

Full Length Research Paper

Enhanced production of subtilisin of *Pyrococcus furiosus* expressed in *Escherichia coli* using auto-inducing medium

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A subtilisin gene identified in the reported genome sequence of *Pyrococcus furiosus* was amplified and inserted in pET-22b(+) vector to produce the recombinant plasmid pET-SB. *Escherichia coli* BL-21 (DE3) CodonPlus was transformed with this plasmid and the enzyme was expressed up to 30% of the total cell protein on induction with IPTG. The expressed protein appeared at a position corresponding to ~20 kDa on SDS-PAGE as compared to theoretical molecular mass of 17.6 kDa. This aberrant electrophoresis mobility could be due to specific amino acid composition of the protein. Auto-induction with lactose also produced a similar level of expression but the total amount of the enzyme produced was 2.4 fold greater than that when produced with IPTG induction. This was due to a higher cell density obtainable in the auto-inducing medium. The enzyme expressed in the insoluble state could be partially refolded after denaturation with urea at high pH. This study reports for the first time high-level expression of subtilisin of *P. furiosus* in *E. coli* using an auto-inducing medium.

Key words: Subtilisin, *Pyrococcus furiosus*, auto-induction, enhanced production.

INTRODUCTION

Studies have continued to produce commercially important enzymes like proteases with better thermostability and other favorable properties for applications. *Pyrococcus furiosus*, an obligate anaerobic marine archaeon, has been the focus of significant biochemical and physiological studies because of its hyperthermophilic characteristics (Adams, 1993). Several proteins of *P. furiosus*, which function at temperatures greater than 90°C and enzymes displaying novel activities have been isolated and characterized (Leuschner and Antranikian, 1995). Many novel protease genes were identified by the whole genome sequencing project of *P. furiosus*. These proteases were reported to range in estimated molecular

masses from 66 to 135 kDa, are highly stable at temperatures of 95°C and above and many are resistant to denaturation by sodium dodecyl sulfate (Ilse et al., 1990). Cell extracts from *P. furiosus* were found to contain five proteases, some of which were found resistant to denaturation by sodium dodecyl sulfate, 8 M urea, 80 mM dithiothreitol and 5% β-mercaptoethanol (Halio et al., 1996; Hicks, 1998). A subtilisin-like cell envelope-associated protease was isolated from *P. furiosus*. It was thermostable up to a temperature of 115°C and had a half-life of more than 96 hours at 80°C and 4 h at 100°C. It was found to have the highest homology to the subgroup of the subtilisin-like serine proteases (Eggen et al., 1990; Voorhorst et al., 1996).

We have previously reported cloning and expression of genes encoding carboxypeptidase (Ikram et al., 2008a) and a prolylendopeptidase (Ikram et al., 2008b) of *P. furiosus* in *Escherichia coli*. A subtilisin (SB) gene of *P.*

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furiosus was found at the locus_tag PF0688 in the reported genome sequence. This paper describes for the first time the expression of this gene in an auto-inducing medium and purification and refolding of the enzyme to an active state.

MATERIALS AND METHODS

DNA, enzymes, kits and plasmids

The genomic DNA of *P. furiosus* was obtained from the American Type Culture Collection (ATCC 43587). Taq DNA polymerase, T4 DNA ligase, restriction enzymes and Inst/Aclone™ PCR product cloning kit were purchased from MBI Fermentas (USA) whereas QIAquick DNA gel extraction and QIAprep spin miniprep kits were acquired from QIAGEN, Inc. USA. pET-22b(+) expression plasmid was from Novagen, USA.

Bacterial strains and culture media

E. coli strains DH5 α and BL21 (DE3) CodonPlus (Stratagene, USA) were used as cloning and expression hosts, respectively. *E. coli* were routinely grown in an orbital incubator shaker at 37°C and 150 rpm, unless stated otherwise. The cultivation media used in this study included LB, TB and M9NG and these were prepared as described earlier (Sambrook and Russell, 2001; Sadaf et al., 2007).

