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Innovative fermentation strategies for the production of extremophilic enzymes

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Abstract A new type of microfiltration (MF) bioreactor, developed in our laboratory, was investigated for use in improving efficiency of the production of extremophilic enzymes. In spite of the difficulties in cultivating hyperthermophiles, we achieved, in 300 h fermentation, more than 38 g/l dry weight of *Sulfolobus solfataricus* using a MF technique, and we demonstrated that the activity of alcohol dehydrogenase (ADH), as the reporter enzyme, was not affected by cell density. However, hyperthermophile cultivation is difficult to scale up because of evaporation and the very low growth rate. Thus, to achieve high productivity we cultivated, in the MF bioreactor, recombinant mesophilic hosts engineered for the production of two thermophilic enzymes, namely, trehalosyldextrin-forming enzyme (SsTDFE) and trehalose-forming enzyme (SsTFE) from *Sulfolobus solfataricus*. The traditional Luria-Bertani broth used for recombinant *Escherichia coli* growth was replaced with a semidefined medium. The latter was used in both the batch and the MF experiments, and the ratio of complex components (e.g., yeast extract and tryptone) to a simple carbon source (glycerol) was decreased during the fed-batch phase to further decrease the medium cost in view of industrial applications. The bioprocess developed was able to improve productivity 500 fold for rSsTFE and 60 fold for rSsTDFE with respect to the wild type cultivated in MF mode. Comparisons with another recombinant enzyme, α-glucosidase (rSsαgly), from *Sulfolobus solfataricus* produced in our MF bioreactor are reported.

Key words Microfiltration · Thermophilic enzymes · Fermentation technology · Industrial applications · Recombinant · *Escherichia coli* · *Sulfolobus solfataricus*

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Introduction

Extremophilic microorganisms have gained, in the past few decades, research attention for their unique properties, which are of considerable biotechnological and, therefore, commercial significance (Herbert 1992). Because of the limitations in their cultivation, unless new specific metabolic pathways are evident, there is no reason for using extremophiles for the production of common primary metabolites such as lactic acid, citric acid, and ethanol. However, the enzymatic patrimony of these microorganisms is extremely valuable because of the outstanding characteristics of stability and activity at unconventional temperatures, pH, salinity, and in the presence of organic solvents (Cowan 1992). A number of thermophilic enzymes have been isolated, purified, and characterized, showing enormous potential for the development of novel industrial processes (Canganella et al. 1994; Leuschner and Antranikian 1995; Sunna et al. 1997; Adams and Kelly 1998). So far, these expectations have not been translated into concrete applications because of the major bottlenecks related to the costs of the production process. In fact, two main limitations can be outlined: the remarkably low biomass yields that characterize extremophilic microorganisms cultivation and the low basal enzyme expression level.

The first bottleneck can be overcome by designing and developing innovative bioreactors for achieving high cell density. The approach to this can follow two routes: either employing fed-batch cultures (Lee 1996; Korz et al. 1995) or developing special systems for the removal of toxic metabolites and the replacement of nutrients (Ogbonna and Märkl 1992; Pörtner and Märkl 1998). To date, both these methods have been successfully followed: a fed-batch technique was employed for the cultivation of *Sulfolobus solfataricus* (DSM 1617), reaching 22.6 g/l dry weight (Park and Lee

1997); on the laboratory scale, exploiting a dialysis process, high cell density was achieved in the cultivation of *Sulfolobus shibatae, Pyrococcus furiosus*, and *Marinococcus* (Krahe et al. 1996), and using a novel microfiltration (MF) technique, we obtained high biomass yield of *Sulfolobus solfataricus* (Schiraldi et al. 1999).

However, thermophiles cultivation requires unconventional conditions such as extremes of temperature and pH. These requirements demand the development of expensive apparatuses, and the growth phase is time consuming because the growth rate is very low (e.g., 0.03 h^{-1} for *Sulfolobus solfataricus* Gθ). In addition, high biomass yield does not improve specific enzyme activity. The latter can only be enhanced by employing molecular biology techniques for cloning and expression of thermophilic enzymes into mesophilic hosts (Nordberg Karlsson et al. 1999; Martino et al. 2001; de Pascale et al. 2001). Of course, innovative fermentation strategies applied to recombinant mesophilic microorganisms can overcome the aforementioned limitations by greatly enhancing biomass yield in a shorter period of time. Moreover, enzyme production can be enhanced and recovery can be extremely simplified by exploiting the different thermal stability of host proteins compared to the recombinant product.

