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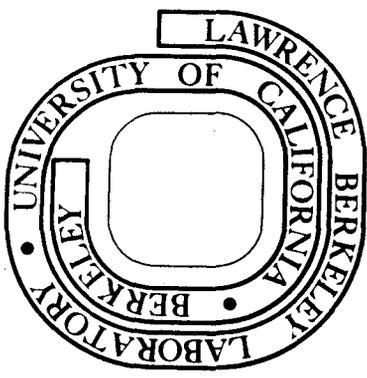
PROCESS DESIGN BASIS FOR ENZYMATI  
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Charles R. Wilke, Urs v. Stockar, and Ren Der Yang

July 1976

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Process Design Basis  
for  
Enzymatic Hydrolysis for Newsprint

by

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Abstract

Relevant laboratory data and critical assumptions for the design of a tentative process for enzymatic hydrolysis of newsprint are discussed. The results support previously developed cost estimates for the production of sugars from cellulose, which indicate that the method may be approaching economic feasibility. However, pilot plant studies are needed to test the concepts under realistic conditions. Also, continued basic research can be expected to lead to further process improvement.

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## INTRODUCTION

Tentative processes for enzymatic hydrolysis of newsprint have been proposed by Wilke, Yang and von Stockar (1), Wilke and Yang (2) and by Wilke and Mitra (3). The most recent scheme will be reviewed in this paper for reference. These processes have been designed on the basis of small scale laboratory data for some of the key steps combined with engineering analysis. A number of assumptions were used to complete preliminary process designs and cost estimates for conversion of newsprint to sugars.

It is the goal of this paper to discuss the more important basic information required to design such processes and to present new laboratory data justifying the respective assumptions made in the most recent design (1). The discussion will also reveal which of the necessary information is still largely based on assumptions and thus necessitates further research.

## PROCESS DESCRIPTION (1)

Figure 1 is a schematic flow diagram of the proposed hydrolysis process. Flow quantities correspond to the most recent design by Wilke, Yang and von Stockar (1). For simplicity the facilities for milling, heat exchange, induction solids sterilization and residual solids combustion have been omitted in the flow diagram, although they were designed and are included in the processing cost analyses.

The primary plant feed consists of 885 tons per day of newsprint containing 6% moisture. By means of moderate shredding and hammermilling the feed is reduced to approximately

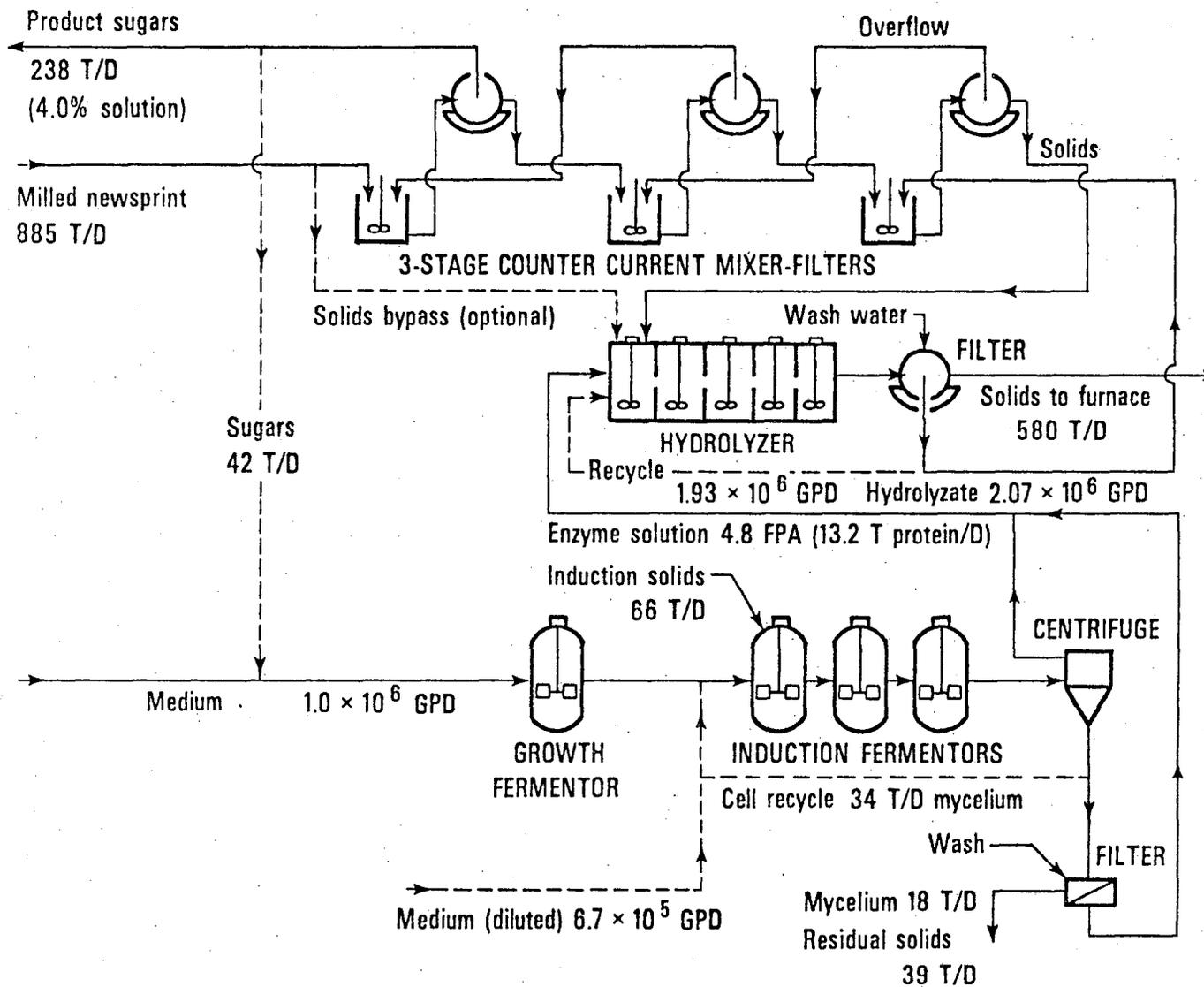


Fig. 1. Flow diagram of process for enzymatic hydrolysis of newsprint.

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Table 1

## Base Design Case Specification

Feed (-20 mesh Newsprint	885 Ton/Day
Cellulose Content <sup>1</sup>	61% (Dry)
Enzyme Activity	3.5 FPA
Cellulose Hydrolysis	50%, 40 hr., 45°C
Enzyme Recovery	34%
Product (As Glucose) <sup>2</sup>	238 Ton/Day
Product Concentration	4%
Cell Recycle Fraction	0.65

<sup>1</sup>Assumed newsprint composition: 61%  $\alpha$ -cellulose, 21% lignin and 16% hemicellulose.

<sup>2</sup>Representative sugar composition: 72% glucose, 22% cellobiose, 4.4% xylose, 1.5% mannose.

-20 mesh. An additional 66 tons per day of feed material is diverted to the first enzyme induction fermentor after sterilization with steam. The product sugar stream from the hydrolyzer is contacted countercurrently in 3 mixer-filter stages with feed solids for enzyme recovery. Each mixer filter stage consists of a mixing tank to provide 30 minutes contact time and a horizontal belt vacuum filter to separate the solids from the liquid.

Hydrolysis is conducted over 40 hours at 45°C at a solid/liquid ratio of 1/20 w/w based on inputs to the hydrolyzer. The latter consists of 5 agitated cylindrical concrete digestors of the type used for solid waste treatment in sanitary engineering. Cellulose conversion of 50% is assumed, at an overall enzyme strength equivalent to 3.5 FPA<sup>1</sup> in the hydrolyzer. Provision is made for recycle of a portion of the product sugar solution (plus enzyme) back to the hydrolysis vessel. A sugar concentration of 4.0% is obtained for the case shown. A range of sugar levels is possible depending on the mode of operation and amount of sugar recycle employed.

