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High level expression of a hydrophobic poly(ethylene terephthalate)-hydrolyzing carboxylesterase from *Thermobifida fusca* KW3 in *Escherichia coli* BL21(DE3)

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ABSTRACT

The gram-positive thermophilic actinomycete *Thermobifida fusca* KW3 secretes a highly hydrophobic carboxylesterase (TfCa) that is able to hydrolyze poly(ethylene terephthalate). TfCa was produced in the *Escherichia coli* strain BL21(DE3) as a fusion protein consisting of a pelB leader sequence to ensure periplasmic localization of the protein and a His_6 tag for use in its purification. To enhance the recombinant enzyme yield, the *tfca* gene from *T. fusca* KW3 was successfully optimized for codon usage in *E. coli*. In addition, the gene expression induction conditions were optimized and the temperature for cell cultivation was lowered to reduce inclusion body formation. The optimized codons and expression conditions yielded 4500-fold higher TfCa activity than the wild-type strain. Using a pH-controlled bioreactor for cultivation, a TfCa protein concentration of 41.6 mg/L was achieved.

1. Introduction

Thermobifida fusca is a thermophilic soil bacterium that degrades plant cell wall polymers in compost and soils. Recently, two extracellular esterases from *T. fusca* that have the ability to hydrolyze poly(ethylene terephthalate) (PET) have been isolated and characterized (Dresler et al., 2006; Chen et al., 2008). The enzymes have been described as cutinases due to their ability to hydrolyze the plant polyester cutin (Fett et al., 1999). While both *Escherichia coli* and *Bacillus megaterium* have been used as expression hosts for these hydrolases, the successful expression in *B. megaterium* required a codon-optimized gene (Yang et al., 2007). *T. fusca* KW3 produces another extracellular enzyme capable of hydrolyzing PET. This carboxylesterase (TfCa) has a molecular mass of 52.4 kDa (Billig et al., in preparation). PET-hydrolases are of interest because they hydrolyze recalcitrant, synthetic, aromatic polymers such as PET (Müller et al., 2005) and therefore have applications in biocatalytic surface hydrolysis and functionalisation of synthetic polymers (Alisch et al., 2004; Alisch-Mark et al., 2006; Guebitz and Cavaco-Paulo, 2007).

2. Materials and methods

2.1. Amplification and cloning of the tfca gene

The *tfca* gene was amplified from *T. fusca* KW3 genomic DNA by standard polymerase chain reaction (PCR) methodologies using Taq DNA polymerase. The cycle programme was performed as follows: 1 cycle of 10 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 2 min at 72 °C; and a final extension step of 5 min at 72 °C. The nucleotide sequence of the homologous putative carboxylesterase from *T. fusca* YX, whose complete genome has been recently sequenced (Lykidis et al., 2007) was used as basis for the primer design to amplify the *tfca* gene. The forward primer used was 5′-TTTTTCCATGGCCGTGGAGATCGTGATCAGA-3′ and the reverse primer was 5′-TTTTAAGCTTGTACAGCGGCACCCCGTCCCACA-3′. NcoI and HindIII restriction sites (underlined) were added to the forward and reverse primer, respectively. The reverse primer eliminated the stop codon of the gene. The PCR product was first cloned into the pJET1/blunt vector (Fermentas, St. Leon-Rot, Germany) by blunt-end ligation. Next, it was amplified in *E. coli* XL1-Blue and sub-cloned into the pET-20b(+) expression vector (Novagen, Darmstadt, Germany) using the added restriction sites. The final pET-20b(+) construct containing the pelB leader sequence, His_6 tag, and *tfca* gene was sequenced using the Big Dye Terminator

Abbreviations: CAI, codon adaption index; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani medium; PET, poly(ethylene terephthalate); *p*-NPB, *p*-nitrophenyl butyrate; TfCa, carboxylesterase from *Thermobifida fusca* KW3; Tris, tris(hydroxymethyl)aminomethane; w/v, mass–volume-percentage.

