Proceedings of the Ninth Annual Biochemical Engineering Symposium

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PROCEEDINGS OF THE NINTH ANNUAL
BIOCHEMICAL ENGINEERING SYMPOSIUM

Report No. 81

INSTITUTE FOR SYSTEMS
DESIGN AND OPTIMIZATION

Kansas State University -- Manhattan

L.T. Fan
L.E. Erickson

April 28, 1979
PROCEEDINGS OF THE NINTH ANNUAL
BIOCHEMICAL ENGINEERING SYMPOSIUM

Kansas State University
Manhattan, Kansas 66506

April 28, 1979

L. T. Fan and L. E. Erickson, Editors
This report presents the proceedings of the Biochemical Engineering Symposium held at Kansas State University, April 28, 1979. Since a number of the contributions will be published in detail elsewhere, only brief reports of each contribution are included here. Requests for further information on work at Iowa State University should be directed to Dr. Peter J. Reilly; at Colorado State University to Drs. V. G. Murphy and A. R. Moreira, and at Kansas State University to Drs. L. T. Fan and L. E. Erickson.

The symposium was attended by Mary M. Frederick, Peter J. Reilly, Carol G. Bohnenkamp, Gbekeloluwa B. Oguntein, Al Fratzke, Juliana C. Shei, and Ricardo Fournier A. of Iowa State University, Rebecca R. Welling, Maya Helfenstein, Fred Blum, Duane C. Ulmer, Antonio R. Moreira, and Vince Murphy of Colorado State University, George I. Kvasitadze of USSR Academy of Sciences, Hirokazu Nishitani, Osaka University, and Yong-Hyun Lee, Bamidele O. Solomon, Vasanti Deshpande, L. T. Fan, Edward R. Hsu, David Beardmore, Pawan Handa, and Larry E. Erickson of Kansas State University.

L. T. Fan
L. E. Erickson
Editors
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INTRODUCTION

In previous reports, a crude enzyme preparation from \textit{Aspergillus niger} with xylanase activity, Rohm and Haas Rhozyme HP-150 Concentrate, had been partially separated by passage through Ultrogel AcA 54 gel permeation resin (Figure 1).\textsuperscript{1,2} One of the major peaks, Fraction B, that attacked larchwood xylan was further subjected to cation exchange chromatography with SP-Sephadex C-25 at pH 4.5 to separate those enzymes with isoelectric points above pH 5.5. Eluate was gathered into Pools 1-5 (Figure 2). This paper will report the purification to homogeneity and the partial characterization of Pool 4.

MATERIALS AND METHODS

Enzyme Assay

Enzyme activity was determined with a Somogyi-Nelson reducing sugar assay.\textsuperscript{3,4} Samples of 50-100 µl of enzyme solution were mixed with 0.25 ml of approximately 1% xylan (2% of Sigma X-3875 larchwood xylan, Lot 97C-0066-1, dissolved in water at room temperature and the undissolved material centrifuged out), and 0.75 ml of 0.075M sodium acetate buffer at pH 5.0. After a 20 min incubation at 40 °C, 1 ml of Somogyi's solution was added, and the mixture was held at 99.5 °C for 20 min. After standing for 30-60 min at room temperature, some of the mixture was transferred to a 1 cm path length cuvette, and its absorbance determined at 500 nm. Activity was measured in units (U), where 1 U was the amount of enzyme that released reducing power equivalent to 1 µmol of xylose in 1 min under the conditions of the assay.

Protein Determination

With column effluents, protein was determined by measuring the absorbance of solution in a 1 cm path length cuvette at 280 nm. With enzyme pools, the Bio-Rad protein assay, based on Bradford's method,\textsuperscript{5} was used.

Disc Gel Electrophoresis

Disc gel electrophoresis was conducted on purified Pool 4 following the method of Laemmli.\textsuperscript{6}

Isoelectric Focusing

Isoelectric focusing on Pool 4 at various stages of its purification followed the procedure of Righetti and Drysdale.\textsuperscript{7}
High Pressure Liquid Chromatography

Samples of xylosides after enzymatic hydrolysis were analyzed with a Waters ALC 201 liquid chromatograph, using a 56 cm long, 0.95 cm o.d. jacketed stainless steel tube packed with 19-25 μm Bio-Rad Aminex Q-15S ion exchange beads in the Ca2+ form, through which 1.0 ml/min water was pumped. The column was maintained at 84 °C; sample size was 80 μl.

Materials

A large number of glycosides were used in the standard assay to determine the specificity of Pool 4. They included arabinogalactan, carboxymethylcellulose, cellulose, chitin, dextran, dextrin, glucomannan, inulin, laminarin, locust bean gum (galactomannan), yeast mannan, p-nitrophenyl-β-D-glucoside, pectin, and polygalacturonic acid. In addition, xylodiose (X2), xylotriose (X3), xylotetraose (X4), and a mixture of xylopentaose (X5) to xylanonaose (X9) were prepared by acid hydrolysis of larchwood xylan, followed by passage of the supernatant through a charcoal–Celite column with a linear butanol gradient.

Xylan free of arabinosyl-initiated side chains was made from larchwood xylan (Sigma X-3875, lot 97C-0066-1) by mixing 50 g with 4975 ml of water, centrifuging out the approximately 20 g of undissolved material, and treating both supernatant and pellet with H2SO4 at pH 2 and 100 °C for 90 min. The supernatant was neutralized with Ba(OH)2, followed by centrifugation of the resulting BaSO4. The pellet was neutralized with NaOH.

RESULTS

Purification of Pool 4 Xylanase

Two 100 ml samples of Pool 4 from SP-Sephadex C-25 column chromatography were evaporated at room temperature to 25 and 31 ml and added separately to a 2.1 x 133 cm Sephadex G-50 column. After elution with 0.2 ml/min of 0.03M citrate buffer at pH 5.0, one peak with xylanase activity was collected from each run (Figure 3).

These two peaks, containing 50 and 68 ml, were pooled and added to a 1.7 x 59 cm SP-Sephadex column, eluted at 1 ml/min with 0.025M acetate buffer, pH 4.6, with a linear gradient constructed with 0.3M NaCl on the high side (Figure 4).

The 76 ml heart cut was pooled and found to be homogeneous by isoelectric focusing and disc gel electrophoresis. Plots of activity and protein concentration of gel slices after isoelectric focusing of Pool 4 from the first SP-Sephadex separation, Sephadex G-50 chromatography, and the second SP-Sephadex separation are shown in Figures 5A–5C. The isoelectric point of the enzyme was found at pH 6.6.

Starting with the crude enzyme, the cumulative yield was 0.41%, with large losses occurring in the Ultrogel AcA 54 and the first SP-Sephadex steps, where other xylanases were being removed. A more moderate loss occurred in the second SP-Sephadex step. The cumulative purification was 9,47-fold, with a final specific activity of 57.3 U/mg.
Specificity of Pool 4 Xylanase

The homogeneous enzyme had little or no activity in a 60-min assay on 0.5% final concentrations of arabinogalactan, CM-cellulose, untreated or crystalline cellulose, chitin, dextran, inulin, laminarin, locust bean gum, mannann, or p-nitrophenyl-β-D-glucoside, or on dextrin at 0.05% final concentration or 0.25% glucomannan. There was significant activity on 0.3% pectin and 0.3% polygalacturonic acid, and the products of these reactions are now being identified to determine whether xylan impurities were actually being attacked, rather than pectin or polygalacturonic acid.

Of the xylosides subjected to attack with homogeneous Pool 4 xylanase, xylobiose was totally resistant, while X₃ was hydrolyzed slowly to X₂ and xylose (Figure 6). Xylotetraose was rapidly broken down to X₂, with some X₃ and xylose, and the X₃ was more slowly hydrolyzed to X₂ and xylose (Figure 7). It is not yet clear which bond in X₃ is broken, or whether both are subject to attack.

Soluble xylan in roughly 1% initial concentration, both untreated and treated to remove arabinosyl-initiated branch points, was hydrolyzed extensively in the regular assay by homogeneous Pool 4 xylanase. Insoluble xylan, both treated and untreated, was almost totally resistant to attack. Extended incubation of xylan yielded large amounts of xylobiose.

Molecular Weight of Pool 4 Xylanase

A 2.5 ml portion of homogeneous Pool 4 xylanase was added to a 1.5 x 100 cm Sephadex G-50 Superfine column, eluted with 0.025M sodium acetate buffer, pH 5, and 0.05M NaCl at 0.25 ml/min. Eluate samples were assayed as described previously.

Standards of 2.5 ml of cytochrome C, lysozyme, chymotrypsinogen A, ovalbumin, and bovine serum albumin in 1 mg/ml concentration (10 mg/ml for lysozyme) were similarly chromatographed. Using these standards, the molecular weight of the homogeneous enzyme was found to be 21,000 daltons.

Amino Acid Profile of Pool 4 Xylanase

The amino acid composition of homogeneous Pool 4 was obtained with a Durrum D-400 amino acid analyzer, using 150 µl samples containing approximately 100 µg of protein. Glycine, glutamic acid, aspartic acid, and alanine were found in high amounts, and methionine, cysteine, phenylalanine, histidine and arginine in low amounts.

Effect of pH and Temperature on Activity and Stability of Pool 4 Xylanase

Homogeneous Pool 4 xylanase was assayed in the standard manner, except at varying pH's with varying buffers. From pH 2.6 to 6.4, the buffer was 0.035M sodium citrate, while from pH 6 to 8, 0.035M sodium phosphate buffer was employed. The buffer from pH 8 to 9 was 0.35M sodium borate. At all pH's the ionic strength was adjusted to 0.5M with KCl. Blanks without enzyme and calibrations with varying amounts of xylose were run at each pH. Highest activity was at pH 5.
The standard assay was conducted at temperatures from 10 to 90 °C with samples of the homogeneous enzyme. Highest activity was found at 55 °C; up to 35 °C the activation energy was 6.10 ± 0.71 kcal/mol, where the range signifies the 95% confidence limits.

After samples of homogeneous enzyme were concentrated four-fold, 1 ml was mixed with 3 ml of 0.035M sodium citrate buffer at pH's between 3.5 and 6, or 0.035M sodium phosphate at pH's 7 or 8, with the ionic strength adjusted to 0.5M with KCl. The mixtures were incubated at 55 °C, and samples were removed at varying times for assay. Decay at all cases was first order, and was slowest between pH's 4 and 6.

Freezing to approximately -60 °C of 100 µl homogeneous enzyme in 0.75 ml sodium acetate buffer of 0.075M at pH 5, either with or without 0.25 ml 1% xylan, followed by thawing after 2 days and subsequent refreezing for another 2 days, led to no loss of activity.

Effect of Metal Ions on Pool 4 Xylanase

Samples of 25 µl homogeneous Pool 4 xylanase were assayed in the normal manner, except that buffer was mixed with 1M solutions of a number of metal chlorides (AlCl₃, BaCl₂, CaCl₂, CrCl₂, CuCl₂, FeCl₃, HgCl₂, KCl, LiCl, MgCl₂, MnCl₂, NaCl₂, NiCl₂ and ZnCl₂) to obtain assay concentrations of the metal ions of 7.5 x 10⁻²M, 7.5 x 10⁻³M, 7.5 x 10⁻⁴M, and 7.5 x 10⁻⁵M. Mg²⁺ and all heavy metals tested were inhibitory at 7.5 x 10⁻²M, and Co²⁺, Cu²⁺, Fe³⁺, Mg²⁺, Zn²⁺, and Hg²⁺ affected enzyme activity at even lower concentrations. The results were somewhat clouded by the effect of the ions themselves on the reducing sugar assay in the absence of enzyme.

DISCUSSION

The xylanase in Pool 4 described here appears to be similar or identical to the enzyme studied by Takenishi and Tsujisaka, after being purified to homogeneity by Fukumoto et al. Unlike β-amylase, a related exo-hydrolase, this enzyme attacked the straight-chain oligomer of DP 3, and therefore produced monomer. In addition, xylotetraose is broken down to a mixture of xylose, xylobiose, and xylotriose, while β-amylase cleaves maltotetraose to maltose only. It is not yet known which asymmetric bond in xylotetraose, or which bond in xylotriose, is attacked, or whether this enzyme is active on other than xylosyl-β-(1→4)-xylosyl bonds in xylans. In addition, whether this enzyme hydrolyzes pectin or polygalacturonic acid itself, or just a xylan impurity in these preparations, is unknown. Also a puzzle at the moment is the lack of activity on insoluble xylan. We expect to resolve most of these questions in the near future.

REFERENCES


**ACKNOWLEDGMENT**

This work was supported by National Science Foundation Grant PFR77-00198 and by the Engineering Research Institute, Iowa State University.
Figure 1. Elution pattern of crude hemicellulase at 119 ml loading and 0.67 ml/min from a 3.1 x 148 cm Ultrogel AcA 54 column at pH 4.5.
Figure 2. Elution pattern of Fraction B at 2805 ml loading and 1.0 ml/min from a 3.3 x 64 cm SP-Sephadex C-25 column at pH 4.5.
Figure 3. Elution pattern of Pool 4 at 25 ml loading and 0.2 ml/min from a 2.2 x 133 cm Sephadex G-50 Superfine column at pH 5.0.
Figure 4. Elution pattern of Pool 4 at 118 ml loading and 1 ml/min from a 1.7 x 59 cm SP-Sephadex C-25 column at pH 4.6.
Figures 5A, 5B, and 5C. Activities and protein stains of Pool 4 after isoelectric focusing of eluates from initial SP-Sephadex, Sephadex G-50, and final SP-Sephadex column chromatography.
Figure 6. Hydrolysis of xylotriose by homogeneous Pool 4.
Figure 7. Hydrolysis of xylotetraose by homogeneous Pool 4.
SOME EXPERIMENTAL OBSERVATIONS ON THE
ADSORPTION OF CELLULASE ONTO CELLULOSE
AND ON THE BEHAVIOR OF ADSORBED CELLULASE

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INTRODUCTION

Cellulose is an insoluble and structured substrate, and therefore, some of the kinetic characteristics of enzymatic hydrolysis of cellulose are different from those of the usual homogeneous enzyme catalyzed reaction. One of these characteristics is adsorption or desorption of cellulase on the cellulose surface. The formation of an enzyme-substrate (E-S) complex by means of adsorption is an essential step for the enzymatic hydrolysis of cellulose. Thus, it is important to obtain information on the adsorption and desorption of enzyme onto cellulose particles. Several papers have been published in this area; however, most of the published papers are concerned with the practical application of adsorbed cellulase in cellulose hydrolysis. Relatively little attention has been given to the kinetic and mechanistic aspects of the adsorption and desorption of enzyme onto cellulose particles.

The purposes of this work were: (1) to measure the amount of adsorbed enzyme during the hydrolysis and then examine the relationship between the hydrolysis rate and the amount of the E-S complex formed, (2) to determine the effect of hydrolysis conditions on the extent of E-S complex formation, and (3) to study the behavior of adsorbed cellulase on cellulose particles.

MATERIALS AND METHODS

1. Enzyme

The enzyme source consisted of a culture filtrate of Trichoderma reesei QM 9414. The medium composition and method used for cultivation were similar to those described by Mandels et al. The soluble protein content of the enzyme broth was 2.25 mg/ml by the Lowry method. The various enzyme activities of the filtrate were measured by the method developed by Mandels et al. The filter paper activity (FPA), CMC activity, and 8-glucosidase activity were 1.56 IU/ml, 14.8 IU/ml, and 0.072 IU/ml respectively. For some experimental runs, the enzyme was concentrated using a model 402 Amicon Stirred Cell, with a type PM 10 ultrafiltration membrane which retains polymers with molecular weights of 10,000 or more.

2. Cellulose Substrate

Solka Floc, a hammer-milled sulfite pulp, was the main substrate used. The Solka Floc was apportioned into particle size fractions using a Ro-Tap test sieve shaker. The fraction, passing 270 mesh and not passing 400 mesh,
was used as a basic substrate in the experiment. It is referred to as the standard substrate.

3. Determination of Structural Features of Cellulose

The specific surface area and crystallinity index were measured to characterize the cellulose structure after the cellulose was solvent dried. The methods used were the same as those employed in our previous work. The water retention volume (WRV), which is defined as the ratio of the weight of water retained per weight of cellulose, was measured as shown in Table 1.