Make-up enzyme is produced in a two-stage fermentation system, employing the fungus Trichoderma viride QM9414. Cell growth is obtained in the first stage at a dilution rate of 0.2 hr<sup>-1</sup> employing a medium containing 1% product sugars plus minerals and protein nutrient given in Table 2. The induction system is operated at an overall dilution rate of 0.017 hr<sup>-1</sup> excluding the cell recycle stream. Both stages employ agitated

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<sup>1</sup>Filter Paper Activity

Table 2  
Medium Raw Materials

<u>Component</u> <sup>1</sup>	<u>GM/Lit</u>	<u>\$/Ton</u>	<u>Tons/Day</u>
$(\text{NH}_4)_2\text{SO}_4$	1.4	90	9.3
$\text{KH}_2\text{PO}_4$	2.0	120	13.2
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.4	33	2.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3	110	2.0
$(\text{NH}_2)_2\text{CO}$	0.3	160	2.0
Protein Nutrient <sup>2</sup>	0.5	300	3.3

<sup>1</sup>Trace elements are assumed supplied by the process water.

<sup>2</sup>Pharmamedia, Traders Protein Corport., Ft. Worth, Texas.

stainless steel vessels operated at 30°C with aeration rates of 0.15 and 0.015 v.v.m. in the growth and induction stages, respectively. The growth stage feed is sterilized in a heat exchanger system (not shown). The induction section effluent is passed through a centrifuge from which a portion of the underflow is fed back to the first induction stage. Ten parallel induction stages are employed. The flow quantities in Figure 1 correspond to a cell recycle fraction of 0.65. Recycle fraction is the fraction of cells leaving the last induction stage which is returned to the first stage. For the case shown, the use of recycle will maintain the cell density in the induction system at 7 gm per liter, assuming negligible growth in the induction system when newsprint is employed. The resultant enzyme production is sufficient to provide an enzyme concentration of 3.5 FPA in the hydrolyzer. A portion of the centrifuge underflow is filtered and the cells are discarded to maintain adequate cell viability.

Spent solids from the hydrolyzer following filtration are fed to a furnace and steam-power plant to provide process steam and electricity for the process.

The economic analysis based on this, as summarized in Table 3, suggests a manufacturing cost of sugar of 5.2¢/lb. Clearly enzyme production is the major cost factor, comprising nearly 60% of the total. The process is highly capital intensive. 35% of the total cost arises from the fixed capital requirement to manufacture the enzyme.

Table 3

	Capital Base	Labor Base	Utilities	Raw Materials	Total
Pretreatment	0.427	0.039	0.084		0.55
Hydrolysis	0.942	0.079	0.153		1.17
Enzyme Production	1.873	0.078	0.284	0.835	3.07
Enzyme Recovery	0.313	0.078	0.017		0.41
Totals	3.56	0.27	0.54	0.83	5.20

## HYDROLYSIS

General Design Criteria

Design of the process described above is based on what is considered an optimum set of hydrolysis and pretreatment conditions. Optimum conditions are those which will yield a maximum cellulose conversion while minimizing, at the same time, the amount of make-up enzyme required per pound of sugar produced. The latter is especially important due to the dominating role of the enzyme make-up in the sugar cost. It is of course directly proportional to the ratio of enzyme concentration to substrate concentration used in hydrolysis and inversely proportional to the conversion achieved, and it also depends on the enzyme concentration remaining in the solution after hydrolysis, because this portion can be recovered by adsorption as previously described. The conversion and cellulase recoverability are both functions of the hydrolysis and pretreatment conditions. Therefore, experiments will now be reported which were designed to measure the conversion and the enzyme recoverability under various hydrolysis and pretreatment conditions.

Hydrolysis was carried out in batches of 300 or 600 ml under continuous agitation with a marine impeller at 300 r.p.m. Enzyme activities were measured in terms of Filter Paper Activity,  $C_1$  and  $C_x$  activity according to Mandels and Weber (4), and international units were estimated from FPA using the information published in the study on measurement of cellulase by Mandels et al. (5). Sugar concentrations were determined as glucose by

Table 4

Effect of Enzyme Concentration or Conversion and Enzyme Requirement  
(Solid concentration 5% w/w ratio)

## ENZYME CONCENTRATION

FPA	IU/ml (estimated)	Hydrolysis duration hrs	Conversion %	Enzyme requirement no recovery <u>g Enzyme</u> <u>g sugar</u>	Enzyme left in solution %	Estimated enzyme requirement with recovery <u>g Enzyme</u> <u>g Sugar</u>
4x3.4	2.16	48	58	0.25	65	0.088
4x2.5	1.12	48	55	0.17	35	0.11
2x2.84	0.7	48	45	0.12	-	-
3.49	0.55	41	42	0.088	22	0.069
3.13	0.44	48	43	0.074	20	0.0588

the DNS test (6).

For hydrolysis runs with high enzyme strength, cellulase protein was precipitated with acetone (3:1 v/v) and redissolved in a smaller amount of acetate buffer.

#### Effect of Enzyme Concentration

Table 4 summarizes a series of experiments conducted at different cellulase concentrations. Conversion may clearly be increased by using higher enzyme strength. However, the conversion increase is less than linear with enzyme concentration, and therefore the amount of enzyme used in hydrolysis per pound of sugar produced increases with the enzyme concentration. The increased enzyme usage is offset in part by an increase in recoverable enzyme left in the filtrate after hydrolysis (6th column), which suggests some sort of saturation effect in the equilibrium between adsorbed and dissolved cellulase. But even when the increased recovery is allowed for in evaluating the enzyme requirement (last column), the most efficient utilization of the enzyme occurs at the lowest enzyme strength and therefore at the lowest conversion.

Too low conversion, however, cannot be used in a hydrolysis process because of the potential raw material cost, and because of the increase of hydrolysis vessel volume required to produce a given amount of sugar. Although no formal optimization was made, a Filter Paper Activity of 3.5 affording a low enzyme requirement, while at the same time permitting reasonable conversions, seems favorable.

Table 5

Effect of Suspension, Concentration and Milling

(based on experiments by B Rubik (17)).

Suspension	Milling	FPA	Hydrolysis duration	Conversion	Enzyme requirement no recovery	Enzyme left in solution	Estimated enzyme requirement with recovery
%			hr	%	<u>g cellulase</u> g sugar	%	<u>g cellulase</u> g sugar
5	Wiley (-20 mesh)	3.5	40	50	0.076	22	0.059
5	Ball (-325 mesh)	4.06	48	86.1	0.055	-	-
10	Wiley (-20 mesh)	4.06	48	24.8	0.076	0.0	0.076
10	Ball (-325 mesh)	4.06	48	72.9	0.032	-	-

