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Hydrolysis of Lactose by β-Glycosidase CelB from Hyperthermophilic Archaeon *Pyrococcus furiosus*

Comparison of Hollow-Fiber Membrane and Packed-Bed Immobilized Enzyme Reactors for Continuous Processing of Ultrahigh Temperature-Treated Skim Milk

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Abstract

Recombinant β-glycosidase CelB from the hyperthermophilic archaeon *Pyrococcus furiosus* was produced through expression of the plasmid-encoded gene in *Escherichia coli*. Bioreactor cultivations of *E. coli* in the presence of the inductor isopropyl-1-thio-β-D-galactoside (0.1 m*M*) gave approx 100,000 U of enzyme activity/L of culture medium after 8 h of growth. A technical-grade enzyme for the hydrolysis of lactose was prepared by precipitating the mesophilic protein at 80°C. A hollow-fiber membrane reactor was developed, and its performance during continuous processing of ultrahigh temperature-treated (UHT) skim milk at 70°C was analyzed regarding long-term stability, productivity, and diffusional limitation thereof. CelB was covalently attached onto Eupergit C in yields of 80%, and a packed-bed immobilized enzyme reactor was used for the continuous hydrolysis of lactose in UHT skim milk at 70°C. The packed-bed reactor was ≈10-fold more stable and gave about the same productivity at 80% substrate conversion as the hollow-fiber reactor at 60% substrate conversion. The marked difference in the stability of free and immobilized CelB seems to reflect mainly binding of the soluble enzyme to the membrane surface of the hollow-fiber module. Under these bound conditions, CelB is essentially inactive. CelB is essentially inactive. Microbial contamination of the reactors did not occur during reaction times of up to 39 d, given that UHT skim milk and not pasteurized skim milk was used as the substrate.

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Index Entries: Reaction engineering; diffusional reactor; extremozyme; lactose process.

Introduction

Enzyme Reactors for Hydrolysis of Milk and Whey

The costs of the β-hydrolase employed in lactose conversion are relatively high compared with the costs of biocatalysts in other classic enzyme-catalyzed processes such as the hydrolysis of starch and sucrose or the isomerization of glucose. Estimates of approx 200 US $/ton of product have been quoted for the costs of the lactose-hydrolyzing enzyme *(1)*. Considering the low value-added margins of a process that converts lactose into its constituent monosaccharides, glucose and galactose, there is a strong economic requirement for recycling the enzyme and making it available for multiple rounds of substrate hydrolysis. Within the limited range of technical options available for commercial practice, a surprisingly large number of immobilized enzyme reactors and ultrafiltration bioprocess reactors have been developed. Several reactor designs have been commercialized and carefully optimized regarding long-term performance during enzymatic conversion of different lactose-containing substrates. These scientific and technologic developments in the field have been reviewed in the literature *(2–4)*. The use of ultrafiltration technology pertains mainly to the deproteination of the substrate solution before the enzymatic hydrolysis step takes place, and the immobilization of the soluble β-hydrolase on one side of the membrane of a hollow-fiber module, thereby avoiding the physical contact of whey or milk proteins and the biocatalyst *(2)*.

Thermostable β-Glycosidases for Lactose Hydrolysis

The advantages of thermostable β-hydrolases lie not only in a reduced rate of thermal denaturation but also pertain to a smaller risk of microbial contamination when the process is carried out at an elevated temperature. The necessity of avoiding contaminating microbial growth is a central and critical problem to nearly all processes dealing with the enzymatic conversion of lactose *(2–4)*. In practice, the growth of spoilage bacteria in rich media such as milk and whole whey and at mild reaction conditions has prevented a more widespread use of "lactase" enzyme technology in the dairy industries and is a major obstacle toward developing a continuous lactose process *(1–4)*. We have shown recently that the β-glycosidases from the hyperthermophilic archaea *Sulfolobus solfataricus* and *Pyrococcus furiosus* (CelB) might be interesting biocatalysts for the hydrolysis of lactose and the synthesis of galacto-oligosaccharides at process temperatures of 70°C or greater *(5–7)*, at which growth of all nonhyperthermophilic microorganisms is prevented efficiently. Therefore, a continuous process would seem to be feasible at such high temperatures.

Here, we report the development of a hollow-fiber reactor and a packed-bed immobilized enzyme reactor for the hydrolysis of lactose in

ultrahigh temperature-treated (UHT) skim milk using CelB. We describe the efficient production of a technical-grade enzyme for use in the lactose process. We also compare the two reactors regarding stability during long-term operation at 70°C and productivity.

Materials and Methods

Materials

UHT skim milk with a fat content of 0.5% (w/v) was obtained from Almlieslfrisch GesmbH (Linz, Austria). Pasteurized skim milk with the same fat content was purchased from local grocery stores. Eupergit C 25 L was from Röhm (Darmstadt, Germany). Isopropyl-1-thio-β-D-galactoside (IPTG) was from Margaritella (Vienna, Austria).