Construction of pET-SB expression plasmid

Based on the published sequence of *SB* gene (locus_tag PF0688), a pair of gene-specific forward (5'-ATAACATATGGCCCTAGG AGGATCTGC-3') and reverse (5'-GATGGATCCAAAAGTTG TAGACC-3') primers was designed to amplify the gene fragment from the genomic DNA of *P. furiosus*. The forward and the reverse primers contained restriction sites for *Nde*I and *Bam*HI (shown in bold), respectively, at their 5' termini to facilitate the directional cloning. The PCR reaction was set up in 50 μ l reaction volume and the conditions used were: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 30 s, extension at 72°C for 50 s and a final extension at 72°C for 20 min in an Applied Biosystem 2720 thermal cycler.

The amplicon was first cloned in pTZ57R/T vector by employing dA.dT tailing technique and then subcloned in pET-22b(+) at *Nde*I/*Bam*HI sites to generate the expression plasmid pET-SB. The recombinant plasmid, thus obtained, was maintained in *E. coli* DH5 α with ampicillin (100 μ g/ml) as the selection antibiotic. The sequence and correct integration of insert were verified by restriction digestion and sequence analysis using Beckman Coulter CEQ™ 8000 Genetic Analyzer.

Expression analysis

For the expression of cloned *SB* gene, the plasmid pET-SB was transformed into CaCl₂-competent *E. coli* BL21 (DE3) CodonPlus cells as described earlier (Ikram et al., 2008a, b). From the positive transformants, selected on LB-ampicillin plate, a single colony was inoculated in 10 ml LB-ampicillin medium contained in a 100 ml Erlenmeyer flask and incubated in a shaking incubator (IRmeco GmbH, Germany) at 150 rpm and 37°C overnight. Fresh 50 ml LB

medium was inoculated with 1% overnight culture and incubated as described earlier. Protein expression was induced with either 1 mM isopropyl- β -D-1-thiogalacto-pyranoside (IPTG) or 10 mM lactose, when the culture OD₆₀₀ reached 0.6 - 0.8, unless stated otherwise. Following induction, cells were allowed to grow at 37°C shaking incubator with constant agitation. During cultivation, 1 ml aliquots were collected after every one hour to monitor growth (OD₆₀₀) and analyze protein expression by 12% SDS-PAGE (Laemmli, 1970).

To analyze the expression of subtilisin-like protease in soluble and insoluble fractions, cells from an aliquot of the culture were harvested, washed with 0.05 M Tris-Cl (pH 7.5), resuspended in the same buffer to a final OD₆₀₀ of 10, lysed by sonication in UP 400S sonicator (dr. hielscher GmbH, Germany) on ice by giving 10 bursts of one minute each with one minute interval between two successive pulses and then centrifuged at 4,500 rpm for 20 min. The soluble and insoluble fractions, thus obtained, were analyzed for the presence of the expressed enzyme by SDS-PAGE.

Enzyme solubility and refolding

Solubility of the recombinant enzyme, expressed as inclusion bodies, was checked in different solubilization buffers containing 0.05 M Tris-Cl (pH 8.0-11.0) and varying concentration of denaturing agent urea (2-8 M). Aliquots of the cell pellet containing equal amounts of the insoluble enzyme were resuspended in each of the solubilization buffer while stirring gently for 30 min at room temperature followed by centrifugation at 10,000 rpm for 10 min. Solubilization of the cell pellet contents was judged by measuring absorbance of the supernatant at 280 nm and turbidity at 450 nm.

For refolding, the solubilized proteins were diluted with ice-cold refolding buffer [0.05 M Tris-Cl (pH 8.0), 5 mM EDTA, 0.05 M L-arginine] to a concentration of ~200 μ g protein/ml. The mixture was incubated at 4°C for 12 h followed by dialysis against 0.05 M Tris-Cl (pH 8.0) overnight with three changes of the buffer during dialysis. Refolding efficiency was evaluated by using 500 μ l of each refolding sample for enzymatic assay.

