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Optically monitoring Baker's yeast (*Saccharomyces cerevisiae*) growing in an air-fluidized/expanded potato starch matrix

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SUMMARY

In order to study cell behavior in solid fermentation processes, model systems using gelatin and starch have been developed to track Baker's yeast growth. The difficulty in estimating the cell concentration within solid materials arises because both the solid material and the cellular material contribute to the measurement (such as optical resistance). In general, however, the two materials cannot be easily separated, hence the need to measure the cells along with the solid supporting material. A simple spectrophotometric method has previously been shown to work well in both aerated submerged batch cultures and aerated static solid cultures. The optical approach is applied here to monitor a more complex solidified system: cell growth in a novel air-fluidized/expanded bed of yeast growing on a starch matrix. Conventional assays for reducing sugar, total extracellular protein, and extracellular lysine were also applied to monitor yeast behavior in this new system.

INTRODUCTION

The objective of our research was a better understanding of the engineering and biochemical relationships of air-fluidized solid fermentation processes. This was achieved by studying the growth of Baker's yeast in and on potato particles. An optical method was developed to monitor the concentration of yeast cells in the solid matrix. Since it is difficult to separate the yeast from the starch, the optical reading was done on the composite particles.

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Previously we have carried out a series of investigations utilizing the so-called "semi-solid" fermentation (SSF) technique in which the growth medium containing the utilizable substrate has been "solidified" by the use of a gelling agent, i.e., the viscous fermentation medium is visually a solid, yet has a very high content of water. This SSF technique was used to study the fermentation of glucose by *Saccharomyces cerevisiae* in Maxon-Johnson medium "solidified" with gelatin, first anaerobically in glass beakers, then aerobically in thin layers on glass microscope slides [10–14].

This present batch study used a gaseous fluidized bed technique in solid fermentation. A fluidized bed

solid fermentation system has been used previously by Moebus and Teuber [8], in work predating 1981, to produce ethanol with solid particles of *S. cerevisiae* in a CO_2-fluidized bed. Akao and Okamoto cultivated *Aspergillus sojae* as early as 1975 in a fluidized bed of wheat bran powder to obtain higher enzyme activities than those obtained in commercial solid culture [1]. Our work differs from the previous studies in that the water level in the semi-solid culture was controlled by the humidity in the air feed, not by the direct addition of liquid water. No stirring device was needed in our fluidized bed fermentor.

MATERIALS AND METHODS

Organism

Baker's yeast (*Saccharomyces cerevisiae*, lot No. Jan. 15–85B) purchased at a local grocery in the form of Fleischmann's brand active dry yeast was used during August, 1985. The inoculum concentration was 3.6 mg dried yeast/g wet potato solids.

Culture medium

Synthetic medium C of Maxon and Johnson (MJ) without glucose was used for the growth medium [7]. For this experiment, concentrated (3.3 ×) MJ medium was used in place of dilute medium to keep the solid substrate within desired water bounds. The culture medium was sterilized with steam at 121°C for 15 min before adding to the solid substrate. Medium was added to give a concentration of 0.15 ml medium/g wet potato solids.

Fermentor and fermentation protocol

A schematic diagram of the fluidized bed fermentor is shown in Fig. 1. Steam was used to sterilize the entire system. Air was filter-sterilized with glass wool, then passed through (at 230 liter/min) a 1-m air saturation column, achieving about 95% saturation at about 50°C. Between 99 and 100% relative humidity was achieved in the in-line separation (middle) column by dropping the temperature to approx. 35°C. A nearly saturated air stream is required to prevent the solid substrate from drying out during the run. The temperature in the fermentation column was controlled at 33 ± 2°C by a thermoregulator located at the top of the 1-m glass fermentation column. The column was equipped with four Teflon plates, each with many large holes (approx. 0.8 cm), at the bottom to permit uniform air flow through the column. A fine grid plate was placed above the bottom, and above the four Teflon air channeling plates, to support the potato solids. The relative humidity of the effluent air stream was determined from the measured wet and dry bulb temperatures. The aerated fluidized bed expanded to about twice the non-gasified volume, both when the bed was relatively dry and when it was mixed with condensed water, hence the usage of the expression "expanded bed" in reference to this process.