In this article, we report the use of recombinant mesophilic hosts for expressing thermophilic enzymes using our microfiltration (MF) bioreactor. We previously published the production of α-glucosidase (Ssαgly) from *Sulfolobus solfataricus* in *Escherichia coli* (Schiraldi et al. 2000). To further explore the flexibility of our MF bioreactor, we extended our research to two other thermophilic enzymes of key importance for the carbohydrate industry, namely, trehalosyldextrin-forming enzyme (SsTDFE) and trehalose-forming enzyme (SsTFE) from *Sulfolobus solfataricus* (Di Lernia et al. 1998; Kato 1999). These two enzymes, SsTDF and SsTF, are of great interest in relation to the production of non-reducing polysaccharides. Moreover, when used in series, they can produce trehalose, an α-1,1 non-reducing disaccharide that was found to have the ability to stabilize biomolecules during dehydration (Roser 1991; Eroglu et al. 2000).

Materials and methods

Materials

Polypropylene capillary membranes were provided by B. Braun Biotech International (Melsungen, Germany). Yeast extract was purchased from Difco (Becton Dickinson, Le pont de Claix, France). Tryptone was obtained from Oxoid (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Microorganisms

Sulfolobus solfataricus Gθ was kindly provided by Dr. R. Cannio (CNR-ISA, Avellino, Italy). Engineered *Escherichia coli* RB-791 expressing either trehalosyl dextrin-forming enzyme (rSsTDFE-Ec) or trehalose-forming enzyme (rSsTFE-Ec) were provided by Dr. D. de Pascale (Organic Chemistry Department, University Federico II, Napoli, Italy) (de Pascale et al. 2001).

Equipment

A Biostat CT (B. Braun Biotech International, Melsungen, Germany) of 2-l working volume was used in these experiments. It was equipped with a digital control unit, and on-line measurements of air-flow rate, acid and base addition, stirring velocity, and temperature were registered by the Multi-Fermenter Control System program (MFCSwin, B. Braun Biotech International). One MF module was inserted into the vessel for *S. solfataricus* Gθ MF experiments, whereas two modules were used for the cultivation of recombinant *E. coli*. The modules were sterilized in situ together with the medium, except for the main carbon sources (e.g., glucose or glycerol), which were filtered or autoclaved separately.

MF fermentation: *Sulfolobus solfataricus* Gθ

During MF experiments, the batch phase lasted 60–80 h; then feeding started according to protocols previously described (Schiraldi et al. 1999). The exchange of exhaust medium (dilution rate, 0.07 h^{-1}) through MF started after 100–120 h of cultivation. Samples were taken every 4 h. To further investigate the metabolism of this thermoacidophilic archeon at very high cell density, we evaluated the production of the reporter enzyme alcohol dehydrogenase (ADH) (Cannio et al. 1996). Cells were separated from the supernatant by centrifugation and then disrupted chemically by using Triton X-100 (0.1% w/w) in a Tris-HCl buffer (50 mM, pH 8.0). Total protein concentration was measured by the dye-binding assay of Bradford (1976). The ADH activity in the cell homogenate was assayed at 70°C using a 25 mM glycine-NaOH buffer, pH 10, using 5 mM benzyl alcohol and 5 mM NAD. The reaction kinetic was followed by measuring absorbance at 340 nm on a spectrophotometer (DU 640; Beckman, Milan, Italy).

Fermentation experiments: recombinant *Escherichia coli* Rb-791

Batch

The preinoculum was always grown overnight in an Aquatron shaker (Infors, Switzerland) on a Luria-Bertani medium at 37°C, pH 6.8, 200 rpm. Fermentation experiments were performed using a semidefined medium (Nakano et al. 1997) with the addition of 20 g/l glycerol, 2.4 g/l yeast extract, and 1.2 g/l tryptone. Fresh medium (1.8 l) was inoculated with 0.1 l preinoculum. Temperature and pH were kept constant during fermentation at 37°C and 6.8, respectively. Stirring velocity and aeration rate were ini-

tially set at 300 rpm and 1.5 l/min and were increased during the experiment to keep the dissolved oxygen partial pressure above 5% saturation.