4. Hydrolysis of Substrate

All samples were dried overnight at 80°C, and 5 grams of the substrate were weighed into each of two 300 ml flasks. Then, 40 mL of distilled water, 5 mL of 1.0-M citrate buffer (pH 4.8) and 50 mL of culture filtrate of Trichoderma reesei QM 9414 were added to obtain a 5% substrate solution. These flasks were placed in a 50°C Lab-Line shaking water bath set at 250 RPM. Samples were withdrawn at different time periods, centrifuged, and the supernatant refrigerated.

5. Analytical Methods

The samples were analyzed for soluble protein in the aqueous phase (unadsorbed) applying the Lowry method. The concentration of adsorbed soluble protein was calculated indirectly as described in the next section. The filter paper activity was measured as described by Mandels et al. The reducing sugar content was measured as glucose by the DNS (dinitrosalicylic acid) method, and the glucose content was measured by the enzymatic method applying 4X Glucostat.

6. Calculation

The residual cellulose concentrations were evaluated indirectly from the measured reducing sugar concentrations. The amount of the adsorbed soluble protein was estimated by subtracting the measured soluble protein concentration in the supernatant from the initial soluble protein concentration in the supernatant. However, the initial concentration of protein in the supernatant might have increased after addition of cellulose because water penetrates into the cellulose particles. In the present calculation, therefore, a concentration factor was introduced to reflect this increase. The factor is based on the assumption that water can penetrate into cellulose particles but the protein molecule can not because it has a large molecule size. The change in the volume of the reaction mixture after cellulose addition was neglected because the quantity of the cellulose added was small (5 wt%). Thus the calculation procedure can be summarized as shown below.

\[ S = S_0 - (\text{DNS} \times 0.9) \]

\[ CF = \frac{1000}{1000 - (\text{WRV} \times S)} \]

\[ E_c = E_0 \times CF \]

\[ E_{ads} = E_c - E \]
where

\[ S_0 = \text{initial cellulose concentration in the reaction mixture, g/\ell} \]
\[ S = \text{estimated residual cellulose concentration in the reaction mixture, g/\ell} \]
\[ \text{CF} = \text{concentration factor} \]
\[ E_o = \text{initial protein concentration in the supernatant, g/\ell} \]
\[ E_c = \text{adjusted initial protein concentration in the supernatant after penetration of water into cellulose particles, g/\ell} \]
\[ E = \text{measured protein concentration in the supernatant, g/\ell} \]
\[ E_{\text{ads}} = \text{amount of protein adsorbed from the unit volume of the supernatant, g/\ell} \]

RESULTS AND DISCUSSION

1. Hydrolysis Rate and Quantity of Adsorbed Soluble Protein

Figure 1 shows the typical hydrolysis curves of Solka Floc by the filtrate of Trichoderma reesei QM 9414. The hydrolysis of cellulose was rapid in the initial stage of reaction. Fifty percent of the total reducing sugar produced was created during the first 12 hours approximately. After this period, the increase in the reducing sugar slowed appreciably. It has been suggested that this is due to the structural changes in the cellulose fibers.\(^{16,17}\)

In our previous work,\(^1\) we measured the changes in the specific surface area and total surface area during 96 hours of hydrolysis along with the changes in the crystallinity index (CrI). The results indicated that these structural changes partially contributed to the enhancement of the reduction in the rate of hydrolysis during the later phase of reaction. However, it was difficult to visualize that the slow-down was caused only by the structural changes, because sufficient surface area was available even after 96 hours of hydrolysis and the increase in CrI was moderate. Therefore, we have postulated that a surface deactivation mechanism and an enzyme inhibition mechanism are also responsible for the slow-down.

Figure 1 also shows the changes in the amount of adsorbed soluble protein during hydrolysis. The amount was determined by taking the average of four different experimental runs. The initial uptake of the soluble protein was very rapid with most of it being adsorbed immediately after the enzyme solution was added. A slow uptake then continued for approximately 1 hour. After this period, the enzyme began to return to the solution phase. According to Mandels et al.,\(^3\) who carried out a similar experiment, the maximum adsorption occurred at about 8 hours, and most of the enzyme still existed in the adsorbed state even after 24 hours. About 50% of the soluble protein was still being adsorbed after 70 hours of hydrolysis. Our result was slightly different from that of Mandels et al.,\(^3\) in that the maximum adsorption occurred earlier than that observed by Mandels et al. Furthermore, the quantity of enzyme adsorbed decreased more rapidly in our work than of Mandels et al.
One of the causes, which contribute to the desorption of enzyme, may be the decrease in the effective, but not simply the total, surface area (or active sites) of the cellulose fiber for adsorption. This is based on the observation that the maximum adsorption occurred at approximately 1 hour; however, the specific surface area reached a maximum at around 8 hours. Therefore, it is difficult to attribute the extent of the enzyme adsorption solely to the availability of surface area. Another possible interpretation is that the products also contribute to the desorption of cellulase, which will be elaborated in the next subsection.

A comparison of the hydrolysis and enzyme adsorption curves indicates that the rate of the hydrolysis was apparently proportional to the amount of adsorbed soluble protein throughout the reaction, except for the relatively short initial period. This observation implies that the slow-down at the later phase of hydrolysis might be due to the difficulty for the E-S complex to be formed, which might have been caused by the decrease in the active site surface area and enzyme inhibition by the hydrolysis products such as cellobiose and glucose.

2. Effect of Addition of Glucose and Cellobiose on E-S Complex Formation

It was reported that hydrolysis products, such as cellobiose and glucose, inhibit the enzyme reaction of cellulose hydrolysis. Cellobiose is especially known to be a strong cellulase inhibitor. To understand the effects of glucose and cellobiose on the E-S complex formation and the hydrolysis, five experiments, including one control, were performed as summarized in Table 2. In each of these experiments, a different amount of glucose or cellobiose was added to the hydrolysis mixture at the onset of the hydrolysis. Table 2 shows the average protein adsorbed during the initial 4 hours and reducing sugar produced after 4 hours. The hydrolysis products significantly affected the E-S complex formation as well as the extent of hydrolysis. As the concentrations of sugar increased, the quantity of protein adsorbed decreased. The extent of E-S complex formation was not affected appreciably by the difference in types of sugar. Meanwhile, the different types of sugar do significantly affect the hydrolysis; cellobiose was a more severe inhibitor than glucose.

3. Effect of Enzyme Concentrations

The amount of enzyme adsorbed increased as the initial enzyme concentration in the solution increased as shown in Fig. 2; however, this increase ceased at the initial soluble protein concentration of 1.2 g/l. This may be explained in two ways as follows:

One possible explanation is that the enzyme is adsorbed onto the cellulose surface first to form a monolayer, and then the excess enzyme may be adsorbed to form additional layers. Only the enzyme adsorbed in the monolayer plays an active role in the hydrolysis. This leads to the case of surface area limitation. The other possible explanation is that the surface area is composed of two fractions, an active site and an inactive site. The enzyme can be adsorbed on both sites; however, only the enzyme adsorbed to the active site participates in the hydrolysis. This gives rise to the case of active site limitation. Additional experiments need to be carried out to determine the correct mechanism.
4. Nature of E-S complex

An understanding of whether the adsorbed enzyme is in dynamic equilibrium with the enzyme in solution or whether the enzyme is tightly bound to cellulose to form a chemisorption type adsorption is important for process development of an enzymatic hydrolysis of cellulose. Experimental results in Fig. 3 show the behavior of adsorbed cellulase. The control curve shows the original batch reducing sugar production; another curve shows the reducing sugar production when the supernatant was removed after 24 hours of hydrolysis, and a buffer and sugar subsequently added. A third curve shows the reaction mixture centrifuged after one hour of reaction time, with a buffer and sugar added. As seen in Fig. 3, the reducing sugar production rate was nearly equal to that obtained in the original batch. This illustrates that the enzyme adsorbed onto cellulose initially was primarily responsible for cellulose digestion. It also indirectly indicates that the E-S complex may be tightly bound to each other to give rise to a chemisorption type adsorption.

CONCLUSIONS

It was found that the hydrolysis rate of cellulose was closely related to the concentration or amount of the E-S complex in the reactor during the batch hydrolysis reaction. The decrease in the rate of hydrolysis at the later phase of reaction was probably due to the difficulty in the formation of the E-S complex, which might have been caused by the decrease in the active portion of cellulose particles and by the enzyme inhibition of the produced products. The hydrolysis products, glucose and cellobiose, significantly affected the E-S complex formation as well as the hydrolysis rate. The use of concentrated enzyme in hydrolysis did not give rise to the expected increase in the rate of hydrolysis beyond a certain point even though the adsorption of enzyme was enhanced by increasing its concentration beyond this point. This might be due to the multiple layer adsorption or possibly to a limitation of active sites on the cellulose surface area. The adsorbed enzyme could not be easily removed from the cellulose particles indicating that the formation of the E-S complex is due to a mechanism similar to chemisorption. It appeared that the initially adsorbed enzyme was primarily responsible for cellulose digestion.

ACKNOWLEDGMENT

This work was supported by the Agricultural Experimental Station, Kansas State University.

REFERENCES


Table 1. Water retention volume of different cellulose substrates

<table>
<thead>
<tr>
<th>Cellulose, Mesh</th>
<th>Water retention volume*</th>
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<tr>
<td>Solka Floc</td>
<td>5.77</td>
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<tr>
<td>270 - 400</td>
<td>6.73</td>
</tr>
<tr>
<td>140 - 170</td>
<td>10.12</td>
</tr>
<tr>
<td>100 - 120</td>
<td>10.50</td>
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<tr>
<td>60 - 70</td>
<td></td>
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<tr>
<td>Microcrystalline Cellulose</td>
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<td>Sigma Cell 50</td>
<td>2.88</td>
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*Water retention volume = ratio of \(H_2O\) retained per weight cellulose

Table 2. Effect of addition of products

<table>
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<tr>
<th>Sample</th>
<th>Average protein adsorbed 0 - 4 hrs, g/l</th>
<th>4 hrs DNS, g/l</th>
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<tr>
<td>Control</td>
<td>0.78</td>
<td>10.0</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>20 g/l</td>
<td>0.61</td>
<td>6.55</td>
</tr>
<tr>
<td>30 g/l</td>
<td>0.48</td>
<td>6.52</td>
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<tr>
<td>Cellobiose</td>
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<tr>
<td>20 g/l</td>
<td>0.57</td>
<td>4.6</td>
</tr>
<tr>
<td>30 g/l</td>
<td>0.46</td>
<td>3.3</td>
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Cellulose was mixed with unconcentrated enzyme solution, which resulted 5% cellulose concentration, 1.19 g/l of initial soluble protein (enzyme) concentration, and FPA of 0.8 IU/ml in the hydrolysis vessel.
Figure 1. Changes in the reducing sugar concentration (○) residual cellulose concentration (△), and the amount of enzyme adsorbed (△) during the 5% Solka Floc Hydrolysis.
Figure 2. Effect of the initial soluble protein concentration of 5% Solka Floc hydrolysis.
Figure 3. Effects of the removing the supernatant and replenishing it with buffer and glucose.
PROPERTIES OF A HOMOGENEOUS ENDO-XYLANASE FROM ASPERGILLUS NIGER

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Ames, Iowa 50011

INTRODUCTION

In the two previous symposia, work that had separated a crude hemicellulase preparation from Aspergillus niger into different fractions was reported.\(^1,2\) This paper presents the purification to homogeneity of one of these fractions, known as Pool 8, which had been separated by Ultrogel AcA 54 gel filtration chromatography and then by DEAE-Sephadex A-25 anion exchange chromatography at pH 5.4 (Fig. 1). Many of the properties of this homogeneous xylanase were also determined, and are reported here.

MATERIALS AND METHODS

Materials

Larchwood xylan solutions were prepared by adding 20 g of either Sigma xylan lot 62C-2820 or lot 97C-0066-1 to 1 liter of distilled water and centrifuging at 4°C and 10,000 rpm in a Sorvall RC-5 centrifuge for 30 min. The supernatants were vacuum filtered to give approximately 1% (w/v) xylan (Lot 1 or Lot 3) solutions.

Xylobiose, xylotriose, xylotetraose, and a mixture of xylopentaose to xylo-nonaose were prepared by acid hydrolysis of xylan followed by separation in a charcoal-Celite column with a linear butanol gradient. Xylan preparations free of arabinosyl-initiated side chains were made by dissolving 1% xylan in water, centrifuging, and treating both pellet and supernatant with H\(_2\)SO\(_4\) at pH 2 and 100°C for 90 min. The pellet was neutralized with NaOH and the supernatant with Ba(OH)\(_2\).

Roughly 1% solutions of yeast mannan, laminarin, inulin, polygalacturonic acid, chitin, dextran, dextrin, arabinogalactan, pectin, carboxymethylcellulose, cellulose, locust bean gum (galactomannan), p-nitrophenyl-β-D-glucoside, and glucomannan were prepared in much the same way as with xylan.

Enzyme Assay

The activity of xylanase was determined by quantitatively measuring the production of reducing sugar by the Somogyi-Nelson assay.\(^3,4\) A 1 ml solution composed of 0.25 ml of 1% xylan-lot 3 and 0.75 ml of 0.075M sodium acetate buffer at pH 5.0 was mixed with 100 ml of enzyme solution and incubated for 20 min at 40°C. Immediately after the incubation, 1 ml of Somogyi's reagent was added and the mixture was heated for 20 min at 99.5°C. After cooling for 5 min in a water bath at room temperature, 1 ml of Nelson's reagent was added.
The optical density of the mixture in a 1 cm pathlength cuvette at 500 nm was measured after 30-60 min. Activity was measured in units (U), where 1 U was the enzyme that released reducing power equivalent to 1 μmol of xylose in 1 min under the conditions of the assay.

**Protein Determination**

In column chromatography, elution of proteins was followed with an on-line ISCO UA-5 ultraviolet monitor with a 0.5 cm cuvette and a 280 nm filter, or by manual sampling followed by determination of absorbance at 280 nm. With enzyme pools, the Bio-Rad protein assay, based on Bradford's method, was used. Very low protein quantities were determined using Spector's and Bearden's modifications of Bradford's method.

**Disc Gel Electrophoresis and Isoelectric Focusing**

The electrophoresis method was that of Davis modified for use with six 100 mm tubes. Isoelectric focusing was performed using the Bio-Rad modification of the Righetti and Drysdale method.

**RESULTS**

**Enzyme Purification**

In work previously reported, a crude *Aspergillus niger* xylanase, Rhozyme HP-150 Concentrate from Rohm and Haas, was separated into a number of fractions with activity on at least one xylan preparation by Ultrogel AcA 54 gel filtration chromatography. One of these pools, Fraction C, was further separated by SP-Sephadex C-25 ion exchange chromatography at pH 4.5, which allowed proteins with isoelectric points below approximately pH 5.5 to pass through the column unretarded. The material that was not retarded was loaded on a DEAE-Sephadex A-25 column at pH 5.4 and separated into different activity peaks labelled Pools 8 to 13 (Figure 1). This paper reports the purification to homogeneity and partial characterization of Pool 8.

After unsuccessful attempts to purify Pool 8 with hydrophobic and hydroxylapatite chromatography, 175 ml was dialyzed against distilled water. The sample was then concentrated with an Amicon PM10 membrane in a Model 402 stirred cell to 42 ml, and 25 ml were loaded on a 2.2 x 125 cm Sephadex G-50 column. The enzyme was eluted at 0.18 ml/min with 2 column volumes of 0.03M sodium citrate buffer at pH 5.15 (Figure 2). A narrow cut, which reduced the yield of activity but eliminated as much protein as possible, was pooled, yielding 40 ml.

A 37 ml sample of this pool was added to a 2.4 x 40.5 DEAE-Sephadex A-25 column, and eluted at 1 ml/min with the same buffer, to which a linear NaCl gradient was imposed (Figure 3). The fractions with highest activity were pooled, yielding 145 ml, and dialyzed against the buffer without salt. A 100 ml portion was then concentrated with an Amicon UM2 membrane to 15 ml for further work. Disc gel electrophoresis showed the enzyme preparation to be pure (Figures 4A, B, C).
Starting with the crude enzyme, Pool 8 was obtained in 0.12% yield, the major losses being sustained in the Ultragel ACA 54 and first DEAE-Sephadex steps, where other xylanases were separated, and in the second DEAE-Sephadex step. The cumulative purification was 3.60, and the final specific activity was 21.4 U/mg.

