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Process Development Studies for the
Production of β -Glucosidase from
Aspergillus Phoenicis

Mary Jane Howell and C. R. Wilke
M.S. thesis

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PROCESS DEVELOPMENT STUDIES FOR THE PRODUCTION OF
 β -GLUCOSIDASE FROM *ASPERGILLUS PHOENICIS*

Mary Jane Howell* and Charles R. Wilke

ABSTRACT

This work is concerned with the production of β -glucosidase from *Aspergillus phoenicis* for use in the enzymatic hydrolysis of cellulose. Kinetic growth data indicate that two distinct periods of growth exist. The results support the hypothesis that the observed growth kinetics result from a biochemical differentiation of the filament which is independent of the substrate concentration. The optimum temperature for cell mass and β -glucosidase production was found to be 30°C. The optimum pH for β -glucosidase production is 5 and the highest specific cell growth rate was observed when the growth medium was controlled at pH 4.5. The highest levels of β -glucosidase were obtained when the pH of the growth medium was adjusted initially to pH 5 and not controlled during the fermentation. The β -glucosidase activity was partly intracellular with about half the enzyme activity retained in the mycelia after 100 hours of growth. The most economical substrate was 0.75 g/l of Solka Floc, a spruce wood pulp, plus 0.25 g/l of *Trichoderma viride* cellulase. The addition of cellulase was required because *A. phoenicis* does not produce all the enzymes required to solubilize cellulose.

When freeze-dried A. phoenicis enzyme was added to the hydrolysis of acid treated corn stover by Tricoderma viride cellulase, the total sugar yield was increased by 4 g/l of hydrolysate over the yield of 20 g/l obtained without β -glucosidase addition. In addition, the cellobiose, which previously accounted for about 10 per cent of the sugar concentration, was converted to glucose, a more widely useable product.

Preliminary designs of several processes for the production of β -glucosidase were made. The most economical processes were continuous production schemes which included methods of increasing the rate of release of intracellular enzymes. Ball milling was the most cost effective method, but the use of an elevated temperature stage was economical enough to warrant further study. The cost of production of β -glucosidase was found to be too high to justify its addition to a process for enzymatically hydrolyzing cellulose at this time. Further optimization of the enzyme production process and the hydrolysis reaction is required.

PROCESS DEVELOPMENT STUDIES FOR THE PRODUCTION
OF β -GLUCOSIDASE FROM ASPERGILLUS PHOENICIS

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1. INTRODUCTION

The diminishing reserves of oil and natural gas have made solar energy appear increasingly more attractive as a supplementary energy source. Solar energy has the advantages of being evenly distributed over the earth and inexhaustible. Conversion of solar energy to electricity requires a large collection area and adequate storage capabilities to assure availability of power at night. Solar energy collected in the form of biomass bypasses the storage problem in that it can be stored on the field until ready for use. The low level of technology required for production of energy from biomass also makes it an attractive energy source for developing countries. In the United States alone more than 21 quads ($1Q = 10^{15}$ BTU) of energy in the form of biomass is produced each year.¹ (For comparison, the national energy consumption in 1975 was 71Q, mostly in the form of fossil fuels.) Of this biomass about one-third is left on the land as residue, with wheat straw and corn stover the largest contributors. Methods of collecting and transporting these residues could be worked into the established agricultural system.

Electric or steam power from direct solar energy collection and biomass could potentially satisfy many industrial and domestic energy needs. However, unless an economical and efficient rechargable battery for an electric automobile is developed these sources can not fulfill our needs for a transportation fuel. The dependence of our economy and way of life on the automobile establishes the importance of finding an alternative fuel that can be used in automobiles, preferably with

little or no design changes required. Researchers in Brazil have shown that ethanol produced from sugar cane can be mixed with gasoline to make a fuel that burns much more cleanly than gasoline alone.² Mixtures containing up to 20 per cent ethanol did not require engine modifications.

Work has been done on processing schemes for the production of ethanol from newsprint and corn stover.^{3,4} In these schemes the cellulosic biomass was enzymatically hydrolyzed to glucose which was then fermented to ethanol with yeast.

This study was concerned with increasing the efficiency of glucose production from corn stover. The goal of this research was to develop a process for production of a supplementary enzyme to be used in the hydrolysis of cellulosic biomass to glucose and to evaluate the economic feasibility of the process.

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2. PROJECT BACKGROUND

This work is part of a project for which the goal is to develop and optimize a process to enzymatically hydrolyze cellulosic materials to glucose. The glucose may then be fermented to ethanol for use as a fuel or chemical intermediate. Single cell protein is produced as a byproduct. A simplified diagram of the process is shown in Figure 2.1. The cellulosic material, corn stover, is milled and pre-treated with dilute sulfuric acid. The acid pretreatment releases most of the pentosan fraction leaving the remaining solid material more accessible to enzyme hydrolysis.

In the hydrolysis step cellulase enzyme reacts with the pre-treated corn stover to produce soluble sugars and a residue which may be burned for power generation. The composition of the soluble sugars may vary with the cellulase preparation.

Cellulase enzyme is not one enzyme but a mixture of several enzymes whose specificities have not been fully determined. The enzymes can, however, be grouped by function. For Tricoderma koningii and T. viride, common fungal sources of cellulase, the cellulase components, C_1 , C_x and β -glucosidase have been identified.

The C_1 components are generally considered to be cellobiohydrolases.⁶ They act by removing cellobiose, a β -dimer of glucose, from the end of a cellulose chain. Cellobiose has been found to be a competitive inhibitor of C_1 .² The amount of inhibition of C_1 by cellobiose depends on the relative concentrations of the substrate (cellulose) and the inhibitor. The inhibitory effect of cellobiose is most evident at

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Figure 2.1. A simplified diagram of the process to produce sugars and ethanol from agricultural residues.

low cellulose concentrations.

C_1 components have been found to display different activities toward different cellulose structures. Some investigators¹ have reported that C_1 acting alone is effective in hydrolyzing crystalline cellulose. In general though, C_1 can not degrade cellulose, especially amorphous cellulose without the presence of other cellulase components. C_1 activity is often defined in terms of its ability to increase the activity of the C_x components on cellulose.

The C_x components are most often randomly acting endoglucanases but they may also include exoglucanases which remove single glucose units from the non-reducing end of the cellulose chain. C_x working alone can not extensively solubilize cellulose, but some of the C_x components are able to work in synergism with C_1 to reduce the cellulose primarily to cellobiose with some larger oligomers and glucose also formed.⁶

The addition of β -glucosidase, a cellobiase, to the hydrolysis of cellulose by C_1 and C_x components results in the conversion of cellobiose and higher oligosaccharides to glucose. Even without the presence of C_x , the degree of hydrolysis of cellulose by C_1 can be greatly enhanced by the addition of β -glucosidase due to reduction of the inhibiting effect of cellobiose.

When the cellulase of T. viride is used to hydrolyze corn stover, glucose and cellobiose account for roughly 90 per cent of the total sugars produced.⁵ The cellobiose concentration increases rapidly then falls with longer reaction time as it is converted to glucose.

The reduction of cellobiose in the hydrolysis step would be advantageous for the following two reasons:

1. As described above, cellobiose inhibits the action of the cellulase enzymes. If the cellobiose concentration is lowered the rate of glucose production will increase.
2. The organism currently under consideration for fermentation of hydrolysate sugars, Saccharomyces cerevisiae, can not catabolize cellobiose.³ Therefore cellobiose produced in the hydrolysis step passes through the ethanol fermentation, thus reducing the overall carbohydrate utilization and requiring further treatment to reduce the B.O.D. of the effluent to an acceptable level.

A reduction in cellobiose may be accomplished by supplementing the T. viride cellulase with β -glucosidase. Yamanaka⁷ studied the hydrolysis of newsprint with mixed enzyme systems. T. viride enzyme was mixed with enzyme from Botriodiplodia theobromae which has higher β -glucosidase activity, but lower C_1 and C_x activities. When the mixture was 15 to 30 per cent B. theobromae enzyme solution, the amount of glucose produced was 20 to 40 per cent higher than when all T. viride cellulase was used and the total sugars produced were increased by 10 to 20 per cent. When Solka Floc, in which the cellulose is more accessible to attack than in newsprint, was hydrolyzed by a mixed enzyme system the improvement in glucose yield was even more pronounced.

Previous workers have studied the effect of adding Aspergillus phoenicis and Aspergillus niger β -glucosidases to hydrolysis reactions

containing a fixed concentration of T. viride cellulase.⁴ It was found that the two Aspergillus β -glucosidases were equally effective in enhancing both glucose and total sugar production. For the substrates studied, crystalline cellulose (Avicel) and -200 mesh ball-milled Solka Floc, the amount of time required to reach any sugar concentration was reduced by at least 50 per cent at the optimum β -glucosidase concentration.

Although it has been established that addition of β -glucosidase to T. viride cellulase increases the hydrolysis yield, the substrates previously studied were more accessible to enzyme attack than corn stover. It was therefore of interest to determine if the addition of β -glucosidase would improve the enzymatic hydrolysis of corn stover, both practically and economically.

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3. PREVIOUS WORK

3.1. Sources of β -Glucosidase

There are many different sources of β -glucosidase. Plant sources include sweet almond,⁷ the enzyme extract of which is sold commercially, and barley flour.² There are also hundreds of species of bacteria and fungi that produce β -glucosidase. The plant sources were not pursued in this study as it was decided to restrict the area of inquiry to microbial sources.

One of the most thorough studies of a fungal source of β -glucosidase was done by Yamanaka with Botriodiplodia theobromae.¹⁵ The enzyme was stable at pH 5, the pH of the enzymatic hydrolysis of cellulose, but was not very heat stable, losing half of its activity in 40 hours at 50°C.

