Communications to the Editor

*Fermentation of Lactose to Ethanol with Recombinant Yeast in an Immobilized Yeast Membrane Bioreactor**

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A novel concept of membrane bioreactor in which living cells are sandwiched between ultrafiltration (UF) and reverse osmosis (RO) membranes was applied for lactose fermentation to ethanol by genetically engineered yeast cells. The productivity of the Lactophile 13B strains was higher than that of the Lactophile 13D strains. In both cases performance data similar to those for glucose fermentation to ethanol by *Saccharomyces cerevisiae* were obtained. However, the operational stability of recombinant yeast cells was improved in the new bioreactor in comparison to the stability of these cells in a shake flask.
Key words: *Saccharomyces* • lactose fermentation • glucose fermentation • reverse osmosis membrane

Yeasts are used in commercial fermentation to convert carbohydrates to ethanol. General biomass such as agricultural crop residues, municipal, forest, and food wastes are the available carbon resources for fermentation. Cheese whey is a byproduct of the dairy industry, but one-half of cheese whey is not utilized profitably. A great deal of research activity has been directed toward utilizing lactose from cheese whey as a raw material for ethanol production by fermentation.[1-3] Therefore, genetically engineered yeast cells which ferment the lactose portion of the cheese whey as a carbon source were developed for the production of ethanol. One alternative considered was to develop the genetically engineered yeast cells which were able to survive at high ethanol concentrations.[4]

We have recently proposed a new concept for bioreactors in which living cells are sandwiched between an ultrafiltration (UF) membrane and a reverse osmosis (RO) membrane. The UF membrane separates the cells from the feed volume, while providing free passage for all nutrients to the cell mass below. The RO membrane, while immobilizing cells, also helps in separating the nutrients (lactose and salts) from the product stream preferentially. This improves product purity and concentration. The permeate disengages at the bottom surface of the RO membrane and is collected below. The above reactor concept has been tested for the fermentation of glucose to ethanol by *Saccharomyces cerevisiae*.[5]

The objective of this work is to test the applicability of the new reactor concept for the genetically engineered yeast cells. The stability of the cells in the sandwiched bioreactor is also investigated.

MATERIALS AND METHODS

Strain Development

Two kinds of transformant strains were developed to convert lactose into ethanol by inserting plasmid pSH096 and pSH206 into a yeast integrating vector and *S. cerevisiae* DBY745, respectively.

Plasmid pSH096 was constructed by isolating lactose utilization genes from *Kluyveromyces lactis* and then inserting that DNA into a yeast integrating vector, pRY296. Plasmid pSH096 contains the lactose utilization genes LAC4, LAC12, and LAC13 from *K. lactis;* a region of the HO (homothallism) gene; a region containing the yeast CRC1 (iso-1-cytochrome C) promoter; the G418^r gene; an *ori* (origin of replication) region; the pBR322-derived *amp*^{r} (ampicillin resistance) gene; and a second HO region. The strains transformed were DBY745. Transformants of the strains grew weakly on minimal lactose medium, and untransformed strains were unable to grow on lactose.

The other plasmid pSH206 contains regions of the naturally occurring yeast 2-μm circle for autonomous replication, including REP1, FLP, REP2, and yeast origin of replication. In addition, pSH206 contains the regions of *E. coli* plasmid pBR322 and LEU2-d gene. Plasmid pSH206 was constructed as follows. First, the SmaI site was modified by insertion of an 8-bp SalI linker to give pSH200. Next, the 10.5-kb XhoI fragment from pSHL6 that contains LAC4, LAC12, and LAC13

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was ligated into the SalI site of pSH200 to give pSH206. Then, pSH206 was transformed into *S. cerevisiae* DBY745 (α-adel-100, leu2-3, leu2-112, and ura3-52).

Plasmid pSH206 differs from pSH096 primarily in that pSH206 is capable of autonomously replicating in constitutively high copy number in yeast cells. Replicating plasmids (multicopy plasmids) often have the disadvantage of being unstable, so they are readily lost from a population of cells in the absence of specific selection for the plasmid. Two recombinant yeast strains, called Lactophile 13B (13B) and Lactophile 13D (13D) were developed. Lactophile 13B contains an integrated plasmid, and 13D contains an autonomously replicating plasmid.