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v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, USA).

2.2. Optimization of the tfca gene

The codon usage of the *tfca* gene was adapted to the codon bias of *E. coli* (Geneart GmbH, Regensburg, Germany). To monitor the success of the codon usage optimization, shake flask cultures (50 mL) of *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) containing the non-optimized or the codon-optimized *tfca* gene were grown at 37 °C in Luria–Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl) containing 100 μg/mL of ampicillin. The expression of the *tfca* gene was induced at an optical density of 0.1 at 600 nm by adding 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were lysed using the PeriPreps Periplasting Kit (Epicentre Biotechnologies, Madison, WI, USA).

2.3. Expression of recombinant TfCa

Cultivations of *E. coli* BL21(DE3) containing the codon-optimized *tfca* gene were performed in shake flasks (150 mL) or in a bioreactor (Minifors, Infors GmbH, Einsbach, Germany) with 5 L of working volume. Starting conditions of the reactor were 500 rpm stirrer speed, pH 7.5, and 1 L/min of airflow. The pH was controlled by adding either 1 M NaOH or 1 M H_3PO_4. The level of dissolved oxygen was kept between 70 and 90%. *E. coli* cells were grown in LB medium with 100 μg/mL of ampicillin to an optical density (600 nm) of 3.5 at 37 °C and for 14–20 h at 18 °C. After cultivation to an optical density (600 nm) of 1.5, 5 mM of IPTG was added. The cell suspension was centrifuged at 10,000 × *g* for 2 min and the pellet was resuspended in Tris–HCl buffer (pH 7.0). The cells were disrupted by three cycles of sonication (90 s, 120 W, 60% power, and 60% pulse) on ice using a KE76 tip (Bandelin, Berlin, Germany). The suspension was centrifuged at 11,000 × *g* for 30 min. Esterase activity was determined as described above.

2.4. Purification of recombinant TfCa by affinity chromatography

After 15 h of cultivation at 18 °C with 5 mM IPTG, the *E. coli* cell suspension was centrifuged, resuspended in Tris–HCl buffer (pH 7.0), and disrupted by sonication on ice as described before. The suspension was centrifuged at 11,000 × *g* for 30 min. The TfCa in the supernatant containing the soluble protein fraction and in the insoluble protein fraction of the pellet was purified using a 5 mL Ni-NTA gravity flow column (Ni-NTA Superflow, QIAGEN, Venlo, The Netherlands). An aliquot of the supernatant containing the soluble protein fraction (40 mL) was loaded onto the column. The column was washed with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole at pH 8). Non-specific proteins bound to the column were eluted thrice using buffer containing 50, 70, and 90 mM of imidazole, respectively. Finally, TfCa was eluted with buffer containing 250 mM of imidazole. The pellet containing the insoluble protein fraction was resuspended in denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, and 8 M urea at pH 8.0), incubated for 60 min at room temperature, and centrifuged. An aliquot of the supernatant obtained (40 mL) was loaded onto a Ni-NTA column. The column was washed twice with denaturing buffer adjusted to pH 6.3 and the TfCa protein was eluted using the same buffer adjusted to pH 4.5.

2.5. SDS-PAGE analysis of TfCa purification

Approximately 6 μg of the purified protein was loaded onto a polyacrylamide gel. The protein concentration was determined using bovine serum albumin as a standard (Bradford, 1976). A 12% (w/v) separating gel and a 4% stacking gel were used. The gels were stained with Coomassie Blue. A protein marker kit (LMW-SDS, GE Healthcare, München, Germany) was used.

2.6. Esterase activity assay

Esterase activity was determined using *p*-nitrophenyl butyrate (*p*-NPB) as substrate. A mixture of 940 μL phosphate buffer (100 mM, pH 7.5), 10 μL of *p*-NPB solution (10 mM in absolute ethanol), and 50 μL of enzyme solution was added into a cuvette and mixed well. The increase of absorbance at 410 nm was measured at 25 °C using a Cary 50 Bio spectrophotometer (Varian, Darmstadt, Germany). One unit of esterase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute (Donnelly and Crawford, 1988; Alisch et al., 2004).