Assays

The β-hydrolase activity of free CelB was measured at 80°C using lactose as the substrate and reaction conditions reported recently *(5)*. One unit of enzyme activity corresponds to 1 μmol of glucose released/min. Protein was determined by using the Bio-Rad dye-binding assay with bovine serum albumin (albumin fraction V; USB, Cleveland, OH) as the standard. Glucose was measured enzymatically.

Bioreactor Cultivation for Production of CelB

Composition of Medium

The standard culture medium for growing *Escherichia coli* contained 10.0 or 30.0 g/L of glucose, 10.0 g/L of tryptone, 5.0 g/L of yeast extract, 5.0 g/L of sodium chloride, 1.0 g/L of ammonium chloride, 3.0 g/L of dipotassium hydrogenphosphate, 6.0 g/L of potassium dihydrogenphosphate, 0.25 g/L of magnesium sulfate, heptahydrate, 1 mL/L of solution of trace elements, and 30 mg/L of kanamycin. For the solution of trace elements, the following components were dissolved in 1 L of 5 *M* HCl: 4.0 g of ferric sulfate, heptahydrate; 1.0 g of manganese sulfate, monohydrate; 1.0 g of aluminum chloride, hexahydrate; 4.0 g of ferric sulfate, heptahydrate; 1.0 g of manganese sulfate, monohydrate; 0.4 g of cobalt chloride, hexahydrate; 0.2 g of zinc sulfate, heptahydrate; 0.2 g of sodium molybdate, dihydrate; 0.15 g of copper sulfate, pentahydrate; and 0.1 g of boric acid.

Cultivation Conditions

The plasmid pLUW511 *(8)* which contains the structural gene of the β-glycosidase CelB from *P. furiosus* and delivers a kanamycin resistance, was kindly supplied by Drs. T. Kaper and J. van der Oost (Institute of Microbiology, Wageningen Agricultural University, The Netherlands). It was transformed into competent cells of *E. coli* BL21 (DE3). Stock cultures of the bacterial strain harboring the plasmid were prepared with 50% (w/w) glycerol and stored at –70°C.

For the microbial production of recombinant CelB in a 15-L bioreactor, the inoculum was made in two steps. *E. coli* from the glycerol stock was cultivated at 37°C in six 100-mL baffled Erlenmeyer flasks each of which contained 20 mL of culture medium. Glucose (10 g/L) was used as the carbon source. Agitation was at 125 rpm using an Infors (Bottmingen, Switzerland) model Multitron reciprocal incubator. After 16 h of growth, the contents of each flask were transferred into a 1-L baffled Erlenmeyer flask containing 250 mL of medium. Incubation continued as just described for approx 13 h, after which the shake-flask cultures were used as inoculum of the bioreactor.

The bioreactor cultivation of *E. coli* took place in an MBR stirred-tank reactor (MBR Bioreactor AG, Wetzikon, Switzerland) with a working volume of 15 L and equipped with a six-blade Rushton turbine impeller and a pH and an oxygen electrode. The previously described medium supplemented with 30 g/L glucose was used. The operational parameters for the cultivation were 37°C; pH 7.0, controlled by automatic addition of NH_4OH (28% [v/v]); initial impeller speed of 300 rpm; 30% oxygen saturation, regulated by variation of the impeller speed; and aeration rate of 1 vvm. Polypropyleneglycol was added as required to prevent foaming.

Cultivation was carried out in fed-batch mode. It started with a volume of 14.4 L, and glucose was added at a rate of 1.25 mL/min from a concentrated sugar solution (450 g/L). Samples of 20 mL were taken hourly and worked up as described subsequently. The induction of heterologous gene expression was done with IPTG which was added to the culture medium in a final concentration of 0.1 m*M* after a 2-h incubation time. After another 6 h, the cells were harvested, centrifuged for 20 min at 6000*g* and 4°C, washed with 20 m*M* sodium citrate buffer (pH 5.5), and stored at –30°C.

Analytical Procedures to Monitor Cell Growth and Enzyme Formation

Growth

Samples were taken from the reactor and tested immediately for the following parameters: optical density (OD), measured spectrophotometrically at 600 nm; biomass dry wt, measured by using an MA 30 moisture analyzer (Sartorius, Göttingen, Germany); [glucose] in the medium, measured by using an EBIO® compact glucose analyzer (Eppendorf, Hamburg, Germany). The weight of NH_4OH consumed for pH control was also recorded.