Sternberg et al.¹² screened over 200 strains of fungi and 15 of bacteria for β -glucosidase production by growing them for two weeks in shake flasks at 28°C. The black aspergilli were the best producers with Aspergillus phoenicis outstanding. The black aspergilli (named for the color of their spores) have previously been acknowledged for their capabilities in manufacturing β -glucosidase.^{3,11} Preliminary work in this laboratory has shown that A. phoenicis produces an enzyme solution with more than twice the β -glucosidase activity of that obtained from B. theobromae.

3.2. Production of β -Glucosidase from the Black Aspergilli

In the Thom morphology A. phoenicis is grouped with several other members of the genus Aspergillus in what is called the A. niger group.¹³

These species differ in size of conidia and length of primary sterigmata but share many common characteristics. Members of the A. niger group are used in the production of numerous commercial products. As a result of this industrial interest an abundance of information has been obtained on them, especially in the area of growth kinetics. Included in the enzymes industrially produced by A. niger are amylase, catalase and glucose oxidase. The fermentations of glucose to citric acid and gluconic acid also employ strains of A. niger. Because there is little published work on A. phoenicis while a considerable amount exists on A. niger, the latter may be useful in indicating possible characteristics of A. phoenicis.

Early studies of the cellulolytic abilities of the black aspergilli were done during the 1940's when strains of the A. niger group were used by government and commercial labs to test the mold-proofness of a variety of manufactured goods.¹⁴ These strains were chosen because they were found growing on cellulosic materials and it was assumed that they were responsible for the degradation of the materials. However, White et al.¹⁴ in a study performed in 1948 determined that while certain species of Aspergillus exhibited cellulolytic activity, none of the members of the A. niger group were capable of cellulolytic action when cultured on cotton duck fabric.

A more detailed investigation was performed by Reese and Downing¹¹ in which 422 Aspergillus isolates were grown on wool, cotton duck and bleached cotton sheeting. The result was that while all but one member of the A. niger series were unable to hydrolyze cellulose, they could all hydrolyze β -1,4 glucosidic bonds (i.e. they produce

β -glucosidase). The cellulolytic ability was determined by measuring the loss in tensile strength of strips of cloth on which a strain was cultured. A subjectively chosen minimum of 15 per cent reduction in tensile strength was required for the organism to be classified as possessing cellulolytic ability. As a result, whereas A. phoenicis was termed unable to degrade cellulose, a 10 per cent and 8 per cent reduction in the tensile strength of bleached cotton sheeting and grey cotton duck respectively was observed.

Preliminary work on the production of β -glucosidase with A. phoenicis QM329 has been performed by Sternberg et al.¹² Submerged growth on starch in shake flasks was compared to surface culture on bran (Koji method). The shake flask cultures produced a higher specific activity of β -glucosidase and a higher enzyme yield per gram of carbon source. When grown in shake flasks on starch, β -glucosidase levels could be increased by the addition of an inducer. Yields when grown on simple sugars such as glucose were lower than when grown on starch, even with the addition of an inducer. It was reported that addition of a surfactant such as Tween 80 doubled the β -glucosidase yields.

The characteristics of the purified β -glucosidase of A. phoenicis were also studied. Good activity was obtained on all β -linked dimers of glucose. Action was less rapid on tetramers than on dimers. The K_m value, as determined by a Lineweaver-Burk plot, was 0.75 mM for cellobiose. The maximum velocity was 164 μ moles glucose released per minute per mg. of β -glucosidase. Substrate inhibition was observed even at relatively low cellobiose concentrations. The optimum pH for activity of the β -glucosidase at 50°C was 4.3 with more than

50 per cent activity observed at pH 3 and 6. The enzyme was stable at pH 4.8 and 50°C with 85 per cent of the activity retained after four days.

3.3. Kinetics of Fungal Growth

The growth rate of cellular organisms is often related to the cell concentration, the concentration of the growth-limiting substrate and inhibitors in a complicated manner. The specific growth rate, μ , is defined as:

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (3.1)$$

where

X = cell weight concentration

t = time

If μ is constant, the resulting growth is called exponential growth. When dX/dt is constant, linear growth occurs.¹

Bacterial growth can often be described by an exponential relationship. However, fungal growth is usually more complex. Two modes of growth have been described for the fungi in submerged culture, pellet growth and filamentous growth.⁹

When fungal growth is in the form of a pellet, there is apical growth only at the tips of the hyphae. This may be caused by a limited rate of diffusion of oxygen or another nutrient through the pellet mass. Pellet growth may be expressed as:

$$X^{1/3} = kt + X_0^{1/3} \quad (3.2)$$

where

k = a constant

x_0 = cell weight concentration at $t = 0$.

In studies with the freely branching filamentous organisms such as the ascomycetous fungus Neurospora this relationship was found to apply.⁸ It was observed that growth was restricted to the tips of the hyphae and that the bulk of the tissue formed did not continue to grow. Because the radii of the pellets increased at a constant rate, the cube root of their volumes (masses) increased at a proportional rate. Pellet growth is often accompanied by a hollowing of the center.⁶ Severe limitations of nutrient diffusion by dense pellet structure may result in autolysis of the center.

The other mode of fungal growth, filamentous growth, occurs when all the hyphae can branch without restriction. A tangled mass of hyphae that is homogeneously dispersed throughout the medium is formed. In this mode the growth is exponential and takes the form

$$x = x_0 e^{\mu(t-t_0)} \quad (3.3)$$

where

x_0 = cell weight concentration at $t = t_0$

e = base of natural logarithms

which is identical to Equation (3.1).

A. niger has been observed growing exponentially when in a filamentous form, but when in a pellet form the growth was determined to follow this equation:⁴

$$x^2 = x_0^2 + 2k'(t - t_0) \quad (3.4)$$

where

k' = a constant.

Another investigator found that A. niger displays exponential growth even when in pellet form.⁵ It was later supposed that the pellets were loose in texture and not compact enough for cube root growth to occur.⁹

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4. EXPERIMENTAL PROCEDURES

4.1 Fungus Strain and Culture Medium

The Aspergillus phoenicis culture used in this study, QM 329, was obtained from the University of Massachusetts, Amherst, Mass. Potato Dextrose Agar (Difco) slants were inoculated from this culture and incubated at 30°C until sporulation occurred. The slant cultures were then stored at 4°C.

Inoculum for the fermentation experiments was prepared by transferring a small amount of mycelia and spores from a slant to a 250 ml shake flask containing 100 ml of sterile medium of the composition shown in Table 5.1.^{4,5} The flasks were incubated for five days at 30°C on a rotary shaker.

The medium shown in Table 4.1 was used in all fermentation experiments unless otherwise noted. Sternberg et al.⁵ reported good production of β -glucosidase from A. phoenicis using this medium.

4.2 Flask Experiments

4.2.1 Constant Temperature Experiments

Temperature optimization experiments were conducted with 500 ml Erlenmeyer flasks containing 200 ml of sterile medium with starch as the carbon source. The culture flasks were kept in water baths that were controlled at constant temperatures within 1°C variation. Agitation was supplied by magnetic stirrers. Periodically the culture flasks were removed to a sterile hood where samples were taken aseptically with disposable plastic pipettes. For the remaining flask

Table 4.1. Culture Medium

Component	grams/liter H ₂ O
soluble starch	10.00
KH ₂ PO ₄	0.20
K ₂ HPO ₄	0.15
NaH ₂ PO ₄ ·H ₂ O	2.00
Na ₂ HPO ₄	1.50
NH ₄ NO ₃	0.60
NaNO ₃	3.80
MgSO ₄ ·7H ₂ O	0.30
trace metal solution*	1.0 ml

* Trace Metal Solution

Component	grams/liter H ₂ O
Fe ₂ (SO ₄) ₃ ·6H ₂ O	0.054
(NH ₄) ₂ P(Mo ₃ O ₁₀) ₄	0.024
ZnSO ₄ ·7H ₂ O	0.050
CuSO ₄ ·5H ₂ O	0.0025
MnSO ₄	0.0055
H ₃ BO ₃	0.057

experiments, the cultures were agitated with a gyratory shaker inside an incubator maintained at 30°C.

4.2.2 Constant pH Experiments

The pH was held constant at 3.5, 4.25, 4.75, 5.25 and 5.75 by adding 0.2 M sodium citrate buffer to the growth medium. Citrate buffer was chosen because it is effective in the entire pH range of interest and because it is unlikely to be harmful to the fungus because citric acid is a growth product of members of the A. niger family.

Periodically during the experiments the pH of the buffered medium was measured and adjusted if needed with 4 N H_2SO_4 or 4 N NaOH.

The pH of samples was adjusted to pH 5 with acid or base to assure that all assays were performed at a consistent pH.

4.3 Fermentor Experiments

A five liter Microferm fermentor (New Brunswick Scientific Co.) was used in experiments to observe growth kinetics, rate of β -glucosidase production and oxygen uptake rate. The fermentor was charged with three liters of medium and sterilized in a vertical autoclave. Filter-sterilized air at the rate of 0.75 vvm was sparged through the fermentor. The oxygen tension of the broth was measured with a New Brunswick Scientific oxygen probe connected to a Beckman recorder. The temperature was regulated by circulating water through hollow baffles that were submerged in the liquid. Agitation was supplied by two turbine impellers on a shaft magnetically coupled to the drive. The pH was adjusted and controlled at the desired value with the addition of 4 N H_2SO_4 and 4 N NaOH by a New Brunswick Scientific pH controller

and an Ingold combination pH electrode. Sampling of the broth was accomplished by drawing the liquid through a sampling tube into a removable sterile flask. To assure a representative sample, the lines were cleared with filtered air before removal of liquid. The sample was centrifuged and the enzyme activity of the supernatant measured. The enzyme activity remaining in the mycelium was also determined. A known weight of cells was filtered, washed, and resuspended in water to make a 0.75 per cent suspension. The cells were then disrupted by ultrasonication while cooled in an ice bath, and after centrifugation the enzyme activity of the supernatant was measured.