A second purification using the same steps also yielded a homogeneous preparation, as tested by isoelectric focusing. Yields and specific activities were similar to the first purification. The isoelectric point was at pH 3.75.

**Enzyme Characterization**

Assays were conducted with the substrates listed earlier, using 0.1 ml of enzyme solution in double the usual assay volume at pH 4.5 for 40 min, but otherwise with the usual assay procedure. The homogeneous enzyme was strongly active on the xylopentaose-xylonaose mixture, on xylan-lot 3, and on debranched xylan, but only slightly active on xylan-lot 1. All other substrates were resistant to xylan attack.

The products of those substrates strongly attacked by the xylanase preparation were analyzed by a Beckman ERA-2001 enzymatic glucose analyzer and by a Waters ALC-201 analytical liquid chromatograph using a column packed with Aminex 50W-X4 in the Ca\(^{2+}\) form eluted with water. No glucose was present in any of the samples. The product found in largest amounts when the xylan samples were hydrolyzed was xylotriose, followed by xylopentaose, xylobiose, xylohexaose, and xylotetraose. With the xylopentaose-xylonaose mixture only low amounts of xylopentaose were found in the product, but otherwise the product profile was little changed. Large amounts of starting material remained unreacted in each case. No xylose was found in any product sample.

Enzyme assays conducted on the homogeneous enzyme at different pH's (with Sorensen's buffer from pH 1.95 to 2.86 and with McIlvaine's buffer from pH 3.68 to 8.04) yielded the highest activity near pH 5.

The enzyme was assayed at pH 5.0 and at temperatures from 15°C to 90°C. Highest activity was found at 42°C. Below that temperature the activation energy was 2.18 ± 0.22 kcal/mol, where the second value was the 95% confidence range.

Stability at various pH's was determined using the standard enzyme assay with Sorensen's buffer at pH 2.6 and McIlvaine's buffer at higher values. The samples were incubated for various times up to 128 min at 50°C. Highest stability was at pH 5.0, where the half-life was approximately 100 min.

The enzyme was activated approximately 30% by the addition of 75 mM CaCl\(_2\). HgCl\(_2\) was inhibitory above 1 mM. p-Chloromercuribenzoate (PCMB) did not affect enzyme activity.

Molecular weight was estimated by SDS gel electrophoresis using the method of Laemmli to be 28,000 daltons, and by gel permeation chromatography using Sephadex G-50 to be 26,500 daltons.
Carbohydrate content by the phenol sulfuric acid method of Dubois et al.\textsuperscript{12} was 22%. The enzyme was high in serine, glycine, glutamic acid, and aspartic acid, as determined by a Durrum D-400 amino acid analyzer. Little cysteine, arginine, methionine, lysine, and phenylalanine were present.

**DISCUSSION**

The enzyme whose purification and characterization have been presented in this paper is a typical endo-hydrolase, in that only molecules having at least five xylose units were hydrolyzed, while no xylose was formed from any substrate. A further indication of this was the ability of Ca\textsuperscript{2+} to activate the enzyme, as it does with α-amylase.

It is not clear why the enzyme had different activities on two different xylan preparations. A possibility is that one is more branched, shielding much of itself from enzymic attack.

This xylanase is not particularly stable, but its optimum pH for activity and stability are similar to those found for other xylanases from *A. niger*, as is its low molecular weight. It has no sulfhydryl group in the active center, as it is insensitive to PCMB addition.

**REFERENCES**

ACKNOWLEDGEMENT

This work was supported by National Science Foundation Grant PFR77-00198 and by the Engineering Research Institute, Iowa State University.
Figure 1. Elution pattern of Fraction C at 2340 ml loading and 1 ml/min from a 3.3 x 57 cm DEAE-Sephadex A-25 column at pH 5.4.
Figure 2. Elution pattern of Pool 8 at 25 ml loading and 0.18 ml/min from a Sephadex G-50 column at pH 5.15.
Figure 3. Elution pattern of Pool 8 at 37 ml loading and 1 ml/min from a 2.4 x 40.5 cm DEAE-Sephadex A-25 column at pH 5.15.
Figures 4A, 4B, 4C. Activities, protein, and protein stains of Pool 8 after gel electrophoresis of eluates from initial DEAE-Sephadex, Sephadex G-50, and final DEAE-Sephadex column chromatography.
SOLID STATE FERMENTATION OF MANURE FIBERS

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ABSTRACT

The feasibility of utilizing steam treated feedlot waste fibers as a substrate for solid state fermentation by the cellulolytic fungus Chaetomium cellulolyticum for the production of a protein enriched ruminant feed was investigated. The fiber fraction was treated at 30% dry matter in an autoclave for ten minutes at temperatures ranging from 170 - 200°C and then fermented at various levels of dry matter, nitrogen supplementation and aeration. Steam treatment increased the amount of soluble reducing sugars as much as 24 fold for the 200°C-ten minute treatment compared to the control, and the cellulase reactivity was increased 30 fold for the 190°C-ten minute treatment compared to the control. Fermentation of the material treated at 170°C increased the TCA precipitable protein 85% and decreased the cellulose content by about 38%. Despite the increased protein content and decreased cellulose content, the in vitro rumen digestibility was decreased by 16%.

INTRODUCTION

There are an estimated 9.3 x 10^7 tons (dry weight basis) of manure produced yearly (3) in the United States which must be properly disposed of to prevent odor and pollution problems. Possible disposal of cattle manure may be through three main channels 1. use as a fertilizer, 2. use as a fuel, or 3. reuse as a livestock feed. An engineering and economic overview of possible livestock waste utilization techniques indicated that feed utilization techniques were generally superior to fertilizer and fuel uses (9). Of the various methods to process whole feedlot waste (WFLW) for refeeding, fractionation with complete recycle appeared to be the most viable alternative.

Many treatments to improve the nutritional quality of feedlot waste (FLW) fibers have been proposed. These include drying and grinding of the fibers (7,8,14), acid or alkaline hydrolysis (8,14,16), or allowing the substrate to undergo microbial fermentation prior to refeeding (1,7,8,). Nesse et al. (15) used steam at temperatures from 130 - 200°C to treat FLW fibers. They found that the fibers released more than 60 mg of reducing sugars per gram as a result of autohydrolysis at 190°C for 30 minutes. The same conditions also resulted in a reactivity to cellulase that was more than ten times that of the untreated fiber.

Our investigation was to determine if steam treated fibers could be fermented aerobically in solid state cultures to increase the nutritional value. Such a process, it was reasoned, could result in an increased protein content and an increase in digestibility.
MATERIALS AND METHODS

Fractionation of Feedlot Waste:

WFLW was collected at random from the Colorado State University research farm, slurried to 15-20% dry matter and then blended in a one-gallon Waring blender. The mixture was placed on a reciprocating screen and the filtrate was allowed to drain through. The collected filtrate was rescreened two times before being discarded. The fiber which was entrapped on the screen was then manually removed, and the overall process was repeated two more times with fresh water utilized each time to reslurry the fiber. After the third screening, fibers were pressed with a potato ricer and spread on a plastic sheet and allowed to air dry. The fibers were then stored at room temperature.

Steam Treatment:

Figure 1 is a schematic representation of the treatment equipment. The steam generator was a five gallon pressure vessel connected to a one gallon stainless steel autoclave by a 1/4 inch stainless steel line. A cylindrical basket constructed from 1/16 inch mesh screen was filled with 200 g fiber and placed in the reaction vessel. The lid was then bolted into place and the steam line opened. The desired temperature was reached in 30 seconds. Thereafter, temperature was controlled within ± 2°C by manually manipulating the valves in the inlet and vent lines. After the fiber had been treated the desired time, the autoclave was rapidly vented and the material removed.

Fermentation:

For fermentation, 25g (dry weight) of treated or untreated fiber was placed in a one quart mason jar and then inoculated with 25 ml of blended mycelia of Chaetomium cellulolyticum (ATCC 32319). The jar was then placed in an incubator held at 37°C for seven days. The inoculum was prepared from cultures initially grown on malt extract agar (BBL) and then transferred to Chahal and Hawksworth medium (4) for a period of 3-4 days.

Analysis:

Total nitrogen was determined by the micro-kjeldahl method (2). Protein was estimated by the amount of trichloroacetic acid (TCA) precipitable nitrogen x 6.25.

Cellulase reactivity was measured by the amount of reducing sugar produced when 1 g (dry weight) of fiber was treated with 20 ml of a cellulase solution (0.05 filter paper units per ml) for one hour at 50°C (10). The amount of soluble reducing sugars was determined by the dinitrosalicylic acid (DNSA) method (13). Cellulose, lignin, and hemicellulose were determined by the method of Goering and Van Soest (6).

In vitro rumen digestibility (IVRD) was determined by the modified method of Melenberger et al. (12). The rumen fluid was mixed with a mineral and buffer mixture at a ratio of 4:1 as described by McDougall (11).
RESULTS

Some of our early results indicated that steam treatment of dry fibers (90-95% dry matter) produced very little increase in reactivity to cellulase. Since the fibers were approximately 20-30% dry matter after the separation process, the effect of treating fibers at 20-40% dry matter on cellulase reactivity was determined.

Table 1 gives the amount of soluble reducing sugars released and the cellulase reactivity of fibers treated at 180°C for ten minutes at various percent dry matter. These results indicate that wetting the fibers to 30% dry matter prior to steam treatment results in higher cellulase reactivity than using relatively dry fibers. Although 30% dry matter appears to be optimal for conditions tested, fibers in the range of 25-40% dry matter could possibly be utilized. Addition of innoculum to treated fibers initially containing 30% dry matter results in a fermentation mixture that is 20% dry matter.

The effect of steam treatment at various temperatures on the production of soluble reducing sugars and reactivity to cellulase is given in Table 2. Heat treatment increased the amount of soluble reducing sugars as much as 24 fold (200°C - ten min) as compared to the control, and the cellulase reactivity was increased up to 30 fold (190°C - ten min).

The composition of the heat treated fibers is given in Table 3. There was considerable decomposition of hemicellulose, but no apparent loss of cellulose following the treatment. The observed increase in soluble reducing sugars with steam treatment is apparently a result of hydrolysis of the hemicellulose fraction. The total crude protein (total kjeldahl nitrogen x 6.25) remained relatively constant which is what one would expect unless there was decomposition of nitrogenous compounds. The percent TCA precipitable protein decreased with higher temperatures, possibly due to Maillard reactions.

The changes in composition and IVRD for FLW fibers treated for ten minutes at 180°C and for untreated fibers, each fermented for one week with C. cellulolyticum, are given in Table 4. Increased protein production and cellulose degradation were obtained by adding an inorganic nitrogen supplement of ammonium sulfate and urea (1.12 g ammonium sulfate and 0.24 g urea per 20 g fiber). The TCA protein content of the fermented heat treated fibers increased approximately 2 fold, from 5.9 to 12.5%. However, a 12.5% TCA protein content was also obtained in cultures with untreated fibers. Thus pretreating the FLW fibers prior to fungal fermentation did not increase the protein content above what is attainable with untreated fibers.

Fermentation of the fibers decreased the cellulose content of the untreated fibers 13% and the steam treated fibers 48%. Despite the fermented fibers having an increased protein content and decreased cellulose content, the IVRD was decreased (compared to unfermented fiber) in almost every instance. Heat treatment of fibers without subsequent fermentation increased the IVRD by 39%.

Chahal et al. (5) growing C. cellulolyticum on cellulosic materials in shake flask cultures were able to obtain a final product of up to 40% crude protein. Since Chaetomium is an aerobic microorganism it was felt that perhaps we were growing the cultures under oxygen limited conditions. Subjecting the
cultures to a sterile air flow of 15 ml per minute produced the changes in composition and IVRD given in Table 5. The TCA protein content only reached 11% for the treated fermented fiber, however; the crude protein content was observed to be 20%. Cellulose degradation was 38% for the treated fermented fiber with a 75% degradation of the hemicellulose content. Again a decrease was observed in the IVRD of treated fermented fibers versus treated unfermented fibers.

Placing the changes in fiber composition on an absolute weight basis as in Table 6, a somewhat different picture of the fermentation process is obtained. During the fermentation process the fungus metabolizes some of the carbohydrate to carbon dioxide, thus the final product contains fewer total grams of material than initially present. It can be seen that the TCA protein content of the untreated fermented fiber was actually the same as that of the treated fermented fiber. However, the untreated fermented fiber contained a larger weight fraction of cellulose than did the treated fermented fibers.

DISCUSSION

The pretreatment and subsequent fermentation reported in this study are novel in several respects. The FLW fibers can be separated from WFLW and then subjected to a steam treatment without drying and size reduction, keeping the cost of pretreatment relatively low. The soluble portion of the WFLW obtained during fractionation is relatively high in protein and can be dried and pelleted as a protein supplement allowing complete recycle of the feedlot waste. In addition to the steam treatment increasing the reactivity of cellulose to cellulase, the process would also destroy potential pathogens. The use of solid state rather than submerged culture also has advantages. Utilizing solid state cultures, the expense of continuous aeration and agitation are reduced; there are no foaming problems; and the final product does not have to undergo an extensive dewatering process.

The results presented here indicated that steam treatment of FLW fibers increased their reactivity to cellulase over 24 fold and their IVRD by 39%. Subsequent fermentation of treated fibers did not result in an increase in TCA protein beyond that obtainable with untreated fibers; however, significant decrease in the cellulose content of the steam treated fibers was observed. Although fermented fibers had higher TCA protein and lower cellulose levels than the corresponding unfermented fibers, their IVRD's were lower. Whether fungal fermentation is a viable means for upgrading FLW fibers will await further testing for optimal pretreatment and fermentation conditions as well as an economic evaluation of the process.

ACKNOWLEDGEMENT

I would like to thank Dr. Gerald Ward for the use of his laboratory in performing the fiber analysis. Funds for this project were provided by the National Science Foundation.
REFERENCES


TABLE 1. EFFECT OF DRY MATTER CONTENT ON REDUCING SUGARS AND CELLULASE REACTIVITY AFTER STEAM TREATMENT 180°C/10 MIN

<table>
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<th>DRY MATTER</th>
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<th>CELLULASE REACTIVITY (MG/G)</th>
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<td>95% DM</td>
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<tr>
<td>40% DM</td>
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TABLE 2. EFFECT OF STEAM TREATMENT ON REDUCING SUGARS AND CELLULASE REACTIVITY(a)

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(a) Fibers initially 30% dry matter prior to steam treatment.
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<th>HEMI-CELLULOSE</th>
<th>LIGNIN</th>
<th>ASH (c)</th>
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<td>190°C/10 MIN</td>
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(a) Fibers initially 30% dry matter prior to steam treatment.
(b) Protein content estimated by nitrogen x 6.25.
(c) Acid insoluble ash.
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<td>34.7</td>
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(a) Fermentations carried out at 20% dry matter.
(b) Protein content estimated by nitrogen x 6.25.
(c) Acid insoluble ash.
(d) 180°C Treated Fiber. Fiber initially at 30% dry matter prior to steam treatment.
TABLE 5. CHANGE IN FLW FIBER COMPOSITION AFTER FERMENTATION WITH \textit{C. CELLULOLOYTICUM} (a)

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<thead>
<tr>
<th>TREATMENT</th>
<th>CRUDE PROTEIN (b)</th>
<th>TCA PROTEIN (b)</th>
<th>SOLUBLE CELLULAR MATERIAL</th>
<th>CELLULOSE</th>
<th>HEMI-CELLULOSE</th>
<th>LIGNIN</th>
<th>ASH (c)</th>
<th>IVRD</th>
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</table>

(a) Fermentations carried out at 20\% dry matter.
(b) Protein content estimated by nitrogen x 6.25.
(c) Acid insoluble ash.
(d) 170°C treated fiber. Fiber initially at 30\% dry matter prior to steam treatment.
<table>
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<tr>
<th>TREATMENT</th>
<th>CRUDE PROTEIN (b)</th>
<th>TCA PROTEIN (b)</th>
<th>CELLULOSE</th>
<th>HEMICELLULOSE</th>
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</thead>
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<tr>
<td>UNTREATED</td>
<td>2.0</td>
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<td>5.2</td>
<td>3.9</td>
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<td>8.1</td>
<td>6.0</td>
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<tr>
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<td>2.0</td>
<td>9.2</td>
<td>4.7</td>
</tr>
<tr>
<td>TREATED (d) FERMENTED-AERATION</td>
<td>3.8 (c)</td>
<td>2.1</td>
<td>3.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

(a) Fermentations carried out at 20% dry matter.
(b) Protein content estimated by nitrogen x 6.25.
(c) Increase in crude protein of fermented fibers due to the addition of inorganic nitrogen as described in materials and methods.
(d) 170C treated fibers. Fibers initially at 30% dry matter prior to steam treatment.
EQUIPMENT FOR PRETREATING FLW FIBER

Figure 1. Schematic representation of the equipment used in the steam treatment of feedlot waste fibers (TC = thermocouple well, PG = pressure gauge).
INTRODUCTION

The high cost of oil and its by-products has discouraged the idea of utilizing hydrocarbons and methanol as substrates for cell growth. There is a shift of emphasis from microbial cultivation on petroleum products to cultivation on renewable resources. These renewable resources include carbohydrates, cellulose, naturally occurring amino acids and fats and oils.