Enzyme Production

Each sample was centrifuged for 20 min at 6000*g* and 4°C, washed twice with 20 m*M* sodium citrate buffer (pH 5.5), and diluted twofold in the same buffer. The bacterial cells were then disrupted by ultrasonication using a Bandelin Ultraschallaufschlussgerät (Berlin, Germany) at 50% continuity and 80% impulse force. The preparation thus obtained was incubated in 2-mL plastic tubes for 30 min at 80°C, using a thermomixer model 5436 (Eppendorf) for the incubation at an agitation of 900 rpm. Most

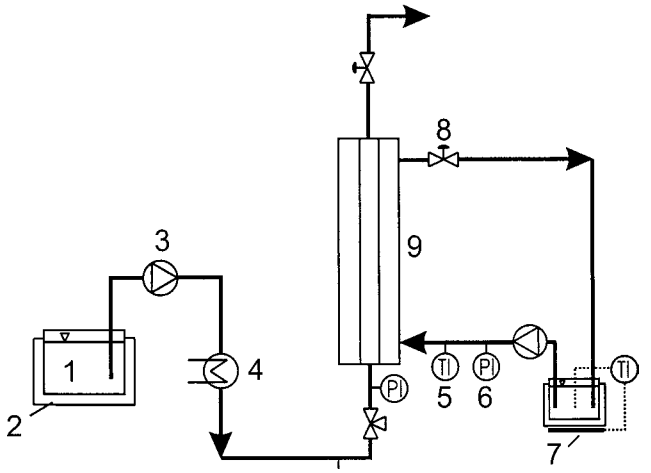

Fig. 1. Scheme of hollow-fiber reactor for hydrolysis of lactose by hyperthermostable β-glycosidase CelB. 1, Storage tank for the substrate; 2, ice box; 3, gear pump; 4, heat exchanger; 5, thermometer; 6, manometer; 7, hotplate; 8, butterfly valve; 9, hollow-fiber module.

nonthermostable protein precipitated under these conditions, and after centrifugation the supernatant was tested for β-hydrolase activity and soluble protein.

Preparation of Technical-Grade CelB

The bacterial cell paste (≈220 g) was thawed and diluted three-fold in 20 m*M* sodium citrate buffer, pH 5.5. To release the intracellularly formed CelB, the cells were disrupted by using a continuously operated Dyna-Mill KDL bead mill (Bachofen, Basel, Switzerland). The cleavage width was 0.02 mm, and beads of 0.1–0.2 mm in diameter were used. The average residence time of the cells was 25 min, and the temperature inside the milling chamber never exceeded 20°C. The product was collected in ≈500 mL portions, subjected to heat treatment, and worked up as already described above. The solution containing partially purified CelB was stored at −20°C until further use. Azide (0.02% [w/v]) was added.

Hollow-Fiber Membrane Reactor

Reactor Setup

Figure 1 shows the process scheme applied to hydrolyze lactose in skim milk at 70°C using CelB. We used an 18-in. hollow-fiber module, model HF1-43-PM5-PB from Romicon (Koch, Wilmington, MA). The module was characterized by the following parameters: molecular weight cut-off of 5000, total surface area of 930 cm^2, fiber diameter of 1.1 mm, feed volume of 40 mL, and shell volume of 90 mL. In the reactor system shown in Fig. 1, diffusion was the driving force for lactose to permeate to the shell side, where hydrolysis occurred, and for the constituent monosaccharides to be delivered back to the product stream.

Reactor Operation

The substrate was UHT skim milk. It was supplied continuously from the storage tank, heated to 70°C by passage through a heat exchanger and pumped through the hollow-fibers by using an Ismatec BVP-Z gear pump equipped with a P181 pump head (distributed by Bennett, Bleadon, Somerset, UK). At the same time, the enzyme solution was circulated at a flow rate of 75 mL/min through the shell side of the reactor via the external storage tank of the enzyme, where it was kept at 70°C (see Fig. 1). The circulation of the enzyme was achieved by means of a 501U peristaltic pump (Watson-Marlow, Cheltenham, UK). The flow rate delivered by the pump was adjusted so as to obtain efficient diffusion rates of sugars and to avoid enzyme deposition on the membrane surface at the same time.

The substrate flow rate and the amount of enzyme activity in the shell volume were varied during the reactor operation, and the effect of these factors on the extent of lactose conversion was monitored. The substrate turnover was measured as the concentration of glucose released in samples taken at the reactor outlet. Enzyme activity in the shell solution was monitored at regular intervals to determine the stability of CelB under operational conditions.

Sanitation of Reactor and Storage of Module

At the end of each experiment, both sides of the hollow-fiber module were emptied completely, filled with water, and operated as previously described until the product flow was clear. Then, the membrane was flushed with water, 0.1 *M* NaOH, water, 0.1 *M* nitric acid and finally again with water, each step lasting for at least 30 min. This was done in both standard operation and backflush mode. The hollow-fiber module was stored in 1% (v/v) formaldehyde.

Packed-Bed Immobilized Enzyme Reactor

Immobilization of CelB onto Eupergit C

A total amount of 12,000 U of CelB with a specific β-hydrolase activity of 1600 U/mg was incubated at room temperature with 15 g of Eupergit C in 1.0 *M* sodium phosphate buffer, pH 6.0, for 96 h. The immobilizate was used without further treatment. CelB attached to Eupergit C has been used recently for the production of nonnatural glycosides *(9)*.