4.4 Hydrolysis Experiments

4.4.1 Pretreatment of Corn Stover

The corn stover was dried and ground to approximately 50 mesh in a Wiley mill. In a 2 liter round-bottom flask 87 g of corn stover and 1 liter of 0.09 M H_2SO_4 were mixed. The suspension was boiled and stirred for 5 1/2 hours. After cooling, the residue was repeatedly filtered and washed with H_2O until the pH of the wash-water rose to 5. The treated corn stover was then air dried at room temperature.

4.4.2 Enzymatic Hydrolysis

In an 800 ml glass hydrolysis vessel 15 g of pretreated corn stover was added to 275 g of T. viride cellulase having a filter paper activity of 3.5 (see p. 19). The enzyme had been previously buffered to pH 5 by addition of 0.05 M sodium acetate buffer. The β -glucosidase activity in each vessel was adjusted to a desired value by adding freeze dried A. phoenicis β -glucosidase. The vessel was stirred

continuously with a marine impeller while incubated at 45°C. To prevent evaporation of the contents the vessel was sealed with a rubber lid. Samples were taken with a pipette that was lowered through an opening in the lid. The samples were immediately centrifuged and the supernatant frozen to prevent further reaction.

4.5. Cell Disruption Experiments

The cells were obtained after 100 hours of batch growth. Washing with water and vacuum filtering removed the growth medium that was entrained with the cells.

4.5.1. Ultrasonication

In 20 ml of water, 1 g of wet cells (dry weight = 0.1 g) was suspended. A Branson model J-17A ultrasonicator delivered 30 watts to the cells with a 3/8-inch probe. The cell suspension was kept at 2°C by resting the metal tube containing the suspension in an ice bath. Periodically a sample was taken and centrifuged and the enzyme activity of the supernatant determined.

4.5.2. Bead Milling

A jacketed reactor vessel with a capacity of one liter was filled with 600 g of 3 mm glass beads, 20 g of wet cells (dry weight = 2g) and 240 ml of water. Ice water was circulated through the jacket to keep the temperature of the cell slurry at 4°C. A marine impeller turning at 500 rpm supplied mixing to the vessel. The slurry was sampled periodically with a pipette. The sample was centrifuged and the enzyme activity of the supernatant was determined.

4.6 Assay Procedures

4.6.1 Fungal Dry Weight Assay

The dry cell mass determination was used to measure the growth rate of the fungus. A known volume of cell suspension was filtered through a preweighed Nucleopore filter with 0.8 micron pore diameter. The filter cake was washed to remove entrained culture medium and oven dried at 70°C. The filter and dried fungal cake were then weighed and the fungal dry weight determined by difference.

4.6.2 Protein Assay

An adaptation of the method proposed by Lowry et al.³ was used to determine protein concentration. The following is a description of the adapted procedure: Reagent A consisted of 3 per cent Na_2CO_3 in 0.1 N NaOH. Reagent B consisted of 2 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 4 per cent sodium tartrate in H_2O . The assay solution was made by mixing 100 ml of Reagent A with 1 ml of Reagent B. After 3 days the mixture was discarded and replaced with freshly prepared solution.

To 0.5 ml of sample containing up to 150 µg of protein, 5 ml of assay solution was added. The mixture was incubated at room temperature for 10 minutes. Very rapidly 0.5 ml of 1 N Folin phenol reagent was added and shaken vigorously. After 10 minutes the absorbance was read at 600 nm against a reagent blank. Several dilutions of a solution of crystalline bovine albumin were used to make a standard curve.

4.6.3 Carbohydrate Concentration Determination

The glucose oxidase-peroxidase (GOP) assay was used to measure the low concentration of glucose produced in the β-glucosidase assay.

The GOP reagent was made by dissolving 2 ml of purified glucose oxidase (Sigma Chemical Co., type V, 1200 units/ml) and 50 mg peroxidase (Sigma Chemical Co., type II, 125-200 purpurogallin units/ml) in 500 ml of 0.89 M trishydroxymethyl methylamine (Tris) buffer, pH 7.0. To this was added 6 ml of a 4 per cent solution of o-dianisidine dihydrochloride in H₂O.

To a test tube holding 4 ml of 0.89 M Tris and 2 ml of sample containing between 10 and 200 µg of glucose, 4 ml of the above reagent was added. The tubes were incubated at 40°C for 1 hour. The reaction was stopped by addition of 3 drops of concentrated HCl. After the tubes were allowed to cool to room temperature the absorbance was read at 420 nm against a reagent blank. The concentration was read from a standard curve prepared for each reagent.

Starch concentration was measured by the anthrone-sulfuric acid method. The reagent was prepared by dissolving 0.75 g anthrone in a liter of 72 per cent H₂SO₄. A 5 ml aliquot of reagent was added to 1 ml of sample containing less than 150 mg/l carbohydrate in a test tube. The test tubes were placed in boiling water for 20 minutes, then cooled in an ice bath. The absorbance was read at 620 nm against a blank of anthrone reagent and water. A standard curve prepared from solutions of known starch concentration was used to determine the concentration of the samples.

Gas-liquid chromatography (GLC) was used to determine the concentrations of cellobiose and glucose in the enzyme hydrolysis product.

A detailed description of the methods used can be found in Lawrence Berkeley Laboratory Report #5967.²

4.6.4 Enzyme Activity Assays

Enzyme activity measurements are used as a practical measure of the effective concentration of the enzyme. For a solution including several different proteins, such as a fungal broth, a protein determination is not as descriptive as an assay for a specific enzyme function.

The activity of β -glucosidase was determined by adding 0.5 ml of enzyme solution containing 3 to 50 units of β -glucosidase to 1.5 ml of 0.02 M cellobiose in 0.05 M sodium acetate buffer, pH 5.0. This solution was mixed and allowed to react at 40°C for 15 minutes. The reaction was stopped by adding 4 ml of Tris buffer (0.89 M, pH 7.0) and mixing. The glucose produced was measured with the GOP glucose assay. One unit of β -glucosidase activity is defined as the amount that will make one μ g of glucose per minute under the above conditions. After addition of the Tris buffer the sample may be frozen and stored for an indefinite period before the glucose assay is performed.

The substrate solution for amylase activity determination contained 1 per cent soluble starch and 0.006 M NaCl in 0.05 M sodium acetate, pH 5. To 0.5 ml of starch solution 0.5 ml enzyme solution was added and incubated at 30°C for 3 minutes. The reaction was stopped by cooling in an ice bath. The reducing sugars produced were measured using the dinitrosalicylic acid (DNS) method⁶ and expressed as glucose equivalents. The amylase activity was expressed as mg of glucose produced per ml of enzyme solution during the reaction period.

Filter paper activity is a measure of the cellulose degrading ability of an enzyme solution. A strip of Whatman No. 1 filter paper 1 cm by 6 cm was rolled and placed in a test tube. After adding 1 ml of enzyme solution and 1 ml of 0.05 M sodium acetate buffer, pH 5, the sample was mixed and incubated at 40°C for 1 hour. The reaction was stopped by cooling in an ice bath. The DNS method was used to determine the reducing sugar concentration which was expressed as glucose equivalents. Filter paper activity (FPA) was expressed as glucose equivalents per ml of original enzyme solution.

4.6.5. Q_{O_2} Measurement

The specific oxygen uptake rate, Q_{O_2} , was measured to determine the amount of oxygen that should be supplied to the growth medium. A material balance on the amount of oxygen at a point in the system can be written as:¹

$$\frac{d\bar{C}}{dt} = k_L a (C^* - \bar{C}) - Q_{O_2} X \quad (4.1)$$

where,

$\bar{C}$ = concentration of dissolved oxygen in bulk liquid

C^* = concentration of dissolved oxygen in equilibrium

with the bulk gas phase

$k_L a$ = volumetric oxygen transfer coefficient, hr^{-1}

Q_{O_2} = specific oxygen uptake rate

X = cell mass concentration

The measurement was made by stopping the air flow and measuring the rate of decrease in $\bar{C}$ with time using a fast response dissolved

oxygen probe. Without aeration, the term describing the transfer of oxygen between the gas and liquid phases drops out leaving,

$$\frac{d\bar{C}}{dt} = -Q_{O_2} X \quad (4.2)$$

A dry cell mass determination was done at the time of each oxygen response measurement. The time without aeration was kept low (only three or four minutes) to prevent oxygen starvation from occurring.

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5. RESULTS AND DISCUSSION

5.1. Growth Rate

Batch fermentations with the five-liter fermentor were used to determine the growth rate of the fungus and the utilization of the substrate and oxygen. It was important to maintain a constant agitation speed from experiment to experiment because a variation in shear stresses would affect the size and form of the pellet growth. Even though a high agitation speed was used, a frequently encountered problem was the growth of a mat of fungal cells along the wall of the fermentor vessel at the liquid level. This was remedied by periodically uncoupling the agitator drive shaft and manually shaking the vessel until the wall growth was dislodged. The frequency of this operation varied between once and five times per day depending on the rate of mycelial growth. The problem was caused by a combination of factors, the major one being the filamentous nature of the organism which resulted in its tendency to form dense mats of mycelia. Also contributing were the high aeration rate which tended to float the pellets to the surface and the design of vessel which incorporated a narrow clearance between the stainless steel baffles and the glass vessel wall. This permitted mycelial pellets to be trapped against the wall where they remained and grew. These problems are not likely to be as serious in a large scale industrial fermentor in which the surface-to-volume ratio is much smaller and the clearance between parts is greater than in the laboratory fermentor.

Measurement of the growth rate is important for estimation of the dilution rate needed for a continuous fermentation. Although β -glucosidase production is not growth-associated, the rate of cell mass production does influence the initial rate at which β -glucosidase is made. This will be discussed in detail later in this chapter.

Soluble starch was chosen as the growth substrate because it is totally soluble in the medium and would not interfere with the dry cell mass determination. The pH was adjusted to 5 at the start of the fermentation and was not controlled. The fermentation temperature was 30°C. Figure 5.1 shows the growth curve which can be broken into five consecutive phases.