Enzyme assay

500 μ l of the appropriately diluted enzyme (~250 μ g protein) and 500 μ l of 0.5% solubilized azocasein (Sigma Aldrich Co., USA) were incubated at 70°C for 30 min in a shaking water bath. Following incubation, the reaction was stopped by adding 2 ml of 10% trichloroacetic acid and the mixture was placed on ice for 10 min. Precipitated proteins were separated by centrifugation at 6,500 rpm for 10 min and the amount of azo dye liberated in the supernatant was determined by measuring OD₄₄₀ (Reichard et al., 1990). One unit of enzyme activity is defined as the amount of enzyme which releases 1 μ g amino acids equivalent to tyrosine per minute under the assay conditions.

RESULTS AND DISCUSSION

Cloning and expression of *SB* gene

The PCR amplified product of the *SB* gene, incorporating restriction sites for *Nde*I and *Bam*HI at the 5' and 3' termini of the *SB* gene, respectively, was electrophoresed. The ~500 bp fragment was gel purified, T/A

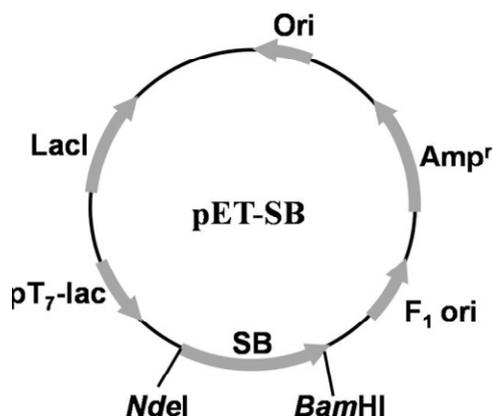


Figure 1. Recombinant plasmid pET-SB showing the position of 501 bp SB gene insert in the expression vector pET-22b.

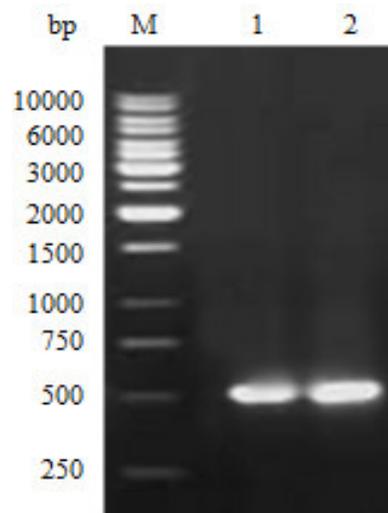


Figure 2. Agarose gel electrophoresis of the PCR product showing the presence of SB insert in the recombinant plasmid isolated from two of the transformed cells. Lane M, size markers; Lanes 1 and 2, PCR product obtained from two different transformed cell colonies.

cloned in pTZ57R/T and subcloned in pET-22b(+) to generate pET-SB expression plasmid (Figure 1). The expression plasmid was first maintained in *E. coli* DH5 α for vector propagation and then transformed into BL21 (DE3) CodonPlus for expression studies. Presence of the insert in the plasmid preparation from the positive transformants was confirmed by colony PCR (Figure 2). A prominent band of ~0.5 kb as analyzed by agarose gel electrophoresis confirmed successful transformation. The correctness of the insert was also confirmed by nucleotide sequencing.

Proteins from the cells of the *E. coli* culture harboring pET-SB, when analyzed by SDS-PAGE, showed a prominent band at a position corresponding to ~20 kDa within initial 2 h of induction with 1 mM IPTG. A band of similar size and intensity, however, was absent in the control and uninduced cells (Figure 3a). The expression levels increased gradually reaching a maximum of around 30% of the total *E. coli* cellular proteins, after 10 hours of induction. A study on the effect of IPTG concentration between 0.2 - 1.5 mM showed that 0.5 - 1.0 mM were the optimal levels for subtilisin expression (Figure 3b). However, it was observed that the size of expressed protein was slightly higher than the theoretical molecular mass of SB (17.6 kDa), as calculated through ExPASy Proteomics Server (www.expasy.ch). The expression analysis, repeated with multiple colonies, displayed similar results each time with a band of recombinant protein at a position corresponding to ~20 kDa. It appears that SB probably exhibits an aberrant electrophoretic mobility on SDS-gel, which could be due to its specific amino acid composition. The contents of the negatively charged amino acids Glu and Asp in SB are around 12%, which are relatively higher than usual contents. A recombinant $\alpha 2/\alpha 8$ hybrid interferon expressed in *E. coli* has also been

described to display aberrant mobility on SDS-gel due to high Glu/Asp content (Platis and Foster, 2003).

