Configuration of a Bioreactor for Milk Lactose Hydrolysis

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ABSTRACT

Permeabilized microbial cells can be used as a crude enzyme preparation for industrial applications. Immobilization and process recycling can compensate for the low specific activity of this preparation. For biomass immobilization, the common support is alginate beads; however, its low surface area and the low biomass concentration limit the activity. We here describe a biocatalyst consisting of a paste of permeabilized *Kluyveromyces lactis* cells gelled with manganese alginate over a semicircular stainless steel screen. A ratio of wet permeabilized biomass to alginate of 50:4 (wt/wt) resulted in a paste with maximum immobilized β -galactosidase activity and maximum gel biomass retention. The biocatalysts retained activity better when stored in milk at 4°C than in 50% glycerol. The unused biocatalysts stored in milk did not lose activity after 50 d. However, repeated use of the same biocatalyst 40 times resulted in almost 50% loss of activity. A bioreactor design with two different conditions of operation were tested for milk lactose hydrolysis using this biocatalyst. The bioreactor was operated at 40°C as packed bed or with recirculation, similar to a continuous stirred tank reactor. The continuous system with recirculation resulted in 82.9% lactose hydrolysis at a residence time of 285.5 min (flow of 2.0 ml/min), indicating the potential of this system for processing low lactose milk, or even in processing other substrates, using an appropriate biocatalyst.

(**Key words:** β -galactosidase, *Kluyveromyces lactis*, bioreactor design, milk hydrolysis)

Abbreviation key: CSTR = continuous stirred tank reactor, PFR = plug flow reactor.

INTRODUCTION

Permeabilized whole microbial cells can be used as a crude enzyme preparation for bioprocesses requiring

lower investment cost. *Kluyveromyces lactis*, a natural yeast of the dairy environment when grown in whey permeate, synthesizes a very active intracellular β -galactosidase (Brandi et al., 1996). An economic immobilization technique and a process design can compensate for the low specific activity of the crude enzyme preparation. Currently, a viable option for immobilization is entrapment in alginate gels (Klein and Wagner, 1993). However, the common spherical bead configuration and bead size results in low surface area, while the interior of the bead has limited mass transfer to and from the reaction site (Passos and Swaisgood, 1993). Furthermore, beads cannot be prepared with high biomass concentrations because the biomass suspension cannot be too viscous to be dropped into the ionotropic solution. All these factors contribute to the low activity of the biocatalyst.

For industrial processes, bioreactors operated in continuous flow are desirable to allow automation, uniformity, and economy (Swaisgood, 1991). In the case of an immobilized biocatalyst, it is important to define the conditions for maximal activity and stability. In this investigation, we describe a bioreactor with higher surface area and higher biomass loading capacity prepared by immobilizing a paste of permeabilized yeast cells. We also present a configuration and an assay of a column bioreactor for lactose hydrolysis that can operate as a plug flow reactor or as a continuous stirred tank reactor, by recirculating the substrate through the column.

MATERIALS AND METHODS

Biomass Production in Cheese Whey

A wild strain of *K. lactis* isolated from Brazilian regional dairy factories was precultured and cultivated in whey permeate (10,000 Da retention membrane) with no nutrient addition. The culture was incubated as a batch, in a shaker at 30°C and 200 rpm, with an initial OD₆₀₀ between 0.08 and 0.1. The maximum specific growth rate of the culture, $\mu_{\max}$, was determined by linear regression of the plot, Ln OD₆₀₀ versus time (h), during the initial exponential growth phase. The generation time during the exponential

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growth phase, t_G , was determined using the relation: $t_G = 0.693/\mu_{\max}$. At maximum β -galactosidase activity, when the culture reached the end of log phase (16 h culture and an $OD_{600} = 2.6$), the biomass was harvested by centrifugation for subsequent utilization. During the measurement of cell growth, all samples with OD_{600} higher than 0.4 were properly diluted to the linear OD range and correction made for dilution.

Biomass Permeabilization and Immobilization

The harvested biomass was permeabilized in 50% ethanol for 5 min and then centrifuged. In preliminary essays, the loss of viability of the cells after permeabilization was tested by plating a sample in yeast peptone dextrose. A paste of 4% sodium alginate and 50% permeabilized biomass was immobilized on the surface of stainless steel screens, as a calcium or manganese alginate gel, by submerging the screens covered with the biomass paste into a 1 M calcium or manganese chloride solution. The immobilized biocatalysts were stored in UHT heat processed milk at 4°C, after determining that the biocatalyst activity was greater and better conserved under these conditions than in 50% glycerol.