(1) Lag phase. This is the time required for the cells in the inoculum to adapt to the new conditions and prepare for growth.

(2) Exponential growth phase. In this phase the mycelia multiplied rapidly and the pH dropped from 5 to 3. The increase in cell mass can be described by the exponential equation:

$$X = X_a e^{\mu(t - t_a)} \quad (3.3)$$

and will produce a straight line when plotted on log-linear coordinates. Although the growth was in pellet form, the "cube root" growth described in section 3.3 did not occur. Pirt⁶ attributes this deviation which has been observed in A. niger^{3,5} to the looseness of the pellets which allows unhindered diffusion of nutrients. The specific growth rate, , was constant during this phase and was determined to be 0.235 hr⁻¹. As is shown in Table 5.1 these results are very similar to those obtained

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Figure 5.1. Growth phases of *A. phoenicis*.

Table 5.1. Comparison of Exponential Growth Phases

	Substrate	Temp. (C)	μ (hr ⁻¹)	Duration of exponential phase (hr)	Reference
<u>A. phoenicis</u>	starch	30	0.235	9	This work
<u>A. niger</u>	sucrose	26	0.13	18 - 20	(2)
<u>A. niger</u>	sucrose	32	0.28	12 - 14	(2)
<u>A. niger</u>	glucose	30	0.278	12	(5)
<u>A. niger</u>	sucrose	--	0.289	8	(4)

by other investigators using various strains of A. niger and different carbon sources.

(3) Growth disturbance phase. In this phase, growth slowed to a stop, then increased again after a short lag. The maximum rate of β -glucosidase production occurred during this phase. The disturbance was obviously not due to depletion of the starch, but may be related to depletion of some other nutrient in the medium. Another possibility is that the growth disturbance was due to a biochemical differentiation of the filaments. Machek and Fenc1 have described a differentiation process occurring in A. niger which depends on the age and length of the filaments. After differentiation was complete unequal synthesis of RNA and protein was observed in different parts of the filament.⁴

(4) Secondary growth phase. The growth rate in this phase can best be described by the following equation:²

$$x^2 = x_0^2 + 2k(t - t_0) \quad (3.4)$$

where t_0 is the time at the beginning of the secondary growth phase. The value of k for A. phoenicis was calculated to be $1.17 \text{ g}^2/\text{l}^2\text{-hr}$. The substrate concentration decreased rapidly during this phase and the pH rose to its original value. Production of a red pigment which colored the mycelia and broth began during this phase.

(5) Production phase. In this phase, growth stopped and the cells began to lyse causing the dry cell mass to decrease slowly. As will be discussed below, most of the β -glucosidase production occurred in this phase, with an increasing amount of enzyme appearing in the culture broth as the cells lysed.

The substrate, soluble starch, was not totally consumed in the fermentation. It was thought that another necessary nutrient was exhausted before all the starch could be used, but experimentation showed that starch was the growth-limiting nutrient in these fermentations. Figure 5.2 demonstrates that up to an initial concentration of 25 g/l of starch, a constant fraction, 32 per cent, of the starch was not consumed after six days.

The specific oxygen uptake rate, Q_{O_2} , was measured by the method described in section 4.5.6. Two maxima were observed, corresponding to the two periods of growth, as can be seen in figure 5.1. The greatest total oxygen demand occurred in the secondary growth phase where 3.55 mmol/l-hr were required. Oxygen was not growth-limiting because the 0.75 vvm of air sparged through the fermentor is equivalent to 422 mmol/l-hr of oxygen.

Amylase activity in the culture filtrate was found to increase linearly with time. The activity rose constantly throughout the fermentation, reaching a final value of 0.8 mg/ml at 240 hours.

5.1.1. Effect of Temperature on Growth Rate

As can be seen in Figure 5.3, the total production of dry cell mass has a maximum at about 30°C when grown in shake flask cultures. The optimum temperature for growth of a cell depends on the temperature optima of the enzymes responsible for cell metabolism. A sharp decrease in cell growth with increasing temperature could be the result of the denaturation of one or more enzymes. A more gradual decrease

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Figure 5.2. Utilization of starch by *A. phoenicis*.

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Figure 5.3. Effect of temperature on cell mass concentration at 36 hrs.

could result if there is more than one enzyme capable of catalyzing a particular reaction or if alternative metabolic paths exist.

5.1.2. Effect of pH on Growth Rate

Figure 5.4 shows the results of the shake flask experiments in which the pH of the growth medium was controlled by addition of sodium citrate buffer. It appears that for the range studied the lower the pH, the shorter the lag phase. But rather than finding that the following phases were shifted proportionately, as might be expected, the growth disturbance phase was found to occur at the same time for all values of pH. This observation seems to confirm the above-mentioned opinion that the growth disturbance phase is due to a differentiation of the filament that is dependent on the age and length of the filaments and is independent of substrate concentration.⁴

The second (exponential) phase was longer for the cultures at the lower pH values. Thus they accumulated more cell mass by the beginning of the growth disturbance phase than did the cultures at the higher pH values. However, as can be seen in Figure 5.5, the specific growth rate of the exponential phase was independent of this phenomenon and had a maximum at pH 4.5.

Several investigators have reported variations in the morphology of A. niger with varying pH, most notably the tendency to grow in pellets at higher pH's and in a filamentous form below pH 5.⁷ This was not observed with A. phoenicis in this work as pellet growth developed at all pH values. The pellets were loose and fluffy at the beginning

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Figure 5.4. Effect of pH on cell mass production.

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Figure 5.5. Effect of pH on specific growth rate during the exponential growth phase.

of growth and became more firm and smooth as time progressed. This may partially account for the difference in growth kinetics between the exponential and secondary growth phases.

5.2. β -Glucosidase Production

The β -glucosidase produced by A. phoenicis when grown in the fermentor was measured in both the mycelium and the culture filtrate. Figure 5.6 shows that the relative amounts varied with the age of the culture.

The relationship between β -glucosidase production and cell mass concentration was not a simple one. Although the maximum rate of β -glucosidase production occurs simultaneously with the maximum growth rate, the enzyme production cannot be called truly "growth-associated". To be "growth-associated" the two rates should be directly proportional,¹ but as can be seen in Figure 5.1, they were not. Nor was the enzyme production rate proportional to the concentration of cells as the total β -glucosidase activity found in the mycelia and in the culture filtrate continued rising as the cell concentration declined. Differentiation in the biochemical function (5.1) of the mycelial cells could account for the complexity of the relationship. If this were so, a measurement of the total mycelial mass would not be meaningful without knowing what proportion of the cells were engaged in β -glucosidase production.

5.2.1. Effect of Temperature on β -Glucosidase Production

An experiment was performed using shake flasks to study the effect of temperature on the β -glucosidase activity occurring in the filtrate

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Figure 5.6. Location of β -glucosidase during batch growth on starch.

when the temperature was held constant for the entire fermentation. Figure 5.7 shows that the activity in the filtrate at 240 hours had a maximum between 29 and 30°C. The maximum is rather sharp as a 2°C variation in either direction results in approximately a 20 per cent reduction in activity produced. The amount of β -glucosidase produced depends not only on the effect of the temperature on the fungus during the production phase but on its effect during the preceding growth phases. As discussed above, the production of β -glucosidase is not directly growth associated so the importance of the effect of temperature during the growth phases is not easily determined. It should be noted, however, that the maxima for both cell mass and β -glucosidase production occur near 30°C.

In another experiment the effect of temperature on β -glucosidase production after cell growth was complete was investigated. Shake flask cultures that were grown at 30°C for 100 hours were mixed together and redivided into the flasks. This assured that the cultures in all the flasks had identical compositions at the beginning of the experiment. The flasks were then cultured at 20, 30, or 40°C. Figure 5.8 shows that the β -glucosidase activity measured in the filtrate was highest at 40°C. It is probable that the high temperature encourages lysis of the cells which results in more rapid accumulation of β -glucosidase in the filtrate.

5.2.2. Effect of pH on Production of β -Glucosidase

The effect of pH on the production of β -glucosidase was studied in two ways, (1) by maintaining the pH constant through the entire

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Figure 5.7. Effect of temperature on β -glucosidase activity of the culture filtrate at 240 hrs.

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Figure 5.8. Effect of temperature on β -glucosidase activity of the culture filtrate at 100 hrs.

fermentation and (2) by adjusting the pH initially, then allowing it to follow its natural course. As described in chapter 4, the pH of all enzyme solutions was adjusted to a standard value, pH 5, before β -glucosidase activity was measured. This removed the influence of pH on the observed β -glucosidase activity.

When the pH of shake flask cultures was controlled throughout the fermentations, a sharp maximum in the β -glucosidase activity measured in the filtrate at 240 hours occurred at pH 5, as can be seen in Figure 5.9. The enzyme activity was lowered by 60 per cent when the pH was increased by 0.25 and was lowered to a lesser extent when the pH was decreased. No β -glucosidase activity was observed in the filtrate of cultures grown at pH 3.5. This may indicate that the enzyme is not produced or that the enzyme is produced but denatured at low pH's. It is unlikely that denaturation is totally responsible for the lack of β -glucosidase activity because Sternberg et al.⁸ reported only a 50 per cent inactivation in 22 hours at 50°C at pH 3.7 for A. phoenicis β -glucosidase.

When the pH of an A. phoenicis fermentation is not controlled the pH decreases from its initial value and reaches a minimum at least two pH units lower during the secondary growth phase. The pH then rises and reaches a constant value. In studies done with the fermentor the initial pH of the growth medium was found to influence strongly the mycelial growth and the production of β -glucosidase. Figure 5.10 shows that for an initial pH of 6.2 the mycelial growth rate was slower and growth continued for a longer time than for an initial pH of 5.

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Figure 5.9. Effect of pH on β -glucosidase activity of the culture filtrate at 240 hrs.

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Figure 5.10. Cell mass production for initial pH's 5 and 6.2.