2.7. Analysis of PET nanoparticle hydrolysis by TfCa

A suspension of PET nanoparticles in phosphate buffer (pH 8) was prepared as described by Pütz (2006). Purified TfCa was added to 500 μL of the nanoparticle suspension (final enzyme concentration 0.01 mg/mL) and incubated at 50 °C on a shaker (600 rpm). Controls were performed by incubating the substrate under the same conditions without enzyme. After 24 h, aliquots (100 μL) of the reaction mixtures were adjusted to pH 6.0 with 1 M HCl and analyzed by reversed phase HPLC (Vertommen et al., 2005). A Chromsep Microsorb 300-5 C18 column (250 mm × 4.6 mm, Varian Inc., Palo Alto, USA) was used with an Äkta Basic 10 chromatography system (GE Healthcare, Uppsala, Sweden). Hydrolysis products were detected at 241 nm. The mobile phase consisted of 0.1% trifluoroacetic acid (99% purity, Acros Organics, Geel, Belgium) in water and acetonitrile (HPLC grade, Roth GmbH, Karlsruhe, Germany). The determinations were performed in duplicate. The identity of the detected terephthalic acid was confirmed by mass spectrometry.

3. Results and discussion

The production of PET-hydrolases in large quantities for industrial applications requires a highly robust and efficient recombinant expression system. *E. coli* is the host organism of choice for this purpose due to its ease of use, fast growth, and high level of recombinant protein expression (Baneyx, 1999; Makrides, 1996). However, the use of *E. coli* as a host for the expression of PET-hydrolyzing enzymes is complicated by several factors. Low translation efficiency occurs in *E. coli* due to a different codon usage by *T. fusca*. This problem can be solved by the partial substitution of *T. fusca* codons with intrinsic codons from the expression host. Codon optimization also allows for the adjustment of the average GC content to approximately 50% which is required in further mutagenesis experiments. Another complication is the formation of inclusion bodies caused by incorrect protein folding. However, the yield of active, correctly folded target protein can be improved by lowering the host cell cultivation temperature (Jana and Deb, 2005). High hydrophobicity of the thermostable TfCa protein causes yet another complication, since hydrophobic proteins are often toxic to their expression host (Gerstein, 2002) (Fig. 1).

We have tried several vectors for the expression of the recombinant *tfca* gene in *E. coli* including DH5α and XL1-Blue (data not shown). However, successful protein expression only occurred using the pET-20b(+) vector in the *E. coli* BL21(DE3) host strain. In addition, we also found that amplification of the pJET1/blunt vector containing a *tfca* gene insertion was only successful in *E. coli* XL1-Blue. One possible explanation for this observation is that the *tfca* gene expression was repressed by the overexpression of the *lac* repressor in the host strain. Overexpression of the *lac* repressor would cause suppression of the *lac* promoter located within

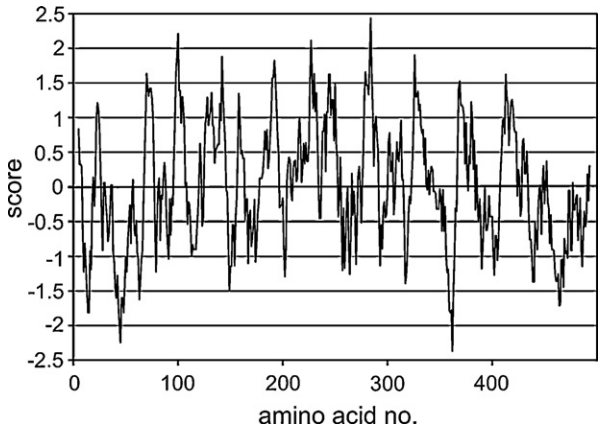

Fig. 1. Kyte–Doolittle hydrophobicity plot of the amino acids of TfCa (Kyte and Doolittle, 1982). Values for the amino acids above 0 indicate the hydrophobic character of the protein. The plot was prepared using ProtScale (Gasteiger et al., 2005).

pJET1/blunt-TfCa and would result in gene amplification but not protein expression (Bullock et al., 1987).