Biocatalyst Optimization

To obtain a biocatalyst with maximum immobilized β -galactosidase activity and maximum gel biomass retention, the following ratios of biomass to alginate were tested: 1:5, 1:7.5, 1:10, and 1:12.5 (g/g) in 1.0 ml of sterilized water and the following ratios of alginate to biomass: 1:50, 1:25, 1:16.7, and 1:12.5 in 1.0 ml of sterilized water. Milk samples were observed under the microscope to verify any biomass released from the screen support. Both calcium and manganese cations were tested to find the best gelling agent. All optimization experiments were conducted using a semicircular stainless steel screen support of 12.5 cm² prepared as previously described, and the hydrolysis was conducted in a batch process. To define the effect of temperature on enzyme activity, the reaction kinetics were studied at temperatures ranging from 4°C to 70°C. For thermal stability studies, the support was first immersed in milk at temperatures from 4°C to 70°C for 2 h, and then the activity was measured at the temperature determined for maximum activity. The half-life of the biocatalyst was determined by the number of reutilizations resulting in a reduction of 50% of initial activity. Before each reutilization the biocatalyst was stored for 6 h in milk, at 4°C. The biocatalyst activity was evaluated by measuring the amount of lactose hydrolysis at 40°C after a 30-min reaction. For

this, glucose concentration was measured by the glucose-peroxidase enzymatic assay (GOD-PAP, Merck, Darmstadt, Germany), using the initial-rate technique. The activity was represented by the inclination of the linear regression of glucose concentration versus time.

Bioreactor Configuration

A bioreactor was designed using the biomass immobilized on a semicircular screen surface in a manganese alginate gel (Genari et al., 2000). The screens were stacked in a glass column with circumferential cuts placed alternately to permit continuous serpentine flow of milk through the column, thus guaranteeing maximum contact between the milk and the biocatalyst surface during the process. The space between the screens was 4.0 mm to allow milk to pass through the entire surface of the biocatalyst units. The bioreactor was operated either as plug flow reactor, with residence time of 130 to 52 min (flow rates between 2.0 ml/min and 5.0 ml/min), or as a continuous stirred tank reactor by recirculating milk through the column from a reservoir at a flow rate of 0.5 L/min (Figure 1). The continuous stirred tank reactor (CSTR) process was tested with residence times of 571 min to 95.2 min, corresponding to flow rates between 1.0 and 6.0 ml/min, respectively. Under steady-state conditions for both types of operation, the percentage of lactose hydrolysis was determined by measuring the glucose concentration as previously described.

RESULTS

Biomass Production

The kinetics of *K. lactis* growth cultured in whey permeate and β -galactosidase activity are presented in Figure 2. The maximum specific growth rate, $\mu_{\max}$, was 0.18 h⁻¹ and the maximum OD_{600} was 6.7. The generation time was estimated as 3.9 h. The β -galactosidase activity was associated with cell growth until it reached the late exponential phase, after 15-h incubation, at an OD_{600} of 2.68, which was just before four complete generation times. The maximal peak for β -galactosidase activity of *K. lactis* batch cultures was obtained at the end of the exponential growth phase. This period lasted less than 3 h and can be monitored by the OD_{600} of the culture, which should be around 2.5, if inoculated with an activated culture at an initial OD_{600} of 0.1. To obtain a biomass with maximum β -galactosidase activity, the cells were harvested at that time.

Figure 1. Diagram of the column bioreactor designed as a continuous stirred tank reactor (A) or as a plug flow reactor (B), with the following parts: (1) column bioreactor; (2) milk feedstock; (3) stirring mask; (4) hydrolyzed milk; (5) water 40°C; (6) peristaltic pump; (7) air filter; (8) transversal section of the bioreactor column.

Biocatalyst Optimization

To maximize biocatalyst activity, several ratios of wet permeabilized biomass to alginate were assayed and the choice was 50:4 (wt/wt), which resulted in a paste with maximum immobilized β -galactosidase activity and maximum gel biomass retention. Microscopic analysis showed that the permeabilized cells were not released from the support into the milk. Samples of milk (1 ml) were pour plated in yeast peptone dextrose. No yeast colonies grew on the agar surface, assuring that the yeast cells were completely nonviable and immobilized. The effect of manganese or cal-

Figure 2. Kinetic growth (Δ) and β -galactosidase activity ($\square$) of *Kluyveromyces lactis* in whey permeate batch culture. The cell growth was followed by OD₆₀₀ and the β -galactosidase activity in mM of ONP (ortho-nitro-phenol) liberated from the hydrolysis of ONPG (ortho-nitro-phenyl-galactosideo) by β -galactosidase divided by biomass. Data are averages from three experiments.

cium alginate on the biocatalyst activity is given in Figure 3. The activity, represented by the slope of the line, was 1.4 mM glucose/min and 0.75 mM glucose/min, for manganese or calcium alginate, respectively. The data showed that the activity using manganese

Figure 3. Time course of lactose hydrolysis by the biocatalyst gelled with calcium ($\circ$) or manganese ($\bullet$) alginate over a semicircular surface screen of 12.5 cm², in a batch process at 40°C. The biocatalyst activity (mM glucose/min) is the inclination of the line estimated by linear regression (line). Data are averages from three experiments.