TABLE 6

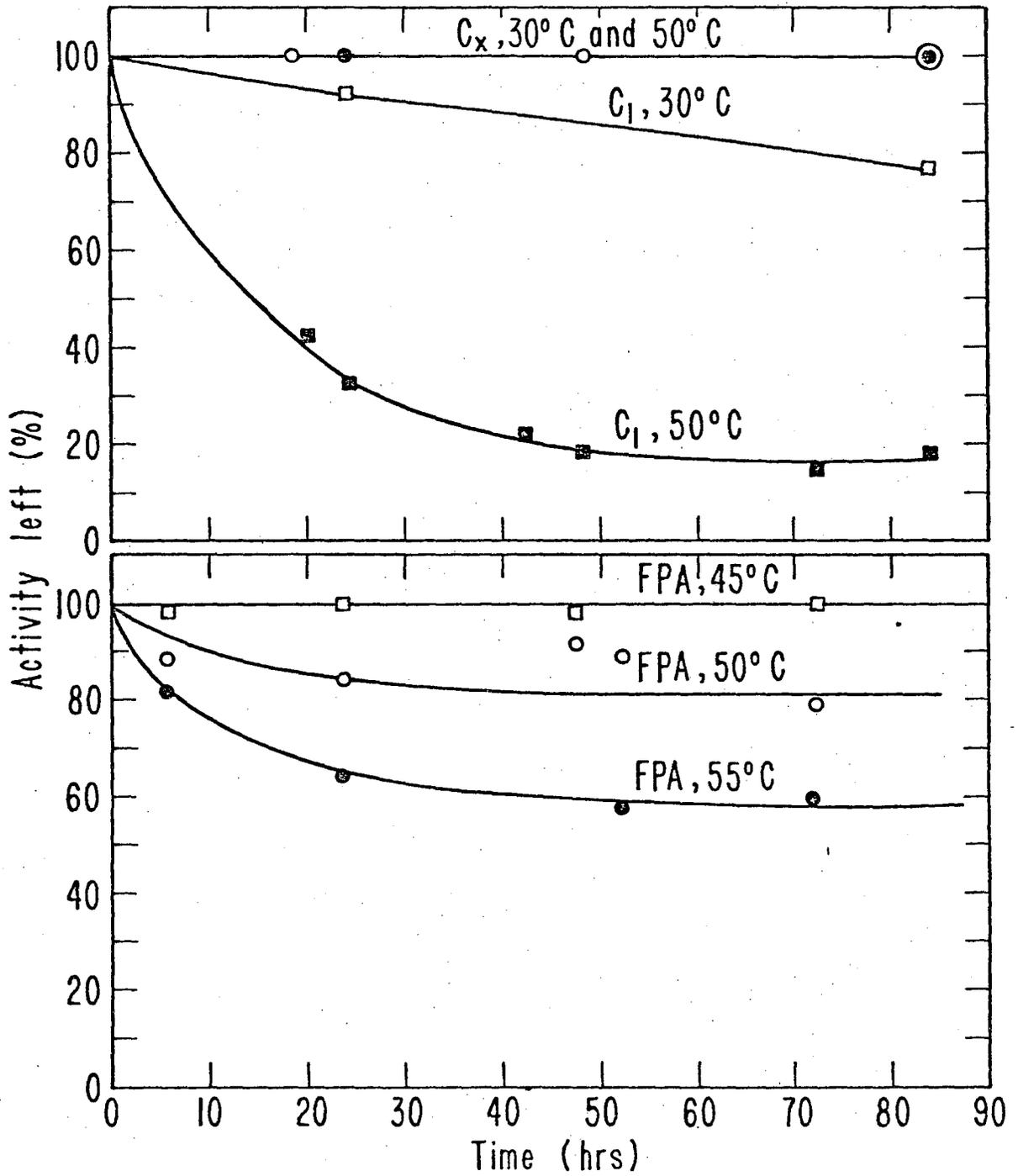
MATERIAL	FPA	ESTIMATED % CONVERSION OF α CELLULOSE	% CONVERSION OF TOTAL SAMPLE	INVESTIGATORS
CORRUGATED FIBERBOARD (MULCHER 1-3CM)	4.5	73	55	MANDELS, ET AL. (29)
NEWSPAPER (MULCHER 1-3 CM)	4.5	74	42	MANDELS, ET AL. (29)
COMPUTER PRINT-OUT (HAMMER MILL)	4.5	73	51	MANDELS, ET AL. (29)
KEY PUNCH HOLES (KEY PUNCH 1MM)	4.5	80	56	MANDELS, ET AL. (29)
NEWSPRINT (WILEY MILL-20 MESH)	3.5	50	31	WILKE & YANG (13)

### Effect of Milling

The effect of milling on the conversion has been reported earlier (2). Ball milling to -325 mesh increases the conversion dramatically, apparently because it reduces the degree of crystallinity of the substrate. The effect of ball milling on enzyme requirement is equally favorable as shown in Table 5. Yet it has been concluded previously that ball milling does not appear practical for large scale due to the high energy costs associated with it. If on the other hand, one accepts a moderate conversion of only 50%, mild shredding obviously suffices as pretreatment. This is demonstrated by Table 6, which compares the conversions obtained from different substrates having been crudly reduced in size.

### Effect of Suspension Concentration

If the concentration of the suspension in hydrolysis could be increased without reduction of conversion, the enzyme requirement, being proportional to the ratio of enzyme to substrate concentration, could be greatly reduced. Experiments summarized in Table 5, however, have shown that conversion percentages are about halved when the suspension concentration is increased from 5% to 10%, so that the amount of enzyme used in hydrolysis per sugar produced stays about the same. Moreover, no enzyme could be detected in the filtrate after a 10% hydrolysis, and no recovery would be possible. Increasing the suspension concentration thus has an adverse effect on both conversion and enzyme requirement. As with enzyme concentration, an optimum value of suspension concentration could probably be established by further research,



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Fig. 2. Thermal stability of cellulase enzyme complex in solution.

but for practical process, 5% appears to be a feasible suspension level.

### Effect of Temperature

Since the enzyme in the free solution is to be recovered, hydrolysis ought to be carried out at the highest temperature at which the free enzyme is still essentially stable. The thermal stability of the free enzyme was determined by incubating batches of enzyme solution at different temperatures in agitated 600 ml jars and measuring the FP,  $C_1$  and  $C_x$  activities as a function of time. The results are summarized in Fig. 2. From these data, 45° was chosen as the hydrolysis temperature.

### Conclusion for Design

On the basis of the foregoing data, hydrolysis conditions were specified as given in the process description. The conversion obtained under these conditions has been measured in many experiments and ranges from 40% to 50%, depending mainly on the batch of enzyme used. Considering these results in comparison with those given in Table 6, 50% conversion appears a reasonable design assumption.

### ENZYME RECOVERY

Although the enzyme requirement for the process may be established approximately by determining the level of cellulase remaining in solution after hydrolysis, the "hydrolysis value" of this recoverable enzyme should be checked. Wilke, et al. (1) determined the hydrolysis value of the enzyme remaining in the sugar solution after hydrolysis at 45°C for 40 hours. They precipitated and redissolved the remaining enzyme in buffer and used the resulting solution instead of plain buffer in a series of Filter Paper tests. As a result it was possible to dilute the

original enzyme by some 35% and still to observe the Filter Paper Activity (FPA) of the original enzyme solution. The hydrolysis value of the recoverable enzyme was concluded to be 35% of the original enzyme at 3.5 FPA. The same tests indicate a final FPA of only 0.6 to 1.2 in the product sugar solution. These values are lower than the figures reported by Wilke and Yang (2). They observed a gradual release of the enzyme after the initial strong adsorption on the cellulosic material, resulting in final activities in the free solution ranging from 50 to 75% of the original levels. But recently conducted hydrolysis experiments (16) showed that no release occurred in most cases after the first, strong adsorption of enzyme on the solids. Rather, the activities in the free solution continued to drop slightly or at best stayed constant. A typical set of curves is shown in Figure 3 for a hydrolysis experiment carried out over 40 hours at 45°C at an enzyme concentration of 3.5 FPA. (16) The substrate, Wiley milled newsprint (-20 mesh) was hydrolyzed in an enzyme solution of an original activity of 3.5 FPA in a 500 ml glass jar equipped with a marine propeller operating at 300 r.p.m. Figure 3 reports the activities obtained after precipitation of the protein in acetone at room temperature (3:1 v/v) and after redissolving the precipitate in acetate buffer of pH 5 in order to eliminate the high sugar concentrations. To calculate from Figure 3 the percentages of original activity remaining in the solution, the enzyme activities were calibrated against protein concentration. Several dilutions of the original enzyme solution with acetate buffer were prepared and the  $C_1$ ,  $C_x$  and FP activities were measured both before and after precipitation with acetone. Protein

concentrations were measured by modified Biuret method (7), which was standardized against Bovine albumin.

The percentages of activities remaining in the solution after 40 hours of hydrolysis, expressed as protein equivalent were: 16% for  $C_x$ , 21.5% for  $C_1$  and 25% for FPA, whereas, 50% of the Biuret protein remained in solution.

The final FPA agrees well with the value reported earlier by Wilke et al. (1) and the experiment is therefore compatible with their dilution test. It is interesting to note that the remaining fraction of FPA in the product sugar solution is higher than the corresponding fractions for  $C_x$  and  $C_1$ , and that the actual hydrolysis value of the remaining enzyme mixture seems to be even greater than the FPA that can be detected in the solution after hydrolysis.

To substantiate these results, the fraction of hydrolysis left in the solution after the hydrolysis ought to be confirmed using various enzyme dilutions in actual hydrolysis runs rather than in FPA tests. Experiments of this sort are now under way actually simulating a continuous countercurrent recovery of the remaining enzyme by adsorption on fresh newsprint. Preliminary results suggest that the actual hydrolysis value might be higher than 35%.

An enzyme recovery of 35% can therefore safely be used in process design studies, and when it is combined with a 50% conversion, the overall enzyme requirement for hydrolysis at a 5% suspension at 3.5 FPA comes out to be 0.047 grams of enzyme protein per gram of sugar produced.