Both strands of the *tfca* gene inserted in the pET-20b(+) vector were sequenced and showed that the amplification and cloning procedure had been successful. This confirmed that the expressed construct consisted of the gene of interest, an N-terminal pelB signal sequence, and a C-terminal His_6 tag. Amplification of the *tfca* gene by polymerase chain reaction (PCR) and subsequent cloning were performed twice independently. The nucleotide sequence of the *tfca* gene was submitted to the European Molecular Biology Laboratory (EMBL) nucleotide sequence database (accession no. FN401519) and used for further optimization of the gene.

Two amino acid sequence variations were detected when the sequence of TfCa was compared to the sequence of the homologous putative carboxylesterase from *T. fusca* YX (Tfu_2427). The complete genome of *T. fusca* YX has recently been sequenced (Lykidis et al., 2007). TfCa has amino acid serine at position 335 and alanine at position 340, in contrast to glycine and threonine at those positions in Tfu_2427, respectively. These data indicate that the enzyme from *T. fusca* KW3 is distinct from the homologous protein of *T. fusca* YX.

The wild-type *tfca* gene contains codons which are rare in *E. coli*. In addition, the gene contains several negatively cis-acting motifs resulting in an inefficient expression in *E. coli*. Therefore, the gene was optimized for an increased expression in *E. coli* by the partial substitution of *T. fusca* codons with intrinsic codons from the expression host (Fig. 2). The non-optimized sequence of the *tfca* gene showed a codon adaption index (CAI) of 0.71 (Fig. 3). The optimized sequence showed a CAI of 0.98 and avoided several cis-acting sequence motifs including internal TATA-boxes, chi-sites, ribosomal entry sites, AT-rich or GC-rich sequence stretches, repeat sequences, and RNA secondary structures. The average GC content was reduced from 69 to 52%. Expression of the optimized *tfca* gene showed a 5-fold higher intracellular esterase activity and approximately 3-fold higher esterase activity in the culture medium (Fig. 4). The pelB leader sequence induced the translocation of the protein into the periplasm after it was produced in the cytoplasm. However, due to a leakage effect of the outer cell membrane, the enzyme was also partially released into the medium (Rinas and Hoffmann, 2004). Leakage of TfCa may have been limited due to its relatively large molecular weight that prevented its passage across the outer cell membrane. A decrease in TfCa enzymatic activity was observed when the host cell concentration at 37 °C was above an optical density of 4.0 at 600 nm. There are several possibilities for this observation. First, a decrease in enzymatic activity could be the result of the toxic effects caused by the exhaustion of the culture medium. Second, cells carrying the pET-20b(+) vector with the *tfca* gene were selected with ampicillin and produced high amounts

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A  GTG GAG ATC GTG ATC AGA ACC GGC TCG GGC GAC GTG CGA GGA AGC AAA GAG AAC GGG ATC GCG GTC TTC CGC GGA    75
B  GTG GAA ATT GTT ATT CGT ACC GGT AGC GGT GAT GTT CGT GGT AGC AAA GAA AAT GGC ATT GCC GTG TTT CGT GGT    75

A  ATT CCC TAC GCT GAG CCG CCT GTC GGC GCC CAC CGC TTT ACC GCA CCC CGC CCG CCC CGC CCC TGG GAC GGG GTA   150
B  ATT CCG TAT GCA GAA CCT CCG GTT GGT GCA CAT CGT TTT ACA GCT CCT CGC CCT CCT CGT CCG TGG GAT GGT GTT   150