The effect of initial pH on β -glucosidase production is shown in Figure 5.11. Enzyme production was delayed in the higher initial pH, run both in the amounts found in the culture filtrate and in the mycelia. The fast initial growth rate for the run at an initial pH of 5 resulted in rapid production of β -glucosidase.

The values of β -glucosidase activity and cell mass found in the pH optimization study using a specially buffered medium in shake flasks were not directly comparable to the results from the fermentor studies because of the differences in the systems. It was therefore of interest to compare a fermentation with the pH controlled at 5 (the pH of maximum growth rate and β -glucosidase production), with one in which the pH was not controlled (Fig. 5.12). The initial pH of the uncontrolled pH run was 5. While the growth rates were equivalent, less total cell weight was produced when the pH was controlled than when it was uncontrolled. The β -glucosidase activity in the culture filtrate was 65 per cent less at the end of the fermentation when the pH was controlled than without pH control.

5.2.3. Effect of Aeration and Agitation on β -Glucosidase Production

An experiment using shake flasks was performed to determine the effect of stopping the aeration or agitation after growth was completed. After growing for 100 hours the A. phoenicis cultures in several shake flasks were mixed and redivided into the flasks to assure a constant initial composition. The flasks were then closed with either a cotton plug which allowed sterile transfer of air or paraffin which sealed out air. The cultures used in the aeration study were incubated on

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Figure 5.11. Production of β -glucosidase for initial pH's 5 and 6.2.

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Figure 5.12. Production of cell mass and filtrate β -glucosidase for uncontrolled and controlled pH.

a rotary shaker. No difference was observed in the amount of β -glucosidase measured in the culture filtrates of the aerated and unaerated flasks. Reducing the aeration after 100 hours of growth did not increase the rate of cell lysis or reduce the rate of synthesis of β -glucosidase.

The cultures used in the agitation study were all sealed with paraffin. When shake flask cultures that had been incubated either with or without agitation were compared, no difference in β -glucosidase production was observed. An implication of these results is that production costs could be reduced without affecting the enzyme yield by elimination of aeration and agitation after 100 hours of growth.

5.2.4. Effect of Substrate on β -Glucosidase Production

The substrate used in the experiments previously described, soluble starch, was chosen because it did not interfere with the dry cell mass determination by filtration. An insoluble substrate would have been caught in the filter and the resulting dry weight of the filter cake would not have been representative of the cell mass concentration. Another reason for the selection of starch was that high levels of β -glucosidase were produced when the fungus grew on starch. However, because soluble starch was available only as a laboratory reagent, it was desirable to find another substrate that would be more economical when used on a large scale, but would still produce an acceptable concentration of β -glucosidase.

Experiments were performed in shake flasks at 30°C. The growth medium was the same as that described in section 4.1 except that

various concentrations of different substrates were substituted for the 10 g/l of starch. The β -glucosidase activity reported was the amount in the culture filtrate at 10 days.

The product of the hydrolysis of acid pretreated corn stover with T. viride enzyme contains about 8 g/l of glucose and 1.5 g/l of cellobiose. It was hypothesized that this would make an economical substrate and that the cellobiose would induce β -glucosidase production. However, when reagent grade cellobiose and glucose were used as substrates cellobiose did not act as an inducer, and β -glucosidase production on glucose, cellobiose, and mixtures of the two was poor (Fig. 5.13). The amount of enzyme made when the fungus was grown on the hydrolysis product was equivalent to the amount made when grown on glucose.

Potatoes were considered as a substrate because of their starch content. Two methods of preparation were used. In the first method the potatoes were peeled, boiled and mashed. In the second they were peeled and ground in a blender with water. The moisture content of the potatoes was determined so that the amount of water added to the medium could be adjusted accordingly. The concentration of the potatoes is expressed on a dry basis. As can be seen in Figure 5.13 the enzyme produced with growth on potatoes was higher than with growth on soluble starch, especially at the lower substrate concentrations. Because the whole potato contains other substances besides starch, some of which are not usable by the organism, an inducer for β -glucosidase production must exist in the potato that is not in the soluble starch preparation.

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Figure 5.13. Effect of substrate concentration on final β -glucosidase activity of the filtrate for various substrates.

Cellulose was also considered as a substrate. The cellulose preparation used was Solka Floc, a spruce wood pulp. Because A. phoenicis does not produce enough cellulase enzyme to grow on cellulose, cellulase from T. viride was added to the growth medium. The amount of water used in making up the medium was reduced accordingly so that the concentration of the minerals remained constant. The filter paper activity of the undiluted cellulase was 3.0 mg/ml.

Figure 5.14 shows the effect of adding various amounts of cellulase on the β -glucosidase activity found in the filtrate. The amount of β -glucosidase added initially in the T. viride cellulase was subtracted from the final β -glucosidase activity to give the net enzyme produced. A maximum is reached at about 35 per cent T. viride enzyme, but very little additional activity is gained above 25 per cent. For this reason 25 per cent T. viride cellulase, which is equivalent to a filter paper activity of about 1.1 mg/ml in the growth medium, was chosen to study the effect of cellulose concentration on β -glucosidase production. As can be seen in Figure 5.13, high levels of β -glucosidase were produced when A. phoenicis was grown on a mixture of cellulase and Solka Floc. The enzyme level decreases rapidly at concentrations above 10 g/l of cellulose.

5.3. Enzymatic Hydrolysis of Cellulose

The effect of adding A. phoenicis β -glucosidase to the hydrolysis of pretreated corn stover by T. viride cellulases was studied. Figures 5.15 and 5.16 show the results of this experiment. The filter paper activity of the original T. viride enzyme solution was 3.6 mg/ml

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Figure 5.14. Effect of addition of *T. viride* enzyme on final β -glucosidase activity of the filtrate.

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Figure 5.15. Hydrolysis of corn stover by *T. viride* cellulase with various concentrations of *A. phoenicis* β -glucosidase: concentrations of glucose and cellobiose.

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Figure 5.16. Hydrolysis of corn stover by *T. viride* cellulase with various concentrations of *A. phoenicis* β -glucosidase: total sugar concentration.

and the β -glucosidase activity was 10 units/ml. Freeze-dried culture filtrate from A. phoenicis was added to the T. viride enzyme to bring the total β -glucosidase activity to 10, 91, 181 and 363 units/ml.

Without supplemental β -glucosidase, a large amount of cellobiose was formed initially which was not totally converted to glucose by the end of the hydrolysis. The remaining cellobiose can not be utilized in the fermentation of glucose to ethanol by Saccharomyces cerevisiae, the organism used in the next step in the cellulose conversion process.

With 91 units/ml of β -glucosidase activity, the cellobiose concentration was significantly reduced and the saturation concentration of β -glucosidase was reached. Above 91 units/ml the initial rate of glucose production was improved slightly but the final concentration of glucose was not increased.

Assuming that glucose and cellobiose account for all the sugars produced, the total sugar concentration was increased by about 4 g/l when supplemental β -glucosidase was added (Figure 5.16). With the addition of β -glucosidase to the hydrolysis, the reaction time could be reduced to 10 hours and still result in more glucose produced than with the original cellulase solution and 48 hours reaction time.

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6. PROCESS DESIGN AND ECONOMIC EVALUATION

6.1. Substrate Evaluation

The substrates tested for β -glucosidase production can be separated into two categories (Section 5.2.4). The enzyme production when A. phoenicis was grown on glucose and on the hydrolysis product was poor and, therefore, these substrates were not considered further. Of the remaining substrates, no single one was outstanding. The differences between the enzyme productivities of the substrates was not substantially larger than what would be expected from experimental error. Therefore, the determining factor became the substrate cost per unit of enzyme produced. The costs of the ground and cooked potatoes were obtained by adding the costs of the processing to the price of the potatoes. Preliminary designs were made of a process to grind potatoes and a process to cook and mash the potatoes. Details of the designs can be found in Appendix B.

Table 6.1 shows the final costs of the substrates. When the unit substrate cost was multiplied by the concentration of the substrate and divided by the enzyme activity obtained at that concentration, a cost per unit of enzyme activity was obtained. This was plotted as a function of enzyme activity as a means of comparing the substrates (Figure 6.1). A desirable substrate would produce high activity at a low cost per unit. The substrate composed of Solka Floc and enough T. viride enzyme to make 1.1 mg/ml filter paper activity in the medium fit this description by having the minimum cost per unit of enzyme at a high β -glucosidase activity. This minimum cost corresponds to

Table 6.1. Substrate costs.

Glucose	10.86 ¢/lb
Corn Starch	8.15
Soluble Starch	16.8
Ground Potato	16.8
Cooked Peeled Potato	23.2
Cooked Unpeeled Potato	17.8
Solka Floc	5.0
Hydrolysate Sugars	5.2
TV Enzyme (0.25 g/l = 1.1 FPA)	1.20 ¢/lb

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Figure 6.1. Substrate cost optimization: cost per unit of β -glucosidase activity vs. β -glucosidase activity.

a β -glucosidase activity of 280 units/ml and, from Figure 5.13, a Solka Floc concentration of 5 g/l. It was this substrate and enzyme yield that were used as a basis for the following process design.

6.2. β -Glucosidase Production

The results described in Chapter 5 have been used to make preliminary designs of several different processes for the production of β -glucosidase. Because the aim was to examine the feasibility of the proposed processes and to determine if future work should be done on one or more of them, no attempt was made to design equipment in detail. Appendix A shows the equipment design and cost estimation procedure used. The design basis was 5.2 tons/day of Solka Floc feed. Fermentation conditions were those determined to give the best β -glucosidase production. The pH was initially adjusted to 5 and not controlled during the fermentation. The temperature during the growth phases was 30°C. Other conditions such as temperature of the production phase and batch or continuous operation were varied and are discussed below for each design case.