## ENZYME PRODUCTION

Design of Enzyme Production Facilities

An enzyme production plant has to be designed to meet the enzyme needs of the hydrolyzers. For the case shown in Figure 7, to produce 280 T/Day of sugars the enzyme requirement is estimated to be 13.2 tons of enzyme protein per day. To permit accumulation of the sugars to a 4% level by recycling the hydrolyzate, enzyme has to be produced at 4.8 FPA to maintain 3.5 FPA in the hydrolyzer. This fixes both the flow through the induction fermentors and the enzyme concentration. The working volume,  $V$ , of the induction stages may then be calculated as

$$V = \frac{F[E]}{k_E \bar{X} \epsilon(\theta)} \quad (1)$$

where:

- $V$  = total working volume of induction fermentors, liters, gals
- $F$  = flow rate through induction system excluding recycle, liters/day, gals/day.
- $[E]$  = enzyme protein concentration, g/l
- $k_E$  = specific cellulase productivity, g/(g)(day)
- $\bar{X}$  = cell concentration, g/l
- $\epsilon(\theta)$  = "effectiveness" factor

As mentioned in the process description, it is assumed possible to maintain a high average cell density, and thus a low working volume by a recycle arrangement. This assumption must be confirmed experimentally in a sustained operation. The specific enzyme productivity,  $k_E$ , must also be determined by experiment. The "effectiveness"

factor,  $\epsilon$ , corrects for the fact that because of a lag phase in enzyme induction not all of the fungal cells present in the system are induced to produce cellulase. Its value comes from an engineering analysis.

The working volume of the growth stage is influenced by the amount of induction solids required for the production of a given amount of enzyme since the undigestible part of the induction solids have to be removed through the bleed stream. This gives rise to a continuous loss of cells which have to be replaced by a growth stage if growth in the induction fermentor is assumed negligible. Thus, the induction solids requirement should also be determined experimentally before the plant can be designed.

#### Enzyme Production Experiments

To provide the necessary information for design identified in the previous section, a series of experiments was conducted. Specific objectives of these experiments were (1) to demonstrate the effectiveness of the cell recycle concept over a prolonged period of time, (2) to measure the specific productivity of recycled cells under various operating conditions and (3) to determine the induction cellulose requirement.

### Experimental Apparatus

Figure 4 shows an example of the experimental systems used. The growth stage was omitted to maintain 100% induction. The central portion of the apparatus consisted of a standard 14 liter fermentor (New Brunswick Scientific Co.). Liquid suspension was pumped during the experiment from this vessel into special devices in which the solids could be separated from the liquid product stream and recycled back into the fermentor. Two principles have been applied and their feasibility tested to achieve separation of the solids from the liquid: centrifugal filtration and settling.

The principle of centrifugal filtration has been developed in theory and tested experimentally by Bhagat and Wilke (8), Sortland and Wilke (9), and Margaritis and Wilke (10). The model built for this series of experiments consisted of a cylindrical filtration chamber of about 2" diameter made of stainless steel.

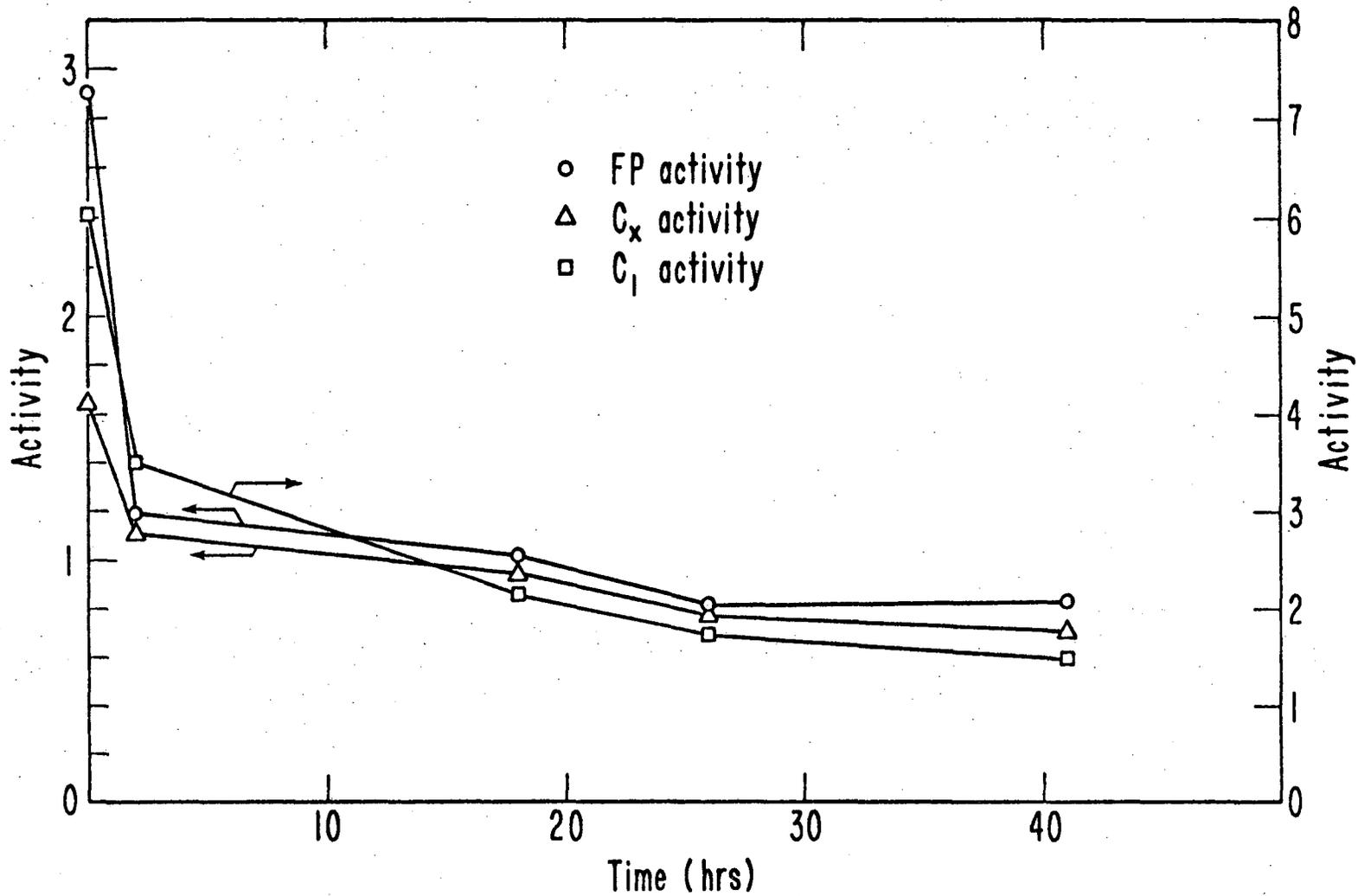
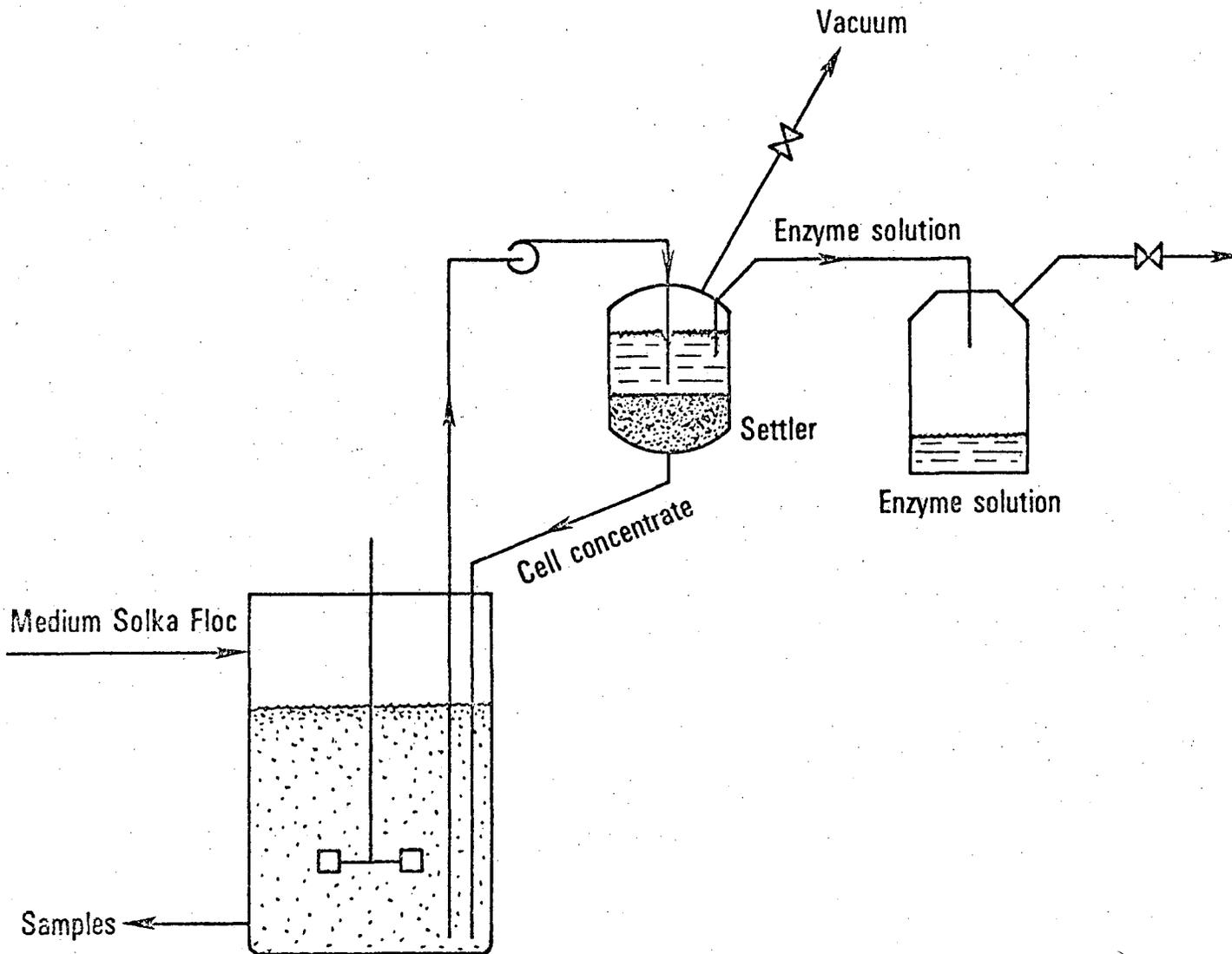