MF fermentation

The experiments were run in batch mode for 7–10 h, then switched to fed-batch for 3–6 h, and finally the MF modules were turned on. The exhaust medium was replaced with fresh salt and trace element solution at the same concentrations as that of the batch medium (D = 0.22 h^{-1}) whereas microorganisms were held in the vessel and were fed through appropriate profiles. The nutrient solution, which contained 570 g/l glycerol, 24 g/l yeast extract, and 12 g/l tryptone, was added at a rate varying from 3 to 18 ml/l per hour to maintain an acceptable doubling time. A cascade control was employed to meet the increasing respiratory requirements by means of stirring, flow rate, and, finally, pure oxygen supplement. Backflushing operations, through inversion of pumping direction, were delayed 3–4 h from the MF module activation so that the dilution rate was kept constant at 0.2 h^{-1}.

Sampling and sample treatment

Samples were collected every hour, and absorbance measurements were checked at 600 nm on a spectrophotometer. Wet weight and dry weight were measured as previously described (Schiraldi et al. 2000). Glycerol and organic acids were analyzed by a Dionex-500 chromatographer (Rome, Italy). For enzyme recovery, wet cells were disrupted by sonication (Sonicator Ultrasonic Processor, Heat Systems Ultrasonic, Plainview, NY, USA) as previously reported (Schiraldi et al. 2000), and the cytosol was separated from cell debris by centrifugation at 13,000 *g* on a microfuge (Microcentrifuge MC-13, Millipore, Milan, Italy).

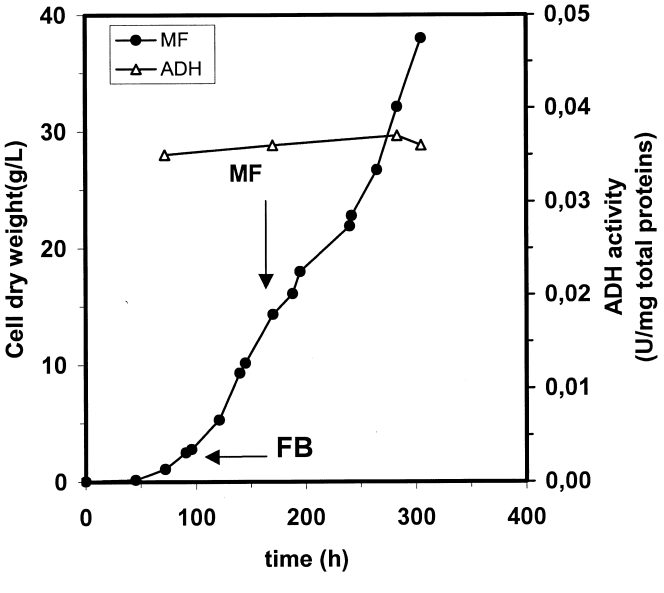

Fig. 1. Growth curve of *Sulfolobus solfataricus* in microfiltration (MF) experiments (Biostat CT 2-l working volume equipped with two MF modules) and corresponding alcohol dehydrogenase (ADH)-specific activity. FB, fed batch

Enzyme assays

The recombinant trehalosyl dextrin-forming enzyme (rSsTDFE) was assayed at 75°C whereas the trehalose-forming enzyme (rSsTFE) was assayed at 75°C and at 85°C in 50 mM citrate phosphate buffer, pH 5.5. rSsTDFE activity was determined by incubating 1–10 µg enzyme with 0.67 mM maltohexaose as substrate for 30 min. The reaction was stopped in an ice-water bath, and the amount of product formed, trehalosylmaltotetraose, was determined by a Dionex Chromatographer equipped with an electrochemical detector, using a Carbopac PA 100 column. One enzyme unit was defined as the amount of enzyme that produces 1 µmol/min of trehalosylmaltotetraose.

rSsTFE activity was determined using 0.1–1 µg enzyme, at 75°C or 85°C, incubating for 30 min with trehalosylmaltotetraose (0.67 mM) as substrate. The final products, maltotetraose and trehalose, were determined as previously described. One enzyme unit was defined as the amount of enzyme that produces 1 µmol/min of trehalose.