Figure 6.2 shows a diagram of the process. For all of the different modes of operation, the design of the equipment preceding the fermentors is identical. Sterilization of the medium prior to addition of the T. viride enzyme is performed continuously. After the sterile medium is cooled by heat exchange with the stream entering the sterilizer, the T. viride enzyme is added and mixed in a holding tank. The β -glucosidase production process is assumed to be located on the site of a cellulose hydrolysis plant so that sterile cellulase enzyme can

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Figure 6.2. Process scheme for production of β -glucosidase by *A. phoenicis* grown on Solka Floc and *T. viride* enzyme. Numbers are flow rates in gal/min.

be diverted into the A. phoenicis growth medium. Most of the cellulase is later returned to the hydrolysis process when the culture filtrate from the A. phoenicis fermentations is concentrated and added to the hydrolysis reaction.

In the following sections, the different process variations are described, the design bases are listed and a compilation of the major equipment is provided for each case. At the end a comparison is made between the economics of the different process variations.

6.2.1. Batch Fermentations

In the batch process, the sterile growth medium enters 20 fermentors of 4.79×10^5 liters arranged in parallel. The aeration rate in each fermentor is 0.08 vvm for the first 50 hours and 0.02 vvm for the remainder of the fermentation. After 200 hours of operation, the fermentor is emptied. A three hour period is allowed for draining and filling. The starting times of the fermentations are staggered so that the flow in and out of the fermentor block is as continuous as possible. Holding tanks before and after the fermentors even out the flow so that the rest of the process can be run continuously. These tanks are not shown in the process flowsheet (Figure 5.1) but have been included in the cost determination. The fermentation broth is then centrifuged and the supernatant, with a β -glucosidase activity of 270 units/ml, is stored. A summary of the design basis for the batch process is shown in Table 6.2. The major process equipment is listed in Table 6.3.

Table 6.2. Batch fermentation design basis.

Solka Floc concentration	5 g/l
Temperature	30 °C
Avg. Aeration rate	0.035 vvm
Fermentation time	200 hr
Down time	3 hr
β -glucosidase activity of product stream	273 units/ml

Table 6.3. Major items of equipment for batch β -glucosidase production process
(capacity 1.00×10^{11} units/hr).

Item	Unit specification/power Requirements	No. of Units	Cost/unit \$
Premix tank	9460 liters, stainless steel	1	21,770
Agitator for Premix tank	2HP	1	2,590
Presterilize heat exchanger	1.43×10^3 ft ² , stainless steel	1	38,300
Sterilizer	insulated stainless steel, 1290 lb/hr steam	1	3,920
Hold tank	4.79×10^5 liters, stainless steel	2	133,410
Agitator for hold tank	28 HP, cost included in Hold tank	2	
Fermentor	4.79×10^5 liters, stainless steel	20	133,400
Agitator for Fermentor	28HP, cost included in Fermentor	20	
Air compressor	3260HP, centrifugal type	1	658,460
Centrifuge	15HP, DeLaval, 40 m ³ /hr throughput	1	34,550
Product storage	1.86×10^6 liters, carbon steel	1	83,030
<u>T. viride</u> enzyme storage	1.18×10^6 liters, stainless steel	1	143,060
Pumps	6.5HP, centrifugal		3,140
Total	3898HP, 1290 lb/hr steam		3,929,510

6.2.2. Continuous Fermentation

The dilution rate of the fermentors was calculated graphically from the batch kinetic data shown in Figures 5.1 and 5.6 by the method of Deindoerfer.¹ Although data obtained from a continuous fermentation is preferable, this method is adequate for the purposes of a preliminary design. In this method, the inverse of the rate of enzyme production with time is plotted versus the enzyme activity. The area beneath the curve is the time required for batch growth, or the residence time if a plug flow reactor is used. The residence time needed for equivalent production in a continuous stirred reactor is determined by multiplying the β -glucosidase activity desired in the product stream by the inverse of the rate of enzyme production at that point in batch growth.

The number of stages of continuous stirred reactors was optimized and it was found that for each growth period the use of two reactors (fermentors) of equal size in series was the most economical design. A total of four fermentors in series was required for the growth stage because of the two distinct periods of growth observed with A. phoenicis (Section 5.1). The use of two fermentors in series was also found to be the best design for the production stage. However, because the β -glucosidase production phase is longer than the growth phase in batch growth, a longer residence time (or a smaller dilution rate) is required. The largest fermentor available has a volume of 1.6×10^6 liters, so three sets of two fermentors in series, each with a volume of 1.57×10^6 liters are required for the production phase.

The aeration rate is adjusted to the needs of each stage, which results in a lower total aeration requirement than in the batch case where the maximum amount required is supplied during the entire growth period. The β -glucosidase activity of the enzyme solution produced in this case is 270 units/ml. The design basis of the continuous fermentation is shown in Table 6.4 and the list of major equipment is in Table 6.5.

6.2.3. Continuous Fermentation with Increased Temperature Stage

In this case, the first stage of the fermentation is carried out at 30°C, as in the previously described process. The second stage is kept at 40°C and no aeration is provided. As discussed in Section 5.2.1 this encourages release of β -glucosidase so that the final enzyme activity of the filtrate is 20 per cent higher than in the continuous process described above. Additional heat exchanger area and steam consumption are needed in the second stage to increase the process temperature to 40°C. A list of the major equipment can be found in Table 6.6. The design basis is in Table 6.7.

6.2.4. Continuous Fermentation with Cell Disruption

As described in Section 5.2, a large amount of β -glucosidase occurs within the mycelia during growth. Most of this activity eventually appears in the culture filtrate, but several days of incubation are required, even when elevated temperatures are used to encourage lysis. Numerous methods of disrupting cells have been described, but most are not practical on an industrial scale. Studies done with A. niger have shown that continuous grinding in a bead mill is an

Table 6.4. Continuous fermentation design basis.

Solka Floc concentration	5 g/l
Temperature	30 °C
Avg. Aeration rate	0.022 vvm
Dilution rate	
Growth fermentors	0.219 hr ⁻¹
Production fermentors	0.010 hr ⁻¹
β-glucosidase activity of product stream	270 Units/ml

Table 6.5. Major items of equipment for continuous β -glucosidase production process (capacity 1.00×10^{11} units/hr)

Item	Unit specification/power requirements	No. of Units	Cost/unit \$
Premix tank	9460 liters, stainless steel	1	21,770
Agitator for Premix tank	2HP	1	2,590
Presterilize heat exchanger	1.43×10^3 ft ² , stainless steel	1	38,300
Sterilizer	insulated stainless steel, 1290 lb/hr steam	1	3,920
Growth fermentor	2.15×10^5 liters, stainless steel	4	90,100
Agitator for Growth fermentor	15.5HP, cost included in growth fermentor	4	
Production fermentor	1.57×10^6 liters, stainless steel	6	238,600
Agitator for Production fermentor	115HP, cost included in production fermentor	6	
Air compressor	1265HP, centrifugal type	1	323,930
Centrifuge	15HP, DeLaval, 40 m ³ /hr throughput	1	34,550
Product storage	1.86×10^6 liters, carbon steel	1	83,030
<u>T. viride</u> enzyme storage	1.18×10^6 liters, stainless steel	1	143,060
Pumps	6.5HP, centrifugal		3,140
Total	2040.5HP, 1290 lb/hr steam		2,451,960

Table 6.6. Major items of equipment for continuous β -glucosidase production process with increased temperature stage (capacity 1.23×10^{11} units/hr).

Item	Unit specification/power requirement	No. of Units	Cost/unit \$
Premix tank	9460 liters, stainless steel	1	21,770
Agitator for Premix tank	2HP	1	2,590
Presterilize heat exchanger	1.43×10^3 ft ² , stainless steel	1	38,300
Sterilizer	Insulated stainless steel, 1290 lb/hr steam	1	3,920
Growth fermentor	2.15×10^5 liters, stainless steel	4	90,100
Agitator for growth ferm.	15.5HP, cost included in growth fermentor	4	
Production fermentor 1	1.38×10^6 liters, stainless steel	1	224,210
Agitator for Product. ferm. 1	80HP, cost included in Production ferm. 1	1	
Production fermentor 2-3	2.00×10^6 liters, s. steel, 300 ft ² heating coils	2	272,600
Production fermentor 4-5	2.00×10^6 liters, s. steel	2	268,920
Agitator for Prod. ferm. 2-5	145HP, cost included in Production ferm. 2-5	4	
Heat exchanger	45 ft ² , 1540 lb/hr steam, for Prod. ferm. 2-3	1	4,180
Air compressor	617HP, centrifugal type	1	189,060
Centrifuge	15HP, DeLaval, 40 m ³ /hr throughput	1	34,550
Product storage	1.86×10^6 liters, carbon steel	1	83,030
<u>T. viride</u> enzyme storage	1.18×10^6 liters, stainless steel	1	143,060
Pumps	14HP, centrifugal		7,440
Total	1370HP, 2829 lb/hr steam		2,201,220

Table 6.7. Continuous fermentation with increased temperature stage design basis.

Solka floc concentration	5 g/l
Temperature	30 °C
Avg. Aeration rate	0.011 vvm
Dilution rate	
Growth fermentors	0.219 hr ⁻¹
Production fermentors	0.012 hr ⁻¹
β-glucosidase activity of product stream	332 units/ml

effective method of releasing intracellular enzymes.⁴ Data taken in this laboratory have shown that while less effective than ultrasonication, a significant amount of β -glucosidase is released when A. phoenicis cells are suspended in water in an 8 per cent suspension and ground in a bead mill (Figure 6.3).

In an industrial mill, pebbles would be substituted for the glass beads used in the laboratory mill because they are more durable. Although ultrasonication releases more activity than milling, preliminary calculations have shown that power costs are 40 times higher and equipment costs 100 times greater for ultrasonication than for pebble milling.