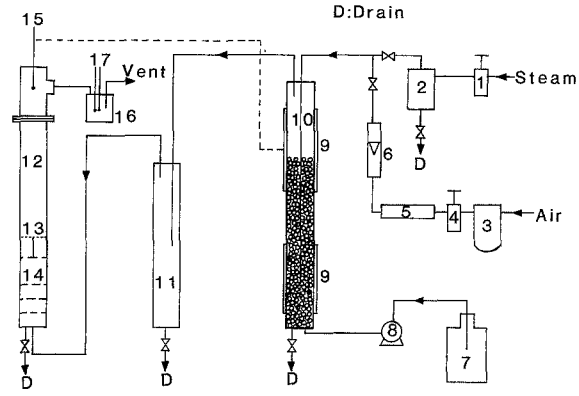

Fig. 1. Schematic diagram of apparatus: 1, steam regulator; 2, steam trap (removes entrained liquid); 3, filter; 4, air regulator; 5, air sterilization filter; 6, air flowmeter; 7, water reservoir; 8, water pump; 9, heating tape; 10, air saturation column with packing material; 11, water separation column; 12, fermentation column; 13, fine grid supporting plate; 14, large hole flow distributor; 15, thermoregulator; 16, humidity measuring chamber; 17, wet and dry bulb thermometers. Here, the "Drain" or "D" represents the effluent port in each of the three columns.

Preparation of solid substrate

Potatoes purchased at a local grocery were steamed at 121°C for 15 min, then homogenized into a paste with a Waring two-speed blender during which time the sterilized culture medium was added. Subsequently, this paste (about 80% water) was well mixed with *Aspergillus oryzae* α-amylase

enzyme (lot No. 31–003, Miles Laboratories). This enzyme was prepared by dissolving the dry enzyme solids in sterilized concentrated MJ medium and added to achieve a level of 0.51 mg per g wet potato. For the 60% water formulation, the mixture (with the enzyme) was prepared by drying at 80°C until it reached a content of approx. 60% water (at approx. 14 h). The 80% water preparation (natural potato water level) was not dried. For that case, the enzyme was added to the starch 12 h before beginning the fermentation.

Part of the enzyme-treated potato paste (+ MJ medium) was used to prepare a standard curve for optical density vs. yeast concentration (see Fig. 2), while the remainder was weighed, mixed with yeast (suspended in concentrated MJ medium), and then placed in the fluidized bed fermentor. The 80.4% water run started with 150 g solids (wet basis), while the 60.5% water run began with 120 g solids (wet basis). An additional 50% initial water concentration run (not pictured) had a total of 100 g solids on a wet basis. The initial pH was about 5 for all runs, uncontrolled for pH, during the fermentation.

Analysis of water content

The moisture content of a sample was determined from a sample portion which was weighed before and after drying at 85 ± 3°C for 30 h. The water content of the fermentation mash increased over the course of the 60% and 80% runs, respectively, from 60.5% to 87.3%, and from 80.4% to 96.2%, primarily because of condensation of water from the air (the glass column temperature was approx. 23°C and the air temperature in the column was approx. 33°C). A third batch fermentation (reported elsewhere) starting at 50% water content also increased to about 90% water over the course of the run. The 60% initial water run started out 'dry' and particles acted as if they were in a gasified solid fluidized bed initially. By 12 h, however, the condensed water changed the characteristics of the mixture such that it acted like an expanded, semi-solid bed. The 80% initial water-case began as an expanded gasified semi-solid bed. While the 50% initial water level run behaved like a fluidized bed initially, the rapid accumulation of condensed water actually led to lower optical density readings than the initial values because of the dilution effect. Since there was little resolution available for these "negative" optical density readings on our spectrophotometer, a different procedure (the sample is diluted to the highest water level and corrected for the changing water content) was used to estimate the cell level for this case than for the 60% and 80% cases. Incidentally, the low water level (less than 80% water) potato samples, in practice, could reflect the potato concentrations due to drying in storage, and these solid fermentation results may offer insight into the fermentation of such stored unprocessed natural materials.