Results and discussion

In novel MF experiments, carried out on a 2-l Biostat C fermenter equipped with two MF modules, we confirmed the possibility of achieving high biomass yield for *Sulfolobus solfataricus* Gθ exploiting the MF bioreactor. The highest cell concentration reached was 38 g/l dry weight (cdw) in 305 h, corresponding to a biomass productivity of 0.125 g/l h. These results were in accordance with those previously achieved (Schiraldi et al. 1999) and proved the consistency of the MF technique. The aim of these experiments was to further demonstrate that the cell basal metabolism was not affected by reaching high cell density. In fact, ADH-specific activity was frequently shown to remain constant at 0.03–0.04 U/mg of total proteins at increasing biomass concentrations (Fig. 1).

TDFE and TFE from *Sulfolobus solfataricus* have great potential for the development of innovative industrial processes in the production of non-reducing carbohydrates and of trehalose in particular. To obtain trehalose, the processes should be used in series, and the optimal process temperature must be 75°C (optimal for rSsTDFE). At this temperature, rSsTFE has still about 65% activity and the expression level is 15 fold that for rSsTDFE (Table 1). The high temperature is extremely important, both for reducing contamination during the process and for decreasing the viscosity of highly concentrated solutions of starch or dextrins (Di Lernia et al. 1998; de Pascale et al. 2001).

We have used recombinant *E. coli* strains developed by de Pascale et al. 2001). Despite use of a vector inducible by

Table 1. Specific activity and throughput derived from the enzymatic assays performed at 75°C

Enzyme	Batch mode		MF mode	
	U/cdw	U/l	U/cdw	U/l
rSsTDFE	46	267	44	792
rSsTFE	700	3,505	667	25,300
	1,129[a]	5,653[a]	1,093[a]	41,458[a]

Data are derived from batch and microfiltration (MF) experiments rSsTDFE, recombinant trehalose-forming enzyme from *Sulfolobus solfataricus*; rSsTFE, recombinant trehalose-forming enzyme
[a] rSsTFE activity was also tested at 85°C, which corresponds to the enzyme optimal temperature

isopropyl-β-thiogalactoside (IPTG), rSsTDFE-Ec did not respond to induction as previously reported (de Pascale et al. 2001), and therefore all the experiments were performed without induction. In contrast, batch cultivations on rSsTFE-Ec showed a final activity of 4,910 U/l with induction at OD_{600} equal to 1, whereas without IPTG the final production was 3,395 U/l (Fig. 2). Because the induction improved the production of rSsTFE-Ec only 25%–27%, and as our purpose was to develop a process suitable for industrial application, we decided to perform all the MF experiments without the addition of IPTG. In fact, the improvement in enzyme productivity did not justify the cost of IPTG in the MF system.

The MF bioprocess was designed to prolong the exponential growth phase by adding nutrients and to diminish the concentration of toxic metabolites in the exhaust medium via microfiltration. Typically, once assembled, the MF apparatus has been used for more than 50 fermentations. The MF experiments lead to a biomass yield for rSsTFE-Ec of 38 g cdw/l compared to about 5 g cdw/l in batch experiments (Fig. 3). Quantitative analyses of glycerol and acetic and lactic acid concentrations showed that catabolites were kept below the toxic level (5 g/l) (Nakano et al. 1997) until very high cell density was reached, while at the end of fermentation the substrate was consumed, up to 2 g/l, in spite of the feeding. After 24 h, the MF modules were unable to further increase the exchange rate of the exhaust medium, and this led to the increase of toxic metabolite concentrations up to the growth inhibition level. The experimental data at 24 and 40 h show that it is most probable that the increase in organic acid concentration occurred because of progressive decrease in transmembrane flux related to fouling of the membrane modules (Fig. 4).