Fig. 3. Enzyme activity remaining in solution during hydrolysis as a function of time.

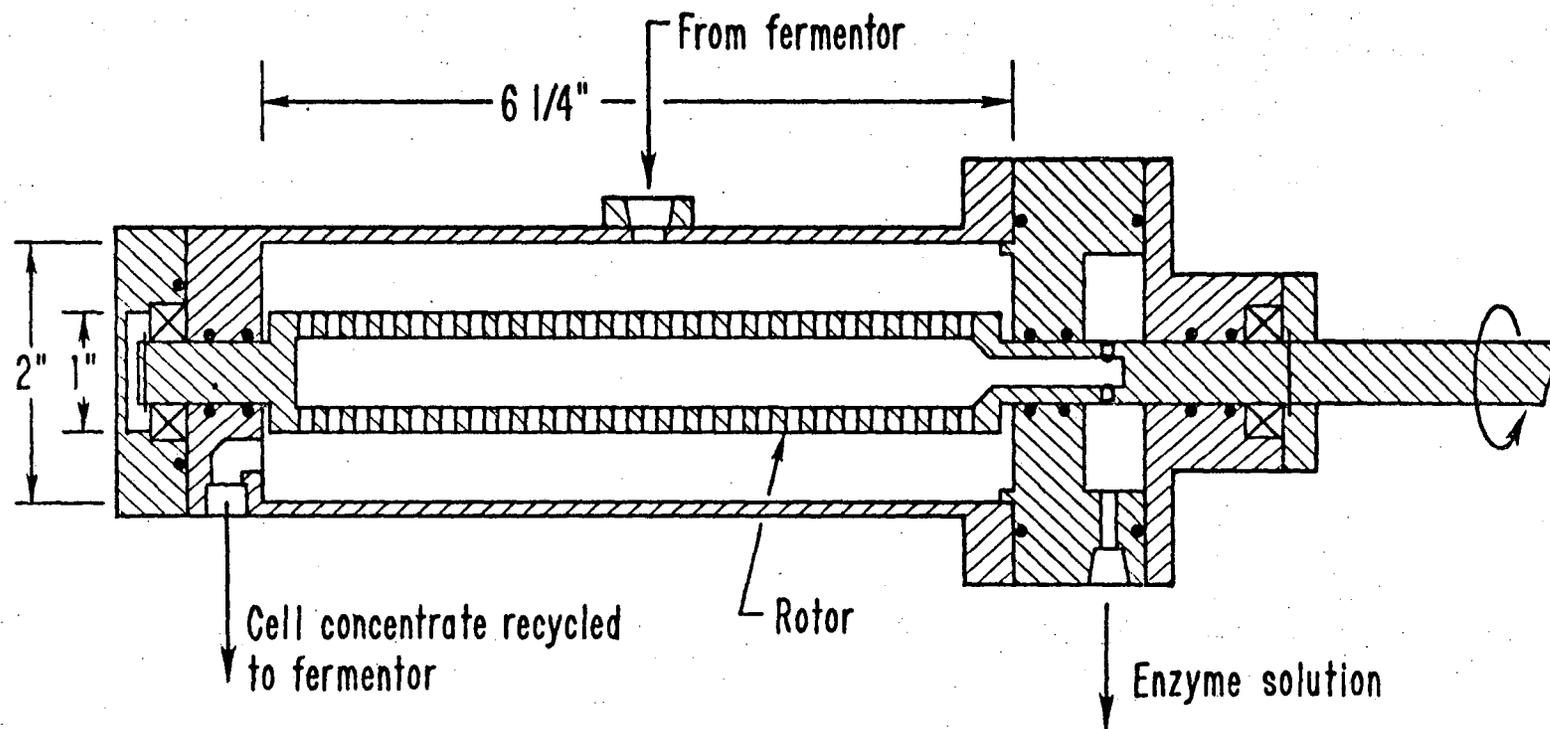
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Fig. 4. Experimental apparatus for continuous cellulase production experiments with cell recycle using a settler as cell separator.



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Fig. 5. Rotary concentrator used for separating fungal cells continuously from product enzyme solution.

(Figure 5). The only moving part was a stainless steel rotor of 1" outer diameter driven by an external motor to speeds up to 6000 rpm. The rotor was hollow and had many 1/8" holes connecting the cavity within the rotor with the filtration chamber. During the operation the cellulose/cell suspension was pumped continuously from the fermentor into the outer filtration chamber at a rate of about 600 ml/hr. The spinning rotor created a velocity field within the annulus whereby most of the solid particles were prevented from reaching the rotor by centrifugal force. This resulted in a concentration of the suspension in the outer filtration chamber which was pumped back into the fermentor. The pressure maintained in the annulus (ca. 3 psi) forced some of the liquid through the holes of the spinning rotor from where it left the device through the hollow shaft and through a discharge chamber. This liquid was collected at a rate of about 140 ml/hr as the crude enzyme product. In a series of preliminary experiments it was found that the rotational speed had to be limited to about 2400 rpm to avoid considerable mechanical disruption and lysis of the fungal cells through the action of turbulence and shear stress. At 2400 rpm the centrifugal concentrator reduced the solid concentration in the enzyme solution to a residual level of 0.7-1 g/l.

To separate the solids from the enzyme product solution by settling a setup similar to the one described earlier in connection with semicontinuous settling experiments (1) was used.

A standard 14 l fermentor was equipped with a settling device mounted above its head plate as shown schematically by Figure 4. It consisted of a spherical glass bulb of about 6 l total volume.

Attached to its lower end was a large diameter glass tube running through the head plate and of the main tank and down through the latter almost to the bottom of the glass fermentor. This tube served to transfer the concentration fungus/ cellulose suspension back into the fermentor by gravitational flow. During continuous cellulase production, the liquid level in the settler was held constant above the level of the fermentor by subjecting the head space of the settler bulb to an accurately controlled constant vacuum. Fermentation broth was continuously pumped into the settler at a rate of 3 to 5 l/hr.

The broth was introduced into the settler several inches below the liquid level, and most of it flowed straight back into the fermentor through the connecting tube. Due to the settling of the solids, the layer just below the liquid surface in the settler always remained clear of solids and enzyme solution could continuously be harvested from this layer. The residual concentration of solids in the overflow normally stayed well below 0.01%.

To maintain the level in the fermentor constant in both the centrifuge and the settling experiments, fresh medium containing delignified cellulose in suspension (Solka Floc, Brown Co.) was blown into the fermentors as needed by pressurizing the medium tanks. Whenever the liquid level dropped below its set point, a level probe energized and opened a magnetic clamp operating on the rubber tubing of the feed line. This mode of operation prevented the clogging problems normally encountered with continuous pumping of cellulose and suspensions.

