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A microfiltration bioreactor to achieve high cell density in *Sulfolobus solfataricus* fermentation

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Abstract A novel technique is proposed to achieve higher cell yield in extremophile fermentation. Because the accumulation of toxic compounds is thought to be responsible for low biomass yields, a bioreactor has been designed based on a microfiltration hollow-fiber module located inside the traditional fermentation vessel. Using the cultivation of the thermoacidophilic archeon *Sulfolobus solfataricus* Gθ as a model, a biomass of $35\,g\,l^{-1}$ dry weight was obtained which proved greater than that of $2\,g\,l^{-1}$ obtained in batch fermentation. The bioreactor was characterized by running several fermentation experiments to check the high stability of the membrane module to sterilization cycles, high temperatures, and acidic pHs, even for prolonged periods of time. It was shown that the exhaust medium is unable to sustain growth for the presence of toxic compounds, and ultrafiltration and ion-exchange techniques were used in all the attempts to regenerate it. The results demonstrated the ability of the method to lower inhibitor concentrations and prolong the growth phase, thus achieving high cell density. Furthermore, they indicated that the toxic compounds are ionic species of less than 1 kDa.

Key words Extremophiles · Fermentation technology · High cell density · Microfiltration · *Sulfolobus solfataricus*

Introduction

During the past few decades, several microorganisms have been isolated in environments such as solfataric fields, mud holes, fumaroles, and surface hot waters usually referred as too "extreme" to permit any life. In 1975, an archeon, subsequently called *Sulfolobus solfataricus*, was isolated in an acidic hot spring in Agnano, Napoli, Italy (De Rosa et al. 1975); this species, which is aerobic, can grow under either autotrophic or heterotrophic conditions (Brock et al. 1972; De Rosa et al. 1984). Among the various strains of *S. solfataricus* isolated that differ in optimal growth parameters (Grogan 1989), the strain Gθ was used here (Cannio et al. 1998). The increasing interest in extremophiles and their biotechnological applications makes the fermentation to high biomass yield of these species highly important because it is also necessary for an efficient cell-related product formation (Bunch 1994). In the fermentation of mesophilic microorganisms, filtration apparatuses have often been used to improve productivity and to obtain a continuous process with an easier downstream (Bailey and Ollis 1977; Hayakawa et al. 1990; Bowen 1993; Frenander and Jönsson 1996). Fairly recently, this approach has also been applied in the fermentation of extremophiles. Krahe et al. (1996) have investigated the most important parameters in high-temperature fermentation and reported on the effectiveness of a dialysis fermenter in the high-cell-density cultivation of *Sulfolobus shibatae*, obtaining a biomass yield that was 10- to 20-fold-higher than the fed-batch process.

We demonstrated that, by using a microfiltration apparatus, it is possible to extend the growth phase of *S. solfataricus* and obtain an enhanced cell yield. This article describes the hollow fiber microfiltration module, the results of cultivation of *S. solfataricus* compared to conventional fermentation processes, and the results obtained on growth inhibition.

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Materials and methods

Materials

Polypropylene and polyethersulfone membranes were provided by B. Braun Biotech International (Melsungen,

Germany). Glucose/GOD-Perid method kit was purchased from Boeringer Mannheim (Milan, Italy). Yeast extract was purchased from Difco and casein hydrolysate acid from OXOID; other chemicals were from Sigma-Aldrich (Milan, Italy).

Microfiltration module design

The chemicophysical stability of several materials was tested. Polyethersulfone and polypropylene were found to fulfill the experimental conditions required in extremophile fermentation at high temperatures and in acidic solutions. However, polypropylene was selected because of its low cost and availability. The polypropylene membrane, Accurel PP, consisted of a long capillary (5–10 m) with an inner diameter of 0.2 mm and a cutoff of 0.22 μm. A number of 15-cm-long capillaries were obtained from the original membrane and were assembled together with a silicon adhesive; one side was closed and the other was sealed to a connector. The module was connected to a peristaltic pump (model 313 U; Watson Marlow, Falmouth, UK) equipped with a silicon tube of inner diameter $\phi = 2$ mm, which provided the driving force for transmembrane flux. Considering the geometry of the module, the total filtering area measured 1.47×10^{-2} m^2.