Culture Maintenance and Characterization

The culture was first streaked and restreaked on minimal agar plate. The dark-blue colonies were obtained after incubation of the plates, which means that the culture is pure and possesses the ability to hydrolyze 4′-bromo-5′-chloro-3′-indolyl-β-D-galactoside (X-gal) due to its β-galactosidase activity. Slants were prepared by pouring approximately 20 mL of minimal agar containing X-gal. Blue colonies from the minimal agar plate were stored in a refrigerator at 4°C after they were incubated for about three days at 30°C.

Culture Media

Minimal medium was used for selection of the strains, and enriched medium was used primarily in the making of solid media for strain storage on slants and plating of reactor samples. Minimal medium components were yeast extract nitrogen base without amino acids (Difco 0919) of 0.67% (w/v) and lactose (reagent grade) of 2% (w/v). Enriched medium components are 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) lactose.

The solid culture medium also consisted of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) lactose, and 2% (w/v) agar. The solid culture medium was used in the isolation of the recombinant yeast strain and in the testing of phenotypical characteristics of the culture in free and immobilized bioreactor systems. In addition to the original solid culture medium constituents, both 40 mg 4′-bromo-5′-chloro-3′-indolyl-β-D-galactoside (X-gal)/L and 70m*M* potassium phosphate buffer, pH 7, were added after autoclaving the medium for the indicator plates. Enriched medium was used for the fermentation medium, but the lactose concentration was changed to 12.5% for the batch-type sandwiched membrane bioreactor.

Immobilization Procedure

The same immobilization technique was used as reported in the previous work.[5]

Reactor Description and Operation

Flask Culture

For the batch experiment in shake flasks, one colony from a slant culture was inoculated into a 300-mL Erlenmeyer flask containing 100 mL enriched medium with 5% lactose initially. Cells in the exponential growth phase, which are usually 14–20 h old, have been used to check stability of the two recombinant yeast strains. To avoid heterogeneity, all the cells from the flask were collected after vigorous shaking. Then, the total cells were measured by a Gilford spectrophotometer (Stasar II model) at a wavelength of 660 nm, and the medium was collected for the analysis of product after filtering the cells. The filtrates were stored in the refrigerator for product and sugar assay. Finally, a known amount of cells from the shake flask was transferred into the fresh flask for the next generation culture.

Batch Type Sandwiched Membrane Bioreactor

Both the batch type bioreactor and the bioreactor operation were described in our previous work in detail.[5] Briefly the membrane sandwich was placed between the flanges at the bottom of a cylindrical reactor. Then, the feed solution containing lactose and other nutrients was loaded and pressurized with nitrogen gas. The product stream was collected over time intervals ranging 20–24 h based on the permeation rate.

Free-Cell Concentration

The free-cell concentration method is the same as that presented in the previous report.[6]

Ethanol and Lactose Concentration

Ethanol concentration was measured by gas chromatograph (Hewlett–Packard 5880A Series) equipped with a 1.82-m glass column. The column was filled with 5% Carbowax 20 M and 80/120 Carbopak B-AW. The lactose concentration was determined by the method suggested by Asensio et al.[7]

Fraction of Plasmid-Containing Cells

The occurrence of plasmid segregational instability in free and cell immobilized bioreactor systems was monitored by evaluating the phenotypical characteristics of the cells. Plating techniques were used for this purpose. Prepared plates were then incubated for 3 days at 30°C and the blue-and-white colonies were counted for the fraction. The plate medium used was enriched media agar plate with X-gal solution.

The recombinant cells possess a *Lac*$^+$ phenotype giving rise to blue colonies, while the *Lac*$^-$ wild-type cells produce white colonies. The recombinant cell fraction was therefore obtained from the ratio of blue colonies

to the total number of colonies on the rich media agar plates. This agar plates method had two distinct advantages.[8,9]

- Both recombinant and wild-type cell populations are counted from the same plate, giving much greater accuracy in calculating the recombinant cell fraction.
- There is no ambiguity in discerning between wild-type and recombinant cells on the same plate.