It proved impossible to sense the level in the fermentors reliably using normal conductivity probes in the vessel. The

reasons were foam formation which could not always be completely suppressed and the high humidity prevalent in the head space which wetted the surface of the insulated probes and thus shortcircuited them after prolonged operation. To sense the liquid level, a special tube was installed ending near the bottom of the ferment jar. Sterile air was constantly fed through it to the broth at a rate slow enough to keep the pressure drops negligible. The hydrostatic pressure at the bottom of the fermentor was measured in this air feed and used as an indication for the liquid level.

Sugar level, enzyme activity, dry weight as well as nitrogen content of the dried solids in the fermentor and in the product was measured frequently in the course of all the experiments.

The Kjeldahl determinations of the nitrogen content of the dried solids were used to estimate the cell density in the fermentation broth. Pure mycelinal mass of T. viride can be shown to contain 6.65% nitrogen if grown on glucose and about 6% nitrogen if grown on cellulose (1), whereas cellulose itself does not appear to contain appreciable amounts of nitrogen. This information served to compute the biomass content of the dried samples and hence to estimate the fungal dry weight in the broth.

## 2.2. Centrifugal Filtration Experiments

A typical long term experiment where centrifugal filtration was used is shown in Figure 6. The experiment was begun much like a normal batch fermentation. The initial medium in the fermentor contained 10 g/l cellulose (Solka Floc) as carbon source and in addition to that 8 g/l of glucose. The vessel was inoculated with a shaken flask culture of Trichoderma viride QM 9414 obtained from the American Type Culture Collection. The glucose present in the

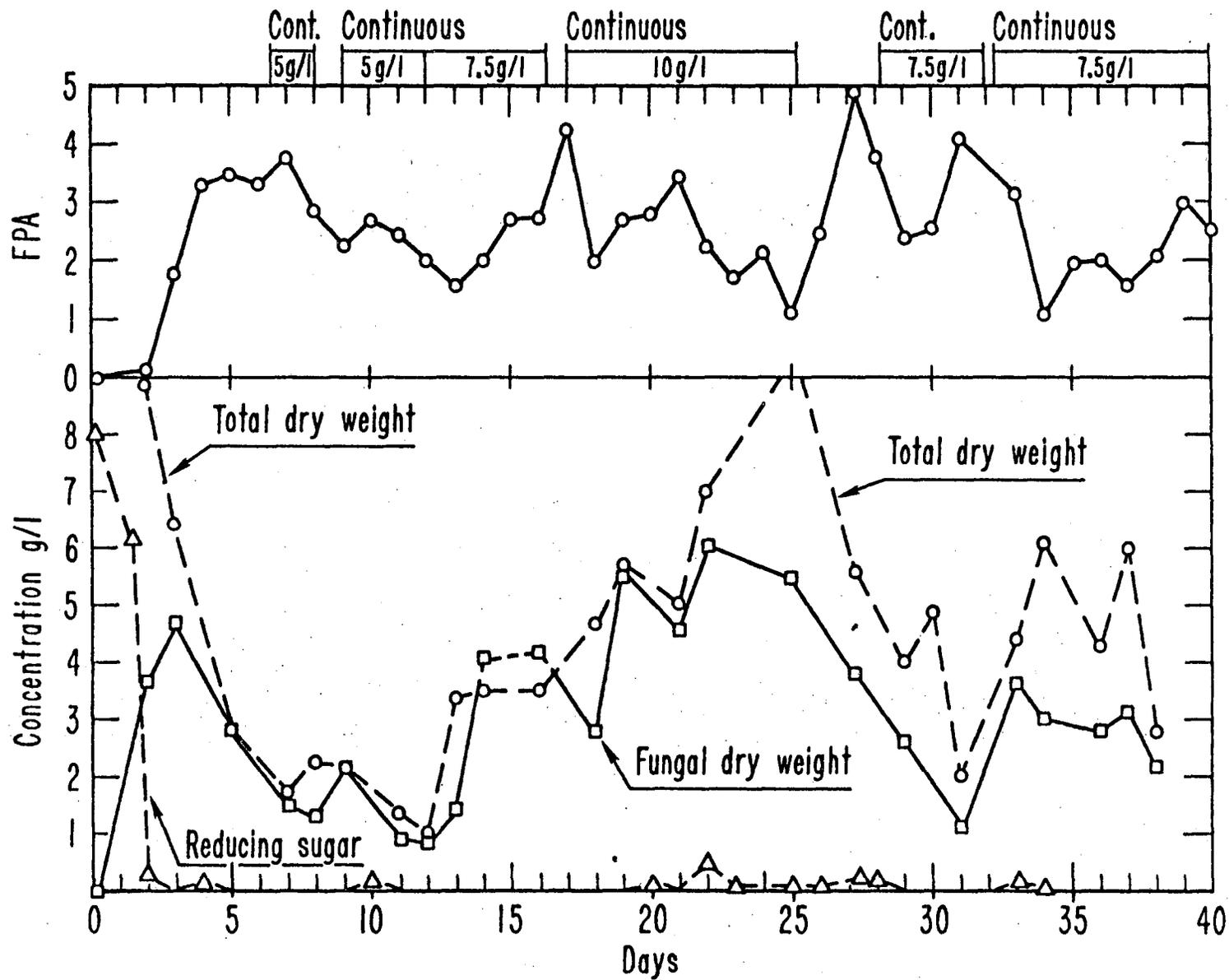


Fig. 6. Continuous production of cellulase with cell recycle using rotary concentrator as cell separator.

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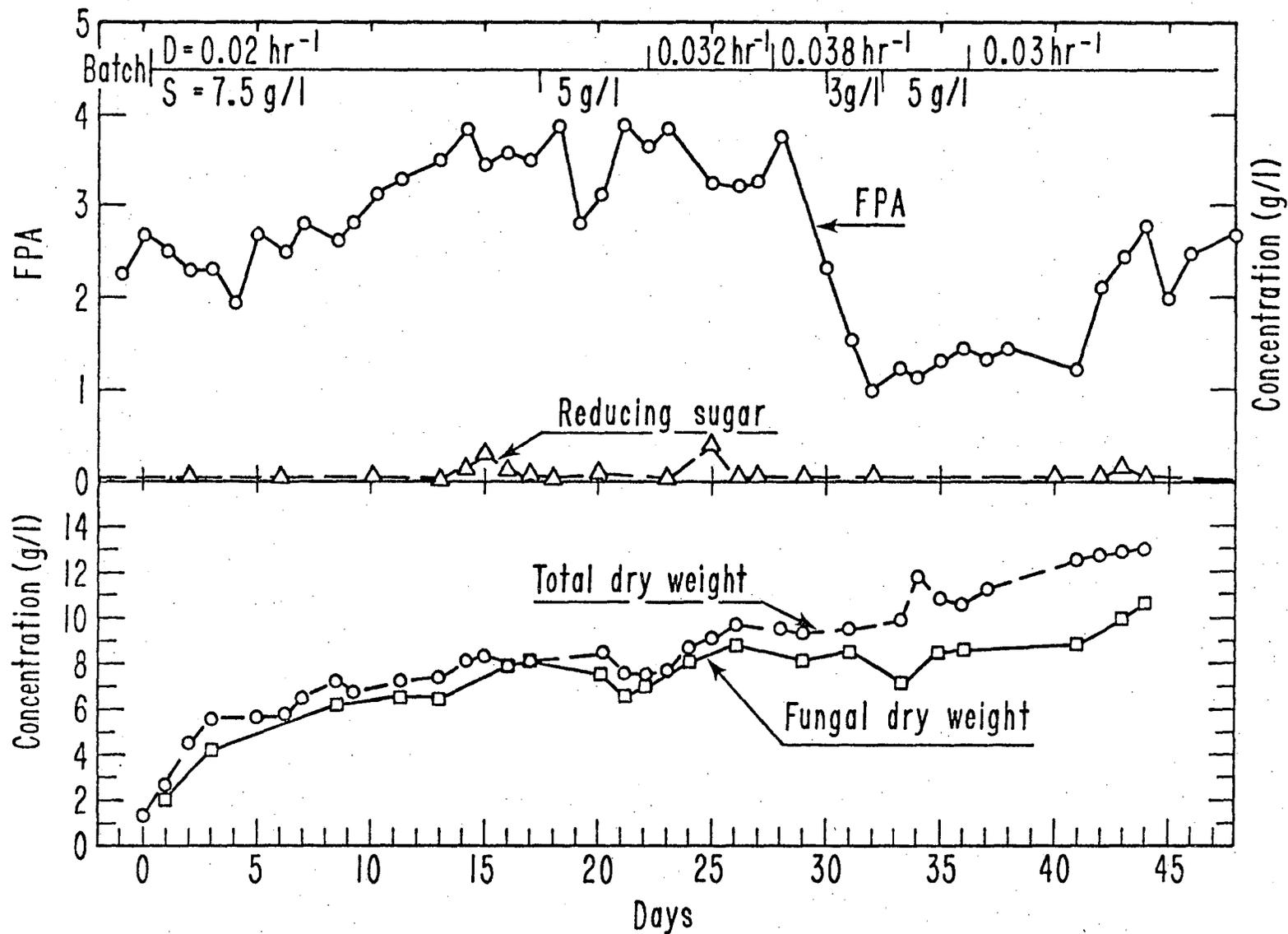


Fig. 7. Continuous production of cellulase with cell recycle using XBL767-3205 settler as cell separator.