Figure 4. Activity of the biocatalyst stored in skim milk at 4°C as a function of number of reutilizations. A semicircular surface screen of 12.5 cm² was used as support and before each reutilization the biocatalyst was stored for 6 h. Data are averages from four experiments.

Figure 5. Effect of reaction temperature on activity of the biocatalyst. Activity was evaluated by measuring the amount of lactose hydrolysis at each temperature, using a semicircular support surface of 12.5 cm², in a 30-min batch process. Data are averages from three experiments.

alginate gels was almost twice the activity in calcium alginate gels.

The best solution in which to store the biocatalyst units was UHT heat processed milk at a temperature of 4°C. Fresh biocatalyst stored in milk did not lose activity after 7 wk of storage. After this time, the activity was 1.6 mM glucose/min, the same activity of the first day. Weekly analyses showed a variation from 1.4 to 1.7 mM glucose/min (data not shown). Operational stability studies indicated that reusing the biocatalyst units 40 times resulted in 50% loss of activity (Figure 4). The influence of reaction temperature on biocatalyst activity and its thermostability are presented in Figures 5 and 6, respectively. The optimal temperature for the reaction was between 40 and 45°C. Temperatures of reaction higher than 50°C or lower than 10°C reduced the activity to about 50% (Figure 5). The test of thermostability showed that 2-h storage in milk, at temperatures higher than 45°C, will result in drastic reduction of enzyme activity (Figure 6). Activity was not detected after 2-h storage at 68°C.

Bioreactor Configuration and Operation

Plug flow reactor (**PFR**) and CSTR designs were tested. Continuous milk lactose hydrolysis in the system without recirculation, resembling a PFR, resulted in a lower hydrolysis rate compared with the system with recirculation, which functions like a CSTR. The productivity per unit surface area or biomass is more than two times higher with recirculation (CSTR) than without recirculation (PFR), as presented in Figure 7.

Increased rates of lactose hydrolysis by reduction of the flow rate, represented by the slope in Figure 7, were similar for both reactor configurations. However, the amount of hydrolysis was higher in the CSTR, ranging from 61.7 to 99%, while hydrolysis for the PFR ranged from 31 to 50%. For a specific flow rate of 30 ml·min⁻¹·m⁻², 32% of the lactose was hydrolyzed in the PFR configuration and 80% in the CSTR. The effect of residence time on the percentage of lactose hydrolysis is presented in Figure 8. According to the data,

Figure 6. Effect of temperature on biocatalyst activity after 2-h of storage in milk. After storage, the activity was evaluated by measuring the amount of lactose hydrolysis at 40°C, in a 30-min batch process. Data are averages from three experiments.

Figure 7. Effect of flow rate per surface area of biocatalyst on milk lactose hydrolysis with the bioreactor operated as a continuous stirred tank reactor (○) or plug flow reactor (●) at 40°C.

the percentage of hydrolysis was higher for the CSTR configuration for all the residence times tested. At a residence time of 100 min, the degree of hydrolysis was 41% for the PFR and 64% for the CSTR.

DISCUSSION

Maximal β -galactosidase activity of *K. lactis* in batch culture at the end of the exponential growth phase was previously observed (Dickson and Martin, 1980; Brandi et al., 1996). The decrease in activity following maximum activity is not well explained, and could be due to several reasons: increasing concentration of an

Figure 8. Effect of residence time on milk lactose hydrolysis with the bioreactor operated as a continuous stirred tank reactor (○) or plug flow reactor (●) at 40°C.

inhibitor, such as glucose, or exhaustion of an activator, such as lactose or even some proteolysis of the enzyme. Dickson and Martin (1980) reported a decrease in β -galactosidase in the stationary phase of *K. lactis* culture even when lactose was detected in the culture medium. The maximal enzyme activity per unit of cell could be a consequence of reaching a balance between a precise concentration of the inducer (lactose or a derivative) relative to the repressor (glucose). The physiological state of the culture rather than its culture time should be monitored. According to Dickson and Martin (1980), a precise number of generation times of the batch culture should be necessary to harvest the cell at its maximal β -galactosidase activity.

Several authors have assayed cell permeabilization with different solvents, and ethanol was suggested by many (Decleire and Van Huynh, 1987; Champhver et al., 1988; Siso et al., 1992; Flores et al., 1994). In our study, ethanol was confirmed to be very adequate and efficient. The use of whole cells whose membranes have been permeabilized to permit access of a substrate to cytoplasmic enzymes of interest has been demonstrated (Decleire and Van Huynh, 1987; Siso et al., 1992). Permeabilized whole cells have been claimed to have an advantage over more pure enzyme preparations due to increased stability, maintained by the intracellular environment; however, the low specific activity of the desired enzyme must be compensated. This can be done by immobilizing high biomass concentration. Immobilization potentially permits recycling of the enzyme preparation, increasing the volumetric productivity of the process. As a matrix, alginate gels allow high biomass retention but gelling in a spherical shape cannot be prepared with high biomass concentrations because the biomass suspension cannot be too viscous to be dropped in a divalent chloride solution.