Assay of Immobilized CelB

The immobilized enzyme (approx 10 mg) was incubated for exactly 5 min at 80°C with 1.5 mL of substrate solution containing 600 m*M* lactose dissolved in 20 m*M* sodium citrate buffer, pH 5.5. Efficient mixing was achieved by using an Eppendorf Thermomixer with instrument settings at 1200 rpm. The enzymatic reaction was stopped by diluting a 100-µL sample into 1 mL of a solution containing glucose oxidase, peroxidase, and 4-aminophenazone *(5)*. This mixture was incubated at room temperature for 40 min before its absorbance at 546 nm was recorded and converted into

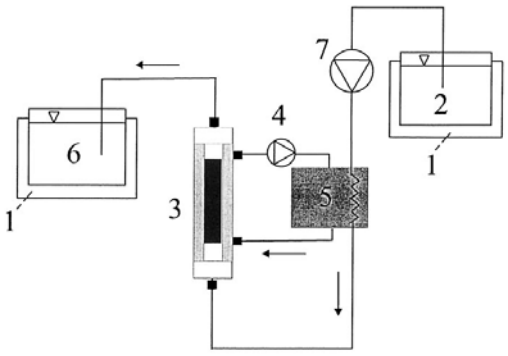

Fig. 2. Scheme of packed-bed immobilized enzyme reactor for hydrolysis of lactose by hyperthermostable β-glycosidase CelB. 1, Ice bath; 2, substrate reservoir; 3, thermostated column reactor; 4, peristaltic pump; 5, hot waterbath; 6, outlet stream of the product; 7, piston pump.

the amount of glucose released using external calibration. Controls lacking the enzyme or the substrate were recorded, and measured values were corrected as required for the corresponding blank readings. One unit of immobilized enzyme activity, U_{imm}, refers to 1 μmol of glucose released/min under the reaction conditions already described. Activities are given in U_{imm}/mL. The binding efficiency (η) relates the measured value of U_{imm} to the value expected from the difference in the activity of the free enzyme before and after the immobilization.

Continuous Conversions in a Packed-Bed Reactor

The insoluble carrier with CelB bound was packed into an Amersham-Pharmacia (Uppsala, Sweden) glass column (1.5 × 18.8 cm) equipped with a jacket for thermostatization (Fig. 2). The storage vessel containing the milk was kept on ice. The substrate solution was brought to reaction temperature by passage through a heat exchanger (at 70°C) and pumped upward through the packed bed at a flow rate of 133 mL/h delivered from an Amersham-Pharmacia piston pump (model 500). The temperature at the outlet of the reactor was monitored continuously, and at certain times, 1-mL samples were taken and used for further analyses. To determine the extent of inactivation of CelB during continuous operation, a sample (≈50 μL) of the immobilized enzyme was taken every 1–3 d at the top of the column, and the residual enzyme activity was measured immediately (see Assay of Immobilized CelB). The constancy of the turnover level, the absence of microbial contamination of the product and the reactor, and the mechanical stability of the carrier were monitored during the whole reaction times, typically between 3 and 4 d. During that time, all other conditions of operation were not changed.

Two different ways of expressing the productivity of the hollow-fiber reactor and the packed-bed reactor system were used: one was grams of glucose released per hour and liters of reactor volume, and the other was grams of glucose released per hour and kilounits of enzyme.

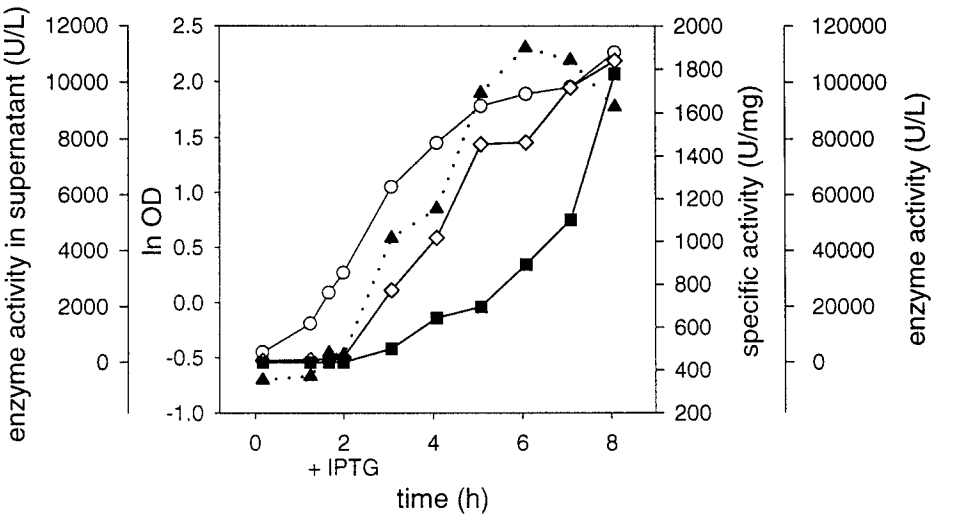

Fig. 3. Bioreactor cultivation of *E. coli* and production of recombinant CelB. ◇, U/L of medium; ▲, U/mg; ■, enzyme activity not associated with bacterial cell mass and found in the culture supernatant; ○, ln OD. After 2 h, 0.1 m*M* IPTG was added to induce the enzyme production. Glucose was fed from the start from a solution of 450 g/L at a rate of 1.25 mL/min. For other conditions, see Materials and Methods.