Sampling procedures

At selected times, the upper flange of the fermentation column was loosened and samples of the fermentation mash (FM) were collected with a long-handled spoon. A 0.5-g portion was weighed and mixed with 10 ml deionized water (mixture DFM, the code for the diluted FM). After vigorous vortex mixing, 3.0 ml of the mixture were diluted with 3.0 ml deionized water and the optical density at 595 nm was measured with a Bausch and Lomb Spectronic 20 spectrophotometer using a standard 1.0-cm-diameter glass cuvette to estimate the cell concentration. The blank for the optical density measurement was prepared from the yeast-free enzyme-treated potato mixture by mixing 0.5 g of it in 20 ml deionized water. The use of the optical density-cell correlation curve in Fig. 2 is described more fully in the Results section. It is noted that the expression, "g amylase treated potato", on the abscissa represents wet starch plus amylase, or g sample less yeast cells.

The rest of the mixture DFM (approx. 7 ml) was centrifuged at 3100 rpm (1850 × *g*) for 1 h. The supernatant was stored in a refrigerator at 4°C for the subsequent analysis of reducing sugar and extracellular protein.

Reducing sugars analysis

The Somogyi-Nelson Method [5] was employed in order to determine the reducing sugar concentration of the sample. Particular attention was paid to

the boiling step: vigorous boiling of the water in the water bath was maintained at 10 min, ± 5 s.

Extracellular protein analysis

The Coomassie blue dye-binding method developed by Bradford [3] was used as previously described in Franklin et al. [6]. The method was calibrated for total protein concentration from a standard curve prepared from ruptured Baker's yeast cells.

Extracellular lysine analysis

A 1.0-ml volume of the extracellular lysine sample was analyzed for L-lysine by a microbiological method [4]. The assay bacterium was *Pediococcus acidilactici* (formerly *P. cerevisiae* P-60) (NRRL B-1116). The endpoint was measured at 595 nm absorbance.

RESULTS AND DISCUSSION

Monitoring yeast cell biomass in fluidized/expanded bed fermentation

Calibration curves between the optical density and the amount of yeast imbedded within the potato mash were developed at two different water levels, as shown in Fig. 2. Varying amounts of dried yeast cells were mixed with equal volumes of the same enzyme-potato starch suspension and optical density (O.D.) measurements were taken for the preparations at 595 nm. It is observed that the O.D. increases monotonically with the concentration of yeast in the diluted suspension of the potato-enzyme matrix (in the range 0.0–36.0 mg yeast/g amylase-potato mixture). For each calibration curve, the blank for the O.D. readings is the same yeast-free enzyme-potato suspension to which the weighed yeast was added. It is not obvious, however, why the calibration curve changes for each change of solids/water ratio, since previously, for gelatin solids, the same calibration curve served all solids levels [13].

The effect of starch (potato solids) upon the optical density of yeast cells is clearly a nonlinear interaction, since subtracting off the starch blank

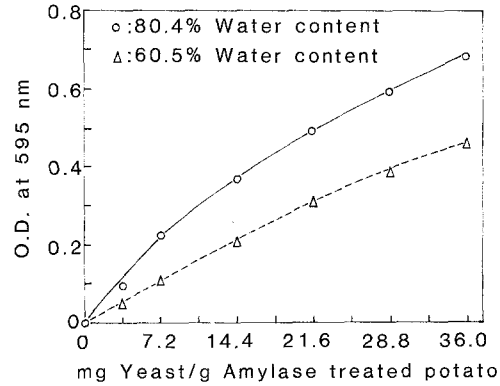

Fig. 2. Standard curves for optical density method for monitoring yeast cell growth in expanded bed cultures.

