Protein Expression and Purification 88 (2012) 26–32

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Studying the expression of a lipase from *Pyrococcus furiosus* using response surfaces

Marcelo Victor Holanda Moura [a], Leticia Dobler [a], Melissa Limoeiro Estrada Gutarra [b], Rodrigo Volcan Almeida [a,*]

[a] Laboratório de Microbiologia Molecular e Proteínas, Instituto de Química, Universidade Federal do Rio de Janeiro, Brazil
[b] Departamento de Engenharia Bioquímica, Escola de Química/Polo de Xerém, Universidade Federal do Rio de Janeiro, Brazil

ARTICLE INFO

Article history:
Received 30 August 2012
and in revised form 12 November 2012
Available online 29 November 2012

Keywords:
Heterologous expression
Lipase
Pyrococcus furiosus
Experimental design
Rare codons

ABSTRACT

The need to find more stable catalysts has encouraged the study of naturally resilient enzymes, such as those found in extremophile organisms. In the present work, the influence of rare codons on the expression in *Escherichia coli* of the lipase Pf2001Δ60 from *Pyrococcus furiosus* was evaluated. Expression was carried out in two *E. coli* strains, BL21(DE3)pLysS and the rare tRNA supplemented Rosetta(DE3)pLysS. 3^2 factorial design was used to appraise the influence of temperature and inducer concentration on enzyme expression every hour for the 4-h expression period. Four response surfaces were constructed for each time, and the statistical parameters were evaluated. Lipase production was twice as high for Rosetta(DE3)pLysS than for BL21(DE3)pLysS. The factorial design indicated that optimal expression occurred at 30 °C after 4 h, with lipase production of 240 U/L. The analysis of statistical parameters during the expression time showed that the velocity at which the enzyme was produced affected cell growth and maximum activity, with a higher speed leading to lower expression and cell growth. The presence of rare tRNAs prevented bottlenecks in lipase expression, and the experimental design was shown to be important for maximizing the production strategies and minimizing the metabolic load to which the host is subjected.

Introduction

The search for sustainable and eco-friendly processes has resulted in a rise in the utilization of biocatalysts in industry. The development of these sustainable and economically viable technologies has led to a quest for new biocatalysts with a better capacity to withstand the often adverse conditions in industrial processes. The archaea domain has many microorganisms with the natural ability to thrive in extreme temperature, pressure, salinity and pH conditions. This makes them potential candidates for study in the search for new approaches in biocatalysis [1,2].

One of the most widely studied archaea is *Pyrococcus furiosus*. This microorganism has an optimal growth temperature of 100 °C and is capable of withstanding high pressures. Its genome has been fully sequenced [3] and some of its enzymes are very important, like Pfu DNA polymerase, a widespread tool for molecular biology [4]. However, the same characteristics that make these enzymes of potential interest also make the microorganisms harder to cultivate on a large scale. For instance, *P. furiosus* is anaerobic and it does not normally reach high biomass concentrations, and it utilizes S° in order to produce H_2S. In this context, molecular biology can contribute greatly to the production and study of hyperthermophilic enzymes on a larger scale, using hosts that are easier to cultivate and can achieve high-level protein expression.

One of these enzymes is the lipase PF2001 from *P. furiosus*. The ORF PF2001 was characterized and the gene was cloned and expressed in *Escherichia coli* without the initial 60 bp, a hypothetical signal peptide coding region. Initially the authors classified the enzyme as an esterase [5]. In another work the authors investigated the stability of the enzyme. The protein retained more than 90% stability when incubated for 6 h at 75 °C in the presence of Triton X-100 when fused to a TRX tag. They also found the enzyme optimal activity temperature to be 80 °C [6]. Furthermore the enzyme was also immobilized on supports with different hydrophobicity levels (butyl Sepabeads and octadecyl Sepabeads) [7]. The work showed that the enzyme was hyperactivated in both supports (140% and 237%, respectively), taking into account these data, the authors reclassified this enzyme as a lipase. This biocatalyst presented high thermal stability (77% activity after incubation at 70 °C for 120 min) and storage time (100% activity after 30 days at room temperature). The optimal pH of the immobilized biocatalyst was found to be between 6 and 7, and the optimal temperature was 70 °C. These data suggest it could be a promising biocatalyst, although before implementation, more studies will be needed for its characterization and to optimize the production of the recombinant enzyme.

* Corresponding author.
E-mail address: volcan@iq.ufrj.br (R.V. Almeida).

1046-5928/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2012.11.008

Highly thermostable lipases, as PF2001, have a variety of potential uses in industry that benefits from its thermostability. One example is pitch control in cellulose and pulp manufactures, where enzymes with optimal temperature of 70–85 °C are used to control pitch deposit in industrial scale [8]. Another example is the synthesis of biodiesel and biolubricants, which benefits from higher temperatures and in these conditions, lipases with higher thermostability can be more efficient than the traditionally used ones in terms of recycling and activity [9].

E. coli is the main host used for heterologous expression. The abundance of tools that exist for its cloning and expression, the low generation time and extensive knowledge of its metabolic processes make this bacterium an attractive host for expressing a large quantity of proteins [10,11]. However, not all proteins are properly expressed in *E. coli*. There can be problems, like differences in the codon usage between the host and the original microorganism, transcription/translation issues, like a lack of post-translational modifications, protein toxicity, and the formation of inclusion bodies, among others [12,13]. The protein is not always folded properly in heterologous expression and one of the factors is the reductive potential inside the *E. coli* cytoplasm, which hampers the formation of disulfide bonds. The overexpression of the heterologous protein in larger quantities than the host can handle is also a factor that lowers the amount of properly folded protein. These factors can often trigger the formation of inclusion bodies, protein aggregates in corpuscular form that accumulate in the cytoplasm. These inclusion bodies cause stress, which activates a series of response mechanisms towards the cell metabolism, including the production of chaperones and proteases, and can alter cell growth and stability [14].

