16 hours after inoculation. Flocked cultures can be added to 24-hour-old cultures of cellulomonas, and a deflocking process will occur. Alcaligenes shows a positive reaction for B-glucosidase and B-galactosidase enzymes.

Cellobiose Agar -

1 gram cellobiose

18 grams bacto-agar

1 liter water

Suspend agar and cellobiose in the water ane put into autoclave for 10 min. at 15 psi and 250°F to dissolve the medium completely. Prepare slants as previously described.

3. Yc13 Yeast

The yeast culture is routinely carried on brain-heart infusion agar slants and is transferred weekly. The colonies have a rough appearance and an off-white-to-gray color. Microscopically, the cells of Yc13 are small and ovate. The culture is B-glucosidase positive. The yeast cultures can be maintained in the cold room for up to three months; but to do so, the cultures should be grown on potato-dextrose agar. Flask cultures of yeast are started in 125 or 250 ml flasks containing 75 ml of 0.6% lactose media directly from the slants. Such cultures are incubated 24 hours in the incubator shaker before transfer.

Potato-dextrose Agar -

39 grams potato-dextrose agar

1 liter water

Suspend agar in the water and put in autoclave for 10 min. at 15 psi and 250°F to dissolve the medium completely. Prepare slants as previously described.

Lactose Flask Media -

$(NH_4)_2SO_4$	- 6 g/l
NaCl	- 1 g/l
K_2HPO_4	- 4.45 g/l
KH_2PO_4	- 3.40 g/l
$MgSO_4 \cdot 7H_2O$	- 0.4 g/l
$CaCl_2$	- 0.1 g/l
Lactose	- 6 g/l
Trace minerals	- 1 ml/l

Assay for B 1-4 Glucosidase Activity -

- Spin down 10 ml of cells in mother liquor.

- Resuspend in 1 ml of 0.1M phosphate buffer (pH 6.8).
- Add 0.1 ml of 0.2% cetytrimethyl ammonium bromide solution for each ml of resuspended cells.
- Incubate at 37°C for 10 minutes.
- Add 1-2 ml of $5x10^{-3M}$ PNPG (p-nitrophenyl B-D-(-)-glucopyranaside).
- After a suitable reaction time (20 min.), the enzyme activity is stopped by adding 2 ml of 1M sodium carbonate.
- The yellow color that developed during the hydrolysis of the substrate is read at 400 nm. The yellow color is due to the release of p-nitrophenol.

B. STANDARD INOCULUM PREPARATION

Cellulomonas - maintained on slant of brain-heart infusion agar

↓ transfer directly with inoculating loop

10 ml screw-cap test tube of flask media with filter paper strip

↓ incubate at 34°C for 3-5 days or positive cellulase activity

Transfer tube culture to a 2-liter flask containing 1 liter of flask media and 10 gms. of filter paper

↓ incubate at 34°C with shaking for 24 hours.

STANDARD CELLULOMONAS

Alcaligenes - maintained on slant of cellobiose agar

↓ inoculate directly

250 ml flask containing 70 ml of lactose media

↓ incubate at 34°C with shaking for 24 hours

STANDARD ALCALIGENES

Yc13 Yeast - maintained on slants of potato dextrose agar or nutrient agar

↓ (preparation is identical to alcaligenes)

STANDARD Yc13

Cellulomonas plus alcaligenes

Standard cellulomonas 1 liter + Standard alcaligenes 70 ml

↓ mix

↓ incubate at 34°C with shaking for 24 hours.

STANDARD MIXED CULTURE OF CELLULOMONAS PLUS ALCALIGENES

Cellulomonas plus Yc13

Standard cellulomonas 1 liter + Standard Yc13 70 ml

↓ mix

↓ incubate at 34°C with shaking for 24 hours.

STANDARD MIXED CULTURE OF CELLULOMONAS PLUS Yc13.

C. CULTURE SAMPLING AND ANALYTICAL TECHNIQUES

Cultures of all stirred fermenters were routinely sampled and determinations made on several important variables. Outlined below is a complete test procedure for determination of cell and substrate concentrations, cellulose content of substrate solids, and reducing sugar and total soluble carbohydrates concentrations.

