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ENZYMATIC HYDROLYSIS OF CELLULOSIC WASTE: The Status of the Process Technology and an Economic Assessment

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INTRODUCTION

The development of a practical and economical technology for the utilization of cellulosic wastes has been a long sought objective of our scientific community. In recent years, the realization of raw material and energy limitations has placed a greater emphasis on recycling and reusing materials and finding greater use for renewable resources. Since cellulose is an annually renewed material,* it has enjoyed considerable attention in the recent past, as witnessed by this and many other symposia and conferences.

Known as a biologically recalcitrant material, cellulose is difficult to react or degrade rapidly owing to its naturally crystalline structure. In addition, its association with lignin complicates both biological and chemical processing. In spite of these drawbacks, several processes for cellulose utilization have shown economic potential. In the area of biological processing, the production of single cell protein (1, 2) and sugars (3 to 5) from enzymatic hydrolysis have produced the greatest interest.

The production of hydrolyzate sugars from waste cellulose using cell free enzyme preparations of *Trichoderma viride* QM 9414 has progressed to the prepilot plant stage at the U.S. Army Natick Research and Development Command. cursory economic evaluations have attempted to elucidate problem areas and indicate the directions of future research.

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*Estimated annual production of 150 billion tons.

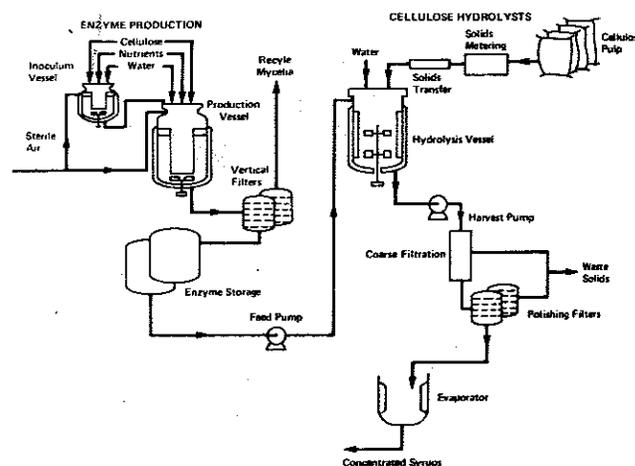


Fig. 1. Flow scheme for pilot plant process.

EXPERIMENTAL METHODS

PRODUCTION OF ENZYME

The cellulase enzyme system used for hydrolysis of waste and pure cellulose is produced by the growth of cellulolytic fungi in submerged culture. The prepilot plant process flow scheme for enzyme production is shown in Figure 1. A highly instrumented concept was adopted in the design of this process in order to effectively evaluate the range of parameters affecting enzyme production. Batch fermentation is used requiring the use of two major vessels, an inoculum or seed vessel (22.5 l working volume) and a production fermentor (280 l working

volume). Temperature, pressure, pH, dissolved oxygen, agitation rate, and sparge are monitored and controlled for both vessels. Also monitored are vessel weight, power delivered to the broth, and inlet and exit gas composition (carbon dioxide and oxygen). Foam is controlled by use of draft tubes, automatic addition of surfactants, and mechanical foam breakers.

Trichoderma viride was chosen as the best organism on the basis of extensive work of Mandels et al. (6, 7). A mutant strain, QM 9414 has been selected because of its increased enzyme production over the parent or wild strain QM 6a.

The major raw materials shown in Figure 1 are cellulose, nutrients, and water. For the production of the inoculum, the mineral salts medium described by Mandels and Weber (8) is used with 1% cellulose (SW40: Brown Company, Berlin, New Hampshire) as the primary carbon source. In addition, 0.075% proteose peptone and 60 p.p.m. polypropylene glycol (M.W. 2 000) are added to enhance enzyme production and control foam, respectively.