A  CGG GAC GCC ACC GAA TTC AGC GCC ACC GCG CCC CGC CCG CCC TAC CCC GAA GCC ATC GGC GCG CTG CTC ATC GAA   225
B  CGT GAT GCA ACC GAA TTT AGC GCA ACC GCT CCG CGT CCT CCG TAT CCG GAA GCA ATT GGT GCA CTG CTG ATT GAA   225

A  CGG TTC ATC CCG GGG GAC GAC TAC CTC ACG CTC AAC GTG TGG ACC CCG GAC CCC AAC GCC GTC GGC CTG CCG GTC   300
B  CGT TTT ATT CCG GGT GAT GAT TAT CTG ACC CTG AAT GTT TGG ACA CCG GAT CCG AAT GCA GTT GGT CTG CCG GTT   300

A  ATG GTG TGG ATC CAC GGC GGG GCG TTC ACC AAC GGC TCC GGC TCA GAA CCC GTC TAC GAC GGC GCT GCT TTC GCC   375
B  ATG GTT TGG ATT CAT GGT GGT GCA TTT ACC AAT GGT AGC GGT AGC GAA CCG GTT TAT GAT GGT GCA GCA TTT GCA   375

A  CGC GAC GGC GTC GTC TTC GTG AGC TTC AAC TAC GGG CTC GGC ATC ATC GGC TTC GCC GAC CTG CCC GAC GCT CCT   450
B  CGT GAT GGT GTT GTT TTT GTG AGC TTT AAT TAT CGC CTG GGC ATT ATT GGT TTT GCA GAT CTG CCG GAT GCA CCG   450

A  TCC AAC CGG GGC CTG CTT GAC CAG ATC GCT GCC CTG GAA TGG GTG CGC GAC AAC ATC GCC CGC TTC GGC GGC GAC   525
B  AGC AAT CGT GGT CTG CTG GAT CAG ATT GCA GCA CTG GAA TGG GTT CGT GAT AAT ATT GCA CGT TTC GGT GGC GAT   525

A  CCC GGC AAC GTC ACC GTG TTC GGG GAA TCC GCG GGC GCC ATG AGC GTG TGC ACC CTC ATG GCC ACC CCG CGA GCC   600
B  CCG GGT AAT GTT ACC GTT TTT GGT GAA AGC GCA GGT GCA ATG AGC GTT TGT ACC CTG ATG GCA ACA CCG CGT GCA   600

A  CGC GGC CTG TTC CGC CGC GCC ATC CTC CAG TCA GGC GCA GGC AAC ATG GCA GTG GCA GCC GAA GAC GCC ACC ACC   675
B  CGT GGT CTG TTT CGT CGT GCA ATT CTG CAG AGC GGT GCA GGT AAT ATG GCA GTT GCA GCA GAA GAT GCA ACC ACC   675

A  ATC GCC GCA GTG ATC GCC CAC CGG CTG GGG GTC GAA CCC ACC GCG GCA GCC CTC GCC CAC GTT CCC GTC GCC CAG   750
B  ATT GCA GCA GTT ATT GCA CAT CGT CTG GGT GTT GAA CCG ACC GCA GCA GCA CTG GCC CAT GTT CCG GTT GCA CAG   750

A  CTC CTC GAC GTC CAA CAA CAG GTC GCC CAA GAA ATC CAG GGA GCC CCC GAC CCT GCA GTG TGG GGG GAA CGG ATC   825
B  CTG CTG GAT GTT CAG CAG CAG GTT GCA CAG GAA ATT CAG GGA GCA CCT GAT CCG GCA GTT TGG GGT GAA CGT ATT   825

A  GCC GGC GGC AGT GTC CTG TTG CCC TTT GCC CCC GTC ATC GAC GGA GAA CTC CTC TCC CAG CGC CCC GCG GAG GCC   900
B  GCA GGT GGT AGC GTT CTG CTG CCT TTC GCA CCG GTT ATT GAT GGT GAA CTG CTG TCT CAG CGT CCG GCA GAA GCA   900