1. A homogeneous sample of the whole culture was drawn from the fermenter; volume of the sample was usually between 30 ml and 100 ml. The total volume of the sample was recorded.

2. The total sample was filtered through a Millipore stainless steel filter funnel with a 200-mesh retaining screen.
3. The liquid filtrate was removed and the solids washed with about 200 ml tap water.
4. The solids retained on the screen were transferred quantitatively to a pre-weighed disposable drying dish and placed in a forced-air drying oven at 95°C for 20 hours minimum drying time.
5. About 5 ml of the liquid filtrate was placed in a 25 ml syringe and filtered through a Millipore syringe filter holder containing one 22mm Millipore glass wool prefilter pad.
6. The resulting filtrate was used for optical density measurement in a Klett-Summerson colorimeter equipped with a ree (#66) filter by dilution with a known volume of deionized water to assure an optical density reading of from 30 to 100 Klett units. The optical density was computed from the Klett reading and the dilution factor and recorded as gross optical density.
7. Duplicate samples of known volume were taken from the liquid filtrate of step 2. The volume of these were usually from 20 ml to 40 ml. The samples were placed in centrifuge tubes and spun at 15,000xg for 20 minutes.
8. The supernatant was decanted from the cell pellet. The optical density of the supernatant was read and recorded as base optical density. The base optical density was then subtracted from the gross optical density to obtain culture optical density. This measurement could be correlated with cell dry weight as shown in steps 10 and 11.

9. The cell pellet was resuspended in de-ionized water and again centrifuged at 15,000xg for 20 minutes.

10. The supernatant was discarded and the resulting cell pellet was dried for 20+ hours in a forced-air oven at 95°C. The cell pellet was weighed and the cell dry weight was divided by the sample volume used in step 7. The result was recorded as the gravimetric cell concentration.

11. A ninhydrin protein determination was carried out on the dried cell pellet of known weight. The results could be used to calculate the protein content of the cells, and the protein concentration in a known volume of culture.

12. The dry solids from step 4 were weighed and recorded. The solids weight divided by the total sample volume gave the solids concentration in the media.

13. The cellulose content of the solids was determined by refluxing 2.0 gms of the dried solids in 100 ml of ethanolamine for 2 hours. The mixture was filtered through a 200-mesh screen in a Millipore filter funnel. The solids retained were washed with water, dried, and weighed. The dry cellulose was then ashed and reweighed. Concentrations of ash and cellulose in the solids could then be calculated.

Correlation of Culture Optical Density With Cell Dry Weight

Culture	Cell Dry Weight per 1000 Klett Units using #66 Filter
Cellulomonas	2.3 grams/liter
Alcaligenes	4.0 grams/liter
Cellulomonas plut alcaligenes	3.0 grams/liter
Cellulomonas plus Ycl3	3.5 grams/liter

D. STANDARD SUBSTRATE TEST PROCEDURE

1. Samples of solid, cellulosic substrates to be tested were dried in a forced-air oven at 95°C for 24 hours.
2. One gram of each sample was added to a 500 ml Erlenmeyer flask containing 250 ml of flask media and 0.25 gm. yeast extract. A test standard flask was prepared with the sample flasks; 1.0 gram of Whatman No. 1 filter paper was used as substrate.
3. All flasks were autoclaved at 15 psi for 30 minutes.
4. Each flask was inoculated with 2.0 ml of Standard Cellulomonas plus Yc13 culture.
5. All flasks were incubated with shaking for 24 hours at 34°C.
6. A liquid sample was aseptically drawn from each flask and the optical density measured and recorded. The flasks were returned to the incubator for 24 more hours.
7. At the end of 48 hours since inoculation, the optical density of each culture was again determined; and the amount of undigested solids remaining in the flask was determined by the gravimetric techniques detailed in the Analysis section of the Appendix.
8. The fraction of solids lost or solubilized during the test was recorded. All values were corrected to 75% digestion of the filter paper standard. The corrected values were recorded as X_s--the fraction of substrate digested in the evaluation test based on 75% filter paper digestion.