As the PF2001Δ60 gene from the *P. furiosus* lipase has codons that are considered rare in *E. coli* – AGG, AGA, AUA, CUA and GGA –, this factor can be a bottleneck in the production of the lipase. Thus, the present work sought to investigate the influence of these codons on the production of lipase PF2001Δ60, using two *E. coli* strains, one with a supplement of tRNAs corresponding to the rare codons, called Rosetta(DE3)pLysS, and the other one without the tRNA supplement: BL21(DE3)pLysS.

The present work also studied the influence of temperature and the concentration of the inducer, isopropyl-β-D-1-thiogalactopyranoside (IPTG)[1], over different times of lipase expression by using 3^2 experimental design.

Materials and methods

Strains

The strains used in the work were: *E. coli* BL21(DE3)pLysS (F^-ompT gal dcm lon $hsdS_B(R_B^- m_B^-)$ λ(DE3)pLysS(cmR)), Rosetta(DE3) (F^-ompT $hsdS_B(R_B^- m_B^-)$ gal dcm(DE3) pRARE (Cam^R)) and Rosetta(DE3) pLysS(F^-ompT $hsdS_B(R_B^- m_B^-)$ gal dcmλ(DE3)pLysSRARE (Cam^R)), from the Laboratório de Microbiologia Molecular e de Proteínas (LaMMP-UFRJ) culture collection. The ORF PF2001 was PCR amplified using the genomic DNA from *P. furiosus*, and specific primers containing BglII and SalI sites. This amplicon was cloned to pET32a generating the plasmid pETPF2001Δ60. The enzyme expressed is fused to TRX tag, totaling 48 kDa [5].

Reagents

The culture medium used to grow the *E. coli* strains was Luria Bertani medium (LB), acquired from Invitrogen®. The antibiotics used were chloramphenicol (Cam) and ampicillin (Amp), and the substrate used for the activity assays was methylumbelliferyl-heptanoate (MUF-Hep), bought from Sigma®. The IPTG used was from Merck™. The Bradford reagent utilized was from BioRad®.

Influence of rare codons

Isolated colonies of *E. coli*, strains BL21(DE3)pLysS pET-Pf2001Δ60 and Rosetta(DE3)pLysS pETPf2001Δ60, were cultivated in solid LB medium, with specific antibiotics, and inoculated in liquid LB under selective pressure. The pre-inocula were incubated at 37 °C under agitation at 170 rpm for 18 h. So the cultures were then inoculated at OD_{600nm} of 0.17 in Erlenmeyer flasks with new LB medium. The cultures were incubated at 37 °C until OD_{600nm} = 0.4 (about 0.075 g/mL dry weight), and induced with 0.75 mM IPTG. Samples were collected at the time of induction and every hour during the 4-h expression time. After the process, the samples were centrifuged for 10 min at 13,000*g*. The supernatant was then discarded and the cells were stored at −20 °C.

To analyze expression, the cells were lysed by ultrasound (300–400 W) in the presence of 300 μl phosphate buffer, pH 7, 0.05 M.

In order to determine the influence of the rare codons on expression, the biomass (mg/mL) and specific activity (U/g of protein) obtained for each strain were compared. The online program GCUA [15] was used to compare the codon usage frequency of *E. coli* and the *pf2001* gene from *P. furiosus*.

SDS–PAGE electrophoresis and zymography

The quantity of cell extracts used in the assays was normalized by OD_{600nm} and the samples were submitted to denaturing conditions using SDS–PAGE electrophoresis with 12% (w/v) polyacrylamide gel, according to [16]. The resulting gel was then incubated in a 2.5% (v/v) Triton X-100 solution for 30 min under mild agitation. After that, the gel was washed with 0.05 mM phosphate buffer, pH 7.0, and then with a solution containing 100 μM of the substrate (MUF-Hep), according to Prim et al. [17]. After the zymogram, the gel was washed with distilled water and then stained with Coomassie solution [18].

Protein assay

Protein concentration was determined according to [19], using Bovine Serum Albumin (BSA) as a standard and a microplate spectrophotometer at 595 nm.

Enzyme activity

The crude extract obtained from the cell lysis was used in all the assays. The activity assay was conducted according to Prim et al. [17], using 100 μM MUF-Hep as a substrate. 10–60 μL samples of the protein extract were used in the analysis at a temperature of 70 °C and in phosphate buffer, 0.05 M, pH 7. One unit of enzymatic activity was defined as the enzyme quantity necessary to catalyze the formation of 1 μmol of MUF a minute.

Experimental design

The statistical analysis used the Rosetta(DE3) pETPf2001Δ60 strain with a 3^2 factorial experimental design, with three replicates at the central point. The variables controlled were inducer concentration (0.25 mM, 0.50 mM and 0.75 mM) and incubation temperature (23 °C, 30 °C and 37 °C), according to Table 1. Experiments were also performed without the presence of the inducer, to act as a control. The response variables were biomass (dry cell weight – g/L) and specific activity (U/g of protein). Response surfaces were

[1] *Abbreviations used:* IPTG, isopropyl-β-D-1-thiogalactopyranoside; Laboratório de Microbiologia Molecular e de Proteínas, LaMMP-UFRJ; LB, Luria Bertani medium; Cam, chloramphenicol; Amp, ampicillin; MUF-Hep, methylumbelliferyl-heptanoate; Bovine Serum Albumin; ALA, 5-aminolevulinate; ANOVA, Analysis of Variance.