Results

Enzyme Production

Course of Bioreactor Cultivation and Enzyme Production

Figure 3 shows the dependence on the incubation time of several parameters characteristic of the growth of *E. coli* in a fed-batch cultivation of the organism and the heterologous production of the recombinant enzyme CelB. The plot of ln OD against time revealed a short lag phase of ≈1 h, after which the culture entered the exponential growth phase. A maximum value for the specific growth rate of approx 0.7 h^{-1} was observed between 1 and 3 h. For incubation times of between 3 and 8 h, the specific growth rate gradually decreased. However, probably since glucose was fed to the medium, growth continued up to and beyond an 8-h-long cultivation. The absence of significant enzyme production before the addition of IPTG at 2 h shows the tight control of the expression of CelB. In the presence of 0.1 m*M* IPTG, β-hydrolase activity was formed in a great amount. As expected for the production of a recombinant enzyme, the appearance of enzyme activity expressed as U/mL and U/mg occurred in parallel over time, up to approx 6 h. Then, the specific activity of CelB started to decrease, whereas the volumetric activity increased further. Interestingly, a significant percentage of the enzyme activity synthesized in the bacterial production system was found to be present in the culture medium. The β-hydrolase activity detected in the supernatant of the cultivation increased

with increasing incubation time, reflecting an increasing degree of cell lysis perhaps because of the mechanical stress imposed by stirring, or other cellular factors. At the time of cell harvest (8 h), it made up approx 10% of the total amount of enzyme activity produced.

However, since the supernatant is diluted regarding the enzyme activity present, downstream processing of CelB not attached to the bacterial cells will be costly. It is likely, therefore, that CelB released to the culture medium would be considered lost. The observed decrease in specific enzyme activity and the release of active enzyme into the medium could serve as criteria for determining an optimum cultivation time of between 6 and 7 h, for the reaction conditions employed in Fig. 3.

Balance of Production of Recombinant CelB

The bioreactor cultivation yielded 220 g of wet bacterial cells (equivalent to 15 g/L), from which 440 mL of a solution of partially purified CelB was obtained. This solution contained 3360 U/mL with a specific β-hydrolase activity of 1628 U/mg. In summary, 1,600,000 U of CelB was produced in a 15-L cultivation. In other words, approx 100,000 U/L of medium was obtained.

Hollow-Fiber Membrane Reactor

Choice of Membrane Module

The hollow-fiber module suitable for the process described herein had to fulfill a number of requirements. The most important points of consideration were that it fully retain the enzyme and be stable at an operating temperature of 70°C. At the same time, adsorption to the membrane and consequent denaturation of CelB should be at a minimum. Furthermore, the commercial availability of various suitable sizes of the module was an issue to be considered in view of a possible scale-up of the process. Contrary to the upper limit for the temperature of operation of polyethersulfone membranes (≈50°C; suggested by the suppliers), we found that these membranes were well suited for long-term use (>1 mo) at temperatures of up to at least 70°C. Part of the membrane screening was carried out with the mesophilic β-galactosidase Lactozym 3000L (Novo Nordisk, Denmark) and showed that a mol wt cutoff of 30,000 was not sufficient to retain completely an enzyme of M_r ≈200,000. Therefore, we decided to use the HF1-43-PM5-PB membrane with a mol wt cutoff of 5000 from Romicon, considering that the active tetramer of CelB has an M_r of 240,000 *(10)*.

Hollow-Fiber Reactor Productivity

Figure 4 shows the results of the continuous hydrolysis of lactose (45 g/L) in UHT skim milk at 70°C using CelB and the hollow-fiber reactor system described in Fig. 1. The enzyme activity and flow rate were varied during a 10-h operation time, as shown in Fig. 4. The aim was to identify the influence of these parameters on product yield and productivity and establish optimal conditions of operation for the hollow-fiber reactor. In the first