For the production of enzyme, a modified and varying mineral salts media is used to accommodate the increased levels of cellulose used in the production stage. Cellulose levels ranging from 1 to 8% have been tried with corresponding increases in the basal media nitrogen and phosphate sources. Several combinations have been tried, with the compositions shown in Table 1. Cellulose is added as either SW-40 or NIB-40 (both Brown Company). Both are Kraft pulps, with the NIB version being unbleached.

The shake flask cultures for use in inoculation of the seed vessel are prepared using the standard salts media with 0.75% cellulose, 0.075% proteose peptone, and 0.2% Tween 80 (Atlas Chemical Industries, polyoxyethylene sorbitan mono-oleate). The Tween 80 is used to stimulate enzyme production. The spore inoculated flasks are incubated on a rotary shaker at 29°C for 4 days.

Cell free enzyme preparations are produced by simple filtration of the culture at completion of the fermentation. The enzyme is stored in refrigerated vessels at 2°C, and biological contamination is discouraged by addition of either 100 p.p.m. (as sulfur dioxide) of sodium meta bisulfite or 0.03% toluene.

Enzyme concentration is measured in International Units

using the standard assay described by Mandels et al. (9). Soluble protein levels are measured using the Lowry (10) Folin procedure after precipitation with acetone.

The solids dry weight is determined by freeze drying the filter cake obtained during the filtering of the spent culture. No attempt was made to distinguish between mycelia and undigested cellulose and lignin. The main purpose of the measurement was to calculate the filtration area required for harvesting and the quality (percent moisture) and quantity of the cake as a function of the fermentation conditions. It is, however, assumed from cursory microscopic observations that very little, if any, cellulose is present in the solids.

HYDROLYSIS OF NIB-40 WOOD PULP

In view of the current factors affecting the economics of cellulose saccharification, it appears that a chemically delignified substrate will eventually prove to be the most practical material for enzymatic conversion to sugars (11). Earlier studies have shown both undried Kraft and sulfite wood pulps to be almost completely hydrolyzable (12). By approaching total conversion, expensive enzyme can be recovered for reuse since losses by adsorption on noncellulosics would be minimized. Prior to conversion of the pilot plant at Natick to handle a wet pulp feed, it was decided to initiate hydrolysis studies utilizing a dry wood pulp NIB-40. This material is an unbleached hardwood Kraft pulp containing about 5% lignin. It is hammer milled to the consistency of a soft, fibrous powder of 70 to 140 μ average fiber length.

To become familiar with the characteristics of NIB-40 saccharification, a series of batch hydrolyses was carried out at various operating conditions. The result was to establish a range of satisfactory operating conditions to be further investigated at the pilot scale. Two liter water jacketed bioreactors were used (1.2 l working volume) with agitation at 600 rev/min. Because of the relatively low bulk density of the substrate and its swelling upon contact with enzyme solution, initial substrate concentrations of greater than 12% could not be adequately mixed. The runs reported were carried out at 50°C and a pH of 4.8 with the enzyme concentration varying from .5 to 1.5 IU/ml (9). Reactions were terminated after 75 hr.

TABLE 1. MEDIA COMPOSITION FOR ENZYME PRODUCTION

Run no.	Cellulose (type) g/l	Urea g/l	(NH ₄) ₂ SO ₄ g/l	KHPO ₄ g/l	Proteose peptone g/l	MgSO ₄ · 7H ₂ O g/l	CaCl ₂ g/l	C/N
1-20	9.4 (SW-40)	0.3	1.4	2.0	0.75	0.30	0.30	8.2
21	26.9 (SW-40)	0.3	2.7	2.0	1.5	0.30	0.30	13.5
23	26.9 (SW-40)	0.3	4.0	2.0	1.5	0.30	0.30	10.3
24	51.1 (SW-40)	0.6	8.0	3.2	2.2	0.30	0.30	10.2
25	80.0 (NIB-40)	0.9	12.7	5.1	3.5	0.50	0.50	8.0
27	25.5 (NIB-40)	0.3	4.0	1.0	1.5	0.30	0.30	9.8
28	25.5 (NIB-40)	0.3	4.0	1.0	1.5	0.30	0.30	9.8

All cultures contained in milligrams per liter FeSO₄ · 7H₂O 5.0, MnSO₄ 1.56, ZnSO₄ · 7H₂O 1.4, CaCl₂ 2.0. Cellulose concentration as metabolizable carbohydrates (includes any C₅ polysaccharides)
Total media weight = 280 kg.