In this paper the method of material and energy balances will be used to analyze and check the consistency of experimental data in the literature. The same method will also be used for the analysis of the results from the experiments now being carried out on the production of useful products from grain dusts.

The material and energy balances are based on the work of Minkevich and Eroshin [1,2]. These same concepts have been widely applied by Erickson et al. [3,4,5,6]. The approach is based on the facts that the heat of reaction per equivalent of available electrons transferred to oxygen ($Q_o$) for a vast variety of organic molecules, the weight fraction of carbon in biomass ($\sigma_b$) and the equivalents of available electrons per quantity of biomass containing one gram atom of carbon ($\gamma_b$) are all constant.

For these regularities, the work of Minkevich and Eroshin [1,10] has shown that $Q_o = 27$ kcal/g equivalent, $\gamma_b = 4.291$ and $\sigma_b = .462$. Their work has also shown that both $Q_o$ and $\gamma_b$ have coefficients of variation of 4% while $\sigma_b$ has a coefficient of variation of 5%.

THEORY

The metabolism of different organic substrates by different microorganisms can be represented by the stoichiometric equation [1,3]

$$\text{CH}_m\text{O}_{n\ell} + a\text{NH}_3 + b\text{O}_2 \rightarrow y_c\text{CH}_p\text{O}_{n\ell} + z\text{CH}_r\text{O}_{s\ell} + c\text{H}_2\text{O} + d\text{CO}_2$$

(1)

In equation (1), $\text{CH}_m\text{O}_{n\ell}$ represents the elemental composition of the organic substrate, $\text{CH}_r\text{O}_{s\ell}$ represents the average elemental composition of the extracellular products while $\text{CH}_p\text{O}_{n\ell}$ denotes the elemental composition of the biomass. An available electron balance on Equation (1) gives [1,3]

$$\gamma_s + 4(-b) = y_c\gamma_b + z\gamma_p$$

(2)
After rearranging and dividing through by \( y \) we obtain

\[
\frac{4b}{y} + \frac{\gamma_b}{\gamma_s} z + \frac{\gamma_p}{\gamma_s} = 1
\]

(3)

The first term is denoted by \( \varepsilon \) and is the fraction of available electrons in the substrate which is transferred to oxygen; the second term which is represented by \( \eta \) is the fraction of available electrons in substrate which is incorporated into biomass and the third term is the fraction of available electrons in substrate utilized in product formation, \( \xi_p \). Thus,

\[
\varepsilon + \eta + \xi_p = 1
\]

(4)

where

\[
\varepsilon = \frac{4bQ_o}{\gamma_sQ_o} = \frac{4b}{\gamma_s}
\]

(5)

\[
\eta = \frac{y_c\gamma_bQ_o}{\gamma_sQ_o} = \frac{y_c\gamma_b}{\gamma_s}
\]

(6)

\[
\xi_p = \frac{z\gamma_pQ_o}{\gamma_sQ_o} = \frac{z\gamma_p}{\gamma_s}
\]

(7)

A carbon balance can also be carried out on equation (1) to give

\[
y_c + z + d = 1
\]

(8)

where

\[
y_c = \text{fraction of substrate carbon incorporated into biomass}
\]

\[
z = \text{fraction substrate carbon utilized in product formation}
\]

\[
d = \text{fraction of substrate carbon evolved as carbon dioxide}
\]

**ANALYSIS OF DATA**

Equations (4) and (8) are utilized in this paper for checking the consistency of data. It is obvious that when \( \eta + \varepsilon + \xi_p < 1 \) and/or \( y + d + z < 1 \) then either an experimental error or unmeasured products resulted. If on the other hand \( \eta + \varepsilon + \xi_p > 1 \) and/or \( y + d + z > 1 \) then it is definite that an experimental error is involved in the data collecting process.

For fermentation processes in which product formation is assumed unimportant we have \( \xi_p = 0, z = 0 \) thus

\[
\eta + \varepsilon = 1
\]

(9)
and

\[ y + d = 1 \]  

The notions above are utilized in checking the consistency and analyzing data from experimental results. For this analysis, however, an allowance of deviation from ideality is provided. This allowance may be statistically predicted based on the coefficient of variation of the regularities; that is, the 95% confidence intervals are \( 0.94 < y + d < 1.06 \) and \( 0.93 < \eta + \varepsilon < 1.07 \). This method of analysis will not only allow the accumulation of good data in the literature but it will allow a better understanding of microbial physiology.

The method of material and energy balance can be used in investigating the efficiency of oxidative phosphorylation in microorganisms. The knowledge of this efficiency permits the theoretical prediction of the energetic yield \( \eta \) for microbial growth.

Usually data collected during fermentation processes include oxygen consumption \( Q_{O_2} \), biomass concentration \( X \), substrate concentration \( S \), product concentration \( P \), carbon dioxide evolution \( Q_{CO_2} \) and occasionally nitrogen consumption rate \( Q_{N_2} \).

From these measured quantities, values of \( \eta \), \( \varepsilon \), \( \xi_p \), \( y_c \), \( z \) and \( d \) can be calculated for both batch and continuous processes using equations already developed by Erickson et al. [3,4,5,6].

The data of S. Nagai et al. [7] was evaluated using the equations below.

\[
\eta = \frac{\sigma_b y_b}{\eta_s y_s (S_o - S_f)}
\]  

(11)

\[
\varepsilon = \frac{480 Q_{O_2} X}{D_0 y_s (S_o - S_f)}
\]  

(12)

\[
y_c = \frac{\sigma_b X}{\sigma_s (S_o - S_f)}
\]  

(13)

\[
d = \frac{120 Q_{CO_2} X}{D_0 (S_o - S_f)}
\]  

(14)

The paper by S. Nagai et al. [7] contains experimental results from glucose limited chemostat culture of Trichoderma sp. The result of the consistency analysis is shown in Table 1. The analysis shows that at low dilution rates \( \eta + \varepsilon < 1 \) and \( y_c + d < 1 \). This signifies either the presence of experimental error or product formation. In this case the author reported the presence of amylase and cellulase enzymes. Also as the dilution rate increased, the values of \( \eta + \varepsilon \) and \( y_c + d \) showed a general upward trend.
This is in accordance with product formation results since higher dilution rates decreased the amount of amylase and cellulase enzymes produced. At high dilution rates, \( D > .092 \), the values of \( (\eta + \varepsilon) \) and \( (y_c + d) \) were both greater than unity. The only explanation for this is the presence of experimental errors. The most likely sources of error which could lead to both \( y_c + d > 1 \) and \( \eta + \varepsilon > 1 \) are inaccurate biomass concentration and/or substrate concentration measurements. The values of \( (\eta + \varepsilon) \) and \( (y_c + d) \) showed a parallel rise until the highest dilution rate \( D = .142 \) was reached.

To illustrate further the application of material and energy balances in data analysis, the data of L. P. Hadjpetrous et al. [8] was analyzed using the following equations

\[
\eta = \frac{\sigma_b Y_b}{\sigma_s Y_s} Y_s \tag{15}
\]

\[
\varepsilon = \frac{3Y_s}{2\sigma_s Y_s Y_c} \tag{16}
\]

The data consisted of molar growth yields and oxygen consumption during aerobic growth for Aerobacter aerogenes on several natural products. The consistency analysis result is shown in Table II. For the growth of Aerobacter aerogenes on mannitol, \( \eta + \varepsilon = .992 \) and for citrate \( \eta + \varepsilon = .950 \). These two results are consistent with the 95% confidence interval. For all other cases \( (\eta + \varepsilon) \) is less than unity. The situation can be accounted for by either the presence of extracellular products or experimental errors. However the authors indicated the accumulation of acetate during most of their experimental work but the amounts were not specified.

L. P. Hadjpetrous [8] reported a value of \( Y_{ATP} = 10.2 \) which is in agreement with values of \( 8 \leq Y_{ATP} \leq 13 \) which have been found for other microorganisms under anaerobic conditions. The efficiency of oxidative phosphorylation was calculated for the growth of Aerobacter aerogenes on glucose, fructose, maltose, sucrose, galactose and mannose. For this analysis, it was assumed that acetate was the extracellular product formed and that for all of these substrates, up to 38 mole of ATP could be produced per mole of the monomer. Also a maximum value of 3.0 was assumed for P/O ratio. Two moles of ATP were assumed to be formed for each mole of acetate produced.

Illustrative calculation: The organic substrate is glucose; \( \eta = .500 \), \( \varepsilon = .190 \), and \( \xi_p = 1 - \eta - \varepsilon = .310 \). The basis is 1 equivalent of available electrons of organic substrate. Thus, \( \eta = 0.500 \) equivalents of available electrons incorporated into biomass, \( \varepsilon = 0.190 \) equivalents of available electrons transferred to oxygen, and \( \xi = 0.310 \) equivalents of available electrons is in acetate. Maximum moles of ATP that can be obtained by oxidative phosphorylation \( A_\text{max} = \varepsilon \times 3/2 = .190 \times 3/2 = .285 \). Moles of ATP generated with acetate \( A = \xi_p \times .25 = .0775 \). Moles of ATP used in forming biomass \( A_b = 12\eta / (\sigma_c Y_c Y_{ATP}) = .030 \). Moles of ATP from glycolysis \( A_g = c/12 = .016 \). Moles of ATP actually obtained by oxidative phosphorylation \( A_\text{max} = A_o = A_b - A_g = .30 - .016 - .0775 = .1955 \). Efficiency of oxidative phosphorylation \( \phi = A_o / A_{\text{max}} = .1955 / .285 = .725 \). Therefore the estimated efficiency of oxidative phosphorylation with respect to the
maximum efficiency in the mitochondria is 72.5% for the growth of Aerobacter aerogenes on glucose. The result of this analysis for the substrates considered is shown in Table 3.

From theoretical calculations Stouthamer [10] found that \( Y_{\text{ATP}}^{\text{max}} = 28.8 \) for the growth of microorganisms with glucose and inorganic salts. When 38 moles of ATP are generated from 1 mole of glucose, the energy required to generate 1 mole of high energy ATP bonds from ADP is approximately 24 Qo/38. Thus, the maximum theoretical efficiency of biomass production under aerobic conditions is [6].

\[
\eta_{\text{max}} = \frac{\sigma_{b} Y_{b} Q_{o} Y_{\text{ATP}}^{\text{max}}}{12} \frac{Q_{o}}{Y_{\text{ATP}}^{\text{max}}} + \frac{24Q_{o}}{38}
\]

We obtain a value of \( \eta_{\text{max}} = 0.88 \) for \( Y_{\text{ATP}}^{\text{max}} = 28.8 \). However experimental findings indicate that the value of \( Y_{\text{ATP}}^{\text{max}} \) frequently lies between 8 and 13. This range of values of \( Y_{\text{ATP}}^{\text{max}} \) yields \( 0.68 < \eta_{\text{max}} < 0.77 \) for 38 moles of ATP/mole of glucose. From Table 3 using the data of Hadjipetrou et al. [8] the average value of the efficiency of oxidative phosphorylation for Aerobacter aerogenes is 0.661 or 23.8 moles ATP generated during oxidative phosphorylation/mole of monomer. Using 25.8 moles ATP/mole monomer and \( Y_{\text{ATP}} = 10.2 \) a value of \( \eta = 0.64 \) is obtained for the growth of Aerobacter aerogenes on the common carbohydrates.

A project involving utilization of grain dust for single cell protein production is currently in progress in our laboratory. In this project, Candida utilis is grown on the glucose obtained by enzymatic hydrolysis of the starch in the dust. The types of grain dust to be evaluated are corn dust, wheat dust and milo dust. So far however, only corn dust has been extensively utilized for the growth of Candida utilis in shake flask experiments. The yields from such experiments are reported in Table 4.

By elemental analysis, this strain of Candida utilis was found to contain 45.6% carbon (i.e., \( \sigma = 0.456 \)). Since the exact composition of the organism is not known, \( Y_{b} = 4.291 \) is assumed and \( Y_{\text{ATP}} = 10.2 \) is also assumed. Using 25.8 moles ATP/mole of glucose, the energetic yield obtained for the growth of Candida utilis on glucose is calculated to be \( \eta = 0.64 \).

The experimental results on the growth of Candida utilis in shake flasks gave an average growth yield \( Y_{S} = 0.50 \) which is comparable to the value \( Y_{S} = 0.455 \) obtained by Paredes-lopez et al. [9] when Candida utilis was grown on juice obtained from Nopa Fruit. Using equation (16) the energetic yield for the growth of Candida utilis on glucose in shake flask experiments is found to be \( \eta = 0.611 \). This value is quite similar to the value of \( \eta \) calculated above. Thus, the combined efficiency of oxidative phosphorylation and ATP utilization in Candida utilis is similar to that for Aerobacter aerogenes when both are grown on glucose and mineral salts.
CONCLUSION

The results of the oxidative phosphorylation efficiency analysis using the results of L. P. Hadjipetrou [8] show that this microorganism appears to obtain less than 38 moles of utilizable ATP from 1 mole of glucose. This inefficiency in ATP generation and utilization leads to $\eta < 0.88$ in real situations.

The sources of error involved in taking data during microbial growth processes are numerous. More disturbing still is the nature of the data which fails to lend itself to accuracy judgment just by the conventional statistical approach. There is therefore a need for a method for checking the consistency of data collected. The method of material and energy balances illustrated in this paper is a straightforward and quick way to do this. The employment of this method will ultimately eliminate or reduce the accumulation of inaccurate data in the literature.

This method of material and energy balances was applied to the data collected during the growth of Candida utilis; however, carbon dioxide production and oxygen consumption data need to be available to fully use the balances presented in this paper.

REFERENCES

NOMENCLATURE

\( A_{\text{max}} \) = Maximum moles of ATP that can be obtained by oxidative phosphorylation

\( A_P \) = Moles of ATP generated with product

\( A_b \) = Moles of ATP used in form biomass

\( A_g \) = Moles of ATP produced from glycolysis

\( A_o \) = Actual mole of ATP obtained from oxidative phosphorylation

\( a \) = Moles of ammonia per quantity of organic substrate containing one atom carbon, (g mole/g atom carbon)

\( b \) = Moles of oxygen per quantity of organic substrate containing one g-atom carbon, (g mole/ g atom carbon)

\( c \) = Moles of water per quantity of organic substrate containing one g atom carbon (g mole/ g atom carbon)

\( D \) = Dilution rate (hr\(^{-1}\))

\( d \) = Moles of carbon dioxide per quantity of organic substrate containing one g atom carbon (g mole/ g atom carbon)

\( \ell \) = Atomic ratio of oxygen to carbon in organic substrate (dimensionless)

\( m \) = Atomic ratio of hydrogen to carbon in organic substrate (dimensionless)

\( n \) = Atomic ratio of oxygen to carbon in biomass (dimensionless)

\( p \) = Atomic ratio of hydrogen to carbon in biomass (dimensionless)

\( Q_o \) = Heat evolution in fermentation per equivalent of oxygen uptake kcal/g equiv.