Before using the module, the membranes had to be treated with a 70% ethanol solution for 1 h to become hydrophilic. The microfiltration module was placed inside the fermentation vessel and fixed vertically to a baffle to have a high turbulence near the filtering surface to minimize fouling. After each experiment, the module was cleaned with a $NaHCO_3$ aqueous solution 10% (w/w) before being treated with the ethanol solution and finally rinsed with deionized water before use. The geometry of the probe developed for a Biostat C fermenter (15 l, B. Braun) permits an increase of its filtering surface to threefold.

Fermentation experiments

All the fermentation experiments were run with an in situ 15-l sterilizable fermenter (Biostat C) equipped with a DCU that was able to control parameters such as temperature, pH, stirring (rpm), level, and dissolved oxygen.

The microorganism, *S. solfataricus* Gθ, was obtained by Cannio (Cannio et al. 1998); its optimal growth conditions were $T = 75$°C and pH = 3.5–4.0. The medium was taken from the DSM catalog (1993) (recipe 182); 1 l of aqueous solution contains 3.1 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 4.5 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CuCl_2 \cdot 2H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, and 0.01 mg $VOSO_4 \cdot 2H_2O$. Nutrients were added to this salt solution in different amounts depending on the experiment. The medium that was more frequently used contained 1 g l^{-1} yeast extract, 1 g l^{-1} casein hydrolysate acid, and 5 g l^{-1} glucose. The pH was adjusted with a H_2SO_4 solution (10% w/w) to 3.5–3.8.

Batch fermentation

Precultures were prepared by inoculating a stock glycerol solution in a fresh medium. The latter was made by autoclaving the salt solution to which the yeast extract and casein hydrolysate acid were added; filter-sterilized glucose was then added to avoid "Maillard" reactions occurring at high temperatures. Flasks were inoculated starting with a cell density of 10^6–10^7, and the cells were left to grow in an incubator for 48–72 h. In the Biostat experiment, approximately 9 l of medium was inoculated with 1 l of culture. Several batch experiments were run at $T = 75$°C, pH = 3.8, with 1 g l^{-1} of yeast extract and 1 g l^{-1} casein hydrolysate acid, and two different glucose concentrations, 1 and 5 g l^{-1}. The air flow rate was varied between 0.2 and 0.5 vvm, and stirring was varied between 80 and 200 rpm.

Fermentation was followed by absorbance measurements at 600 nm; cell density (cells/ml) through direct microscopic analysis using a Büker chamber; cell wet weight obtained by weighing samples after centrifuging 30–50 ml of broth at $7000 \times g$ in a Beckman Avanti-J centrifuge with a 25–50 rotor for 20–30 min and eliminating the supernatant. Cell dry weight was obtained by filtering 5–10 ml broth on a 0.22-μm cutoff membrane (Millipore) and drying overnight at 75°C before weighing. Stirring and air flow rate were adjusted during the experiments to keep the dissolved oxygen above 20% of the saturation. This value was set before each experiment as the dissolved oxygen present in the medium before inoculating under the exact same initial conditions of the fermentation (T, pH, rpm, flow rate).

Fed-batch experiments

The fed-batch experiments began in batch mode and feeding started almost at the end of the exponential phase, thus prolonging the growth phase (Korz et al. 1995). The feeding solution was a concentrated solution containing 250 g l^{-1} glucose and 16 g l^{-1} of both yeast extract and casein hydrolysate. The glucose concentration in the fermentation broth was evaluated off line by the glucose/GOD-Perid method kit (Bergmeyer and Bernt 1974). A profile was then designed for the substrate pump so that glucose feeding varied from 0.15 to 0.6 g l^{-1} h^{-1} during fermentation to keep the glucose concentration constant at about 3–4 g l^{-1}. The initial stirring velocity was 80 rpm and the initial aeration was 0.2 vvm; both were increased during the process to keep the dissolved oxygen above the 20% of air saturation. Growth was followed as in the batch process.