During these tests, there were indications that reaction temperature was affecting the ultimate substrate conversion owing to its effects on enzyme activity. A series of 100 ml shake flask batch experiments were then carried out at various enzyme to substrate concentration ratios and over a 28° to 60°C temperature range to investigate this phenomenon. Merthiolate (0.01%) was used to maintain asepsis for all tests.

ANALYSIS OF SUGARS

The sugar syrups produced were analyzed for xylose, cellobiose, and glucose using high pressure liquid chromatography (13). Prior to injection into the Waters Associates Microbondapack carbohydrate column, it is necessary to prefilter the samples through a 0.45 μ millipore type of HA membrane. This greatly extends the life of the column and results in an analysis time of about 7 min. Samples tested for reducing sugars by the DNS method (9) show consistently 15 to 20% higher values than the totals of the three individual sugars.

PROCESS ECONOMICS

The capital expenditure for the production facility was estimated by using the nomograph of Dryden and Furlow (13), which gives the installed equipment cost. To this cost would be added the contributions due to piping, insulation, and instrumentation.

Utilities cost for the process were estimated at the following rates:

electricity	\$0.04/KWH
steam	\$2.00/1000 lb

The overhead costs were tabulated and calculated according to the following assumptions:

direct overhead	30% of labor and supervision
general plant overhead	65% of operating cost
depreciation	10% of capital costs
interest	12% of working capital

Operating costs for the production facility consists of labor, supervision, and maintenance. Maintenance costs were estimated to be 6% of the capital costs.

DISCUSSION

ENZYME PRODUCTION

In past economic analyses (3, 16, 17), it has become apparent that the process economics depends very much on both the production and utilization of the necessary enzymes. At present, there is conflicting evidence for and against the possibilities of even limited recycling of enzymes. Should it become possible it will most likely involve costly aseptic processing. Raw materials such as expensive nitrogen sources will be saved, but the overall economics are still in question. It is, therefore, imperative to develop a fermentation process which will yield large quantities of high quality enzymes at as low a price per unit as possible.

Past efforts (11, 16) have involved the development of a process for enzyme production using a maximum of 1% cellulose in the submerged culture medium. Higher concentrations of cellulose have in the past produced less enzyme and have, therefore, been neglected. As reported (11, 16), maximum enzyme concentrations of 0.8 to 0.9 IU/ml were achieved in a 400 l vessel using 1% pure cellulose as the carbon source in the fermentation. These fermentations generally took 100 to 120 hr and, as seen in Table 2, resulted in rather low fermentation productivities.

Processing requirements for a 1% cellulose fermentation have been detailed earlier (16), and a full scale processing scheme has been proposed. In general, oxygen transfer requirements never exceed 5.0 mmole oxygen/l-hr. Dissolved oxygen levels are maintained at greater than 10% of the saturation level, with k_{La} values of approximately 60 mmole of oxygen/l-hr-atm. Temperature is controlled at 29°C, and pH is not allowed to drop lower than 3.25.