Microfiltration experiments performed on rSsTDFE-Ec reached 16 g cdw/l, a concentration lower than that of rSsTFE-Ec but still fivefold higher than that of batch experiments (Fig. 5). The biomass productivity improved compared with the corresponding batch experiments for rSs-TFE-Ec and rSsTDFE-Ec by tenfold and fivefold, respectively. Concerning the differences obtained in biomass concentration between the two recombinants, it is possible that there is an interference in the replication cycle of *E. coli* as a result of the heterologous gene expressed. In a preliminary analysis, we compared the thermoactivity curves of these recombinant enzymes and the previously characterized rSsαgly (Martino et al. 2001). From the data plotted in Fig. 6, it can be seen that at 45°C rSsTDFE showed a residual activity of 65%; rSsTFE activity was only about 30%,

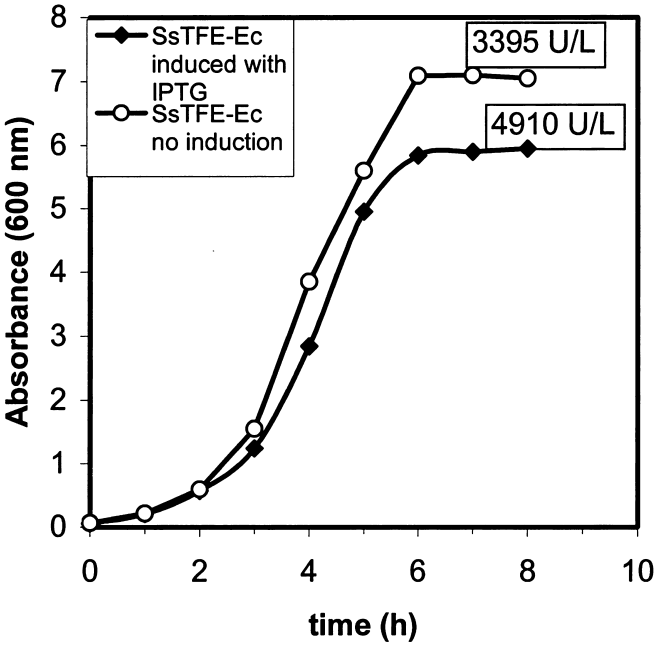

Fig. 2. Growth curves for *E. coli* in batch experiments on SsTFE-Ec (*E. coli*-expressing trehalose-forming enzyme) with induction at OD_{600} = 1 (*closed symbols*) and without addition of isopropyl thiogalactoside (IPTG) (*open symbols*). The values shown refer to the final enzyme production in the two experiments

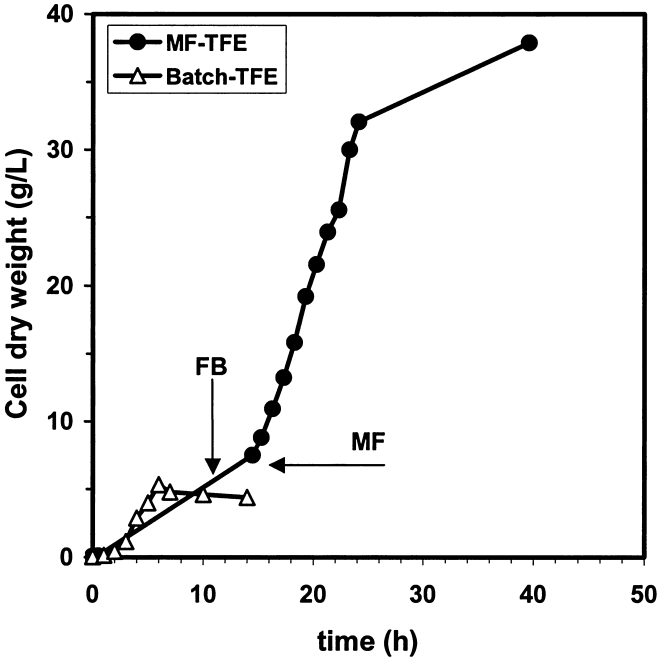

Fig. 3. Comparison between growth curves in fed-batch and MF experiments of recombinant *Escherichia coli* Rb 791 expressing trehalosyldextrin-forming enzyme (SsTDFE)