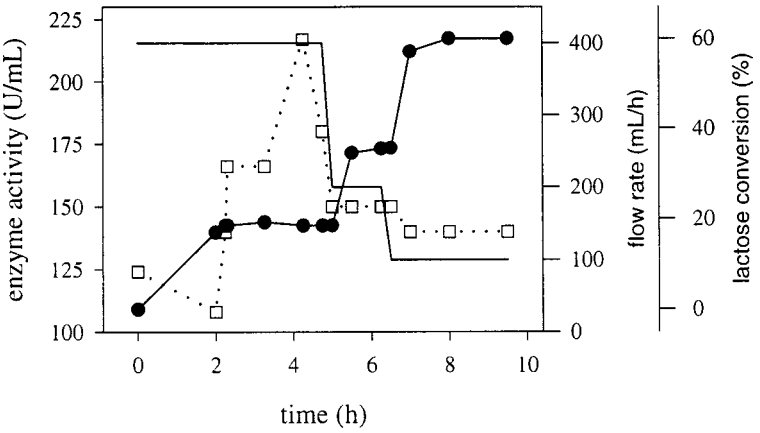

Fig. 4. Optimizing performance of hollow-fiber reactor for hydrolysis of lactose in UHT skim milk catalyzed by CelB at 70°C. ●, Lactose conversion; □, enzyme activity; (—), flow rate. The circulation rate of the enzyme solution on the shell side was constant at 75 mL/min.

phase of the experiment (≤5 h), the flow rate was kept at 400 mL/h, and the enzyme activity was raised in two steps from 110 to 200 U/mL and then decreased to 140 U/mL. The [glucose] released was barely affected by these significant changes in enzyme activity, suggesting that the reactor was working in the diffusional regime under the conditions used. In the second phase of the experiment (5–10 h), the enzyme activity was constant, and the substrate flow rate was decreased in two steps from 400 to 100 mL/h. The substrate conversion increased as the flow rate was decreased and reached a value of ≈60% at a flow rate of 100 mL/h. An analysis of the productivity, expressed as grams of glucose per liter of feed volume and hour, is shown in Fig. 5 and contrasted to the corresponding level of substrate turnover. In spite of the high productivities obtained, it is clear that the observed conversion of lactose at flow rates of ≥200 mL/h are too small for a technical process and commercial practice. If the productivity of 36 g/(L·h) measured at 100 mL/h is further related to the amount of enzyme used in the shell volume (kU/L), a value of approx 0.12 g/(kU·h) can be calculated. A further decrease in flow rate to increase the conversion level was not considered because in that case the productivity would have decreased to unsatisfactory small values.

Long-Term Performance of Hollow-Fiber Reactor and Stability of Enzyme

Considering the high capital costs of integrating a hollow-fiber module into an industrial process, the long-term stability of the reactor under operational conditions is a key issue of the process economy. Apart from the physical stability of the module at the process temperature, microbial contamination and protein denaturation are factors of the lifetime of the module. We found that the membrane was fully stable for a 39-d operation time at 70°C using UHT skim milk as the substrate, which was supplied at a flow rate of 100 mL/h. The necessity of cleaning the module did not arise.

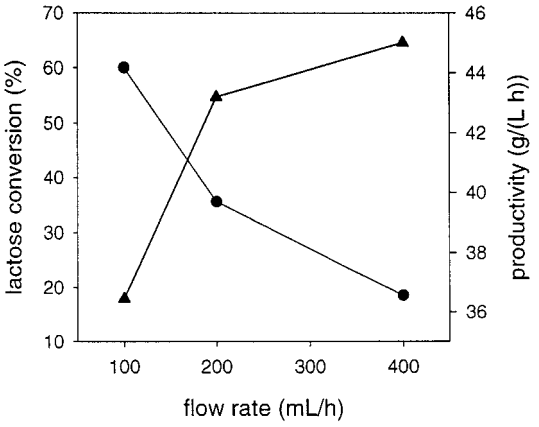

Fig. 5. Analysis of productivity of hollow-fiber reactor designed for hydrolysis of lactose in UHT skim milk at 70°C. ●, lactose conversion; ▲, productivity. The circulation rate of the enzyme solution on the shell side was constant at 75 mL/min.

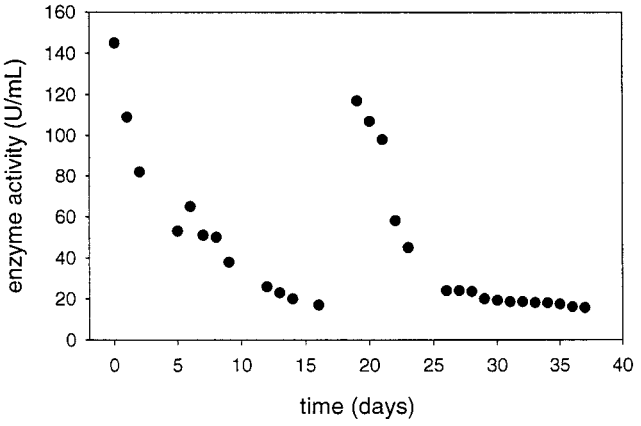

Fig. 6. Stability of CelB in hollow-fiber reactor at 70°C. The conditions used were a substrate flow rate of 100 mL/h, a circulation rate of the enzyme of 75 mL/min, and UHT skim milk as the substrate. After 18 d, fresh enzyme was added.