The possibility of a processing breakthrough was linked to pH control as a result of the work of Sternberg (18). The inactivation of components of the cellulase enzyme system occurs as the pH level drops below 4.0 and becomes extreme at levels below 3.0. However, as reported previously (16), enzyme production is stimulated at the lower pH levels, and at levels higher than pH 3.0 this increase in production offsets

TABLE 2. ENZYME PRODUCTION PRODUCTIVITY

Run no.	Cellulose conc. g/l	Max. enzyme conc. IU/ml	Yield IU/mg of cellulose	Time hr	Productivity IU/ml-hr
19	9.4*	0.93	0.099	121	7.7×10^{-3}
20	9.4*	0.89	0.095	120	7.4
21	26.9*	1.68	0.062	195	8.6
23	26.9*	3.00	0.112	165	18.2
24	51.1*	2.31	0.045	211	11.0
25	80.0†	2.75	0.034	305	9.0
27	25.5†	3.10	0.122	116	26.7
28	25.5†	2.20	0.086	96	23.0

*As cellulose in SW-40.

†As cellulose in NIB-40.

Run No. 21 controlled at pH \geq 3.00

All other runs controlled at pH \geq 3.25

Inoculum: 7.8% of total volume

25 hour inoculum prep.

the increase in enzyme inactivation. The result is higher yields of active enzyme. Growth is slower at the lower pH levels, but optimum conditions for maximum enzyme productivity are somewhere between pH 3.5 and 3.0. At higher substrate concentrations and with enriched media, the pH, if uncontrolled, will drop too low for maximum enzyme production. As a result, shake flask evaluations and uncontrolled fermentor experiments invariably reported low enzyme yields at high substrate concentrations.

Pilot scale fermentations at higher cellulose concentrations were initiated, and pH control at levels higher than 3.0 was employed to minimize pH inactivation and accelerate growth. Table 2 shows the results of these higher concentration runs, and, as expected, higher values of active enzyme concentration were obtained. The results, however, were not consistent, for as cellulose concentration increased, enzyme concentration did not increase proportionally. In fact, as cellulose concentration increased, yields (IU/mg of cellulose) generally decreased (Table 2). Since this was the first series of runs at higher concentrations, it was decided that no hard and fast conclusions could be drawn. It was, however, obvious that there was a great deal to be gained by further investigation. Our current efforts are concentrating on increases in productivity at one cellulose concentration, specifically 25.5 g/l.

In order to increase productivity, it is necessary to decrease the fermentation time without adversely affecting the ultimate enzyme concentration. Increase in cell mass during the early stages of the fermentation has the effect of speeding up the overall process. Two techniques have been tried to accomplish this. The first is a controlling or profiling of the natural pH decline to keep the culture at the more optimum pH levels for growth (4.0 to 4.5) during the early stages (first 20 hr) of the fermentation. This technique was tried on growing cultures with some success as noted by higher than average levels of maximum respiratory activity. However, if the natural dropping pH profile was greatly upset, a deleterious effect was observed on the respiratory activity. Bumping or pulsing of the pH from low levels to more optimum levels (4.0 to 4.5) resulted in a slowing of carbon dioxide production and a decrease in oxygen utilization.

The other technique for building cell population during the initial stages was temperature profiling. As can be seen in Figure 2, the temperature is raised from the usual 29°C (optimum for enzyme production) to 33°C (optimum for growth) shortly after inoculation of the production vessel. Using this technique (run No. 27), a 27% increase in peak culture respiration was observed with a corresponding 30% decrease in total fermentation time. Enzyme activity in the final broth was essentially the same. The overall result was a substantial increase in fermentation productivity. There does, however, seem to be a diminishing return. In a subsequent run (No. 28), the temperature profiling resulted in a 45% increase in respiration over the uncontrolled run and a 42% reduction in fermentation time, but there was a substantial decrease in final enzyme activity. There are perhaps many reasons, but the effect seems to be that more of the carbon source goes into