Microfiltration experiments

Microfiltration experiments were started in batch mode and switched first to fed-batch and then to microfiltration mode. Feeding was performed as described for the fed-batch experiment, increasing the highest glucose feeding rate to 0.9 g l^{-1} h^{-1}. The aeration rate was varied within the experiment from 0.35 to 2 vvm, and stirring was varied from 90 to 400 rpm to meet the culture oxygen demand. The working

volume was kept constant by a level controller which, when the microfiltration module was functioning, switched on a pump to feed a salt solution prepared according to the medium recipe. Until 150 h experimental time ($OD_{600} = 20$), transmembrane flux was set at $0.41 h^{-1}$ and its decline as the resulf of fouling problems was corrected by increasing pump rate; later on the setpoint for the flux was increased to $0.81 h^{-1}$, and in addition to pump rate changes, frequent (about every 3 h) backflushings of 2 min were performed to obtain an average flux of 75% of the set point. Growth was followed as in the batch experiment and, in addition, endocellular α-glycosidase (SsαGly) and β-glycosidase (SsβGly) enzymatic activities were measured and vitality tests were performed.

Plating experiments

The first layer of plate medium was made as previously described by Grogan (1989), while the soft layer contained only half the amount of gellan gum (0.4%, final concentration). The plates were incubated at 75°C under a moist atmosphere in a jar for 7 days.

Cell disruption

The wet biomass, recovered after sample centrifugation, was resuspended in a Tris-HCl buffer 50 mM, pH 8.0, containing Triton X-100 0.1% w/w and mixed for 2–4 min continuously on a vortexer. The supernatant containing cytosolic enzymes was recovered after centrifugation (10000 rpm).

Protein assay

Protein concentration was determined by the dye-binding assay of Bradford (Bradford 1976) using bovine serum albumin (BSA) as standard.

Enzyme assays

SsαGly activity on *p*-nitrophenyl-α-D-glucopyranoside (pNPG) was estimated by adding 50 µl of homogenate to 0.45 ml of sodium acetate buffer (50 mM, pH 5.5) with 4.5 mM pNPG. The reaction mixture was incubated at 75°C for 10 min and stopped with 1 ml of 1 M Na_2CO_3. Absorbance was read at 400 nm and activity was calculated by means of the molar extinction coefficient (18300 M/cm). The activity unit (U) of SsαGly is the amount of the enzyme required to hydrolyse 1 µmol of substrate (pNPG) per minute. SsβGly activity on *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was estimated with the same method as used for SsαGly (Herr 1979).

Toxicity tests

Toxicity tests were performed by inoculating, after sterilization, the following solutions: (1) the microfiltred exhaust medium (permeate) as it was collected during experiments; (2) the permeate solution reintegrated with glucose up to $5 g l^{-1}$ and salts according to DSM recipe; (3) the fresh medium containing twofold salt concentration; (4) the permeate solution ultrafiltred on a 1-kDa cutoff membrane (Millipore) in an AMICON cell, autoclaved, and supplied with glucose up to $5 g l^{-1}$; and (5) the permeate solution treated with the TMD-8 deionizing resin and reintegrated with salts according to the DSM recipe and glucose up to $5 g l^{-1}$. These flasks were incubated at 75°C, and growth was followed for 3–4 days.

Substrate consumption balance

The glucose concentrations in the fermenter and in the permeate were evaluated as previously described, while yeast extract (YE) and casein hydrolysate (CH) concentrations were calculated by assuming that the initial ration to glucose was maintained during the process. The mass balances on substrate consumption were calculated according to the following equations, for both batch and microfiltration experiments:

Batch experiment:
- Initial conditions: $V = 10\,l$; $c_{iG} = 5 g l^{-1}$; $c_{iYE} = 1 g l^{-1}$ $c_{iCH} = 1 g l^{-1}$
- Final conditions: $c_{fG} = 1.6 g l^{-1}$; $c_{fYE} = 0.32 g l^{-1}$; $c_{fCH} = 0.32 g l^{-1}$
- Overall mass balance: substrate consumption = $V \cdot (c_i - c_f)$
- If the batch experiment is repeated *N* times to obtain a certain amount of biomass, the substrate consumption and the substrate waste must be multiplied by *N*.