The stability of the enzyme during long-term reactor operation is another major concern during continuous process development. Figure 6 shows how the activity of CelB in the shell volume of the hollow-fiber reactor changed with dependence on the reaction time. Under the conditions used, CelB had a half-life of about 5 d (Fig. 6). The observed inactivation of CelB in the hollow-fiber reactors occurred at a rate between 10 and 20 times faster than the corresponding inactivation of the enzyme in buffered solution at 70°C. This likely is a product of three factors: the high recirculation rate on the shell side, adsorption of CelB onto the membrane surface, and reactions of CelB with reducing sugars. CelB was shown to be inactivated when a solution of the enzyme was circulated using a peristaltic pump *(11)*. Attachment of CelB to the surface of ultrafiltration membranes was also observed previously *(11)*, and experiments carried out

with mesophilic β-galactosidase showed that an exhaustive preincubation of the hollow-fiber module with a solution of whey proteins can reduce significantly the rate and extent of enzyme inactivation during reactor operation. We reported the inactivation of CelB in the presence of reducing sugars *(5,6)*. A brownish color appeared in the shell volume of the hollow-fiber reactor and became more intense as the reaction time increased. Although this was not studied in a systematic way, the extent of enzyme inactivation seemed to be correlated with the degree of colorization of the enzyme solution. When the substrate flow rate was decreased, the rate of appearance of brown reaction products increased. In principle, when using substrates other than skim milk, a decrease in pH to a value of ≈5.0 might be helpful to prevent, or at least reduce, the Maillard reactions and concomitant inactivation of CelB.

Packed-Bed Immobilized Enzyme Reactor

Immobilization of CelB

Covalent attachment of CelB onto Eupergit C took place with a binding efficiency (η) of 0.79, which is good when compared with the main body of the literature on immobilized β-galactosidases and β-glycosidases. CelB can be immobilized with similar values for η onto controlled pore glass, a carrier often used in connection with enzymatic lactose hydrolysis, and chitosan, another relatively cheap matrix that is quite useful for immobilization of CelB (unpublished results). Although the most expensive among the three carriers, Eupergit C was used here because it provided the most stable immobilized enzyme preparation of CelB. The long-term stability of CelB is a major factor in the process of carrier selection, considering that the covalent enzyme/carrier adduct cannot be regenerated once substantial enzyme activity has been lost during the reaction. The immobilizate of CelB employed for the processing of milk was characterized by a value of 144 U_{imm}/mL.

Lactose Hydrolysis in UHT and Pasteurized Skim Milk

Figure 7 shows results of the continuous hydrolysis of lactose in UHT skim milk using a packed-bed immobilized enzyme reactor containing 65 mL of CelB covalently attached onto Eupergit C. The substrate solution was supplied at a flow rate of 133 mL/h, corresponding to an average residence time of 0.49 h and an axial flow rate of 75 cm/h. The observed turnover level was almost constant during the continuous reaction, the actual measurements varying between 70 and 81% conversion. The operational stability of CelB was good: the enzyme activity showed a slight decrease of ≈10% during a 95-h reaction time. The loss of enzyme activity could be correlated in a qualitative fashion with the occurrence of a brownish color in the packed bed. This indicates again the prevalence of inactivation of CelB by Maillard-type reactions. The observed productivity was between 35 and 40 g/(L·h), or 0.20 and 0.24 g/(kU·h). When pasteurized skim milk replaced UHT skim

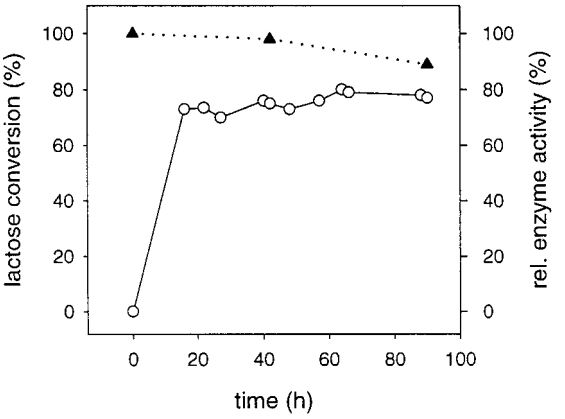

Fig. 7. Continuous hydrolysis of lactose in UHT skim milk using packed-bed immobilized enzyme reactor containing CelB immobilized onto Eupergit C. The packed bed had a volume of 65 mL and a height of 37 cm. The conditions were 144 U_{imm}/mL, 70°C, and a substrate flow rate of 133 mL/h. ○, Lactose conversion; ▲, relative enzyme activity.

milk as the substrate, microbial contamination prevented the operation of the packed-bed reactor for reaction times longer than 12 h; it led to clogging of the packed bed, heavy browning, and inactivation of CelB.