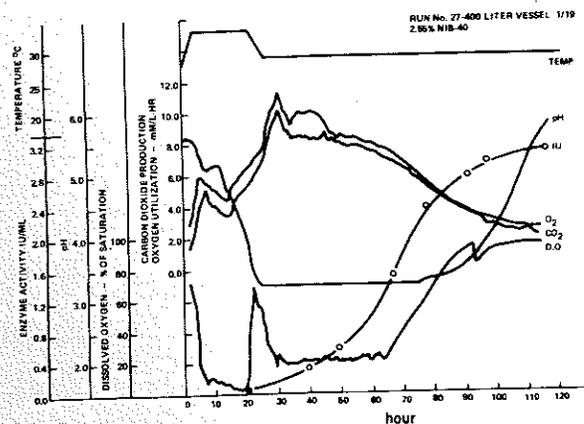


Fig. 2. *T. Viride* batch fermentation with temperature profiling.

cell production and maintenance and less into soluble protein (enzyme) production. It should be noted that the overall productivity was still very high. Specific cellulase activity of the final soluble protein was in all cases between 0.7 to 0.8 IU enzyme/mg protein.

With the higher substrate concentrations, oxygen transfer requirements are substantially higher especially at high growth temperature. A maximum oxygen transfer requirement of 13 mmole oxygen/l broth-hr was observed during the fastest fermentations.

HYDROLYSIS OF NIB-40 DRY WOOD PULP

The initial series of batch hydrolyses of NIB-40 was run under controlled conditions of temperature and pH (50°C and 4.8, respectively) considered optimum for saccharification. On Figures 3 and 4 are plotted the sugar production curve for 8 and 12% slurries at indicated enzyme strengths. Total sugars are expressed as the sum of the xylose, glucose, and cellobiose in the syrups. As has been noted in earlier studies (15), xylose is produced more rapidly initially than the other sugars, and at 70 hr it composes about 25 to 30% of the total. It was also noted that the lowest fraction of xylose occurs at the highest enzyme to substrate (E/S) ratios.

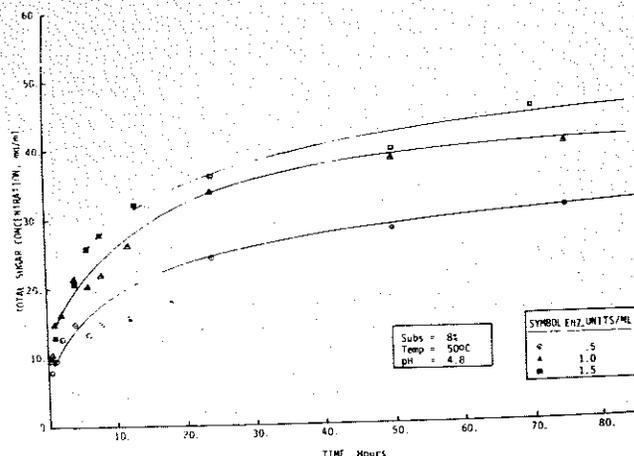


Fig. 3. Total sugars produced during hydrolysis of NIB-40.

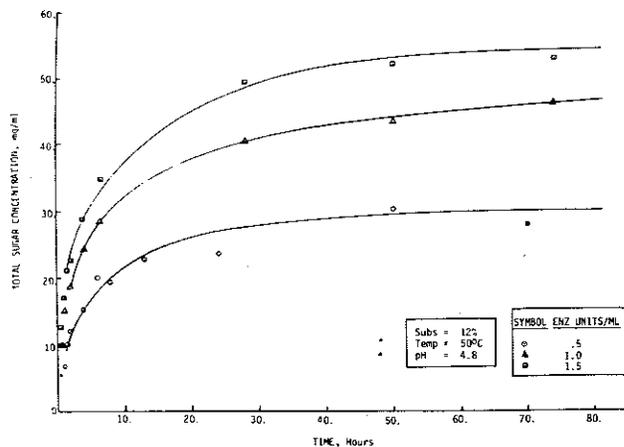


Fig. 4. Total sugars produced during hydrolysis of NIB-40.