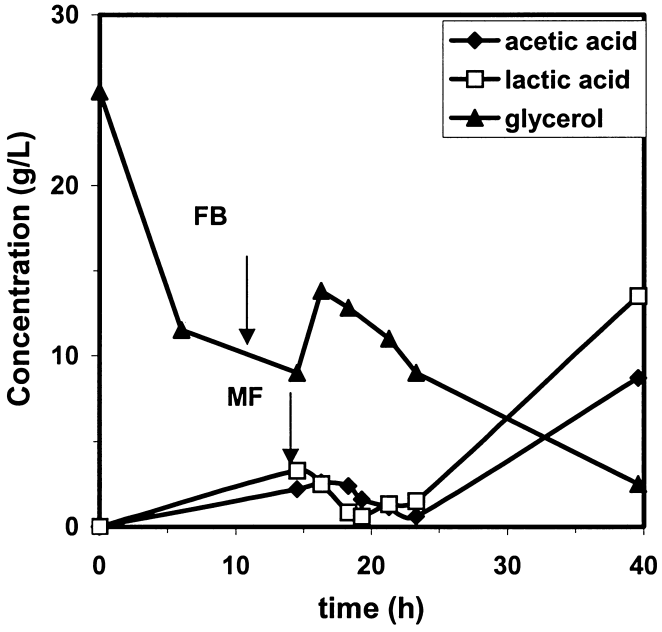

Fig. 4. Profiles of substrate (glycerol, *triangles*) and catabolites such as acetic acid (*diamonds*) and lactic acid (*squares*) during rSsTFE-Ec MF fermentation. *Arrows* indicate the starting of fed-batch (*FB*) and MF phases. Before inoculation, the medium was analyzed to confirm the precise starting points; the samples were taken after overnight growth (14 h). Scatter for glycerol concentration must be ascribed to the step profile of feeding. From 15–24 h experimental time, the medium exchange was efficient in keeping toxic catabolites at low concentrations

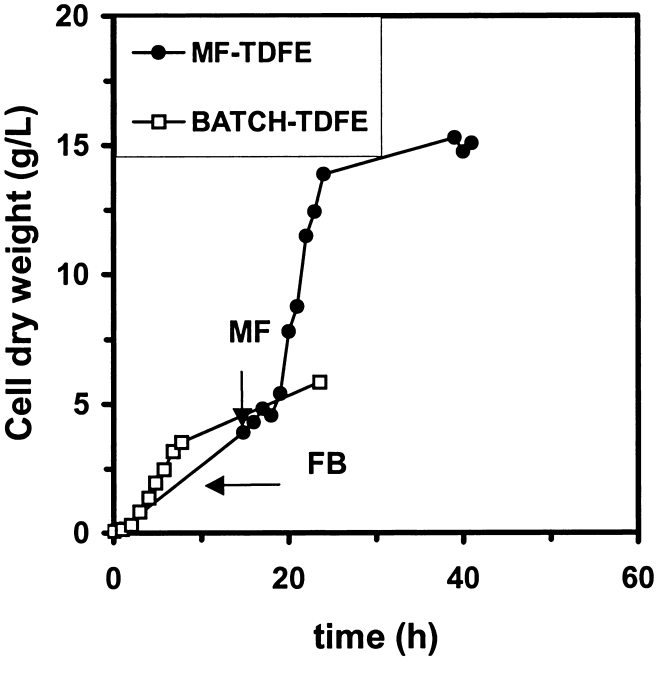

Fig. 5. Comparison between growth curves of recombinant *E. coli* expressing trehalosyldextrin-forming enzyme (rSsTDFE-Ec) in batch and MF experiments. During MF experiments, the fed-batch phase was started after 10–12 h and MF modules were turned on 5 h later (*arrows*)