A  ATC GCC GGC GGC GCC GGA CAC GAC GTC GAC CTG CTG TTC GGC ACC ACC ACC GAC GAA TAC CGG CTG TTC CTC GCC   975
B  ATT GCC GGA GGC GCA GGT CAT GAT GTT GAT CTG CTG TTT GGC ACC ACC ACC GAT GAA TAT CGT CTG TTT CTG GCT   975

A  CCC ACC GGG CTG CTG CCC TTC ATC ACC AGC GAC TAC GTG ACC GCC CAC CTC GCC AAG TCC GGG CTG GAC GCG GAC  1050
B  CCG ACC GGC CTG CTG CCG TTT ATT ACC AGC GAT TAT GTT ACC GCA CAT CTG GCT AAA AGC GGT CTG GAT GCA GAT  1050

A  GCC GCC AAG GCC TAC ACC GCG GAA GGA CGC GGC GAA GAA CCC GGC GAC ATC CTC GCC TCG ATC ATC ACC GAC CAG  1125
B  GCA GCA AAA GCA TAT ACC GCA GAA GGT CGC GGC GAA GAA CCG GGT GAT ATT CTG GCC AGC ATT ATT ACC GAT CAG  1125

A  GTG TTC CGC ATC CCC GCC CTC CGC ATC GCC GAA TCC CGC GTC GAC GCG CCC GCC CGC ACC TTC GGC TAC GAA TTC  1200
B  GTG TTT CGT ATT CCG GCA CTG CGT ATT GCA GAA AGC CGT GTT GAT GCA CCG GCA CGC ACC TTT GGT TAT GAA TTT  1200

A  GCC TGG CGC ACT CCG CAG CTC GAC GGA ATC CTG GGT GCC TGC CAC GCG GTC GAG CTC CCC TTC GTG TTC CGC ACC  1275
B  GCA TGG CGT ACA CCG CAG CTG GAT GGT ATT CTG GGT GCA TGT CAT GCA GTT GAA CTG CCG TTT GTT TTT CGT ACC  1275

A  CTG GAC CGG GCC GCC TCC CTC GTC GGC ACC AAC CCC CCG GAA GAG CTC GCC GAG ACC GTC CAC AAC GCC TGG GTG  1350
B  CTG GAT CGT GCA GCA AGC CTG GTT GGC ACC AAT CCG CCG GAA GAA CTG GCC GAA ACC GTT CAT AAT GCA TGG GTT  1350

A  CGT TTC GCT ACC TCC GGC GAC CCG GGC TGG CCC GCA TGG AAC CCC GAA ACC CGG TCG GTC ATG CGC TTC GAC CAC  1425
B  CGT TTT GCA ACC AGC GGT GAT CCG GGT TGG CCG GCA TGG AAT CCG GAA ACC CGT AGC GTT ATG CGT TTT GAT CAT  1425

A  CCC GTC TCG GAG ATG GTC ACG GAC CCG TAC CCC GCC ACC CGC GCC CTG TGG GAC GGG GTG CCG CTG TAG          1494
B  CCG GTT AGC GAA ATG GTT ACC GAT CCG TAT CCG GCA ACA CGT GCA CTG TGG GAT GGC GTT CCG CTG GGC          1494
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Fig. 2. Comparison of the nucleotide sequences of the non-optimized (A) and the codon-optimized (B) *tfca* gene.