NIB-40 contains about 5% nondigestible lignins. Therefore, there is a potential for 95% saccharification of the substrate. In theory, regardless of the original enzyme concentration, the cellulase enzymes acting in a catalytic role should not be used up during the reaction and should eventually convert all the hydrolyzable substrate. However, Figures 3 and 4 suggest that sugar production is leveling off far below complete conversion. In Figure 5, the percent saccharification is plotted vs. substrate to enzyme (S/E) ratios for 12, 24, and 75 hr. As (S/E) decreases, all of the curves should converge at 95% saccharification, but this does not happen.

Two possible explanations for this are nonproductive complexing and temperature inactivation of the enzyme. The first occurs when the enzyme binds at sites on the cellulose in a way which precludes saccharification or binds to sites on the noncellulosic components of the substrate. If either adsorption is irreversible, the enzymes are essentially removed from the reaction system. It is also possible that higher temperatures cause denaturing of the enzyme protein, rendering it inactive. This is likely a process which occurs to a degree at all temperatures but has a marked effect above some critical value. If the enzyme becomes completely inactivated, total saccharification can never be reached.

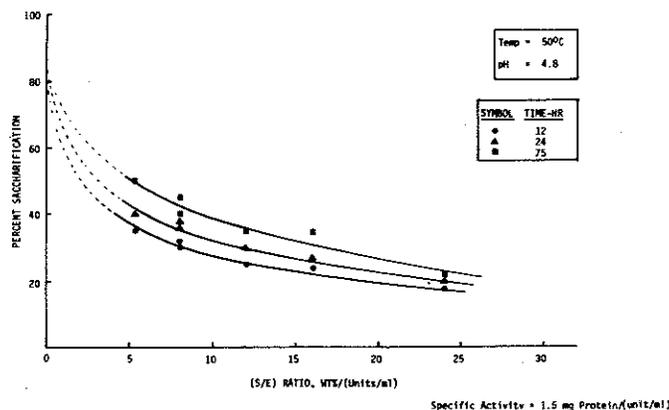


Fig. 5. Saccharification of NIB-40.

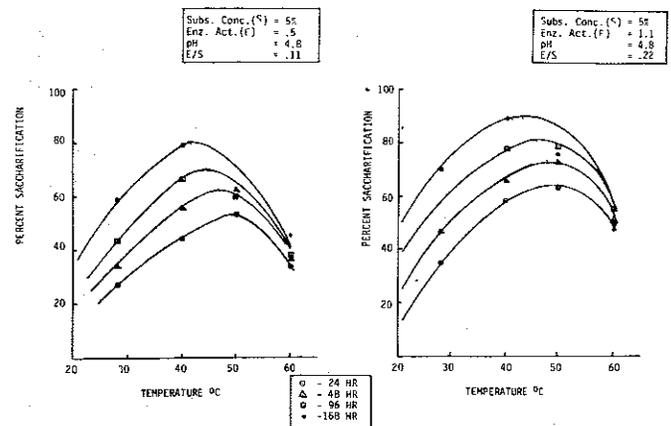


Fig. 6. Batch hydrolysis of NIB-40.

In order to investigate this effect, a series of batch hydrolyses were carried out at 28°, 40°, 50°, and 60°C. The results are plotted in Figures 6 and 7 for times of 24, 48, 96, and 168 hr. At longer hydrolysis times, the results show the maximum degree of saccharification to be shifted toward lower temperatures. This indicates that the enzyme is being inactivated as the temperature increases. At the lower temperatures, the reaction rate is certainly lower; however, the eventual percent conversion will be higher. Points along lines connecting the maxima of each curve represent the temperatures at which it will take the shortest time to get the desired degree of saccharification. The same conversion can be obtained at lower temperatures but will require more time. The shift seems slightly less marked at the higher substrate concentrations, indicating an increased stability of adsorbed enzyme over free enzyme in solution (substrate protection). The saccharification at 50°C did not exceed 85% during any of the runs. Thus, it is more likely that the curves on Figure 5 converge to that value rather than 95%, which would indicate total conversion of hydrolyzable substrate.