Using the batch kinetic data for bead milling, it was determined graphically that a residence time of 45 minutes would be required to obtain the optimum disintegration with a continuously operated mill.² A preliminary design was made of a process in which the fungus is grown continuously at 30°C until the total amount of β -glucosidase in both the mycelium and the filtrate reaches a maximum (equivalent to 120 hours of batch growth). At this point, the culture filtrate is centrifuged. The mycelial cells are then resuspended in water and run continuously through a pebble mill. The pebble mill is jacketed to allow circulation of a brine coolant which prevents the temperature of the cell suspension from rising above 5°C. A summary of the design basis is shown in Table 6.8. The major equipment is listed in Table 6.9.

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Figure 6.3. Effect of time of cell disruption on β -glucosidase released: sonication and bead milling.

Table 6.8. Continuous fermentation with cell disruption design basis.

Solka floc concentration	5 g/l
Temperature	30 °C
Avg. Aeration rate	0.022 vvm
Dilution rate	
Growth fermentors	0.219 hr ⁻¹
Production fermentor	0.017 hr ⁻¹
β-glucosidase activity	
Product stream after fermentors	175 units/ml
Product stream after pebble mill	450 units/ml

Table 6.9. Major items of equipment for continuous β -glucosidase production process with cell disruption (capacity 1.15×10^{11} units/hr).

Item	Unit specification/power requirement	No. of units	Cost/unit \$
Premix tank	9460 liters, stainless steel	1	21,770
Agitator for premix tank	2HP	1	2,590
Presterilizer heat exchanger	1.43×10^3 ft ² , stainless steel	1	38,300
Sterilizer	insulated stainless steel, 1290 lb/hr steam	1	3,920
Growth fermentor	2.15×10^5 liters, stainless steel	4	90,100
Agitator for growth ferm.	15.5HP, cost included in growth fermentor	4	
Production fermentor	1.40×10^6 liters, stainless steel	2	227,370
Agitator for Production ferm.	100HP, cost included in production ferm.	2	
Air compressor	900HP, centrifugal type	1	250,930
Centrifuge 1	15HP, DeLaval, 40 m ³ /hr throughput	1	34,550
Product storage 1	1.86×10^6 liters, carbon steel	1	83,030
Mixing tank	0.5HP, 3900 liters, carbon steel	1	14,080
Pebble mill	200HP, 630 ft ³ capacity, carbon steel	1	187,130
Centrifuge 2	2.5HP, De Laval, 14 m ³ /hr throughput	1	16,670
Product Storage 2	6.32×10^5 liters, stainless steel	1	37,610
Refrigeration	376HP, for pebble mill	1	61,700
<u>T. viride</u> enzyme storage	1.18×10^6 liters, stainless steel	1	143,060
Pumps	8HP, centrifugal	1	4,250
Total	1766HP, 1290 lb/hr steam		1,720,400

6.2.5. Summary of β -Glucosidase Costs

Table 6.10 compares the product costs for the different processes. (The cost estimation procedure is described in Appendix 1.) A significant decrease in product cost is experienced in going from batch to continuous processing. The product cost is reduced even more when the continuous process is modified to increase the rate of enzyme release from the mycelia, as in the last two processes described.

The major reason that the batch process is so expensive is that twice as many fermentors are required as in the continuous process. In the modified continuous processes, the number of fermentors is reduced further resulting in greater savings. The aeration requirements are also lower which means that a lower capacity air compressor can be used.

Even though the lower equipment cost is partially offset by a higher utility cost, the continuous fermentation with cell disruption process is the most economical process. The continuous process with an increased temperature stage also has a low enough enzyme production cost to merit further study.

6.3. Effect of β -Glucosidase Addition on Hydrolysis Costs

To fully assess the value of addition of β -glucosidase to the enzymatic hydrolysis of cellulose, a detailed optimization of the hydrolysis step should be done. However, a preliminary estimate of the economic benefits can be made by determining the effect that addition of β -glucosidase would have on an existing design. The most recent economic design of a process to convert corn stover to glucose is

Table 6.10. Production cost cent/million units.

	Batch	Continuous	Continuous Temp. Increase	Continuous Cell Disruption
Capital related	4.29	2.71	1.97	1.65
Labor Related	0.70	0.41	0.28	0.30
Utilities	0.31	0.48	0.27	0.37
Raw Materials	1.16	1.17	0.95	1.02
General Expenses	0.87	0.41	0.31	0.29
Total	7.33	5.18	3.78	3.63

by Yang et al.³ They reported that after 40 hours of hydrolysis by T. viride enzymes (filter paper activity of 3.5), a sugar solution containing 15.7 g/l glucose is produced at a cost of 0.022 \$/g of glucose.

The cost of β -glucosidase as produced in the most economical process was determined above to be 3.63 \$/million units. However this enzyme solution must be concentrated before addition to the T. viride cellulase to prevent dilution of the cellulase activity. It was calculated that a 10-fold concentration by low temperature evaporation would increase the price of β -glucosidase to 4.79 \$/million units. If 81 units/ml β -glucosidase is added to the hydrolysis, the glucose production will be increased by 52 per cent to 23.8 g/l. The cost of the β -glucosidase would be 0.388 \$/l sugar solution. The cost of a sugar solution produced in the hydrolysis process described by Yang et al. is 0.345 \$/l. Therefore the total cost of making a 23.8 g/l glucose solution is the sum of these two costs or 0.733 \$/l. This is equivalent to 0.030 \$/g of glucose, or a net increase of 0.008 \$/g of glucose over the cost without addition of β -glucosidase. Although addition of β -glucosidase did not improve the profitability in this case, it may prove more valuable if combined with a change in the design of the hydrolysis process. The effect of β -glucosidase on the progression of a hydrolysis is most evident during the first few hours when the rate of glucose production is increased dramatically. An area of future work may be in shortening the residence time in the hydrolysis reactor to reduce process costs.

6.4. Conclusions

Although the profitability of addition of β -glucosidase to the enzymatic hydrolysis of cellulose was not demonstrated, two areas of potential improvement are clear:

- 1) The hydrolysis reaction could be optimized with respect to the addition of β -glucosidase and the length of the reaction time. It is also conceivable that a reduction in the reaction time made possible by addition of β -glucosidase could reduce the cost of glucose production.
- 2) The production of β -glucosidase from A. phoenicis could be further optimized with respect to the growth conditions. The culture medium and oxygen requirements could be optimized and further work on inducers and surfactants should be done.

The ability of β -glucosidase to improve the glucose and total sugar yields in the enzymatic hydrolysis of corn stover has been demonstrated. The yield of β -glucosidase has been improved by optimization of the temperature and pH of growth and of the enzyme production, but several other areas of optimization remain. Further optimization should be done because the large increase in initial rate of glucose production upon addition of β -glucosidase offers promise for a more economical production of glucose.

CHAPTER 6. REFERENCES

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APPENDIX A. DESCRIPTION OF PROCESS DESIGN
AND COST ESTIMATION METHODS

A.1. Equipment Design Equations and Costs

The method of design of the major equipment is described in this section. For all equipment that was in contact with the fermentation both before or during the fermentation, the material of construction was stainless steel. The remaining equipment was carbon steel. Two major sources were used for the equipment costs, Peters and Timmerhaus⁶ and Guthrie.⁴ The graphical data was often used directly, but when convenient it was described by an exponential equation for ease of calculation.

A.1.1. Fermentor Volume

For the batch fermentation, a 200 hour fermentation time with 3 hours to drain and refill the vessel with sterile medium was assumed. A working volume of 80 per cent was used. An approximate fermentor size was chosen and used in the following equation to determine the number of fermentors required.

$$N = \frac{F\theta}{V(0.8)} \quad (A.1)$$

where

F = liquid flow rate, liters/hr

θ = fermentation cycle time, hr

V = fermentor volume, liters (maximum volume = 1.4 106 liters)

N = number of fermentors.

The number of fermentors was rounded off to the next largest whole number. The exact fermentor volume was then calculated by substituting N, F, and θ into equation (A.1) and solving for V.

For continuous fermentations the fermentor volume was determined from this equation:

$$V = \frac{F}{D} \quad (A.2)$$

where

$$D = \text{dilution rate, hr}^{-1}$$

The cost of the fermentor, including agitation, is given by⁶

$$\text{Cost} = \text{MSI } 0.417 V^{0.49} \quad (A.3)$$

where MSI is the current value of the Marshall and Stevens Index (for the first quarter of 1978, MSI = 526.6).

A.1.2. Agitation

The minimum agitation power required to keep cells suspended in an aerated vessel has been described by Calderbank and Moo Young.¹

The power per unit volume can be expressed as

$$\frac{P}{V} = 4.33 \times 10^{-3} \frac{(g\Delta\rho)^{4/3} \mu_c^{1/3}}{\rho_c^{2/3}} \quad (A.4)$$

where

P/V = agitator power, HP/m³ fermentor volume

g = acceleration due to gravity, cm/sec²

$\Delta\rho$ = density difference of dispersed and continuous phases, gm/cm³

μ_c = viscosity of continuous phase, gm/cm-sec

ρ_c = density of continuous phase, gm/cm³

For an A. phoenicis fermentation this becomes

$$P/V = 4.33 \cdot 10^{-3} \frac{[(980.7)(0.028)]^{4/3} (0.008)^{1/3}}{(0.996)^{2/3}}$$

$$= 0.072 \text{ HP/m}^3$$

The cost of the agitator is included in the fermentor cost calculated above.

A.1.3. Fermentor Temperature Control

The fermentors must be maintained closely at temperatures above room temperature. Assuming that the heat of fungal metabolism is negligible, the temperature can be maintained by replacing heat lost through the walls of the fermentor. When an internal coil is used, this heat can be expressed as,⁵

$$Q = A_e U_s \Delta T \quad (A.5)$$

where

A_e = equivalent surface area of the fermentor

U_s = over-all heat transfer coefficient at the sides of the fermentor

ΔT = temperature difference between inside and outside of tank

The equivalent surface area is,

$$A_e = \frac{U_t}{U_s} A_t + \frac{U_b}{U_s} A_b + A_s \quad (A.6)$$

where

A_t = area of top of fermentor

A_b = area of bottom of fermentor

A_s = area of sides of fermentor

U_t = over-all coefficient at top of fermentor

U_b = over-all coefficient at bottom of tank

The minimum room temperature is assumed to be 18°C. Steam supplies the required heat. The cost of the heating coils is included in the fermentor cost.