RESULTS AND DISCUSSION

An important consideration in the analysis and comparison of free- and immobilized-cell bioreactor systems is the ability of each bioreactor type to minimize the deleterious effects of plasmid segregational instability in the absence of any selection pressure. To establish the stability of cell and product formation for free recombinant yeast cells, a cell culture experiment was performed in shake flasks using enriched media.

Figures 1 and 2 show the fraction of recombinant strains and specific productivity, respectively. For the case of 13B cells, the fraction does not change and the average specific productivity was 7.74×10^{-11} g ethanol/cell h. This indicates that after 82 generations (163 h), 100% of the cells still contain plasmid. However, only 53% of the 13D cells contain plasmid after 81 generations (187 h). This represents a substantial loss in the recombinant cell fraction.

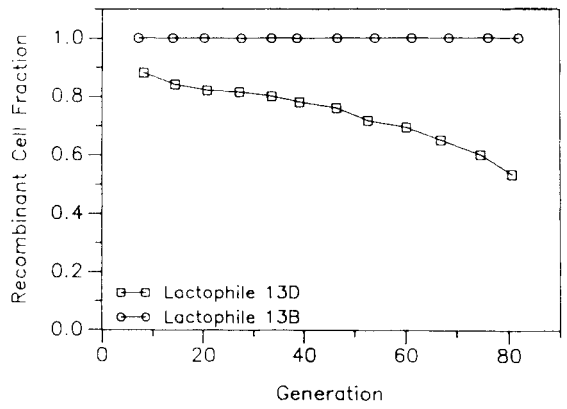

Figure 1. Results of stability experiments in shake flasks.

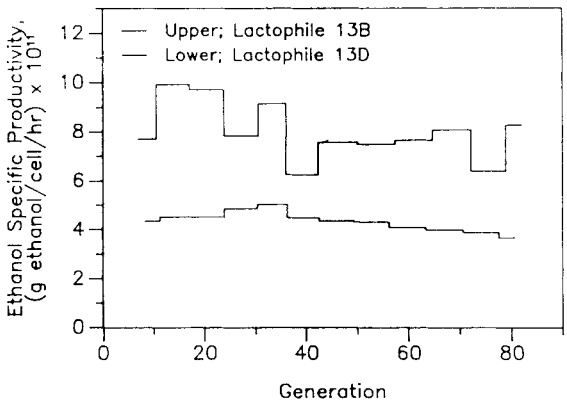

Figure 2. Ethanol specific productivity vs. generation.

After running the batch type membrane bioreactor, 13D recombinant cell stability was compared to the stability in the flask. For the case of the bioreactor, 76% of the cells in the final stage of the bioreaction contain plasmid after an 8-day run. This is a significant improvement over the free-cell flask system. Difference in growth rates between free and immobilized cells decreases the rate of loss of plasmid-containing cells, improving the time profile of the bioreactor. The results from the experiment provide that the immobilized bioreactor system improves the operational stability of recombinant yeast cell fermentations.

Two typical results of the reactor performance are shown in Figures 3 and 4. In both figures there is a maximum in the permeate lactose concentration in the beginning of the run. The permeate ethanol concentration, on the other hand, increases gradually as lactose concentration decreases. The maximum in lactose concentration observed in the beginning of each run was probably due to two reasons. First, a lag was present due to sudden change in environment for the organism in terms of mechanism of metabolism, nutrients media, and cell concentration (the sudden crowding on transfer