Lactose induction

The expression of recombinant SB in *E. coli* was studied by auto-induction with lactose, an inexpensive substitute for IPTG. Lactose at a concentration of 10 mM in LB medium induced SB expression gradually reaching a maximum of ~28% of the total cell proteins after 12 h, as analyzed by SDS-PAGE electrophoresis (Figure 4a). A study on the effect of lactose concentration showed that the expression level increased with increase in lactose concentration up to 10 mM (Figure 4b). Further increase in concentration seemed to have a negative effect on the expression level. 10 mM lactose was, therefore, used as inducer in all subsequent experiments.

The expression of SB, when induced with lactose, was little delayed as compared to that in case of IPTG and also the level of maximum expression was a little lower. However, cell growth achieved in the LB medium following lactose induction was more than twice than observed with IPTG induction (Table 1). Accordingly, the final yield of SB in the lactose-medium was 281.4 mg/L as compared to 117 mg/L from the IPTG induced culture.

In order to enhance the production of SB, auto-induction

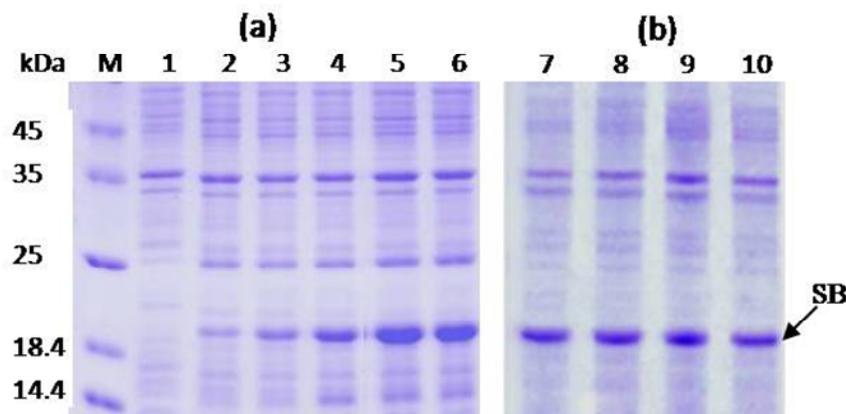


Figure 3. SDS-PAGE of total cell proteins of *E. coli* cells transformed with pET-SB and induced with 1mM IPTG for different time periods (a) and with different concentrations of IPTG (b). Lane M, protein markers; lanes 1 - 6, cells induced with 1 mM IPTG 1, 2, 4, 8, 10 and 12 h, respectively; lanes 7 - 10, cells induced for 10 h with 0.2, 0.5, 1.0 and 1.5 mM IPTG, respectively.