Microfiltration experiment:
- Initial conditions: $V = 10 l$; $c_{iG} = 4 g l^{-1}$; $c_{iYE} = 1 g l^{-1}$; $c_{icH} = 1 g l^{-1}$
- Final conditions: $c_{fG} = 7 g l^{-1}$; $c_{fYE} = 0.46 g l^{-1}$; $c_{fCH} = 0.46 g l^{-1}$
- FEED: $V_F = 4 l$; $c_{FG} = 250 g l^{-1}$; $c_{FYE} = 16 g l^{-1}$; $c_{FCH} = 16 g l^{-1}$
- PERMEATE: $V_P = 70 l$; $c_{PG} = 5 g l^{-1}$; $c_{PYE} = 0.33 g l^{-1}$; $c_{PCH} = 0.33 g l^{-1}$
- Overall mass balance: substrate consumption $= V \cdot (c_i - c_f) + V_F \cdot c_F - V_P \cdot c_P$
- Substrate waste $= V \cdot c_f + V_P \cdot c_P$

Results and discussion

Several fermentation experiments were carried out to find the optimal growth conditions of *S. solfataricus* Gθ. Experiments in batch mode using substrate concentration of $1 g l^{-1}$ and $5 g l^{-1}$ demonstrated that the higher the initial glucose concentration, the better was the final cell yield. As shown in Table 1, aeration and stirring velocity were found to strongly affect the initial doubling velocity ($\mu = (\ln x - \ln x_0) \cdot (t - t_0)^{-1}$ [h^{-1}]); in fact, in the batch 2 experiment, the latter

Table 1. Comparison between experiments at different growth parameters on *Sulfolobus solfataricus* Gθ

Experiment (T = 75°C, pH = 3.8	Aeration (vvm) $t = 0$	Stirring (rpm) $t = 0$	Glucose (gl^{-1}) $t = 0$	OD_{600} (cell/ml)	Cell density (h^{-1})	μ
Batch 1	0.5	200	1	1.1	4.5×10^8	0.032
Batch 2	0.5	200	5	2.4	8.7×10^8	0.035
Batch 3	0.2	80	5	3.0	1.4×10^9	0.081
Fed-batch[a]	0.2	80	4	7.5	3.0×10^9	0.083
Microfiltration[a]	0.35	90	4	57	4.0×10^{10}	0.079

[a] Because of the high cell density achieved during the processes, aeration and stirring were increased up to 0.9 vvm and 120 rpm for the fed-batch and up to 2 vvm and 400 rpm for the microfiltration experiment

was found to be $0.035\,h^{-1}$ (corresponding to a doubling time t_d of 19.4 h), while in batch 3 μ was $0.087\,h^{-1}$ (corresponding to t_d= 8 h).

All the fed-batch experiments began with initial conditions similar to those that gave the best results in batch mode, and feeding started when the doubling velocity was still approximately maximal (OD_{600} = 2). The substrate solution contained casein hydrolysate acid and yeast extract in a ratio of 1:15 to glucose as previously described (Krahe et al. 1996), and this ratio proved to be the most effective in overcoming substrate inhibition. The feeding solution was highly concentrated to keep the working volume constant in spite of the addition of nutrients. During fed-batch experiments, both aeration and stirring were varied to meet the culture oxygen demand: the former was increased up to 0.9 vvm and the latter up to 120 rpm. The doubling velocity decreased from $0.081\,h^{-1}$ to $0.0083\,h^{-1}$ while coming to the stationary phase.

The microfiltration experiment started first in batch mode and soon switched to fed-batch mode; medium exchange began before reaching the stationary phase. When the peristaltic pump connected to the microfiltration hollow fiber module was switched on, the level control started to exchange the exhaust medium present in the fermenter with fresh salt solution. Nutrients were added in the same way as in the fed-batch mode, but with different profiles, depending on the permeate flux and glucose consumption. These experiments lasted about 310 h compared with the batch experiment, which reached its "plateau" in 80–120 h; the fed-batch mode prolonged this growth phase by 40–60 h. The doubling velocity decreased during experiments from 0.045 to $0.01\,h^{-1}$.