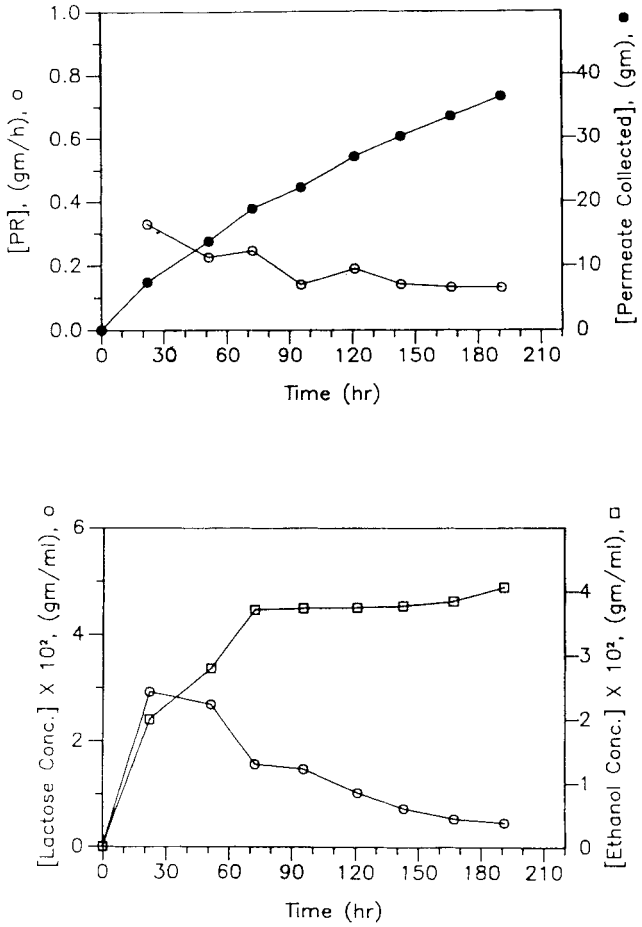

Figure 3. Some performance data of sandwiched bioreactor using strain 13B; operating pressure was 2068 kPa (= 300 psig); initial cell number was 1.0×10^9; initial feed lactose concentration was 12.5 wt %.

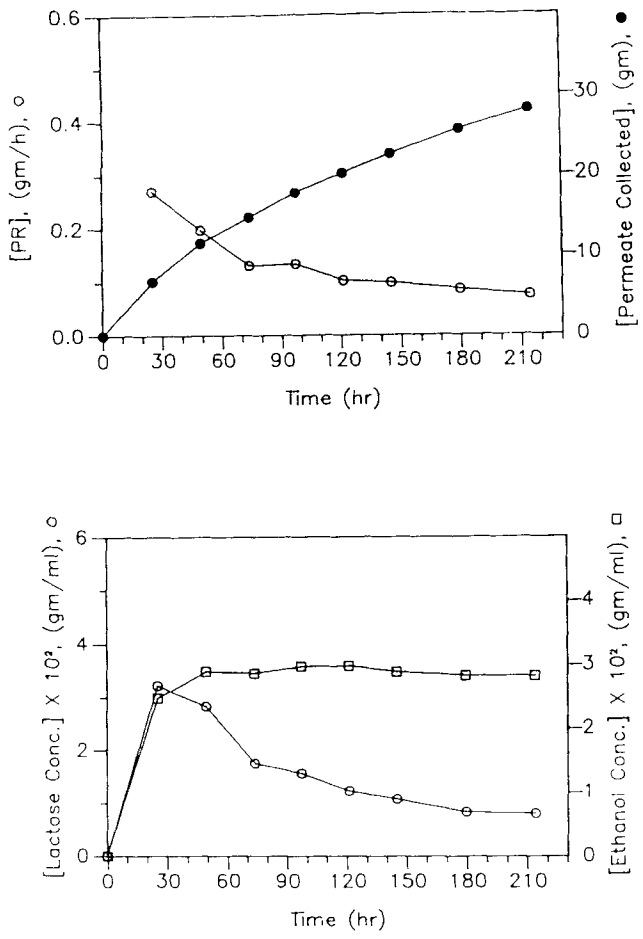

Figure 4. Some performance data of sandwiched bioreactor using strain 13D; operating conditions are the same as in Figure 3.

to the microporous filter) and, second, the fact that the membranes were not pressure treated before their use in the experiment and hence they will not separate effectively until an equilibrium pore size reached. A gradual decrease in the permeation rate has also been observed. This is probably due to compaction of both living cell layers and the membrane polymer.

Comparing two different recombinant strains, 13B shows an ethanol productivity higher than that of 13D, which result coincides with that obtained from the shake flask experiment. The higher growth rate of 13B strains was also confirmed by 13B's final cell number (6.74×10^9) which was higher than that of 13D (5.42×10^9). Thus, we may conclude that 13B strains grow faster and are more stable than 13D strains under the same circumstances. It has been found that the sandwich whole-cell membrane bioreactor system improves the operational stability of recombinant yeast cells for the production of ethanol from lactose.

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