At all of the temperatures investigated, xylose composed 25 to 30% of the total sugars. Cellobiose reaches a maximum, which occurs earlier as temperature is increased, followed by a

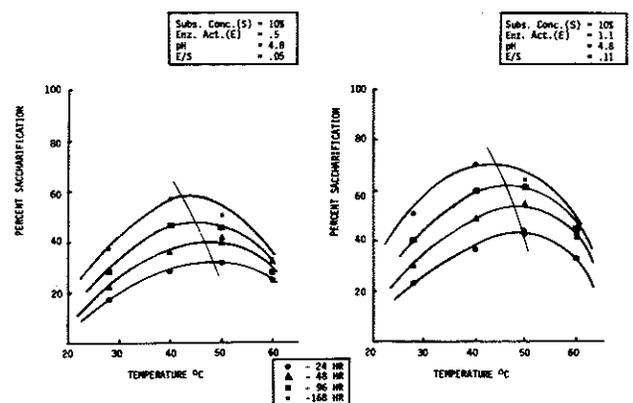


Fig. 7. Batch hydrolysis of NIB-40.

steady decrease with time. After 168 hr, cellobiose still represents 10 to 15% of the total sugars at 28°C but has virtually disappeared from the 60°C hydrolyzates by 48 hr. This may indicate that the β -glucosidase enzyme, which converts cellobiose to glucose, is less sensitive to higher temperatures than the exo-glucanases responsible for production of the cellobiose.

ECONOMICS OF SCALE BASE ON PROJECTED PROCESS

Economic evaluation of the cellulose to glucose process is based on the prepilot work carried out at the U.S. Army Natick Research and Development Command. The evaluation is a preliminary analysis of the process; although many assumptions have been made, it gives some insight as to where the cost intensive areas exist and therefore, suggests where optimization and improvement of the process is needed.

The cost for enzymatic sugar production is based on a projected process. The fermentation process described previously by Nystrom and Allen (16) is operated in a semicontinuous manner, where the product is a 4 IU/ml enzyme broth produced from a 100 hr fermentation. For economical storage of enzyme, 75% of the water is removed from the broth using an ultrafilter. With this scheme, it is possible to hydrolyze a wet waste, such as from a paper mill, without first having to remove any water and affecting susceptibility of the substrate to enzymatic degradation.

The hydrolysis facility operates on a continuous basis. The principal pieces of equipment, in addition to the reactor, are an evaporator for concentrating the sugar solution, a rotary drum filter for removal of the lignin and unhydrolyzed cellulose, and a bucket elevator for transporting the substrate to the reactor. Other pieces of equipment in the process consist largely of holding tanks, service vessels, pumps, etc. The proposed process uses an enzyme broth of 1 IU/ml. It is assumed that a 33% conversion of a 15% substrate feed will occur with a 48 hr residence time, producing a 5% sugar syrup. The total process, however, will include bringing the 5% solution to a 50% syrup. The substrate used in this evaluation is a paper mill waste, characterized by a low lignin content and a relatively high degree of susceptibility. In this analysis, there has been no charge for the substrate, nor is there any credit assumed for any by-product. Thus, the sugar costs reflect only those related to sugar production.

If we neglect water removal costs to bring the 5% sugar solution to a 50% syrup, about two thirds of the total cost of sugar production is attributable to fermentation or enzyme production. The other third of the total price is the cost for hydrolysis.

At present, it appears that there is very little active enzyme left in the hydrolyzate to warrant enzyme recycle. Bench scale experiments have shown, however, that operating the hydrolysis reactor at a lower temperature, while increasing the hydrolysis time, will improve the chances for enzyme recycle. The overall effect on sugar production costs would obviously lower fermentation costs while increasing hydrolysis costs. This will hopefully result in a lower total cost for sugar production.