A comparison between the typical batch, fed-batch, and microfiltration experiments, presented in Fig. 1, validates the microfiltration technique as a sound approach to improve biomass yield by approximately 15 to 20 fold with respect to the batch and 6 fold with respect to the fed-batch process. To our knowledge this is the highest biomass yield ever obtained in *S. solfataricus* fermentation. During microfiltration experiments transmembrane flux was monitored (Fig. 2). The starting exchange flow rate decreased within 18 h because of fouling problems. At the beginning an increase in the peristaltic pump rate was sufficient to restore the set flux correcting this decline; at higher cell densities (e.g., OD_{600} >20) frequent backflushing was necessary to keep the average flux at 75% of the maximum value (Bubbico et al. 1996).

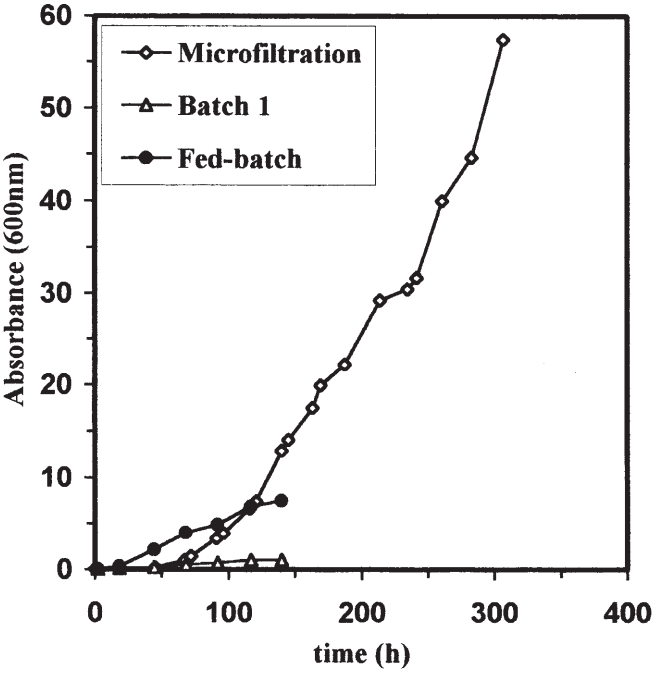

Fig. 1. Comparison between growth curves in batch 1, fed-batch, and microfiltration (MF) experiments. In the first 100 h, the fed-batch experiment resulted in a better biomass yield compared to the MF experiment because of a better doubling velocity due to different initial conditions (aeration and stirring; see Table 1) and an higher inoculum (OD_{600} = 0.08 in fed-batch and 0.03 in MF). The former gave a different slope to the growth curves while the latter moved the fed-batch curve up with respect to the MF curve. Therefore, the OD_{600} = 2 needed to start the feeding phase was reached earlier for the fed-batch experiment (44 h) than for the MF experiment (72 h). The microfiltration module began functioning at 101 h of experimental time

After each experiment, the microfiltration module was cleaned as previously described (Materials and methods), and this proved sufficient to completely restore the maximal flux. Each hollow fiber module was sterilized five times and used for more than 2000 h without being damaged. Vitality tests performed during microfiltration experiments consisted of plating samples taken from the fermentation vessel

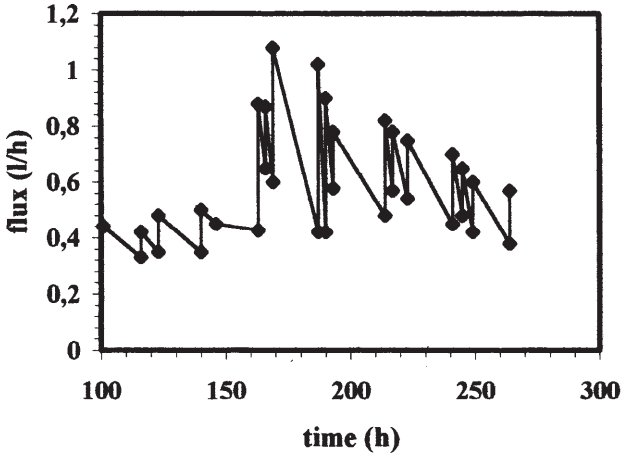

Fig. 2. The setpoint for transmembrane flux until 150 h was 0.4 $l h^{-1}$, and fouling problems were corrected by increasing peristaltic pump rate; later, the setpoint was increased to 0.8 h^{-1} and in addition to pump rate increase, 2-min backflushings were performed every 3 h