does not lead to the same calibration curve in both starch level cases. Since the optical density reading drops at a given cell level, as the concentration of starch increases, this seems to indicate that the partially crystalline starch globules mirror the reflected light from the cells back toward the photocell reading the transmission. Since the spectrophotometer reads "transmission" and calculates "absorption" (our optical density, O.D.) as the negative logarithm of that transmittance, the "absorption" (O.D.) decreases as the increased starch crystals enhance the reflected light transmission. A simple (linearized in cell level) model, which describes this second-order interaction as a modification of a "Beers" law type of equation, is therefore of the form:

$$\text{O.D.} = \alpha(X) - \beta(X)\ (\text{starch})$$

Calibrating α and β from the two cell concentration points at (14.4 mg yeast)/(g amylase treated potato) gives $\alpha = 0.035$ O.D. units/mass of yeast cells (in mg) and $\beta = 0.0524$ O.D. units/cell concentration units in mg yeast per g amylase treated potato. Here, in the above equation, the "starch" (potato solids) concentration is expressed as a mass fraction, g starch/g solution, where "solution" represents water plus "starch". The interactive term, $\beta(X)$ (starch), expresses the enhanced transmission effect which, when added to the O.D. (absorbance), gives a constant, at a given cell level, equal to $\alpha(X)$.

The effect of variation in water content upon the growth of yeast in fluidized/expanded bed fermentation

The growth of yeast in the expanded aerated bed at two different water contents is shown in Fig. 3. The maximum yeast cell biomass attained was higher at 60.5% initial water content (39.5% solids) than at 80.4% initial water (19.6% solids), reflecting the fact that the initial "starch" ratio between the 60% and 80% water cases is 1.60 (48 g/30 g). The built-in correction or normalization factor of 1/1.25, the ratio of the initial sample sizes (120 g/150 g), doesn't completely correct for this discrepancy.

The equally rapid yeast growth and, hence, reproduction at the lower water content suggests that either there were no serious diffusional limitations for the nutrients or the metabolizable sugars were extracted from the enzyme-treated potato solids into the liquid phase slowly enough not to cause substrate inhibition, yet rapidly enough to provide sugar at the same rate as the higher water content starting material.

The effect of varying water content upon the concentration of reducing sugars in the fluidized/expanded bed fermentation

The profiles of reducing sugar levels are shown in Fig. 4 for the 60.5% and 80.4% water contents. A certain amount of reducing sugar still remains after the yeast stops growing (1 day). This remaining reducing sugar may be regarded as non-fermentable sugars, such as certain dextrins.

In Figs. 3 and 4 the average cell yield is about 25–30%, based on the consumed amount of reducing sugar, demonstrating aerobic growth, as expected from the high air flow rate and the resultant vigorous mixing of sufficiently small starch particles.

The effect of varying water content upon the production of extracellular protein in the fluidized/expanded bed fermentation

In Fig. 5 a lower initial water content (higher solids level) seems to lead to a modest increase in the rate of production of extracellular protein (the maximum values are higher and the proteins are

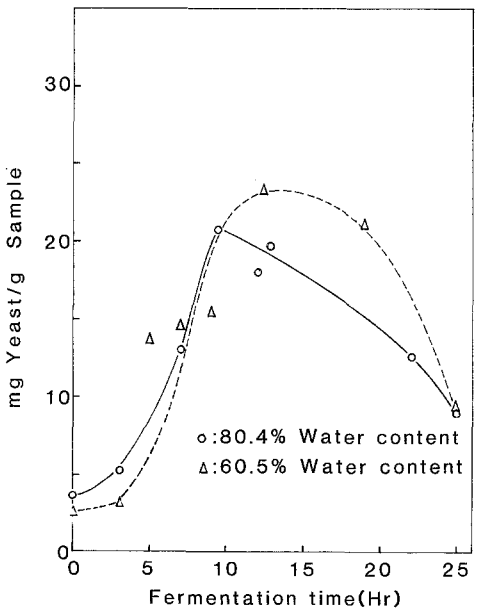

Fig. 3. Yeast cell growth in the expanded bed at two levels of mash moisture.