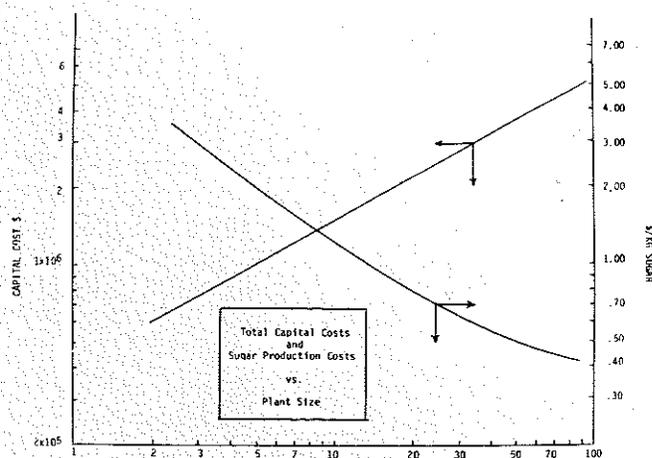


Fig. 8. Capacity in metric tons/day.

Figure 8 shows the capital expenditure and total sugar production cost as a function of plant size. The capacity is expressed in metric tons per day of cellulose throughput. For example, in a plant capable of processing 100 metric tons/day of cellulosic materials, the plant cost would be about 4 million dollars, and the cost for sugar production would be around \$0.40/kg sugar. At present, the cost for producing a kilogram of sugar in a 50% syrup from a 100 metric tons/day plant is not competitive with the conventional starch hydrolysis process. Improvements in the cellulose to glucose process, such as enzyme recycle and chemical and/or physical substrate pretreatment schemes, are not unrealistic and should favorably affect the overall economic picture.

CONCLUSION

The production of large quantities of cellulase enzymes via submerged fermentation has been proven practical (11, 16) but has yet to be optimized. Increases in yields and productivities through increases in substrate concentration and cell density indicate that researchers are far from realizing the ultimate process. The effects of pH control and temperature profiling are just beginning to be investigated, and the initial results are impressive.

Utilization of the cell mass from the enzyme production process deserves more consideration, since a good portion (approximately 20%) of the carbon source goes to production of *T. viride* mycelia. Recycling, as suggested by Wilke (19), has perhaps the greatest potential but must be applied to a continuous or semicontinuous fermentation. Partial utilization is possible (11) in batch fermentations if the filter cake is simply recycled as a source of protein. In either case, more serious attention should be paid to this potentially valuable by-product.

At the present time, the fermentation costs represent about two thirds of the total costs for producing sugars from waste cellulose. Improvements in the process, directed at reducing this contribution to the production costs, can be from two directions. The first approach, as mentioned above, is to improve the fermentation process. Although it is not certain

how much more improvement in the fermentation is possible, the goal is easily identified.

The second approach should be aimed at optimizing enzyme usage in the hydrolysis reactor. Enzyme usage can be minimized either by an enzyme recycle scheme, multistage processing, more complete hydrolysis, increased residence times, or an improved pretreatment process. Initial efforts in this direction have attempted to redefine the temperature optimums (Figure 6 and 7) for batch operations. Much more work is needed in this area before this and other results can be applied to continuous processing.

In particular, more attention should be paid to nonproductive complexing of enzymes to both substrate and nonsubstrate impurities and the role of cellobiose as an inhibitory product (20). A possible method of preventing this and speeding up the reaction would be through the addition of β -glucosidase to the hydrolysis mixtures to hydrolyze the cellobiose.

Another problem requiring more attention is contamination of syrups during hydrolysis. A population of thermophiles has established itself at the Natick pilot plant which, at 50°C, readily metabolize the sugars produced. As saccharification temperatures are lowered, the problem will become more acute.

In general, optimization of the entire process is only beginning, but the areas or unit processes requiring the most attention are clearly defined. In order for enzymatic hydrolysis of cellulose to become a competitive alternative to starch hydrolysis, we must continue to work toward more economical techniques for substrate pretreatment and production and utilization of the necessary enzymes.

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