The flat surface configuration designed here allows increased biomass immobilization. Also, the diffusion limitation is reduced. Although manganese is last on the cation alginate affinity scale (Salter and Kell, 1992) at the same concentration of calcium (2% wt/vol), its gel was stable and consistent. Therefore, manganese is the best choice as an ionotropic cation compared with the common calcium, which has been reported to inhibit β -galactosidase activity, while manganese favors activity and stability (Richmond et al., 1981; Voget et al., 1994). Although milk has a high concentration of calcium and potentially could inhibit β -galactosidase or replace manganese in alginate matrix, it seems not to have occurred here. According to Garman et al. (1996), the calcium in milk is maintained strongly attached to protein, thus it is only in whey, where calcium is free, that it should be removed

to prevent enzyme inactivation. The manganese helps to create an appropriate ionic environment for the enzyme.

Barrozo et al. (2000) observed that the stability of the biocatalyst could be maintained for 5 or more days when stored in 50% glycerol between uses. However, the work presented here indicates that storing the biocatalyst in milk further improves its stability. The stability of the biocatalyst is a result of the intracellular environment, which protects the enzyme; in addition, milk and its colloidal properties helps to maintain enzyme conformation. Furthermore, it is very convenient, is highly available, and has a low cost. Assuming good process techniques, the storage milk can be incorporated into the final product. The stability exhibited by the biocatalyst assures its potential applicability for lactose hydrolysis. The low activity exhibited by the crude preparation of the enzyme, and accentuated by the diffusion limitations typical of gel entrapment, can be compensated by the capability of biocatalyst reuse. A limiting factor is the susceptibility to formation of a protein or lipid film over the screen surface as well as potential microbial contamination. This requires routine cleaning and preservation of the biocatalyst using components that do not denature the enzyme, depolymerize the alginate gel, or interfere with product quality.

The operation of the bioreactor at 40°C assures maximum activity but favors germination of spores remaining from a previous thermal process. Although 4°C results in a biocatalyst activity about one third its optimum at 40°C, it may be more desirable for processing a pasteurized milk to avoid contamination. A thermostable enzyme that functions at 70°C has been reported (Saito et al., 1994) and would seem to be very convenient for simultaneously pasteurizing milk and hydrolyzing lactose. However, this enzyme comes from *E. coli* and thus requires an extra pure preparation to be accepted by the food industry. In this study, enzyme activity decreased substantially at temperatures higher than 50°C. In fact, enzyme activity was reduced after being stored for 2 h at temperatures above 45°C (Figure 6), and activity was not detected after 2-h storage at temperature of 68°C.

In spite of some drawbacks, such as microbial contamination and colloidal deposition over the biocatalyst surface, immobilization has great potential in food bioprocessing. An important factor favoring the higher efficiency of the system with recirculation is the higher milk velocity through the column, which imparts a turbulent flow ($Re = 479.000$) that favors mass transfer. Recirculation also increases volumetric productivity in a similar system. Siso and Doval (1994) reported 80% hydrolysis with a flow of 6 ml/min and a 137-ml

work volume. Tomaska et al. (1996) obtained the same percentage of hydrolysis with a flow of 0.3 ml/min and a 25.45 ml work volume. This indicates that the results presented here can be improved. Obviously a more pure enzyme preparation would improve productivity, but also the system may be further improved by increasing the size of the column and reducing the space between the biocatalyst units. The biocatalyst preparation and the bioreactor configuration described here offer some advantages compared with the conventional methods because it allows a higher concentration of biomass to be immobilized and a larger surface area per unit mass. Such a system should be useful for hydrolysis of whey lactose to give a more fermentable substrate.

CONCLUSIONS

This study showed that a ratio of wet permeabilized biomass to alginate of 50:4 (wt/wt) resulted in a paste with maximum immobilized β -galactosidase activity and maximum gel biomass retention and that the hydrolysis activity using manganese alginate gels was almost twice the activity using calcium alginate gels. The best solution in which to store the biocatalyst was UHT heat processed milk at a temperature of 4°C. Fresh biocatalyst stored in milk did not lose activity after 50 d of storage. The optimal reaction temperature was between 40 and 45°C and the biocatalyst did not lose activity after being stored for 2 h in milk at temperatures from 4 to 45°C. Finally, the experimental results indicated that, for the conditions tested, the CSTR design presented better performance when compared with the PFR design.

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