\( Q_{\text{CO}_2} \) = Rate of evolution of carbon dioxide (g moles/ g dry wt (hr))

\( Q_{\text{O}_2} \) = Rate of consumption of oxygen (g moles/ g dry wt (hr))

\( Q_{\text{N}_2} \) = Rate of consumption of nitrogen (g mole/ g dry wt (hr))

\( q \) = Atomic ratio of nitrogen to carbon in biomass (dimensionless)

\( r \) = Atomic ratio of hydrogen to carbon in products (dimensionless)

\( S \) = Organic substrate concentration (g/litre)

\( S_f \) = Final organic substrate concentration (g/litre)
\( S_0 \) = Initial substrate concentration (g/litre)
\( s \) = Atomic ratio of oxygen to carbon in products (dimensionless)
\( t \) = Atomic ratio of nitrogen to carbon in products, (dimensionless)
\( X \) = Biomass concentration (g/litre)
\( Y_{s} \) = Biomass yield on organic substrate, (g dry wt/ g substrate)
\( Y_{o} \) = Biomass yield based on oxygen (g dry wt/ g mole \( O_2 \))
\( Y_{ATP} \) = Biomass yield based on ATP (g dry wt/ g mole ATP)
\( Y_{\text{max}}_{ATP} \) = Maximum biomass yield based on ATP (g dry wt/ g mole ATP)
\( Y_{c} \) = Biomass carbon yield (fraction of organic substrate carbon in biomass (dimensionless)
\( z \) = Fraction of organic substrate carbon in products (dimensionless)
\( \gamma_{b} \) = Reductance degree of biomass, equivalents of available electrons per biomass containing 1 g atom carbon
\( \gamma_{p} \) = Reductance degree of products
\( \gamma_{s} \) = Reductance degree of organic substrate
\( \xi \) = Fraction of energy in organic substrate which is evolved as heat (dimensionless)
\( \eta \) = Fraction energy in organic substrate which is converted biomass or biomass energetic yield (dimensionless)
\( \eta_{\text{max}} \) = Maximum biomass energetic yield (dimensionless)
\( \xi_{p} \) = Fraction of energy in organic substrate which is converted to products (dimensionless)
\( \sigma_{b} \) = Weight fraction carbon in biomass (dimensionless)
\( \sigma_{s} \) = Weight fraction carbon in organic substrate (dimensionless)

ACKNOWLEDGMENT

This work was partly supported by the USDA Grain Marketing Research Laboratory.
Table 1. Results of consistency analysis on the data of S. Nagai et al. [7].

<table>
<thead>
<tr>
<th>D (hr⁻¹)</th>
<th>𝑛</th>
<th>𝜀</th>
<th>𝑛 + 𝜀</th>
<th>𝑦_c</th>
<th>d</th>
<th>𝑦_c + d</th>
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<td>0.383</td>
<td>0.408</td>
<td>0.791</td>
</tr>
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Table 2. Biomass energetic yield, $\eta$ and fraction of energy evolved as heat, $\varepsilon$, for the data of L. P. Hadjipetrous et al. [8].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\eta$</th>
<th>$\varepsilon$</th>
<th>$\eta + \varepsilon$</th>
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<td>Maltose</td>
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<td>.7258</td>
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<td>.2233</td>
<td>.8172</td>
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<td>D-Mannitol</td>
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<td>.9919</td>
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<tr>
<td>D-Sorbitol</td>
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<td>.2762</td>
<td>.8849</td>
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<tr>
<td>L-Rhamnose</td>
<td>.3111</td>
<td>.1912</td>
<td>.5023</td>
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<td>D-Glucose</td>
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Table 2 Continued

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<td>Pyruvate</td>
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<tr>
<td>Acetate</td>
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<td>.5250</td>
<td>.7420</td>
</tr>
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Table 3. Calculated and reported efficiency of oxidative phosphorylation of aerobacter aerogenes relative to mitochondrial efficiency from the data of J. P. Hadjipetrous et al. [8].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\eta$</th>
<th>$\epsilon$</th>
<th>Calculate Efficiency</th>
<th>Reported Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose*</td>
<td>.5004</td>
<td>.1899</td>
<td>.725</td>
<td>1.033</td>
</tr>
<tr>
<td>D-Fructose*</td>
<td>.5239</td>
<td>.2233</td>
<td>.683</td>
<td>.933</td>
</tr>
<tr>
<td>Maltose*</td>
<td>.5131</td>
<td>.2127</td>
<td>.724</td>
<td>.967</td>
</tr>
<tr>
<td>Sucrose*</td>
<td>.5939</td>
<td>.2233</td>
<td>.864</td>
<td>1.067</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>.5059</td>
<td>.3224</td>
<td>.495</td>
<td>.633</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>.4777</td>
<td>.2951</td>
<td>.476</td>
<td>.633</td>
</tr>
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</table>
Table 4. Growth yield, \( Y_s \), for the growth of *Candida utilis* on glucose obtained by enzymatic hydrolysis of starch in corn dust.

<table>
<thead>
<tr>
<th>TRIAL</th>
<th>( Y_s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.4570</td>
</tr>
<tr>
<td>2</td>
<td>.4503</td>
</tr>
<tr>
<td>3</td>
<td>.4827</td>
</tr>
<tr>
<td>4</td>
<td>.4826</td>
</tr>
<tr>
<td>5</td>
<td>.5235</td>
</tr>
<tr>
<td>6</td>
<td>.5010</td>
</tr>
<tr>
<td>7</td>
<td>.5110</td>
</tr>
<tr>
<td>8</td>
<td>.5000</td>
</tr>
<tr>
<td>9</td>
<td>.5239</td>
</tr>
<tr>
<td>10</td>
<td>.5139</td>
</tr>
<tr>
<td>11</td>
<td>.5285</td>
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</table>
INTRODUCTION

Enzyme regulation is the basis for metabolic control. Enzymatic activity is regulated by controlling its catalytic mechanism and by adjusting the intracellular concentrations of the enzymes. The enzymes of the cell's primary metabolism, the constitutive enzymes, are generally found in constant concentrations. However, they may exist as active or inactive forms, such as the intracconversion of phosphorylase a to phosphorylase b in the metabolism of starch or glycogen. Each enzyme of a metabolic sequence may be subject to product inhibition and/or cofactor activation. Allosteric enzymes, frequently found at the branch points of metabolic pathways, are regulated by feedback inhibition. There is evidence that the adenine nucleotides, as common intermediates to the anabolic and catabolic pathways, may take part in an overall control to the cell's metabolism.

The regulation of the cell's secondary metabolism, which includes those processes not strictly necessary for survival, or which serve to adapt the cell to a changing environment, is less clear. Production of antibiotics is effected by substrate induction—the forcing of the cell to produce a certain product by the presence of its precursor—and by feedback regulation. The production of extracellular enzymes is regulated at the level of the gene. There are three primary mechanisms controlling their synthesis which will be discussed in detail in the following sections.

REGULATION OF PROTEIN SYNTHESIS

The regulation of enzyme synthesis seems to occur mainly at the level of mRNA transcription. However, there is some evidence of translational control. For example, the translation of the lac operon mRNA, the three enzymes coded therein are produced in ratios of 1:1/2:1/5, rather than in equimolar amounts as would be expected. However, this is of minor importance in regards to the extracellular enzymes. These are subject to control by environmental factors. This control may take the form of induction, repression, or catabolite repression.

Induction

Induction occurs when a food source enters the environment of the cell and forces the cell to produce enzymes to metabolize this substance. The best known example of this is the lac operon of E. coli, an inducible enzyme system though not an extracellular one. Little expression of the lac operon is found when E. coli is grown on a carbon source other than lactose. If these cells are then placed in a medium containing lactose, great amounts of β-galactosidase activity will be found within minutes along with the other enzymes that are
coinduced. From a great deal of genetic studies, Jacob and Monod prepared the model shown in Figure 1. The i locus produces a constitutive protein—the repressor molecule. In the absence of inducer molecules, it binds to the o locus, the operator gene, preventing the transcription of the z,y,a genes, which is the operon. Further studies identified the p locus, the promoter gene, as the site at which the RNA polymerase binds. Thus the repressor forms an actual physical block to prevent transcription. When inducer is present in the cell, it binds to the repressor forming an inert complex unable to bind to the DNA.

Repression

The mechanism of repression is similar to that of induction, as seen in Figure 2, though essentially of opposite nature. As an example, the his operon of E. coli codes for the enzymes necessary to synthesize histidine. If the bacteria is in a medium containing ammonia as the only nitrogen source, this operon will be fully expressed. If histidine is added to the culture, the entire sequence of enzymes for histidine synthesis from ammonia will rapidly disappear. This is known as coordinate, or end-product, repression. In this case the repressor molecule produced by the regulatory gene i is inactive in the absence of the corepressor histidine. When histidine binds to the repressor molecule, an active repressor-corepressor complex is formed which binds to the DNA at the operator gene preventing transcription of the operon.

The first quantification of the Jacob-Monod model was presented by Yagil and Yagil. These authors based their quantification on the three equilibrium relations implicit in the model,

\[ nE \rightleftharpoons k_1 R \rightarrow RE_n \]  
\[ O \rightleftharpoons k_2 R \rightarrow OR \]  
\[ O \rightleftharpoons k_4 RE_n \rightarrow ORE_n \]

Equations (1) + (2) hold for induction and equations (1) + (3) hold for repression. From a mass balance

\[ [O_t] = [O] + [OR] \]  
\[ [R_t] = [R] + [RE_n] \]

where [OR], the number of repressor molecules bound to the operator, is assumed negligible compared to the total amount of repressor molecules. There are a maximum of four operators in a cell which validates the assumption.

Defining

\[ a = \frac{[O]}{[O_t]} \quad , \quad 1-a = \frac{[OR]}{[O_t]} \]

where a is the ratio of free operators to the total number of operators, equations (1), (2), (4), (5), and (6) lead to the following expression for induction:

\[ \frac{\alpha}{1-\alpha} = \frac{k_2}{k_1[R_t]} + \frac{k_n}{[E]} + \frac{k_2}{[R_t]} \]
Equation (7) gives the ratio of free operators to bound operators. Letting $[E] \rightarrow 0$, so induction is minimal, we have

$$\frac{\alpha}{1-\alpha} = \frac{k_2}{[R_t]} = a_B,$$

(8)

where $a_B$ is the number of free operators at the basal level of enzyme synthesis. Rewriting equation (7) and linearizing leads to

$$\ln \left( \frac{\alpha}{1-\alpha} - a_B \right) = n \ln [E] + \ln \frac{a_B}{k_1},$$

(9)

which enables to determine $n$. Values of $\alpha$ may be measured as the ratio

$$\frac{(\Delta A/\Delta B)}{(\Delta A/\Delta B)}_{\text{max}}$$

where $\Delta A/\Delta B$ is the change in enzyme activity relative to the change in cell numbers and $(\Delta A/\Delta B)$ is the same ratio at full induction.

Equations (1), (3), (4), (5), and (6) lead to the following expression for repression:

$$\ln \left( \frac{\alpha}{1-\alpha} - a_B \right) = -n \ln [E] + \ln \left[ \frac{a_B}{k_1} \right]$$

(10)

where $a_B = k_4/[R_t]$ and is the basal rate of enzyme synthesis at full repression as $[E] \rightarrow \infty$.

This derivation has five assumptions: 1. all systems are at equilibrium so that rate factors do not play a role, 2. all repressor molecules are saturated with the effector molecules, 3. $[E] > [R] > [O]$, 4. the rate of enzyme synthesis is proportional to the number of free operators, and 5. intracellular concentration of effector is related to the external concentration in some known way.

Toda prepared a dual control model summarized in Figure 3. Although his suggestion of $Q = Q_1 - Q_2$, where $Q$ is the ratio of enzyme activity to its theoretical maximum, $Q_1$ is the number of operator gene 1 free from repressor 1, and $Q_2$ is the number of $O_2$ free from $S^2 R_2$, was a valuable one, there is no biochemical evidence for the existence of two operator sites.

**Catabolite Repression**

When *E. coli* is grown on a medium containing glucose and lactose, it will preferentially use the glucose until it is exhausted, and, after a short lag period, continue growing on lactose. This is known as diauxic growth and is due to catabolite repression. Catabolite repression is the repression of enzymes used in secondary catabolism by the presence of glucose in the culture medium.
Glucose may repress not only enzymes normally thought of as inducible, such as those of the lac operon, but also those normally thought of as constitutive, such as those of the electron transport system. Thus, when glucose is present as a fuel, bacteria prefer to use the most primitive catabolic pathway.

In 1968 both Pastan and Perlman, and Ullman and Monod noticed that exogenous c-AMP partially overcame catabolite repression of the lac operon in E. coli. Since then many other systems have been discovered in bacteria that are under the control of c-AMP. It seems a safe generalization to say that c-AMP in prokaryotes stimulates the synthesis of proteins which are not required under all conditions of growth, and that its role is clearly regulatory but not essential.

Cyclic AMP effects this regulation at the level of transcription. Though termed catabolite repression, it is really a form of positive control, in that the higher the concentration of c-AMP the greater the rate of mRNA transcription. For c-AMP to have an effect a protein known as catabolite gene activator protein, CAP, or c-AMP receptor protein, CRP, must be present in the cell. In E. coli, c-AMP and CAP form a bi-molecular complex which then interacts with the DNA at the promoter site. This causes the DNA to be destabilized to its open form. This may effect transcription in two ways - 1. it may effect the binding of the RNA polymerase to the DNA, with the open form being more favorable to the binding of the polymerase, or, 2. it may cause an inert form of RNA polymerase bound to DNA to shift to an open form and begin transcription. Either type of control is an equilibrium reaction and has not been distinguished to the author's knowledge.

An overall picture of this model is shown in Figure 4, including induction, and is modified from Aiba. This may be expressed mathematically, after Aiba, as $Q = Q_1 \cdot Q_2$ where $Q$ is the same as in Toda's formulation, and $Q_1$ is the fraction of free operator gene and $Q_2$ is now the fraction of the promoter gene bound to CAP·cAMP.

There is no conclusive evidence as to the mechanism of regulation of intracellular levels of c-AMP. Adenylate cyclase forms c-AMP from ATP. Glucose does not seem to inhibit this enzyme in vitro, however the enzyme was disassociated from the membrane with which it is found in the cell. Evidence has been presented which shows glucose to inhibit adenylate cyclase in vitro. It is well known that addition of glucose to a culture growing on another carbon source causes a rapid drop in intracellular c-AMP with a concurrent increase in c-AMP in the medium. Prolonged incubation with glucose causes no further increase in c-AMP in the medium. It has been argued and evidence presented that this secretion is the primary mode of c-AMP concentration regulation. Another enzyme found in E. coli concerned with c-AMP is c-AMP phosphodiesterase. It converts c-AMP to 5'-AMP. Strains of E. coli defective in this enzyme show more resistance to catabolite repression than the wild type, and have consistently higher levels of c-AMP. However, levels of c-AMP are still dependent upon the carbon source utilized.

CONCLUSION

Fermentation processes producing extracellular enzymes are subject to control by induction, repression, and catabolite repression. The mathematical correlations developed for these biochemical processes may be incorporated into overall mathematical models which predict the relation between cell growth and enzyme production.
for various reactor designs. A recent paper (15) utilized the qualitative schematic shown in Figure 5. to develop simple differential equations to model the influences of catabolite repression and induction on enzyme production. Figure 5. depicts pictorially most of what was discussed in previous sections. Other recent papers (16,17) have been devoted to developing mathematical models of similar biochemical processes.

Mathematical models are useful for both understanding the processes they relate to, and also as aids in designing optimization and control strategies. Mini-computers have found wide usage in the chemical process industries, especially the petrochemical industry. The use of computer control in the fermentation industries depends upon the development of simple mathematical models that quantitatively describe the behavior of the biochemical systems in relation to their environment. In light of this need, in our research we will be growing Trichoderma reesei on various soluble substrates in both batch and continuous culture. It is hoped that by measuring intracellular concentrations of c-AMP and ATP in relation to dilution rate, cell mass, cellulase system enzyme activity, and other growth parameters, a simple mathematical model, amenable to the use of on-line computer control in an industrial fermentation, may be developed.

NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-AMP</td>
<td>adenosine 3', 5' - cyclic phosphate</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>adenosine 5' - monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ΔA/ΔB</td>
<td>change in enzyme activity relative to the change in cell numbers</td>
</tr>
<tr>
<td>CAP, CRP</td>
<td>catabolite activator protein, c-AMP receptor protein</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid - chromosomal material</td>
</tr>
<tr>
<td>E,S₁,S₂</td>
<td>effector molecules; inducer, or corepressor</td>
</tr>
<tr>
<td>i</td>
<td>inhibitory gene, codes for repressor protein</td>
</tr>
<tr>
<td>k₁</td>
<td>equilibrium constants</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>n</td>
<td>number of effector molecules</td>
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<td>o</td>
<td>operator gene</td>
</tr>
<tr>
<td>O</td>
<td>operators</td>
</tr>
<tr>
<td>p</td>
<td>promoter gene</td>
</tr>
<tr>
<td>Q</td>
<td>(ΔA) / (ΔA) max, ratio of enzyme activity to its theoretical maximum</td>
</tr>
<tr>
<td>Q₁</td>
<td>fraction of free operator gene</td>
</tr>
<tr>
<td>Q₂</td>
<td>fraction of promotor gene bound to CAP·c-AMP</td>
</tr>
<tr>
<td>R</td>
<td>repressor protein</td>
</tr>
<tr>
<td>t,u,v,y,z,a</td>
<td>genes</td>
</tr>
<tr>
<td>a</td>
<td>ratio of free operators to the total number of operators</td>
</tr>
<tr>
<td>αᵦ</td>
<td>number of free operators at basal enzyme synthesis</td>
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BIBLIOGRAPHY


Fig. 1. Jacob - Monod Induction

Fig. 2. Jacob - Monod Repression
\[ Q = Q_1 \times Q_2 \]
\[ Q_1 = \frac{[O_1]}{[O_1]_t} \]
\[ Q_2 = \frac{[O_2]}{[O_2]_t} \]

Fig. 3. Toda's Dual Control Model, after (6)
Fig. 4. Model of Catabolite Repression, after (12)
Fig. 5. Schematic of induction and catabolite repression modified, after (15)
THE USE OF CELLULOSE STRUCTURAL PARAMETERS TO EVALUATE PRETREATMENTS FOR ENZYMATIC HYDROLYSIS

David H. Beardmore, Yong-Hyun Lee, and L. T. Fan

INTRODUCTION

Enzymatic hydrolysis is a promising means of conversion of waste cellulosics to glucose, from which ethanol is produced by fermentation. However, the hydrolysis of cellulose is slow and difficult because of the structural complexity of the cellulose. The susceptibility of cellulose to enzymatic hydrolysis is affected mainly by the structural features of cellulosic materials. The major structural features of cellulosic materials which affect enzymatic hydrolysis are well summarized in the works of Fan et al.\textsuperscript{1} and Cowling and Kirk.\textsuperscript{2} Their works show that the important structural features include (1) degree of water swelling, (2) crystallinity, (3) molecular arrangement, (4) content of associated material, such as lignin, and (5) capillary structure of fibers. However, the crystallinity and the surface area of cellulose fibers may be the most important structural features. In our previous works,\textsuperscript{3,4} we investigated in depth the effects of these two structural features upon the enzymatic hydrolysis of cellulose and determined their relative importance.

To increase the susceptibility of cellulosic material, structural modification by means of various pretreatment schemes is essential. The purpose of this work was to use cellulose structural parameters to evaluate the effectiveness of pretreatment techniques for enzymatic hydrolysis. Six different cellulose pretreatment techniques, three physical and three chemical, were examined. These techniques include ball milling, gamma radiation, low temperature (170°C) pyrolysis, soaking in 1% NaOH, dissolving in CMCS solvent, and dissolving in concentrated H\textsubscript{2}SO\textsubscript{4}. The cellulose samples were pretreated and dried by applying a solvent drying technique. The technique was applied to preserve to the greatest extent possible the water swollen cellulose structure.\textsuperscript{3,4} Two structural parameters, crystallinity and specific surface area, were measured. In addition, the pretreated cellulose was hydrolyzed with the culture broth from \textit{Trichoderma reesei} QM 9414. The extent of hydrolysis was determined from reducing sugar production data. The effects of each treatment technique on the cellulose structure and the hydrolysis rate were established. The relationship between the structural parameters and the hydrolysis was also examined. The magnitude and the mechanism of the effects of each pretreatment were evaluated to give added insight to the development of a practical pretreatment scheme. Knowledge of effects of the structural parameters on the hydrolysis rate adds to the understanding of the mechanism of the complex enzyme reaction.

MATERIALS AND METHODS

\textbf{Substrate.} Solka Floc SW40 (Brown Co.), a hammer-milled sulfite pulp, was the principal pure cellulose substrate used in this work. The cylindrical
fibers were typically 20 μm in diameter and 100-140 μm in length. The Solka Floc was sieved and the fibers passing 270 mesh and not passing 400 mesh were designated the standard substrate.

Cellulase. The source of the enzyme used was the culture filtrate from batch fermentations of Solka Floc by the fungus *Trichoderma reesei* (viride) QM 9414, which was supplied by the U.S. Army Natick Research and Development Command. The medium composition for shaker flask cultivation was similar to that used by Mandels et al. The soluble protein content of the enzyme broth ranged from 2.2 to 2.5 mg/ml by the Lowry method. The filter paper activity was between 1.3 and 1.5 IU/ml.

Pretreatments. The ball milling method consisted of a 5 % porcelain jar mill with a 50 volume percent charge of 1 inch diameter porcelain spheres. The jar was rotated at about 52 rpm for various time periods. The dry substrate was added in an amount that filled only the void volume of the balls.

The gamma radiation treatment was accomplished by two methods. In the first, the cellulose was sealed in sample jars in the presence of air. The jars were placed in a Co⁶⁰ gamma-cell and irradiated at the rate of 800 rad/min until various total dosages were reached. The second method was employed for higher dosages. The cellulose was packed in polyethylene sample tubes that were dropped to the core of an operating Triga Mark II research nuclear reactor. A small amount of neutron radiation was absorbed by these samples.

The pyrolysis treatment was performed at 170°C for 24 hrs. The cellulose was contained in a glass jar in the presence of either air or helium during the pyrolysis.

The NaOH treatment was performed at a concentration of 1% (0.25 N) NaOH. One liter of the NaOH solution was mixed with 50g of cellulose. One half of the mixture was allowed to stand for 30 minutes at room temperature and the other half was autoclaved for 20 minutes at 256°F and 17 psig. In both cases, the treated cellulose was washed until the wash water was neutral.

CMCS is made by dissolving 20g of sodium tartrate, 15.5g of ferric chloride, and 14.5g of sodium sulfite in 1000g of a 5% sodium hydroxide solution. Twenty grams of the substrate were dissolved in 500g of the CMCS solvent and the mixture was allowed to stand for 6 hours. The dissolved cellulose was reprecipitated on the addition of water and the residue was washed until the wash water was neutral.

A 60% H₂SO₄ solution was prepared and allowed to come to room temperature. Twenty grams of substrate were dissolved in the solution and allowed to stand for 15 minutes. Acetone was added until the reprecipitation of the cellulose was complete. The precipitate was filtered and washed with acetone and then water until the wash water was neutral.

Solvent drying. Water-swollen cellulose was placed in an extraction thimble and mounted in a Soxhlet extraction apparatus. First, 400 ml of methanol was allowed to cycle for 3 to 4 hours. The cycled methanol was replaced with 400 ml of fresh methanol and the extractor was again cycled for 3 to 4 hours. The methanol was then replaced with 400 ml of benzene
which was cycled and replaced with a final 400 ml charge of benzene. The
two methanol and two benzene extractions ensured that the water was completely
removed from the cellulose. The benzene was removed from the sample by air
drying at 80°C overnight.

Parameter measurement. The crystallinity was measured employing the
powder method of x-ray diffraction using a Norelco Diffractometer. The
specimen was mounted horizontally while the Geiger counter moved in a vertical
arc. A CuKα target with a nickel filter was used. The substrate sample was
dried overnight at 80°C and stored in a desiccator. The specimens were
prepared by the method of McCreeery. The samples were scanned for values
of 2θ ranging from 30° to 10°. The crystallinity index proposed by Segal et
al.8 was employed in this work:

\[ \text{CrI} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \]

where \( I_{002} \) is the intensity of the 002 peak (at about \( 2\theta = 22° \)), and \( I_{am} \) is
the intensity at \( 2\theta = 18° \). The \( I_{002} \) peak corresponds to the crystalline
fraction and the \( I_{am} \) intensity corresponds to the amorphous fraction. The
intensities were measured above an approximate baseline representing back­
ground intensity.

The specific surface area of each sample was measured using a Perkin­
Elmer Sorptometer and applying the BET equation. The sample, 0.2 to 0.7 grams,
was placed in a U-tube and degassed by passing helium at 80°C slowly through
the tube. The U-tube containing the sample was attached to the sorptometer
and a nitrogen-helium mixture was passed over the sample. The sample tube
was then submerged in liquid N₂ (77°K) and the nitrogen in the gas stream
was adsorbed on the surface of the cellulose sample. The desorption was
effected by submerging the sample tube in room temperature water. The
changing concentration of nitrogen in the gas stream causes a change in the
conductance of the gas which is recorded on a strip-chart recorder as a
peak. The area of the desorption peaks was measured and compared to a peak
area measured from the release of a known volume of nitrogen. This compari­
on ratio was used in the calculations. Desorptions at three different nitro­
gen partial pressures were performed in order to plot a three-point BET
graph. The slope and intercept were used to calculate the specific surface
area.

Enzyme hydrolysis. The hydrolysis was carried out using the T. reesei QM 9414
culture filtrate. Two 250 ml flasks were used for each different substrate. The
substrate samples were dried overnight at 80°C and 5 grams were weighed into
each flask. Forty ml of H₂O, 5 ml of 1.0 citrate buffer and 50 ml of enzyme
filtrate, all at 50°C, were added to each flask to give a 5% substrate solu­
tion. The stoppered flasks were placed in a 50°C incubator shaker set at
250 rpm. Two ml samples were taken from each flask and combined to make
one 4 ml sample for each different substrate. These samples were taken
at 2, 4, 8, 12, 24, 48, 72 and 96 hours. The samples were placed in a
boiling water bath for 5 minutes to denature the enzyme. The samples were
then centrifuged and the supernatant refrigerated. The DNS method9 was
performed on the samples to measure the reducing sugar concentration.
RESULTS AND DISCUSSION

Effects of Pretreatments on Structural Parameters and Hydrolysis Rate.

Ball milling. Microscopic observation revealed considerable particle size reduction, but there was some evidence of agglomeration or fusion. Figure 1 shows the dramatic reduction in crystallinity caused by the shearing effect of the ball mill. Figure 2 shows the effect of ball milling on the specific surface area. The low surface areas of the solvent dried samples were not expected but may be due to recrystallization or fusion. The hydrolysis rate is increased with ball milling time as Fig. 3 indicates. Although ball milling requires a great deal of energy, it appears to be a simple, clean, effective method of cellulose pretreatment.

Gamma radiation. High dosages of gamma radiation produced a yellowish color and a sugary smell indicating depolymerization in the cellulose. Figure 4 shows that crystallinity is not greatly affected by radiation until very high dosages. The specific surface area of the cellulose increases with increasing dosage as shown in Fig. 5. The relative hydrolysis rate is substantially increased with a high dosage of gamma radiation as depicted in Fig. 6. If high dose rates of gamma radiation are readily available, this pretreatment shows great potential.

Pyrolysis. The air-pyrolyzed sample was dark brown and had a very distinctive sweet odor while the helium-pyrolyzed cellulose was only light brown and possessed only a faint sweet smell. Table 1 lists the specific surface areas of the air- and helium-pyrolyzed cellulose as 2.07 and 3.61 m²/g, respectively. These values are lower than the 8.18 m²/g listed for the standard substrate. The heating apparently reduces surface area and the presence of oxygen may enhance the effect. The crystallinity was slightly decreased from 77.4 for the standard substrate to 74.9 and 74.6 for the air- and helium-pyrolyzed samples, respectively. The hydrolysis rate was greatly improved for this pretreatment as shown in Table 1. The 1.25 relative hydrolysis rate for air-pyrolyzed cellulose is low compared to the 2.18 relative hydrolysis rate for helium-pyrolyzed cellulose probably because of enzyme inhibiting by-products generated in the presence of oxygen. Further research on similar methods may increase the effectiveness of the treatment.

1% Sodium hydroxide. The swelling effect of the NaOH technique is evidenced by the increased surface areas of the two different NaOH-treated samples. The room temperature NaOH treatment raised the specific surface area from 8.18 to 12.18 m²/g, while the heated NaOH method raised the area to 23.16 m²/g. In both cases the crystallinity was only slightly decreased, from 77.4 to 75.5 and 75.9 for the room temperature and heated treatments, respectively. It appears that the heating of the mixture enhances the NaOH swelling effect as the relative hydrolysis rate is 1.45 compared to 1.37 for the room temperature treatment. Physical treatments are more effective on pure cellulose but the NaOH techniques have been shown to be highly effective on native cellulosic wastes.

CMCS. Dissolving the standard cellulose substrate in CMCS at room temperature increased the specific surface area of the cellulose to 27.40 m²/g.
The swelling was expected since the CMCS base is 5% NaOH. However, the crystallinity actually increased to 79.3. The relative hydrolysis rate of 1.44 was similar to those established for the dilute NaOH.

Concentrated H$_2$SO$_4$. The acid solvent treatment resulted in an increase in surface area to 29.21 m$^2$/g. The CRI slightly decreased to 74.3. The relative hydrolysis rate was 1.41 which adds to an interesting result. All of the chemical treatments generated hydrolysis rates between 1.37 and 1.45 times the untreated standard cellulose substrate.

Examining the entire spectrum of pretreatments reveals their magnitude of effect as well as their mode of action. Ball milling had the greatest impact on crystallinity while the 500 Mrad γ-radiation and the chemical treatments increased the specific surface area dramatically. The three most effective treatments were 500 Mrad γ-radiation, helium-pyrolysis, and 96 hour ball milling. It should be noted that the structural parameters of the pyrolyzed samples did not predict their high effectiveness. This suggests that another structural parameter can be important.

Effects of Structural Parameters on the Rate of Enzymatic Hydrolysis

The influence of crystallinity on hydrolysis rate is depicted in Fig. 7. Several points lie along the line representing the functionality for the majority of samples. Some of the points lie above the line due to the higher specific surface areas that they exhibit. Only the 100 Mrad and helium pyrolysis samples lie above the line and have small surface areas.

The influence of specific surface area on hydrolysis rate is shown in Fig. 8. All of the points lying along the line have a relatively constant CRI (70-79). The ball milled samples lie above the line because they possess low crystallinities. The same two samples, 100 Mrad and helium pyrolysis, lie above the line while their crystallinities are between 70 and 79.

CONCLUSIONS

It appears that different pretreatments have different effects on cellulose. In general, physical treatments inflict greater damage to the cellulose crystallinity and chemical treatments expose more surface area. Ball milling, helium pyrolysis, and γ-radiation are the most effective in increasing pure cellulose digestibility. The data largely support the premise that both specific surface area and crystallinity are important factors to hydrolysis rate. It is difficult to conclude that one parameter is more important than the other.

These results are important but further research is needed. Examining natural substrates and employing lignin concentration as another structural parameter may lead to the characterization of the hydrolysis potential of cellulosics through physical parameters alone. Refinement of the experimental techniques herein may clarify the results. Continued study of pretreatments and combinations thereof also deserves consideration in the search for a practical cellulose utilization process.
ACKNOWLEDGMENT

This work was supported by the Agricultural Experiment Station, Kansas State University.