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fermentor resulted in a rapid synthesis of a substantial amount of fungal mass, as indicated by the lower solid curve on Figure 6. When glucose was exhausted after two days the enzyme activity started to appear in the broth (upper solid curve). Therefore, the cellulose content of the system decreased rapidly as demonstrated in the lower part of the diagram by the difference of total dry weight (circles, broken curve) and fungal dry weight (squares, solid curve).

When the cellulase concentration had levelled off the continuous operation has begun at dilution rates ranging from 0.016 to 0.02  $\text{hr}^{-1}$ . Although no growth stage was used to replenish lost mycelium, wash-out of the culture could be prevented and the fungal density maintained at a reasonably high value by the action of the recycle operation. Cellulase could, therefore, be produced continuously at an average activity of 2.6 FPA for over one month.

Three levels of cellulose concentration in the feed have been tested in this experiment. During the first period with only 5 g/l cellulose in the feed both the cell mass and the activity dropped constantly. As indicated by Figure 6, cellulose was more or less exhausted in the fermentor during this period. Only after changing to 7.5 g/l cellulose the latter appeared in the fermentation broth and cell mass and activity rose. This suggests a substrate limitation at 5 g/l cellulose in the feed. A further rise in fungal mass was observed at 10 g/l but a drop of activity occurred. Towards the end of the period with 10 g/l considerable amounts of unhydrolyzed cellulose appeared in the system and the sugar level became abnormally high. The situation might be explained assuming that the accumulation of cellulose is unfavorable to

induction in that enough easily accessible cellulose is present to afford fast sugar production even at low enzyme strengths. The excess of cellulose disappeared partially and the production of cellulase increased when the cellulose concentration in the feed was again reduced to 7.5 g/l.

In continuous operation, a certain starvation might be more favorable to induction than an excess of substrate. This is also indicated in Table 7. The productivities have been estimated from Figure 6 accounting for depletion and accumulation effects in each of the periods of the experiment since no true steady states were obtained. The highest specific productivity of 0.074 units/mg-day was reached at the lowest cellulose concentration. The volumetric productivity, however, was limited because with the prevailing cell loss in the product stream averaging 0.8 g/l the low cellulose feed could not sustain a dense cell culture. This could be corrected by feeding more induction solids, but the specific productivity dropped drastically. An optimum volumetric productivity seemed to exist at 7.5 g/l cellulose in the feed.

An especially rapid production of cellulase was obtained in the fermentor when the continuous operation was interrupted and the culture forced to utilize the remaining cellulose in the fermentor for two brief periods on the 16th and on the 25th day (Figure 6). This again suggests that a certain limitation of the cellulose supply might improve induction.

#### Settling Experiments

Cellulase was also produced continuously over a period close

to two months using the settler as a device to separate the solids from the enzyme product solution. The results of this long term experiment are summarized in Figure 7. The initial start-up phase during which the culture was grown in batch mode is not shown in Figure 7. The productivities under the various conditions were again estimated and included in Table 7. In two cases the days just preceding a change in operating conditions were believed to reflect special steady state values, whereas the values at  $D = 0.02 \text{ hr}^{-1}$  and 5 g/l cellulose were based on the transient behavior accounting for accumulation and depletion rates. A direct comparison of the results is in principle only possible when assuming the productivities are the same as in the steady state.

As with the centrifugal concentrator the maximum specific productivity with the settler again occurred when cellulose supply was limited to only 5 g/l in the feed. (Table 7). But since the settler was capable of keeping the residual solid content in the product stream substantially lower than the centrifugal concentrator, a high cell density could be maintained even at a cellulose concentration of only 5 g/l. Thus the high specific productivity was combined with the advantage of a high cell density, resulting in a better volumetric productivity than was possible with the centrifugal concentrator.

Since the loss of substrate, too, was small compared with the centrifugal concentrator, some cellulose was always present in the system even at 5 g/l cellulose concentration in the feed, as indicated in Figure 7 by the difference between total dry and fungal

TABLE 7

## ENZYME PRODUCTIVITIES

Investigator	FPA	Productivity Units/ml-day	X g/l	Specific Productivity Units/mg-day
Mitra, continuous	1.4	0.0384	4.4	0.016
This work, continuous, cell recycle				
<u>Centrifugal Concentrator</u>				
5.0 g/l cellulose	3.0	0.109	1.3	0.074
7.5 g/l cellulose	2.2	0.112	2.49	0.045
7.5 g/l cellulose	2.48	0.090	2.3	0.039
10 g/l cellulose	2.56	0.050	4.96	0.010
Wilke, et al, semi- continuous, cell recycle				
<u>Settler</u>				
5.7 g/l cellulose	3.95	0.375	5.15	0.072
This work, continuous, cell recycle				
<u>Settler</u>				
D = 0.02 hr <sup>-1</sup> 7.5 g/l cellulose	3.61	0.293	6.9	0.043
D = 0.02 hr <sup>-1</sup> 5 g/l cellulose	3.65	0.434	6.85	0.063
D = 0.033 hr <sup>-1</sup> 5 g/l cellulose	3.25	0.379	8.5	0.045

dry weight.

The effect of dilution rate was studied in the experiment in a limited range. The best productivity seemed to occur at  $0.02 \text{ hr}^{-1}$  but the dilution rate could be increased to  $0.03 \text{ hr}^{-1}$  without significant loss of productivity or enzyme activity. But when the dilution rate was further increased, the activity dropped drastically.

In the last stage of the experiment, the dilution rate was reduced again to the lower settings, but it proved impossible to restore the high productivities observed earlier. Increasing amounts of unused cellulose up to 4 g/l began to appear in the fermentation broth. The color of the fermentation broth had meanwhile turned from the normal bright yellow to a pale yellowish white. Although, the direct relation of enzyme production to cell density can still be seen in the last third of the experiment by comparing the respective curves on Figure 7, it is obvious from the mentioned observations that the metabolic activity of the culture had dropped drastically. Further research is necessary to decide whether such conditions could be caused by some kind of limitation at the high solid concentrations prevailing during the final phase of the experiment or by a loss of viability.

Another advantage of the cell recycle concept is illustrated in Table 8. Especially when high cell densities can be maintained, as during the settler experiments, the induction cellulose requirements seem to be low compared with typical values published for batch experiments (11) (12). When the fermentation is carried out batchwise, the fungal mass is resynthesized completely in every batch. In contrast, the recycle system uses the fungal cells

TABLE 8

## INDUCTION CELLULOSE REQUIREMENT

Investigator	<u>mg cellulose</u> enzyme unit	<u>mg cellulose</u> mg enzyme protein
Nystrom and Allen		
batch, without Tween 80 average	15.3	8.9
Mandels, <u>et al.</u>		
batch, average	8.9	4.65
continuous	8.93	4.35
Wilke, <u>et al.</u> , semi-continuous, cell recycle		
Settler	7.32	3.81
This work, continuous, cell recycle		
<u>Settler</u>		
D = 0.02 hr <sup>-1</sup> 7.5 g/l cellulose	12.29	6.25
D = 0.02 hr <sup>-1</sup> 5 g/l cellulose	5.56	3.14
D = 0.033 hr <sup>-1</sup> 5 g/l cellulose	10.24	4.81

repeatedly and thereby offers the advantage of reducing the induction solids required to synthesize new fungal biomass

#### Conclusion for Design

It may be concluded from the cell recycle experiments that the employment of a cell recycle does indeed improve the productivity drastically in continuous cellulase production. As a basis for design, the specific productivity of recycled cells may be expected to be 0.07 IU/mg-day. Further research is needed to determine whether a loss in viability caused the observed drop in specific productivity at higher cell densities, but it should be possible to maintain adequate viability by constantly supplying fresh cells from a growth stage.