A.1.4. Air Compression

The air compressors for aeration of the fermentation broth are single-stage adiabatic compressors with 80 per cent efficiency. The power required for compression is,⁶

$$HP = \frac{1}{0.8} \frac{0.376 K}{1 - K} P_1 Q \left[\left(\frac{P_2}{P_1} \right)^{\frac{K-1}{K}} - 1 \right] \quad (A.7)$$

where

HP = compressor power, horsepower

K = ratio of specific heat of gas at constant pressure to specific heat of gas at constant volume

P_1 = intake pressure, atm.

P_2 = outlet pressure, atm.

Q = volumetric flow rate of gas at inlet conditions, m³/hr

Compressor costs are given by the equation,⁴

$$\text{Cost} = \text{MSI } 2.9 (\text{HP})^{0.75} \quad (A.8)$$

A.1.5. Heat Exchangers

The areas of the heat exchangers in the process were calculated from,

$$A = \frac{Q}{U_o \Delta T_{lm}} \quad (A.9)$$

where

A = heat exchange area

Q = rate of heat transfer

U_o = over-all heat transfer coefficient

ΔT_{lm} = log-mean temperature difference.

The rate of heat transfer, Q, was determined from a heat balance on one of the streams.

$$Q = w C_p \Delta T \quad (A.10)$$

where

w = weight rate of flow

C_p = heat capacity of the stream

ΔT = difference between the temperatures of the stream entering and leaving the exchanger.

Typical values of U_o were used because this is a preliminary design.

For steam/liquid exchange, $U_o = 450 \text{ Btu}/(\text{hr})(\text{ft})^2(^{\circ}\text{F})$; for liquid/liquid exchange, $U_o = 160 \text{ Btu}/(\text{hr})(\text{ft})^2(^{\circ}\text{F})$. The cost of the heat exchangers was found with this equation:⁴

$$\text{Cost} = \text{MSI } 0.696 (A)^{0.64} \quad (A.11)$$

where the heat exchange area is in units of ft^2 .

A.1.6. Low Temperature Evaporation

A ten-fold concentration of the A. phoenicis enzyme was needed. The maximum temperature that the enzyme can withstand without a significant amount of denaturation is 40°C. The temperature of the refrigerant, CaCl brine, determined the temperature of the enzyme solution in the final effect of the multi-effect evaporator. The brine was assumed to enter the final effect at -15°C and to leave at -4°C. With an 11°C heat transfer driving force in each effect, four effects were required. The heat duty, supplied by steam at atmospheric pressure, was therefore assumed to be one-fourth of the amount required to evaporate all the water removed. The cost of the all steel evaporator was determined from a graph of cost versus total heating surface.⁶ The refrigeration cost was calculated from the following equation:⁶

$$\text{Cost} = 1998 (R)^{0.708} \quad (\text{A.12})$$

where

R = refrigeration, tons.

A.1.7. Pebble Mill

The residence time required for a continuous-flow pebble mill was calculated from the batch data (Figure 6.3) to be 46 minutes. The necessary volume was determined from this value and the flow rate by using Equation (A.2) (the residence time is the inverse of the dilution rate). The cost of the pebble mill was read from a plot of cost versus flow rate,⁶ and adjusted for the current market value with the Marshall and Stevens cost index.

A.1.8. Auxiliary Equipment

Pumps. A 30 ft head was assumed in determining the size of the process pumps. The pump costs and power requirements were found from a plot of head versus flow rate.⁶

Tanks. Storage tanks were needed for the product enzyme solution and for the T. viride enzyme solution. A product storage capacity of 2 days was assumed. The tank for the T. viride cellulase feed was large enough to accommodate a 5 day supply in case the cellulase production was interrupted.

Centrifuges, Air Filter and Mycleium Filter. The costs of these equipment were calculated by applying a scaling factor to those previously designed by Wilke and Yang for the T. viride process.⁷

$$\text{Cost}_a = \text{Cost}_b \left(\frac{\text{Capacity}_a}{\text{Capacity}_b} \right)^{0.6} \quad (\text{A.13})$$

The resulting costs were then brought up to date by multiplying by the Marshall and Stevens cost indexes.

Media Sterlizer. The cost of the media sterlizer was determined by applying Equation A.13 to the cost of a sterilizer designed by Cysewski for the ethanol fermentation process.³

A.2. Estimation of Product Cost

A.2.1. Capital Investment

The fixed-capital investment (FCI) is the money required to purchase and install all the necessary parts of the process. It can often be expressed as the product of a factor and the purchased cost

of the major equipment. For this process, the factor was calculated to be 3.8.

The total capital investment (TCI) is the sum of the FCI and the working capital. The working capital is usually about 15 per cent of the TCI.

A.2.2. Total Product Cost

The total product cost (TPC) includes the manufacturing cost and the general expenses. Peters and Timmerhaus have suggested typical values of the per cent contributions of the factors involved in the TPC.⁶ These values were combined into a workable form and are shown in Table A.1.

The utility costs were based on a 8500 hr-year. Unit prices for the utilities are shown in Table A.2. The base labor cost was \$6/hr. It was assumed that 0.25 men per fermentor were required and that one man would run the auxiliary fermentation equipment.

Raw material costs included the cost of the minerals for the medium and the cost of the substrate. The mineral costs are shown in Table A.3. The prices of the various substrates studied are listed in Table 6.1. The substrate used in the processes that were designed was 0.75 g/l Solka Floc plus 0.25 g/l T. viride enzyme. This substrate cost amounts to 0.122 \$/liter of A. phoenicis enzyme solution produced.

Table A.1. Factors contributing to the total product cost

-
-
- I. Manufacturing cost = 1.95 (Operating labor) + 0.1875 (FCI)
+ Utilities cost + Raw materials cost
- II. General expenses = 0.08 (TPC) + 0.05 (TCI)
- III. Total product cost = I + II

FCI = Fixed capital investment

TCI = Total capital investment

Table A.2. Utility rates

Utility	Cost
Cooling water	20¢/1000 gal.
Electric power*	3¢/kw
Steam**	32.5¢/1000 lb.

* Self generated from fuel oil

** Produced from burning of waste ligno-cellulosic materials

Table A.3. Cost of mineral salts for medium

Chemical	Price, ¢/g*	Cost, ¢/l medium
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.030	0.066
Na_2HPO_4	0.029	0.047
NH_3NO_3	0.010	0.007
NaNO_3	0.016	0.057
KNO_3	0.021	0.007
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.019	0.006
Total		0.190

* Chemical Marketing Reporter, May 8, 1978

APPENDIX B. PROCESS DESIGN AND COST
ESTIMATION OF THE POTATO PROCESSING SCHEMES

The design and costing of a process to prepare potatoes for use as a growth substrate was performed to determine the total cost of the potatoes. Two processing methods were examined, grinding of raw potatoes and mashing of cooked potatoes. The material of construction of all equipment is stainless steel to prevent metal contamination of the A. phoenicis growth medium to which the potatoes are added.

B.1. Ground Raw Potato Process

In this process, the potatoes are washed and fed to a rasp which shreds the raw potatoes into small pieces. Water is added and the slurry is run through a hammer mill to further reduce the size of the potato particles. The final suspension (approximately 3 per cent potato) is then pumped to the A. phoenicis process for mixing with the mineral salts and sterilizing. The major pieces of equipment for a scheme that processes 6500 lb/hr of potatoes are listed in Table B.1. The equipment costs were determined from the graphs provided in two major sources, Peters and Timmerhaus⁶ and Zimmerman,⁸ and were adjusted to current prices.

B.2. Cooked Potato Process

In the cooked potato process, the potatoes are washed and sliced for quick cooking. The slices are conveyed continuously through the cooker where atmospheric steam cooks them. A ricer, consisting of a spiral screw conveyor that forces the potatoes through 3/8 inch slots at the discharge end, follows the cooker.

Table B.1. Major items of equipment for the ground potato process (capacity 6500 lb/hr)

Item	Power Requirements	Cost (\$)	Reference
Washer	2HP	8590	(2)
Conveyor	1HP	3800	(2)
Feed hopper		1030	(6)
Rasp	20HP	7210	(8)
Mixer	0.25HP	4010	(6)
Pump	2HP	2020	(6)
Hammer mill	10HP	2510	(8)
Pump	2HP	2020	(6)
Total	37.25HP	31.190	

Water is then added and the potato slurry is pumped to the A. phoenicis process where it is sterilized after addition of the mineral salts.

Table B.2 is a list of the major pieces of equipment needed for a process that handles 6000 lb/hr of potatoes. The equipment used in this process is most often used in food processing. The equipment costs were obtained by applying equation A.13 to the costs described by Claffey for equipment used in a process to make dried potato flakelets.²

B.3. Estimation of Product Cost

The product cost was determined by the method described in Section A.2, using the same utility and labor rates. Table B.3 is a summary of the design bases and total product costs for the two potato processing schemes.

Table B.2. Major items of equipment for the cooked potato process (capacity 6000 lb/hr)

Item	Power Requirements	Cost (\$)
Washer	2HP	8590
Conveyor	1HP	3800
Slicer	3HP	7320
Cooker	7600 lb/hr steam	88,140
Ricer	1.5HP	9680
Pump	10HP	4510
Total	17.5HP 7600 lb/hr steam	122,040

**Table B.3. Summary of design bases and product costs
for two potato processing schemes**

	Ground raw	Cooked
Product, lb/hr dry basis	1350	1250
Total capital investment, \$	139,500	545,500
Raw materials cost, ¢/lb dry basis	13.1	13.1
Total product cost, ¢/lb dry basis	16.8	16.8

APPENDIX REFERENCES

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