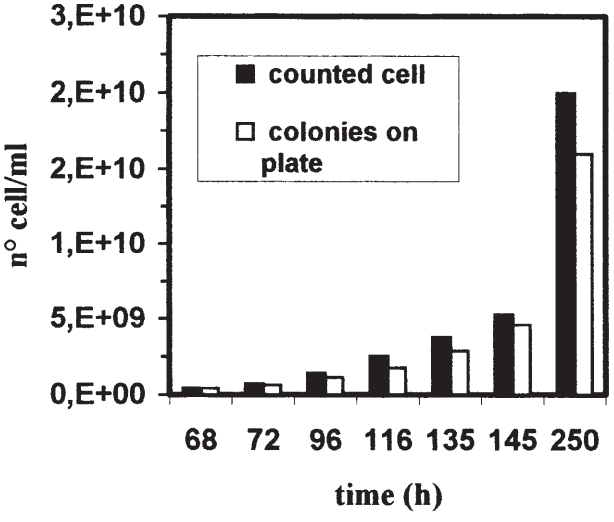

Fig. 3. Vitality tests, comparison between cells counted through direct microscopic analysis and colonies formed on plates after 7 days incubation, for several samples taken at different experimental times from the fermenter

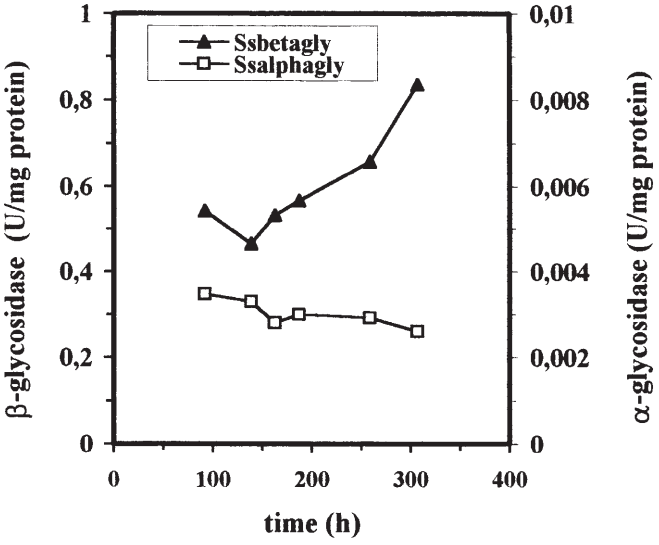

Fig. 4. Specific activities of endocellular Ssαgly and Ssβgly in microfiltration experiments. At different experimental times, cells were collected and enzymatic activities were measured as reported in Material and methods

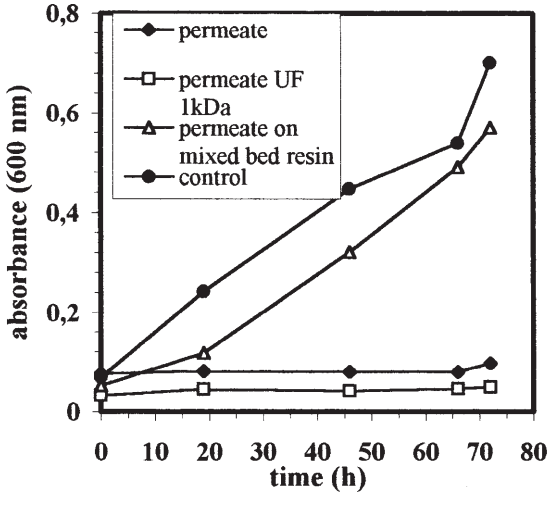

Fig. 5. Toxicity tests. In the attempt to regenerate exhaust medium, the latter was either ultrafiltered on a 1-KDa membrane or deionized on a TMD-8 resin. After these treatments, the permeate was reintegrated with salts and nutrients before inoculating (see Materials and methods). The control is fresh medium (DSM 182) incubated with tests