The design of the process by Wilke et al. (1) evaluates the cost for two values of induction cellulose requirements: 2 and 4 g/g. In view of the experimental findings reported here the first value is definitely on the low side, although it might be possible to realize it if absolutely no cellulose is used for growth in the induction stage, as was assumed in the design.

### The Effectiveness of Cells in the Induction System

When fungal cells are grown on glucose and then transferred into the induction fermentors, they will not produce cellulase immediately. Only those cells will be productive at any given moment which have resided in the induction system for longer than the time required to induce cellulase production. The fraction of productive cells in the induction system may be called the "effectiveness"  $\epsilon$  and depends on the residence time distribution (RTD) and on the ratio  $\theta$  of the time required for induction to the total average residence time of the cells in the recycle system:

$$\theta \equiv \frac{t_{\text{ind}}}{\bar{t}} \quad (2)$$

As shown elsewhere (13), analytical solutions for the effectiveness can be obtained as follows:

$$\epsilon(\theta) = 1 - \theta + \int_0^{\theta} F(\theta) d\theta \quad (3)$$

where  $F(\theta)$  is the fraction of cells leaving the induction system younger than  $\theta$ , i.e. the cumulative external residence time distribution. For  $n$  tanks in series with a recycle, such as the induction system under consideration, the integration in Eq. 3 becomes involved. In a practical case it is, therefore, advisable to approximate the real situation by two extreme configurations: (1) one single well-mixed fermentor with recycle and (2) a plug flow reactor with recycle.

In the case of the well-mixed tank with recycle, the residence time distribution  $F(\theta)$  is the same as for a CSTR without recycle:

$$F(\theta) = 1 - e^{-\theta} \quad (4)$$

The introduction of a recycle does not affect the residence time distribution, but because it increases the cell concentration and thus the hold-up of cells, the value of  $\theta$  and also of  $F(\theta)$  will be reduced. Consequently, the effectiveness of a one-tank system can be enhanced by using a recycle, although the residence time distribution remains unchanged.

Substituting Eq. 4 into Eq. 3 yields

$$\epsilon = e^{-\theta} \quad (5)$$

The analytical solution for  $\epsilon$  in a plug flow fermentor with recycle is obtained by dividing the reactor up into two parallel plug flow devices: One is fed with the recycled cells. Since they have passed through the system at least once they will be 100% induced if  $\bar{t}_1 > t_{ind}$ , and the effectiveness of this partial plug flow device is unity.  $\bar{t}_1$  is the time it takes the cells to travel through the plug flow reactor only once.

The other subsystem is fed with the fresh cells from the growth stage, its effectiveness is the same as for a plug flow reactor without recycle. Since the RTD of a plug flow reactor is

$$F(\theta) = 0 \text{ for } 0 \leq \theta < 1, \text{ and}$$

$$F(\theta) = 1 \text{ for } 1 \leq \theta < \infty$$

Equation 3 yields for this subsystem

$$\epsilon(\theta_1) = 1 - \theta_1 \quad (6)$$

with

$$\theta_1 \equiv \frac{t_{ind}}{\bar{t}_1}$$

The effectiveness of the whole plug flow reactor is equal to the sum of  $\epsilon$  for the subsystems, weighted in proportion to the respective amount of cells flowing through each subsystem:

$$\epsilon = (1 - \theta_1)(1 - R) + R \quad (7)$$

where  $R$  = recycle fraction: fraction of cells leaving induction system which are recycled into induction fermentors. The two extreme values for the working volume of the induction are obtained by substituting Equations 5 and 7 respectively into Equation 1. The resulting equations are implicit in  $V$  because both  $\theta$  and  $\theta_1$  themselves depend on the working volume.

The value of  $t_{ind}$ , the lag in cellulase induction, must be found experimentally. Based on two stage batch culture experiments of Rosenbluth (18), Mitra and Wilke (14) concluded that there is a 30 hour induction lag. In view of the experiment reported in Figure 6, this is probably a conservative value. But it has been used in the following calculations pending more accurate experiments.

The results of the calculations are shown in Table 9 for four different cell concentrations. At the high cell recycle ratios,  $R$ , used in this system, the plug flow and the complete backmixing flow assumption yield virtually the same results and either assumption can be used to obtain a very accurate answer.

It is interesting to note that, in principle, the plug flow reactor has the lower effectiveness and thus the operation contrasts sharply with many classical chemical reactions. The difference becomes quite large at low values of  $R$  (13).

#### Installed Capital Cost of Induction Fermentors

Installed capital costs of the various items of processing

Table 9

Design of Induction System, Comparison  
of Backmixing and Plug Flow

			Complete Backmixing Assumed in Induction System			Plug Flow Assumed in Induction System		
X g/l	R	$\alpha$	Working volume in millions of gals.	$\theta$	$\epsilon$	Working volume in millions of gals.	$\theta_1$	$\epsilon$
2.5	0.63	0.065	10.30	0.083	0.920	10.30	0.216	0.920
5	0.63	0.075	5.543	0.157	0.854	5.566	0.403	0.851
7	0.65	0.087	4.171	0.209	0.811	4.177	0.543	0.810
10	0.64	0.097	3.129	0.279	0.757	3.192	0.717	0.742

equipment are necessary design information. Fermentor cost makes up a large portion of the final sugar manufacturing cost, but cost data for reactors in the scale visualized here cannot be found in the literature. The basis for cost estimation of large scale fermentors will, therefore, be discussed here.

Industrial fermentors are essentially well sealed agitated tanks provided with air sparger and heat exchange coils. The unit cost of 304 stainless steel agitated tanks was extrapolated to the size of the growth fermentors, 58,000 gallons, from graphs given by Peters and Timmerhause (15). In further scaling up similar vessels to the induction fermentor size of 500,000 gallons, the unit cost was assumed to be proportional to the 0.6 power of the volume. Since for the induction stages there will be only mild aeration and agitation at atmospheric pressure, it is assumed that fermentors of this size can be built. Costs were updated employing a Marshall and Stevens Index of 444 corresponding to the first quarter 1975. The results are summarized in Table 10.

The fixed capital investment for the entire plant was estimated by applying a multiplier of 3.1 to the purchased cost of equipment. This value is somewhat lower than the one used for standard carbon steel equipment, but it can be justified by the fact that the cost of stainless steel tanks, which are used in this case, is approximately twice that of equivalent carbon steel units.

#### CONCLUSIONS

The economic analysis based on the outlined process design studies shows at least some promise for an economically feasible process to hydrolyze newsprint enzymatically. The experimental and computational studies reported in this paper support this conclusion

Table 10

	GROWTH FERMENTORS (58,400 GALS)	INDUCTION FERMENTORS (500,000 GALS)
mixing tank (includes agitator and motor	\$ 82,340	\$298,600
heat exchange coil	\$ 1,000	\$ 5,200
purchased fermentor cost	\$ 83,340	\$303,800

and show that the underlying design assumptions are probably realistic. The experiments demonstrate that cellulase can be produced continuously at a high productivity by recycling the fungal cells. Further research is needed to determine to which level the cell concentration can be increased and to learn more about the relationship between specific productivity, cell density, and growth rate. Further data on the kinetics of hydrolysis would also be desirable and would permit a further optimization of the hydrolysis conditions.

From the laboratory data available thus far, it is not yet possible to predict reliably the behavior of the integrated process. Inhibiting substances could, for instance, accumulate in the recycle of the sugar solution or the continuous recovery of the enzyme complex could lead to a deficiency in a specific component.

The design assumptions ought, therefore, be verified by operating the proposed process in an integrated way over a prolonged period of time, preferably in the pilot plant scale.

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List of Symbols

[E]	Enzyme protein concentration, g/l
F	Flow rate through induction system excluding recycle, l/day, gals/day
F( $\theta$ )	External residence time distribution
$k_E$	Specific cellulase productivity, g/(g)(day)
R	Cell recycle fraction: fraction of cells leaving induction fermentors which is recycled to induction fermentors
$t_{ind}$	Time lag in induction
$\bar{t}$	average overall residence time in induction system
$\bar{t}_1$	Time required to travel through induction fermentors only once
V	Working volume of induction fermentors, gals, liters
X	Cell concentration, g/l

Greek Symbols

$\alpha$	Volumetric recycle fraction: fraction of F recycled to induction fermentors
$\epsilon(\theta)$	"Effectiveness" factor
$\theta$	Dimensionless time

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