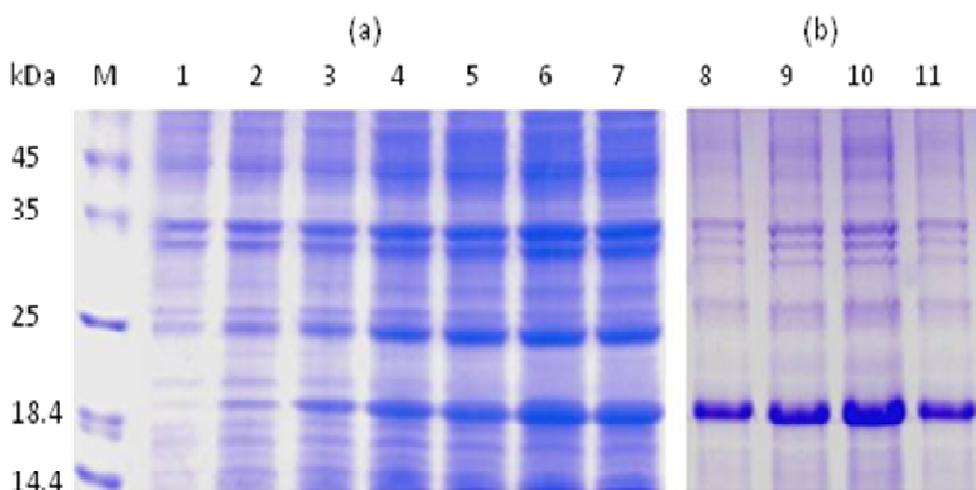


Figure 4. SDS-PAGE analysis of the total cell proteins of *E. coli* transformed with pET-SB and induced with 10 mM lactose for different time periods (a) and with different concentrations of lactose for 12 h (b). Lane M, protein markers; lanes 1 - 7, cells induced with 1 mM lactose for 2, 4, 6, 8, 10, 12 and 14 h, respectively; lanes 8 - 11, cells induced for 12 h with 6, 8, 10 and 12 mM lactose, respectively.

with lactose was done in TB and M9NG media. Maximum OD_{600} attained in these media was 16.9 and 13.4, respectively, which was significantly higher than that observed in LB (Figure 5 and Table 2). Accordingly, the amount of SB obtained was 2 to 3-folds higher with 478 and 540 mg SB per liter of cultivation media. Higher cell growth in M9NG and TB seems to parallel with the richness of nutrients. A little lower yield of SB from M9NG medium seems more attractive as it is comparatively cheaper cost.

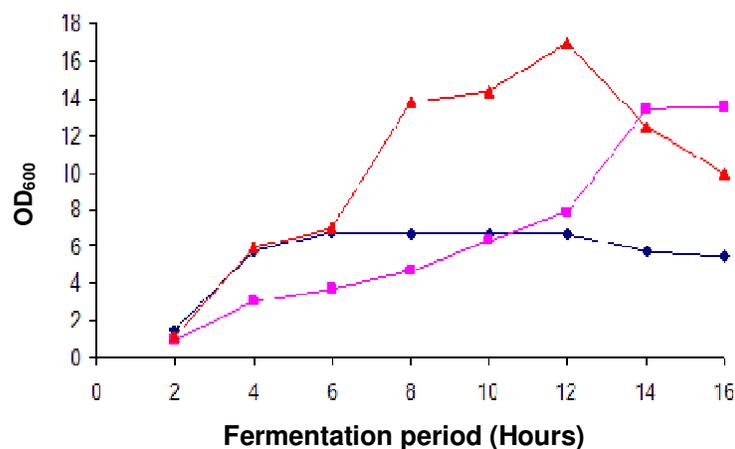
Refolding and purification

An aliquot of subtilisin expressing *E. coli* cells was lysed by ultrasonication and both the supernatant and the insoluble pellet were analyzed by SDS-PAGE. Almost all the expressed SB was in the insoluble form (Figure 6, lane 2). A protein from an extreme thermophile when expressed in *E. coli* would be encountered with an environment, which is much different from that of the original organism especially with respect to temperature,

Table 1. Comparison of SB production when induced with 1 mM IPTG and 10 mM lactose in LB medium.

Time (h)	IPTG				Lactose			
	OD ₆₀₀	TCP* (mg/l)	Expression (%)	SB (mg/l)	OD ₆₀₀	TCP* (mg/l)	Expression (%)	SB (mg/l)
4	1.2	180	6	10.8	1.6	245	5	12.3
8	2.5	385	15	57.8	3.2	480	13	62.4
12	2.6	390	30	117.0	6.7	1005	28	281.4

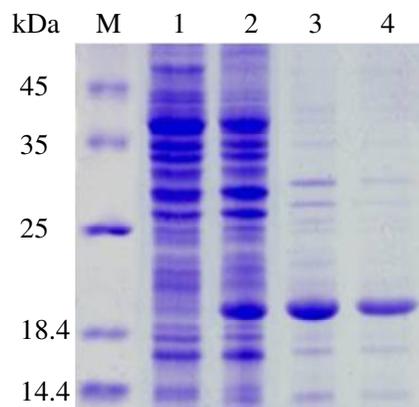
*Total cell proteins.