at different experimental stages. The results (Fig. 3) showed that even at the highest absorbance values the colony-forming cells represented 75%–80% of the cells counted. Furthermore, the enzymatic activity measurements (Fig. 4) confirmed that biomass with an active protein synthesis was produced even in fermentation to high cell density; in fact, the trend of the two specific activities was shown to be only slightly decreased for SsαGly and increased for SsβGly.

As the permeate exhaust medium was shown not to sustain growth, a screening of toxic compounds was performed. The permeate solution was treated in the same way as that explained in Materials and methods, and growth on the treated permeate was followed. It was demonstrated that exhaust medium, even when integrated with salts and glucose, is unable to sustain growth. It was also found that the ultrafiltration treatment was not sufficient to regenerate the permeate solution and therefore we can assume that the molecular weight of inhibitors is lower than 1 kDa. However, the deionizing treatment with the TMD-8 mixed-bed resin proved successful. In fact, the treated medium was able to sustain growth to the same extent as the fresh medium (Fig. 5). In conclusion, we can assume that ionic catabolic compounds of low molecular weight play a key role in growth inhibition.

Substrate consumption balance: comparison between batch and microfiltration experiments

Considering that about 18 batch processes (RB) are necessary to obtain the same amount of biomass of the microfiltration experiment (MF), the two different approaches were compared by mass balance calculations on substrate consumption and waste according to the equations showed in Materials and methods:

RB glucose consumption = 612 g; RB YE consumption = 122.4 g; RB CH consumption = 122.4 g
Glucose waste = 288 g; YE waste = CH waste = 57.6 g
MF glucose consumption = 420 g; MF YE consumption = 33.5 g; MF CH consumption = 33.5 g
Glucose waste = 420 g; YE waste = CH waste = 27.7 g
RB glucose consumption/MF glucose consumption = 1.5
RB YE (CH) consumption/MF YE (CH) consumption = 3.6
RB glucose waste/MF glucose waste = 0.7; RB YE (CH) waste/MF YE (CH) waste = 2

These data indicate that the microfiltration approach, allowing a more uniform medium composition, permits a better yield of biomass on substrates. In terms of nutrient losses, the repeated batch process would waste a twofold greater amount of yeast extract and casein hydrolysate and only 30% less glucose than in the microfiltration experiment.

Conclusions

The microfiltration module proved highly resistant to the extreme working conditions typical of thermoacidophile fermentation. Moreover, in spite of the high cell densities reached during the process, repeated backflushing was shown to be sufficient to maintain the transmembrane flux at an average value of 75% of maximum. The latter is a sound achievement, especially when compared to the usual cross-flow filtration, whose flux shows a decline to 20% of the maximum value within 30 h experimental time (Hayakawa et al. 1990). The assembled membrane bioreactor allowed a cell production that was 15- to 20 fold greater than the best batch experiment. The biomass quality was evaluated by monitoring cytosolic specific enzymatic activities and by performing vitality tests. The results of these experiments confirmed the active metabolism of the biomass produced. These data also validate the microfiltration technique as a sound approach for industrial-scale production of biomass-related product. In fact, 18 batch processes should be carried out to produce an amount of biomass equal to that produced in microfiltration experiments. The overall glucose input is 2.6 g/g of dry biomass for the repeated batch process, while it is 2.4 g for the microfiltration experiment. In repeated batch experiments, the glucose consumption that resulted was 1.5-fold greater and the yeast extract consumption was 3-fold higher than in the microfiltration experiment. However, the most important difference is the number of working hours needed to reach the same amount of biomass: repeated batches will require more than 2000 h without considering time for cleaning and sterilization of the fermenter between processes, while microfiltration needs only 310 h. Experiments on exhaust medium showed that toxicity was related to ionic compounds less than 1 kDa. Further investigation of inhibitory species is necessary to suggest a suitable treatment for exhaust medium recycling to lower nutrient waste.

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