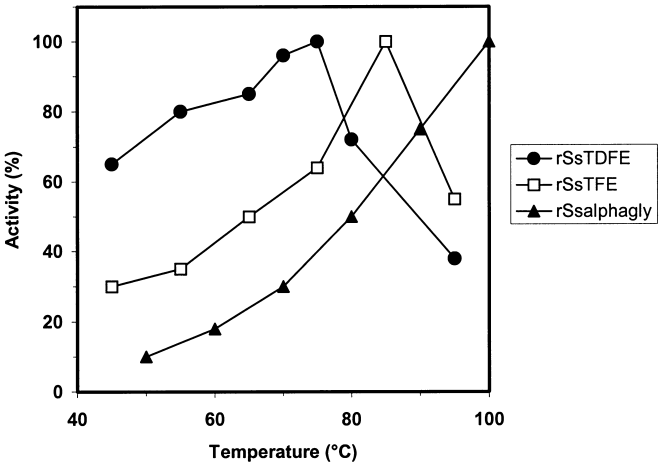

Fig. 6. Thermoactivity curves for the three recombinant enzymes from *Sulfolobus solfataricus*: recombinant trehalosyldextrin-forming enzyme (rSsTDFE) and recombinant trehalose-forming enzyme (rSsTFE) expressed in *Escherichia coli* Rb-791 and α-glucosidase (rSsαgly) expressed in *E. coli* BL21 (DE3)

whereas rSsαgly showed less than 5% activity at 40°C (Fig. 6).

Comparing the biomass yields of rSsTDFE-Ec and rSsTFE-Ec with the data obtained on rSsαgly-Ec (Martino et al. 2001; Schiraldi et al. 2000), and considering the different bacterial strains engineered (*E. coli* Rb-791 for rSsTDFE and rSsTFE, but *E. coli* BL21 for rSsα–gly), we hypothesize a crucial role of recombinant enzyme activity for maximal achievable cell density. In Table 1, we show a comparison of enzyme production (U/l) in a traditional batch experiment and in MF using the recombinant *E. coli* strain indicating that application of the MF technique substantially improved the throughput of the enzyme production process. Specifically, when assays were performed at 75°C there was an eightfold enhancement for rSsTFE and a threefold enhancement for rSsTDFE (see Table 1). The final throughput, tested at the optimal temperature (85°C) for rSsTFE, was 41,458 U/l in MF experiments.

The results are even more impressive if we compare expression levels in the wild-type microorganism and in the recombinant host. In Table 2, it is clearly evident that the combination of genetic engineering and innovative bioprocess/bioreactor design substantially improved enzyme production compared to that of the wild type, i.e., 7 fold for SsTDFE and 65 fold for SsTFE. These data are interesting in terms of industrial application if we evaluate the productivity. In fact, the productivity (Φ = U/l h) obtained in MF experiments was 60 fold for SsTDFE and 500 fold for SsTFE (see Table 2). These achievements can be ascribed to (1) a higher basal level of enzyme expression as the result of genetic engineering; (2) a better controlled environment (substrate and inhibitors concentration) through medium exchange; and (3) a higher biomass concentration obtainable in a time frame comparable to that of batch growth.

Table 2. Comparison between specific activity, volumetric yield, and productivity of the enzymes in MF experiments using the wild-type and the recombinant strains

Enzyme	Wild type			Recombinant		
	U/cdw	U/l	Φ = U/l h	U/cdw	U/l	Φ = U/l h
rSsTDFE	3.3	115.5	0.38	45	792	20.2
rSsTFE	11.6	406.0	1.35	667	25,300	640

Assays were performed at 75°C

Conclusions

The predominant reasons for exploiting thermostable enzymes in novel industrial processes are their higher activity and stability, resulting in major cost savings. Additional benefits can be cited, such as improved mass-transfer rates, lower viscosity, and reduced risk of contamination. As already mentioned, there are several limitations to a broader application of these unique biocatalysts related to their production costs.

Our results demonstrated that a strategy combining molecular biology techniques and bioreactor design can greatly improve thermophilic biomass yield and corresponding enzyme productivity. Therefore, some of the main bottlenecks responsible for the limited applications of extremophilic enzymes have been successfully overcome with this type of bioreactor. Moreover, the MF bioreactor can be easily adapted to meet custom-tailored goals, and therefore it becomes a valuable tool for evaluating, on a laboratory scale, whether it is feasible for the production of other extremophilic enzymes.

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