Discussion

General Evaluation of Enzyme Reactors

The aim of the present study was to demonstrate the utility of a hyperthermostable β-glycosidase, recombinant CelB from *P. furiosus*, for the continuous hydrolysis of lactose in skim milk. Two different process schemes were developed, and the central components of the reaction engineering were a hollow-fiber membrane reactor and a packed-bed immobilized enzyme reactor. The two reactor systems have been widely used and optimized for the processing of whey and milk by using mesophilic β-glycosidases *(2–4)*. They have now been adapted for use with a thermophilic hydrolase, here at 70°C. Both reactors support the physical separation of milk proteins from the biocatalyst and provide a complete retention of the β-glycosidase during continuous operation, thus allowing ≥200-fold reuse of a single batch of enzyme. The results show that the hollow-fiber membrane and the packed-bed reactors perform in a satisfactory fashion at 70°C regarding long-term stability of the enzyme, avoidance of microbial contamination, and constancy of lactose conversion when UHT skim milk is used as the substrate. Whole whey or ultrafiltered whey might be interesting alternative substrates to be processed in these enzyme reactors. The pH dependences of activity and stability of free and immobilized CelB (not shown) suggest that sweet whey with a pH of ≈6.0 would be the better substrate than acid whey with a pH of ≈4. However, CelB attached to

Eupergit C might be useful for the conversion of lactose in acid whey as well (unpublished results), and acidic reaction conditions could prevent Maillard reactions from taking place. It is anticipated that the extent of deproteination of the substrate solution will have a positive effect on the overall stability of the enzyme reactors under conditions of continuous operation. This conclusion is in line with the observation that because of heavy contamination problems during the continuous reaction, pasteurized skim milk was not a suitable substrate of immobilized CelB.

Availability of the biocatalyst in large amounts is a prerequisite for conducting experiments in a hollow-fiber membrane reactor, even on a laboratory scale. The efficient production of CelB by using a bioreactor cultivation of *E. coli* expressing the structural gene of the *P. furiosus* enzyme and downstream processing of the recombinant protein by a single step of heat treatment provided a technical-grade biocatalyst in very good yield (ref. *8*). Further developments in enzyme production will certainly be necessary in order to obtain approval for this enzyme to be used for the production of food ingredients.

Comparison of Enzyme Reactors Regarding Enzyme Stability

We observed marked differences in the half-life of CelB used at 70°C in a hollow-fiber membrane reactor and a packed-bed reactor. While the immobilized enzyme was practically stable during 4 d of continuous operation, approx 50% of the original soluble β-hydrolase activity on the shell side of the hollow-fiber reactor was lost in about the same time. Enzyme stability, therefore, favors the packed-bed reactor. This finding corroborates the experiences of other researchers *(2)* with mesophilic β-hydrolases: the enzyme was generally much less stable in a hollow-fiber membrane reactor, compared with the immobilized enzyme in a packed-bed reactor. Part of the inactivation of CelB might be avoided by preincubating the hollow-fiber module with a proteinaceous solution (e.g., whey), thereby saturating the membrane surface. Another probable cause of inactivation of CelB pertains to glycation of the protein through reaction with reducing sugars. The diffusion of sugars through the ultrafiltration membrane from and to the shell side implies a concentration gradient for glucose and galactose that is reciprocal to the concentration gradient for lactose. A relatively high concentration of monosaccharides and perhaps a broad residence time distribution for sugars on the shell side may account partly for the observed fast inactivation. A possible solution to increase the diffusion rates would be to increase the circulation rate of the enzyme solution. However, that too inactivates the enzyme activity.

Comparison of Enzyme Reactors Regarding Productivity

An essential question of reaction engineering to be answered during process development pertains to the extent to which the activity of the biocatalyst is utilized in the biotransformation reaction: Is the observed

reaction rate limited by the amount of enzyme present or physical mass transfer? For the hollow-fiber reactor, it was shown that the process was operated in an intermediate regime in which both diffusion and the enzymatic reaction are major factors of productivity. A detailed analysis of the packed-bed reactor has not been carried out, but preliminary results suggest the prevalence of external mass-transfer limitation under the conditions used for continuous hydrolysis of milk (unpublished results). Therefore, a meaningful comparison of the productivities measured for the hollow-fiber and packed-bed reactors is quite difficult. However, we point out that the packed-bed reactor delivers about the same productivity as the hollow-fiber reactor at a conversion level of 80%, while the corresponding substrate turnover of the membrane reactor was only 60%. These data clearly favor the packed-bed reactor. Considering the technical parameters of the membrane module chosen, one could envision several modifications of the hollow-fibers that have the potential to improve the performance of the diffusional enzyme reactor in terms of volumetric and specific productivity: a greater mol wt cutoff; a smaller fiber diameter; a higher ratio of membrane surface to feed volume, which was 23 (=930/40) for the laboratory-scale module. In fact, it was possible to show an increase in productivity by a factor of ≈3 when using another hollow-fiber module for which the surface/volume ratio was 75 (unpublished results).

In summary, both reactor systems are technically suitable for the long-term hydrolysis of lactose by using CelB and an elevated reaction temperature. In practice, the upper limits of temperature and average residence time will have to be determined precisely so as to avoid unwanted modifications of the hydrolysis product regarding taste and nutritional factors.

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