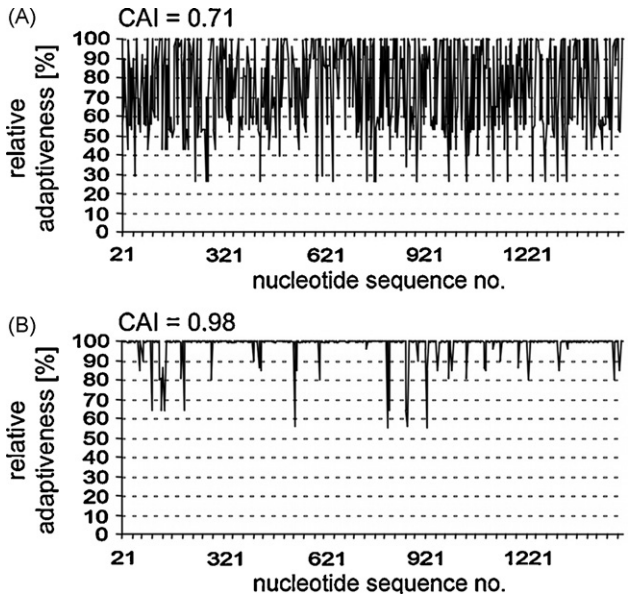

Fig. 3. Graphical representation of the relative codon adaptiveness and total codon adaption index (CAI) of the nucleotide sequence of the *tfca* gene before (A) and after (B) codon optimization.

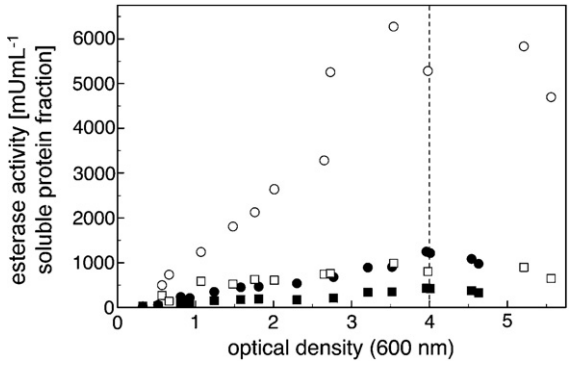

Fig. 4. Effect of codon optimization of the *tfca* gene from *T. fusca* KW3 on the esterase yield obtained with recombinant *E. coli* BL21(DE3). Esterase activity of whole cell protein without (●) and with (○) a codon-optimized gene; esterase activity in the culture medium without (■) and with (□) a codon-optimized gene. The dotted line indicates the optical density of the cells at 600 nm where a decrease in esterase activity was observed.

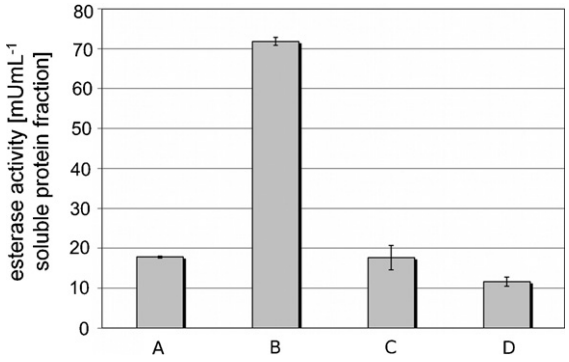

Fig. 5. Effect of cultivation time and temperature of *E. coli* BL21(DE3) cells on recombinant TfCa yield. (A) Cultivation at 37 °C (optical density of 3.5 at 600 nm); (B) cultivation at 18 °C for 16 h; (C) cultivation at 18 °C for 18 h; (D) cultivation at 18 °C for 20 h. The activity in each sample was determined in triplicate. The standard error is indicated for each bar.

of β-lactamase that was released into the medium. High levels of this enzyme could have destroyed the antibiotic and allowed for other *E. coli* cells not carrying the *tfca* gene to overgrow the TfCa-producing cells after prolonged cultivation time. Third, TfCa could have also been partially degraded by proteases during longer cultivation times, since extracellular proteases produced by *E. coli* have recently been reported (Nandakumar et al., 2006).