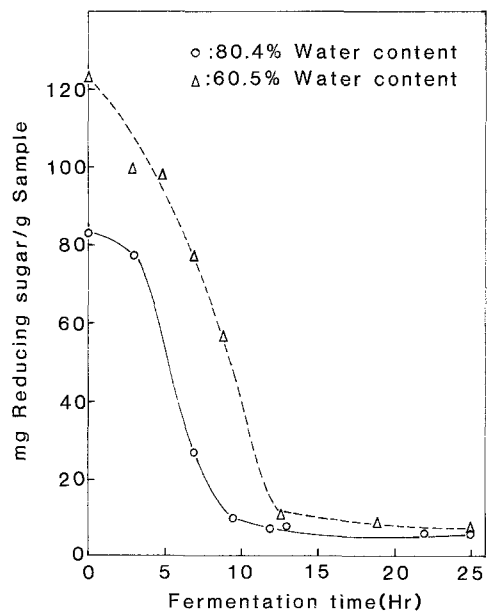

Fig. 4. Reducing sugar level in the expanded bed at two levels of mash moisture.

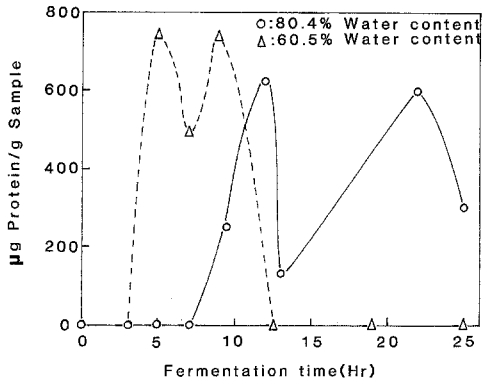

Fig. 5. Extracellular protein production from cells growing in an expanded bed at two levels of mash moisture.

expressed earlier) over that at the higher water content. The previous study by Park et al. [9] indicated similarly increasing production of extracellular proteins resulting from moderate CO_2 activation (at CO_2 addition rates of 57 ml/min) when compared to no gasification of a liquid yeast fermentation culture (600 ml broth volume). Furthermore, as in the CO_2 case, the extracellular protein concentration profile also exhibited double peaking. Additionally, here, a total disappearance of extracellular protein is noted at 13 h for the 60.5% water content case.

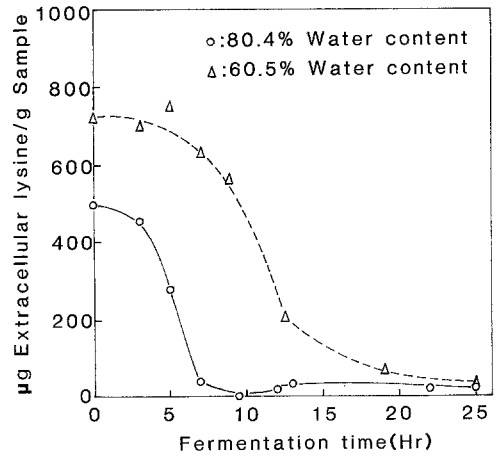

Fig. 6. Extracellular L-lysine production within cells growing in an expanded bed at two levels of mash moisture.

The effect of varying water content upon the production of extracellular lysine in the fluidized/expanded bed fermentation

The measured extracellular L-lysine levels for the two runs are shown in Fig. 6. On an absolute basis the initial points are 85 and 75 mg lysine for the 60.5% and 80% initial water content runs, respectively. This seems to indicate that the initial value is about 2.13 ± 0.38 mg lysine/g starch for the potato yeast mixture on a dry basis (1.75 and 2.5 mg lysine/g starch for the 60.5% and 80.4% cases, respectively). Earlier work [2] indicated that only about 1–2 mg lysine is excreted within the first minute (the "zero time" reading) from the cell for a 3 g yeast cell level (here, approx. 0.2 g yeast) so that the lysine levels shown in Fig. 6 primarily represent the potato lysine concentration. Fig. 6, therefore, depicts the uptake of extracellular lysine during the course of the aerated solid/semi-solid fermentation process. Only for late times (e.g., greater than 10 h for the 80.5% run) do the extracellular lysine levels rise, presumably by excretion from the yeast cells. Such increases may in fact infer moderate cell lysis.

CONCLUSIONS

The feasibility of estimating the concentration of yeast cells optically in a mash growing in a fluidized/expanded bed, without separating the cells from the mash, has been demonstrated. The more usual analytical methods (here, lysine, protein and sugar) also carry over to this fluidized solid system from the standard liquid fermentation processes.

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