**Figure 5.** Growth profile of *E. coli* cells transformed with pET-SB and induced with 10 mM lactose, when cultivated in LB (◆), M9NG (■) and TB (▲) media.**Table 2.** Total cell proteins and the amounts of SB expressed in *E. coli* cells when cultivated in different media.

Media	Maximum OD ₆₀₀	TCP (mg/l)	SB (% of TCP)	SB (mg/l)
LB	6.70	990	28	277
M9NG	13.4	1840	26	478
TB	16.9	2250	24	540

thus preventing its correct folding.

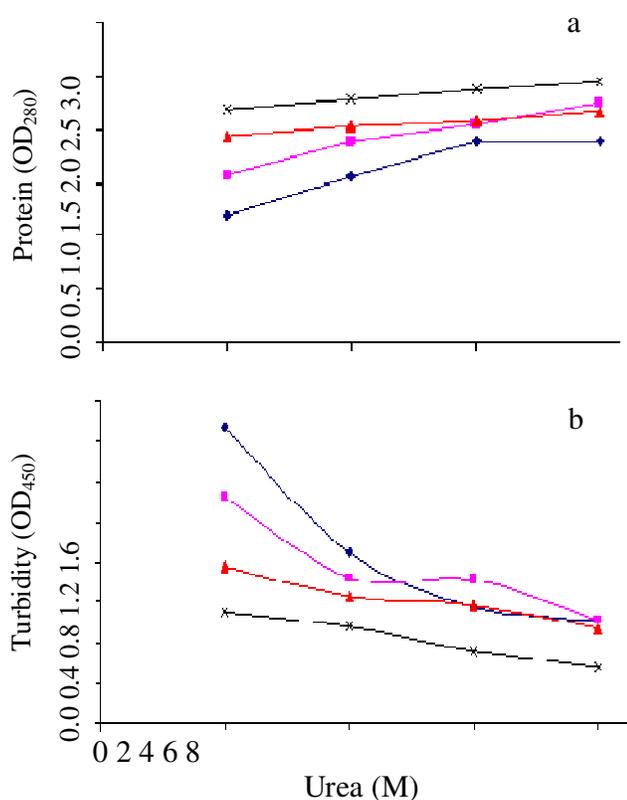
The misfolded SB in the cell pellet was suspended in solution having increasing concentrations of urea. The proportion of solubilized protein (Figure 7a) and reduction in turbidity (Figure 7b) showed that, the solubility of inclusion bodies in Tris-Cl (pH 11.0) containing 2 M urea was higher than in Tris-Cl solution (pH 8.0) and 8 M urea. The solubilized protein solution (~2 mg/ml) was diluted ten-fold with the dilution buffer, incubated overnight at 4°C and dialyzed as described previously for refolding. Samples were collected during the entire expression, washing, solubilization, refolding and dialysis steps and

**Figure 6.** Refolding of subtilisin expressed in *E. coli*. Lane M, protein markers; lane 1, supernatant of the cell lysate; lane 2, insoluble pellet from the cell; lane 3, insoluble pellet washed with buffer containing Triton X; lane 4, refolded subtilisin.

subjected to SDS-PAGE to examine the purity of SB, which reached a level of around 92% without any

Table 3. Summary of SB purification from 5.2 g wet weight of *E. coli* cells.

Steps	TCP (mg)	SB (mg)	Recovery (%)	Purity (%)
Cell lysate	650	169	100	26
IBs	280	162	96	58
WIBs	195	138	82	71
Solubilization	110	89	53	81
Dialysis	90	83	49	92

**Figure 7.** Effect of urea concentration on protein solubilisation (a) and turbidity (b) in the lysate of *E. coli* cells expressing subtilisin, at pH 8.0 (◆), 9.0 (■), 10.0 (▲) and 11.0 (x).

chromatographic purification procedure (Figure 6 and Table 3).

Protease activity of the preparation thus obtained, as assayed using azocasein as substrate, was 114 U/mg protein. It appears that for expressing and obtaining proteins from extreme thermophiles like *P. furiosus* in the correctly-folded form, one would need to match the conditions the protein is likely to come across in the parent organism.

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