To further optimize the expression of the *tfca* gene in *E. coli* BL21(DE3), we examined how different host cell concentrations and amounts of IPTG used to induce gene expression influenced the recombinant esterase activity. No correlation between IPTG concentrations (1–5 mM) and esterase yield was observed. Similarly, varying concentrations of *E. coli* cells when inducer was added (optical densities between 0.5 and 1.5 at 600 nm) produced similar amounts of esterase activity. We next assessed the effect of a lower cultivation temperature on esterase activity. By lowering the temperature to 18 °C, the activity in the whole cell protein fraction detected after 16 h of cultivation was nearly 4-fold higher when compared to cells grown at 37 °C (Fig. 5). These results suggest that the enzyme partially formed intracellular inclusion bodies despite correct folding and expression of TfCa in *E. coli*. The extent of inclusion body formation decreased when the cells were cultivated at a lower temperature. The time of cultivation also strongly influenced the enzyme activity obtained. The activity in the whole cell protein fraction reached a plateau from 14 to 16 h of cultivation and decreased rapidly with longer cultivation times. Using the optimized expression conditions, the esterase activity of TfCa (U/mL) was approximately 4500-fold higher than the activity of TfCa pro-

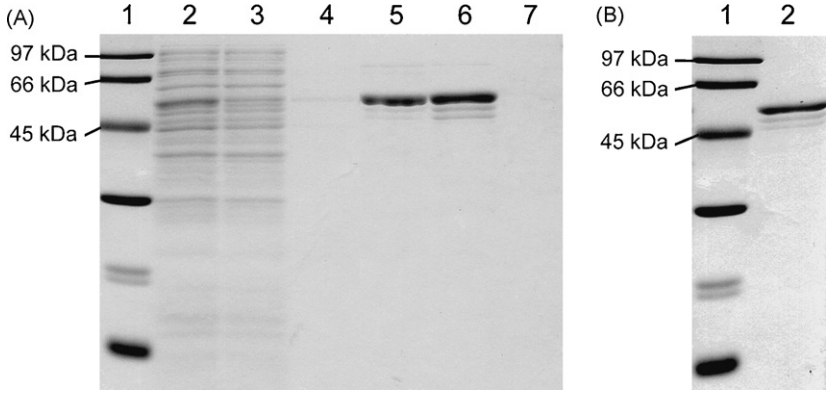

Fig. 6. SDS-PAGE analysis of recombinant TfCa produced by *E. coli* BL21(DE3) and purified by Ni-NTA affinity chromatography. (A) Purified TfCa from the soluble protein fraction. Lane 1, protein marker; lane 2, crude cell extract; lane 3, flow-through; lane 4, washed fraction; lanes 5 and 6, eluted fractions; lane 7, post-elution. (B) Purified TfCa from the insoluble protein fraction. Lane 1, protein marker; lane 2, eluted fraction.

duced in *T. fusca* KW3. The specific activity (U/mg protein) increased more than 30-fold compared to the production in *T. fusca* KW3 (Billig et al., in preparation).

The recombinant TfCa in the whole cell soluble protein fraction as well as in the insoluble protein fraction containing inclusion bodies could be highly purified in a single step by Ni-chelate affinity chromatography, as indicated by SDS-polyacrylamide gel electrophoresis (Fig. 6). Using a pH-controlled bioreactor for cultivation, a TfCa protein concentration of 41.6 mg/L corresponding to an enzyme activity of 3000 U/L could be achieved.

The ability of TfCa to hydrolyze PET nanoparticles was demonstrated by incubating the purified enzyme with the substrate for 24 h at 50 °C. Under these conditions, TfCa added in a concentration of 0.01 mg per mL released 7 nmol of the PET-hydrolysis product terephthalic acid per mL.

In conclusion, we have successfully expressed a highly hydrophobic enzyme from the thermophilic actinomycete *T. fusca* KW3 in *E. coli* as a fusion protein with a pelB leader sequence for periplasmatic localization of the protein and a His_6 tag for use in purification. The recombinant esterase yields were significantly enhanced by performing codon usage optimization and by lowering the cultivation temperature of the transformed *E. coli* cells to 18 °C as a way of reducing the formation of inclusion bodies.

Acknowledgment

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