REFERENCES


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Fig. 1. The effect of ball milling on cellulose crystallinity.
Fig. 2. The effect of ball milling on the specific surface area of cellulose.
Fig. 3. The effect of ball milling on the extent of enzyme hydrolysis.
Fig. 4. The effect of gamma radiation on the crystallinity.
Fig. 5. The effect of gamma radiation on the specific surface area of cellulose.
Fig. 6. The effect of gamma radiation on the extent of enzyme hydrolysis.
Fig. 7. The influence of crystallinity on the extent of enzyme hydrolysis.
Fig. 8. The influence of specific surface area on the extent of enzyme hydrolysis.
USE OF IMMOBILIZED β-AMYLASE/GLUCOAMYLASE MIXTURES TO PRODUCE
HIGH MALTOSE SYRUPS

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INTRODUCTION

Hydrolysates of corn starch high in maltose have been articles of commerce for many years. Usually the last step in their production is a process where dextrin or corn syrup, which can be produced from corn starch by either acid or α-amylase hydrolysis, is further hydrolyzed with glucoamylase and a fungal amylase mixture that gives high yields of maltose.

β-Amylase, which cleaves maltose units from the nonreducing ends of α-(1→4) linked glucosides, might be used here instead, but is is a much more expensive enzyme than fungal amylase preparations that give similar products. Recently, however, β-amylase was successfully immobilized in this laboratory to alkylamine porous silica, leading to the possibility that it could be employed at lower cost than before. Therefore, immobilized β-amylase and immobilized gluccamylase were mixed in different ratios and then packed into tubular reactors, where they hydrolyzed different dextrin or syrup preparations to syrups whose composition changed with increasing process time. These results were compared with preliminary data obtained from dextrin hydrolysis by immobilized glucoamylase and fungal amylase, in research similar to that conducted on the soluble enzymes by Hurst and Turner.

MATERIALS AND METHODS

Materials

Glucoamylase from Aspergillus niger (Lot PPAG 12) was donated by Novo. It had an activity of 1125 AGU/g, where 1 AGU was the enzyme necessary to hydrolyze 1 μmol of maltose/min at 25°C and pH 4.5.

β-Amylase from wheat (Lot 3431-21) was donated by Miles. Its activity was 170 MMU/mg, where 1 MMU would liberate 1 mg of reducing sugar from soluble starch in 30 min at 40°C and pH 5.

Fungal amylase (Claraé Concentrate, Control No. J23-54744) was also donated by Miles. It was obtained from Aspergillus oryzae, and had an activity of 99,200 SKBU/g, where 1 SKBU dextrinizes 1 g/h of β-limit dextrin at 30°C and pH 4.8.

Maltrin-15 dextrin was obtained from Grain Processing. It had a Dextrose Equivalent (DE) value of 15.8, where DE is a measure of the reducing value of the resulting solution, pure glucose having a DE of 100 and pure starch a DE near zero.
R-420 corn syrup was also supplied by Grain Processing. Its DE was 38.6.

Alkylamine Controlled Pore silica was donated by Corning. It had a particle size of 30/45 mesh (354-595 μm), and a pore diameter of 40 ± 10% nm.

Glutaraldehyde (50% aqueous solution, Lot J8A) was purchased from Eastman.

**Immobilization Procedure**

On a number of occasions, 5 g of silica beads were activated at room temperature for 2 h in an evacuated flask with 100 ml of glutaraldehyde that had been diluted to 2.5% with 0.1M phosphate sulfur, pH 7.0. After washing with water and phosphate buffer, the activated beads were reacted at 4°C overnight with one of the three enzymes dissolved in phosphate buffer. With β-amylase, 0.05 g of enzyme was dissolved in 25 ml of buffer and reacted with 5 g of beads. Glucoamylase (0.1 g) dissolved in 25 ml buffer and mixed with 5 g of beads, while with fungal amylase the quantities were 0.2 g of enzyme, 25 ml of buffer, and 10 g of beads. The resulting enzyme-carrier complexes were washed with phosphate buffer and then with 0.02M acetate buffer at pH 4.8.

**Reactor System**

Columns of 1.0 cm i.d. and 2.2 cm length were packed with approximately 1.3 g dry weight of beads that had been uniformly mixed to give specified volume ratios of enzyme-carrier complexes. From this reactor, 50 ml of 30% (w/v) dextrin or syrup solution flowed at 16 ml/min to a 100 ml stirred reservoir and then through a Cole-Parmer 7014 peristaltic pump back to the reactor. All parts of the system except the pump were submerged in a 42°C water bath.

**Analytical**

Samples of 25 μl taken from the reservoir were analyzed with a Waters ALC-201 liquid chromatograph using one of three columns held at 84°C: two were 9.5 mm o.d. 560 mm long jacketed stainless steel tubes packed with Bio-Rad Aminex 50W-X4 or Q15S cation exchange resin in the Ca²⁺ form, while the third was a prepacked 7.8 mm i.d., 300 mm long Bio-Rad HPX-42 column. Flow rate of water through the first column was 0.5 ml/min; with the other two it was 1.0 ml/min. Peaks were integrated with an Infotronics CRS-204 integrator. Variation of results among the three columns was within the reproducibility obtained from any one column.

**RESULTS AND DISCUSSION**

Maltrin-15 dissolved in 0.02M acetate buffer at pH 4.8 was reacted with pure immobilized glucoamylase and with mixtures of 1/1, 1/3, and 1/10 immobilized glucoamylase to immobilized β-amylase. Its hydrolysis is shown in Fig. 1. Similar experiments were conducted on R-420 corn syrup with pure glucoamylase and 1/3 and 1/10 glucoamylase/β-amylase mixtures (Fig. 2) and on Maltrin-15 dextrin with pure fungal amylase and with 1/2 and 1/3 glucoamylase/fungal amylase mixtures (Fig. 3).
As would be expected, higher ratios of β-amylase or fungal amylase to glucoamylase led to higher maximum concentrations of maltose but to much slower rates of reaction, especially after the maltose maximum had been passed. Also as expected, use of lower DE starting materials gave higher maximum concentrations of glucose, confirming the work of Lee et al. Reaction profiles of immobilized glucoamylase with Maltrin-15 varied somewhat from those previously reported, indicating that pore diffusion was slightly limiting (lower amounts of enzyme were immobilized to carrier than would be the industrial practice to lessen the effects of pore diffusion limitation).

Perhaps the most noteworthy result was that mixtures containing fungal amylase gave higher maximum amounts of maltose than did those containing β-amylase, a function of ability of the former to attack closer to the branch point of malto-oligosaccharides. Thus, while immobilized glucoamylase/β-amylase mixtures can be employed successfully, in that they have optimum pH's sufficiently close to each other, there seem to be few reasons to use them in preference to immobilized glucoamylase/fungal amylase mixtures, which also performed satisfactorily.

ACKNOWLEDGMENT

This work was supported by the Engineering Research Institute of Iowa State University and by Iowa Comprehensive Manpower Services.

REFERENCES

Fig. 1. Hydrolysis of 30 wt.% Maltrin-15 (DE 15.8) with immobilized glucoamylase/\(\beta\)-amylase mixtures.
Fig. 2. Hydrolysis of 30 wt.% R-420 corn syrup (DE 38.6) with immobilized glucoamylase/β-amylase mixtures.
Fig. 3. Hydrolysis of 30 wt.% Maltrin-15 (DE 15.8) with immobilized glucoamylase/fungal amylase mixtures.
INTRODUCTION

In submerged aerobic cultures of microorganisms, a large body of liquid is agitated by sparging air into it. The rising swarm of bubbles supplies the oxygen that is necessary for cell growth. In large scale industrial operations, this poses the problem of minimizing mass transfer costs by controlling the air flow rate. For efficient operation of such a system, the importance of high interfacial gas-liquid areas cannot be overstressed. In addition, the physical properties of the liquid medium play an important role in achieving good mass transfer rates. It is, therefore, necessary to account for these in designing an airlift fermentor.

An airlift fermentor consists of a draft tube which is enclosed by a tall cylindrical vessel. A sparger at the bottom supplies air to the microorganisms which grow in the liquid. As the bubbles rise, some oxygen in them dissolves in the liquid. Smaller bubbles provide good interfacial areas, but they rise slowly. As a result, there may be exhaustion of oxygen in the bubble, and it may remain in the medium without serving any useful purpose. Large bubbles rise faster, but they reduce the contact area. Further, they usually escape into the atmosphere, instead of being carried into the annular region, to be recirculated. The small bubbles, which are entrained in the annular region are carried downward by the circulating liquid. The difference in dispersion densities provides the driving force for liquid circulation.

It thus appears that bubble behavior plays an important role in oxygen transfer in an airlift system. The rise velocities of the bubbles are largely governed by density differences, bubble size and liquid viscosity, and to a lesser extent, by the surface tension of the liquid in which they rise. Literature on bubble rise velocities in low viscosity liquids is abundant. [e.g. Haberman and Morton (1956), Garner (1954), Van Krevelen and Hoftijzer (1950)]. Peebles and Garber (1953) suggested different correlations for bubble rise velocities in different ranges of Reynolds numbers from their experiments on air bubbles in water. For highly viscous liquids, however, experimental data are relatively sparse. Kojima et al (1968) made an extensive study of bubble rise velocities as a function of bubble diameter in liquids with viscosities from 1.57 to 103.8 poise. Their data show that the rise velocity of a bubble is dependent upon liquid viscosity. Redfield and Houghton (1965) reported the rise velocities of carbon dioxide bubbles in dextrose solution. Angelino (1966) studied the velocities of air bubbles in glycerol solutions. Recently, Grace et al. (1976) presented a generalized correlation for terminal velocities of liquid drops and bubbles. Acharya et al. (1977) recently reported experimental results on drag coefficients for isolated bubbles and velocity transition (beyond Stokes region) in non-Newtonian media. However, there appears to be a need for more experimental data and empirical correlations
to predict rise velocities in highly viscous liquids, especially beyond the Stokes region. The purpose of this work is to investigate bubble behavior in airlift fermentors by performing some preliminary calculations.

**METHODS OF CALCULATION**

The region of bubble rise velocity varies considerably with bubble Reynolds number. The following equations, which are available in literature, were examined. Region I: Very small bubbles follow Stokes’ eqn.

$$\frac{g \Delta \rho d_B^2}{18 \mu}$$

For low viscosity fluids, this equation is valid up to about $Re_b = 2$. (Peebles and Garber, 1953). The bubbles are spherical and rigid, and viscous forces dominate inertia and surface tension forces. Medium-size bubbles in viscous liquids are also likely to follow this equation at low bubble Reynolds numbers. Acharya et al. (1977) have shown that the bubble radius at which transition occurs may be predicted for viscous liquids. Region II: At higher Reynolds numbers, the bubble interface is freed and there is a transition to the Hadamard-Rybczynski region. Many workers have reported a jump in the bubble rise velocity in this region. The Hadamard equation is

$$\frac{g \Delta \rho d_B^2}{12 \mu}$$

Peebles and Garber defined this region by

$$2 \leq Re_b \leq 4.02 \left[ \frac{8 \mu}{\rho L \sigma} \right]^{0.214}$$

and suggested an empirical correlation for rise velocity, from their experiments on several liquids.

$$v_r = 0.33 g^{0.76} \rho^{0.52} \frac{L}{\mu} \left( \frac{d_B}{2} \right)^{1.28}$$

This indicates weaker influence of viscosity. It does not, however, account for the velocity jump. In the absence of more experimental data in the transition region, it appears more appropriate to use eqn. (2a) than eqn. (2c).

Region III: At higher Reynolds numbers, bubbles are deformed, and the rise velocity is independent of viscosity. Mendelson (1967) used the wave analogy to derive the following eqn.

$$v_r = \left( \frac{2 \sigma}{\rho d_B} + g \frac{d_B}{2} \right)^{1/2}$$

For small bubble diameters, the second term is negligible in comparison with the first term, and bubble rise velocity decreases with increasing bubble diameter; however for large bubbles, the second term is more important (Acharya et al. 1977).
In the present work, eqn. (3) has been used throughout region III. It is not possible to define the boundary between regions II and III, as the inequality (2b) is no longer valid for highly viscous liquids. The equation giving lower rise velocity was chosen beyond Stokes region.

Bubble size (and hence the rise velocity) is governed by two factors - 1) conditions at the orifice, and 2) the hydrodynamic field in which it moves. Bubbles in a turbulent flow field break up rapidly, forming smaller segments and greater contact area. In mechanically agitated tanks, bubble breakup is induced by the shear stress created by the impeller, but in an airlift fermentor it depends on the turbulence of the fluid flow field which depends on the power per unit volume associated with the airflow.

Turbulence is present if the scale of the energy containing eddies is much larger than the scale of the energy dissipating eddies. The ratio of the two microscales is defined as

\[ \frac{\lambda}{\eta} = \frac{d_{\text{BM}}^{1/2}}{\frac{1}{3}\mu L^{1/4}} \frac{P}{\rho L^{3/4}} \]  

The ratio \( \lambda/\eta \) is a measure of the degree of turbulence. Bhavaraju et al. (1978) from their experiments on 0.1% carbopol solution (viscosity \( \sim 0.3\mu \)) have suggested that \( \lambda/\eta \) should exceed 200 for turbulent conditions. The bubble size in this case is given by (Calderbank 1967)

\[ d_{\text{BM}} = C_1 \left( \frac{\rho L}{\mu G} \right)^{0.6} \left( \frac{P}{\rho L} \right)^{0.4} \]  

where \( C_1 = 0.7 \) and \( C_2 = 0.1 \) (for 0.1% carbopol) (Bhavaraju et al. 1978).

At high viscosity, when turbulence is absent, bubble size is governed by the conditions at the orifice (Bhavaraju et al. 1978).

\[ \frac{d^0}{\rho L^{0.1}} = 3.23 \left( \frac{\mu}{\rho L^{0.1}} \right)^{0.31} \]  

Eqn. (7) indicates that the bubble size is governed mainly by the gas flow rate per orifice. The dependence on orifice diameter is low. It may, therefore, be possible to reduce bubble size by increasing the number of orifice holes in the sparger, or by having smaller holes. A large number of bubbles, however, increases the probability of coalescence, which may counteract the increase in the interfacial area thus achieved through sparger design.

Other quantities of interest are the gas holdups, the number of bubbles, and the pressure drops in the two regions. The friction factors in the two regions are calculated by using the appropriate equations for laminar flow or turbulent flow:

\[ f = \frac{16}{\text{Re}_L} \quad \text{or} \quad f = \frac{0.0791}{\text{Re}_L^{0.25}} \]
For the draft tube

\[ \text{Re}_d = \text{Re}_d \frac{\rho L L_d}{d} \]

and for the annular region

\[ \text{Re}_a = \text{Re}_a \frac{\rho L L_{eq}}{d} \]

where \( d_{eq} \) is twice the hydraulic radius. The pressure drop in the draft tube and annular regions may be estimated using standard equations based on the liquid circulation velocity (Bird et al., 1960). The turnaround pressure drop is estimated by using the following equations (Hatch 1973)

\[ \Delta P_t = 2f_d \rho L Q_1 (Q_1 + Q) L_e / \left( D_h b A_b^2 g_c \right) \]

\[ Q_1 = V_L \pi (d_i^2 / 4) \]

where the equivalent length was assumed to be 1500 cm for the given fermentor dimensions, and \( D_h b \) (height of the draft tube above the base) was assumed to be equal to the diameter of the draft tube. The appropriate value of gas flow rate is the gas flow rate in the lower part of the annular region; however, the feed gas flow rate is used instead.

The total pressure drop is then written as

\[ \Delta P_{TOTAL} = \Delta P_{fa} + \Delta P_{fd} + \Delta P_t \]

The gas holdups were then calculated as follows:

\[ \phi_d = \frac{V_s}{V_s + V_r} \]

\[ \phi_a = \phi_d - \frac{\Delta P_{TOTAL}}{\rho L H_0} \]

The number of bubbles in each region can be calculated following Resnick and Galor (1968):

The average residence time of bubbles is given by

\[ \bar{\theta} = H_1 \phi/V_s \]

The number of bubbles is, therefore

\[ N_b = \frac{30 \bar{\theta}}{d_B^3} = \frac{60 \bar{\theta} H_1}{\pi d_B^3 V_s} \]
Since
\[ v_s = \frac{4Q}{\pi d_i^2} \]

therefore,
\[ N_{b,d} = \frac{1.5\phi d_i^2}{d_B^3} \]

and
\[ N_{b,a} = \frac{1.5\phi a_{10}(d_0^2 - d_1^2)}{d_B^3} \]

Calculations were done for a 5 meter tall airlift fermentor, with a diameter of 50 cm, which contained a 3 meter tall draft tube 35 cm in diameter.

**DISCUSSION OF RESULTS**

The variation of bubble rise velocity with diameter, with viscosity as parameter is shown in Fig. 1. For a given bubble diameter, the rise velocity decreases with increasing viscosity. Stokes region is assumed to be present up to \( Re = 2 \) (Peebles and Garber, 1953). The range of this region increases with viscosity. For \( \mu = 10p \), it extends up to \( d_B = 1.5 \) cm; however, the critical diameter predicted by Archarya et al., 1977 is much smaller (about 0.5 - 0.6 cm). At the end of this region, there is a discontinuity in the curve, the extent of which appears to increase with viscosity. A jump in the rise velocity at the end of Stokes region has been reported by many workers (e.g. Acharya, 1977). In Fig. 1, the jump is shown for viscosity of 5p, at the critical bubble diameter (0.54 cm). The rise velocities were calculated using equation 2c (Peebles and Garber) and 2a (Hadamard-Rybczynski). Eqn. 2a predicts higher rise velocities, which agree with the experimental data observed elsewhere (Angelino, 1966). The equation due to Peebles and Garber predicts lower rise velocities. More experimental data on rise velocities are necessary in this range of Reynolds numbers for high viscosity liquids.

The effect of viscosity on equilibrium diameter of a bubble is shown in Table I. The listed values of \( l/\eta \) indicate that bubble diameter is governed by turbulence up to viscosities of 1 poise, and by the orifice diameter at higher viscosities. The variation of bubble diameters at an air flow rate of 8000 cm\(^3\)/s and 1000 orifice holes (Table I) indicates that bubble diameter is much smaller in the case of turbulence-generated bubbles than for orifice-generated bubbles. In both cases, the diameter increases with viscosity.

Table III shows the effect of reducing the number of orifice holes. Comparison of Tables I & III shows that the turbulence-generated bubble-diameter remains unchanged, but the orifice-generated bubbles are larger. The effect of reducing the air flow rate is seen, on comparing the bubble diameters in Tables I and IV. A reduction in the gas flow rate by 1/2
results in larger turbulence-generated bubbles, whereas the orifice-generated bubbles decrease in size. In all these calculations, Hadamard eqn. (2a) has been used. It appears that in most cases Stokes region is absent, and Hadamard region is present for very highly viscous liquids only, for the range of mean bubble diameters studied. In practice, however, one may expect to also generate smaller bubbles so that region I is also present. For high bubble Reynolds numbers which occur in the case of turbulence-generated bubbles, region III is predominant; in region III viscosity is an unimportant factor with regard to rise velocities. Table II includes similar calculations, using eqn. 2c (Peebles and Garber) in region II. Comparison of these values with those in Table I suggests that for highly viscous liquids, the rise velocities thus predicted are smaller by about 28-35%.

The effect of viscosity on gas holdup is shown in Fig. 2 for two different airflow rates. In region II, the Hadamard-Rybczynski equation is used. A liquid circulation velocity of 15 cm is assumed. This is less than the bubble rise velocity for bubbles with the Sauter mean diameter. The estimate of gas holdup in the annular region decreases as liquid velocity and pressure drop due to liquid circulation increase. The difference in gas holdups in the draft tube and annular regions appears to increase with viscosity. At higher airflow rates, the gas holdups in both regions are higher. For viscosities between 1.5p and 5p, the gas holdup in the draft tube is almost constant, because the rise velocity is independent of viscosity (region III). For very high viscosities (> 5p) the Reynolds number falls in the Hadamard region, thus resulting in low bubble rise velocities, and higher holdups. At viscosity of 1.5p, there is a sharp change in gas holdup, due to the transition from turbulence-generated to orifice-generated bubbles. The turbulence-generated bubbles are smaller, and hence they rise slower, thus giving rise to higher gas holdups, than the orifice-generated bubbles.

Liquid Reynolds numbers and frictional pressure drops for the draft tube and annular regions are listed in Table V. The frictional pressure drop increases linearly with viscosity. As one may expect, liquid circulation is turbulent at very low viscosities only (0.01 and 0.1p). This does not exactly coincide with the boundary between bubbles generated by turbulence and orifice diameter.

The effect of viscosity on the number of bubbles in the two regions, is shown in Table VI. The number of bubbles in each region decreases with viscosity, and a higher percentage of bubbles remain in the draft tube.

CONCLUSIONS

It appears that region III is predominant, in the calculation of rise velocities in the case of low viscosity liquids, and either region II (Hadamard) or region III for highly viscous liquids. For the Sauter mean diameter, Stokes region is absent, however smaller bubbles in highly viscous liquids are likely to fall in this range of bubble Reynolds number.

One may conclude that viscosity does not significantly affect bubble rise velocity per se, when bubble Reynolds number falls in region III. However, the rise velocity depends on bubble diameter, viscosity is an
Table I. Estimated values of Sauter mean bubble diameter and bubble rise velocity for airlift tower with 1000 orifice holes and an airflow rate of 8000 cm$^3$/s using Hadamard equation (2a) in Region II.

<table>
<thead>
<tr>
<th>$\mu$ (p)</th>
<th>$\frac{L}{\eta}$</th>
<th>$D_B$ (cm)</th>
<th>$Re_B$</th>
<th>$V_{rise}$ (cm/s)</th>
<th>Remarks</th>
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<tr>
<td>0.01</td>
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<td>0.35</td>
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<tr>
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<td>26.96</td>
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<td>57</td>
<td>1.66</td>
<td>3.74</td>
<td>22.55</td>
<td>o, II</td>
</tr>
</tbody>
</table>

* t - turbulence-generated bubbles
0 - orifice-generated bubbles
Numbers refer to Regions I, II, and III.

Table II. Estimated values of Sauter mean bubble diameter and bubble rise velocity for airlift tower with 1000 orifice holes and an airflow rate of 8000 cm$^3$/s using Peebles and Garber's equation (2c) in Region II.

<table>
<thead>
<tr>
<th>$\mu$ (p)</th>
<th>$\frac{L}{\eta}$</th>
<th>$D_B$ (cm)</th>
<th>$Re_B$</th>
<th>$V_{rise}$ (cm/s)</th>
<th>Remarks*</th>
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<tr>
<td>0.01</td>
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<td>1.0</td>
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<tr>
<td>2.0</td>
<td>190</td>
<td>1.41</td>
<td>19.6</td>
<td>27.72</td>
<td>o, II</td>
</tr>
<tr>
<td>5.0</td>
<td>96</td>
<td>1.55</td>
<td>6.0</td>
<td>19.36</td>
<td>o, II</td>
</tr>
<tr>
<td>8.0</td>
<td>68</td>
<td>1.62</td>
<td>3.27</td>
<td>16.10</td>
<td>o, II</td>
</tr>
<tr>
<td>10.0</td>
<td>57</td>
<td>1.66</td>
<td>2.45</td>
<td>14.75</td>
<td>o, II</td>
</tr>
</tbody>
</table>

* t - turbulence-generated bubbles
0 - orifice-generated bubbles
Numbers refer to Regions I, II, and III.
Table III. Estimated values of Sauter mean bubble diameter and bubble rise velocity for airlift tower with 500 orifice holes and an airflow rate of 8000 cm$^3$/s; eq. (2a) was used in region II.

<table>
<thead>
<tr>
<th>$\mu$ (p)</th>
<th>$\frac{D}{n}$</th>
<th>$D_B$ (cm)</th>
<th>$Re_B$</th>
<th>$V_{rise}$ (cm/s)</th>
<th>Remarks*</th>
</tr>
</thead>
<tbody>
<tr>
<td>.01</td>
<td>$1 \times 10^4$</td>
<td>0.35</td>
<td>793.4</td>
<td>22.69</td>
<td>t, III</td>
</tr>
<tr>
<td>0.1</td>
<td>$1.8 \times 10^3$</td>
<td>0.44</td>
<td>97.3</td>
<td>22.10</td>
<td>t, III</td>
</tr>
<tr>
<td>1.</td>
<td>$3.2 \times 10^2$</td>
<td>0.55</td>
<td>12.25</td>
<td>22.10</td>
<td>t, III</td>
</tr>
<tr>
<td>2.</td>
<td>190</td>
<td>1.75</td>
<td>26.7</td>
<td>30.47</td>
<td>0, III</td>
</tr>
<tr>
<td>5.</td>
<td>96</td>
<td>1.92</td>
<td>12.18</td>
<td>31.70</td>
<td>0, III</td>
</tr>
<tr>
<td>8.</td>
<td>68</td>
<td>2.01</td>
<td>8.14</td>
<td>32.36</td>
<td>0, III</td>
</tr>
<tr>
<td>10.</td>
<td>57</td>
<td>2.06</td>
<td>6.73</td>
<td>32.68</td>
<td>0, III</td>
</tr>
</tbody>
</table>

*t - turbulence-generated bubbles
0 - orifice-generated bubbles
Numbers refer to Regions I, II, and III.

Table IV. Estimated values of Sauter mean bubble diameter and bubble rise velocity for airlift tower with 1000 orifice holes and an airflow rate of 4000 cm$^3$/s; eq. (2a) was used in region II.

<table>
<thead>
<tr>
<th>$\mu$ (p)</th>
<th>$\frac{D}{n}$</th>
<th>$D_B$ (cm)</th>
<th>$Re_B$</th>
<th>$V_{rise}$ (cm/s)</th>
<th>Remarks*</th>
</tr>
</thead>
<tbody>
<tr>
<td>.01</td>
<td>$8.6 \times 10^3$</td>
<td>0.46</td>
<td>1018</td>
<td>22.05</td>
<td>t, III</td>
</tr>
<tr>
<td>0.1</td>
<td>$1.5 \times 10^3$</td>
<td>0.58</td>
<td>129</td>
<td>22.17</td>
<td>t, III</td>
</tr>
<tr>
<td>1.</td>
<td>270</td>
<td>0.73</td>
<td>16.7</td>
<td>22.87</td>
<td>t, III</td>
</tr>
<tr>
<td>2.</td>
<td>160</td>
<td>1.140</td>
<td>14.7</td>
<td>25.78</td>
<td>0, III</td>
</tr>
<tr>
<td>5.</td>
<td>81</td>
<td>1.25</td>
<td>6.39</td>
<td>25.55</td>
<td>0, II</td>
</tr>
<tr>
<td>8.</td>
<td>57</td>
<td>1.31</td>
<td>2.87</td>
<td>17.54</td>
<td>0, II</td>
</tr>
<tr>
<td>10.</td>
<td>48</td>
<td>1.34</td>
<td>1.97</td>
<td>14.67</td>
<td>0, I</td>
</tr>
</tbody>
</table>

*t - turbulence-generated bubbles
0 - orifice-generated bubbles
Numbers refer to regions I, II, and III.
important factor in determining whether bubble diameter is governed by
turbulence in the liquid, or by the conditions at the orifice. In the
latter case, that is, at high viscosities, sparger design becomes import­
tant. It is desirable to have smaller orifice holes and a large number
of holes in order to generate smaller bubbles. Very small holes create
plugging problems, and very large number of holes increase the probability
of bubble coalescence, especially for slowly rising bubbles.

Viscosity also affects gas holdup in the two regions of the fermentor.
The difference in holdup increases with viscosity, but the estimate of gas
holdup in the draft tube is independent of the liquid circulation velocity.

ACKNOWLEDGMENT

This work was partly supported by NSF Grant ENG 77-16999.

NOTATION

d_B = Diameter of a single bubble

d_{BM} = Sauter mean diameter of bubbles

d_{eq} = Equivalent diameter of annular region

d_1 = Diameter of draft tube

d_0 = orifice diameter

g = acceleration due to gravity

H_i = height of the draft tube

\lambda = scale of the energy containing eddies

(P/V_l) = Power per unit volume

\Delta P = pressure drop

Q = volumetric gas flow rate

Q_l = volumetric liquid flow rate

Re_b = Bubble Reynolds number

V_L = liquid velocity

V_r = Bubble rise velocity

V_s = Superficial gas velocity
Greek letters:

\[ \eta \] = Kolmogoroff microscale
\[ \mu \] = liquid viscosity
\[ \mu_G \] = gas viscosity
\[ \rho_L \] = liquid density
\[ \Delta \rho \] = density difference between liquid and gas
\[ \sigma \] = surface tension
\[ \phi \] = gas holdup

REFERENCES

13. D. W. Van Krevelen, P. J. Hofijzer, Ch.E. Prog. 46, no. 1, p. 29 (1950).
Table V. Estimated values of liquid Reynolds numbers and pressure drops in the draft tube and annular regions of an airlift fermentor with 1000 orifice holes and an airflow rate of 8000 cm³/s, for a liquid circulation velocity of 15 cm/s.

<table>
<thead>
<tr>
<th>μ (p)</th>
<th>Re_{ld}</th>
<th>Re_{la}</th>
<th>ΔP_{fd}</th>
<th>ΔP_{fa}</th>
<th>ΔP_{t}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>5.25 x 10⁴</td>
<td>1.12 x 10⁴</td>
<td>20.2</td>
<td>230</td>
<td>6.3</td>
</tr>
<tr>
<td>0.1</td>
<td>5.25 x 10³</td>
<td>1.12 x 10³</td>
<td>35.8</td>
<td>427</td>
<td>11.2</td>
</tr>
<tr>
<td>1.0</td>
<td>525</td>
<td>113</td>
<td>118</td>
<td>4270</td>
<td>36.8</td>
</tr>
<tr>
<td>2.0</td>
<td>263</td>
<td>563</td>
<td>235</td>
<td>8530</td>
<td>73.5</td>
</tr>
<tr>
<td>5.0</td>
<td>105</td>
<td>22.5</td>
<td>588</td>
<td>2.13 x 10⁴</td>
<td>184</td>
</tr>
<tr>
<td>8.0</td>
<td>65.6</td>
<td>14.1</td>
<td>940</td>
<td>3.41 x 10⁴</td>
<td>294</td>
</tr>
<tr>
<td>10.0</td>
<td>52.5</td>
<td>11.3</td>
<td>1.18 x 10⁴</td>
<td>4.27 x 10⁴</td>
<td>368</td>
</tr>
</tbody>
</table>

Re_{ld} = Liquid Reynolds number in draft tube
Re_{la} = Liquid Reynolds number in annulus
ΔP_{fd} = Frictional pressure drop in draft tube
ΔP_{fa} = Frictional pressure drop in annulus
ΔP_{t} = Turnaround pressure drop.
Table VI. Estimated values of Sauter mean diameter and bubble rise velocity for airlift tower with 1000 orifice holes and an air flow rate of 8000 cm$^3$/s.

<table>
<thead>
<tr>
<th>$\mu$ (p)</th>
<th>$N_{b,d}$</th>
<th>$N_{b,a}$</th>
<th>% in draft tube</th>
<th>% in annulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>3456100</td>
<td>3590142</td>
<td>49.05</td>
<td>50.95</td>
</tr>
<tr>
<td>0.1</td>
<td>1765401</td>
<td>1830967</td>
<td>49.09</td>
<td>50.91</td>
</tr>
<tr>
<td>1.0</td>
<td>884884</td>
<td>890638</td>
<td>49.84</td>
<td>50.16</td>
</tr>
<tr>
<td>2.0</td>
<td>44778</td>
<td>42946</td>
<td>51.04</td>
<td>48.96</td>
</tr>
<tr>
<td>5.0</td>
<td>33066</td>
<td>27466</td>
<td>54.63</td>
<td>45.37</td>
</tr>
<tr>
<td>8.0</td>
<td>30333</td>
<td>21915</td>
<td>58.06</td>
<td>41.94</td>
</tr>
<tr>
<td>10.0</td>
<td>32419</td>
<td>22454</td>
<td>59.08</td>
<td>40.92</td>
</tr>
</tbody>
</table>

$N_{b,d}$ = number of bubbles in draft tube

$N_{b,a}$ = number of bubbles in annulus
Fig. 1. Bubble rise velocity vs. Bubble diameter using equations (1), (2a), and (2c) with viscosity as a parameter.
Fig. 2. Variation of gas holdup in the draft tube, Δ, and annulus, Θ, with liquid viscosity for a liquid circulation velocity of 15 cm/sec and a sparger with 1000 holes; solid line corresponds to a sparger gas flow rate of 8000 cm³/s and dashed line to 4000 cm³/s.