OPTIMIZATION OF EXPRESSION CONDITIONS OF THE ACETYLESTERASE CE16 FROM *Hypocrea jecorina* ENCODED BY A SYNTHETIC GENE AND EXPRESSED IN *Escherichia coli* CELLS

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Abstract: Acetylesterase CE16 was identified as a part of the enzymatic cocktail secreted by fungus *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) during its growth on cellulose. Later it was classified as the first member of a newly organized carbohydrate esterase family CE16. Further studies showed that acetylesterase is crucial for complete deacetylation of naturally acetylated xylans enabling their saccharification by xylanases. To study the relationship between structure and function of acetylesterase, highly purified recombinant enzyme produced by *Trichoderma reesei* Rut C-30 was prepared. The enzyme was composed of 348 amino acid residues from which the 1 - 19 formed a secretion signal peptide. Determined molecular mass of purified recombinant acetylesterase (Aes1) was 45 kDa which was more than molecular mass calculated from amino acid sequence. As it has been proved later, the difference was caused by the enzyme glycosylation. Glycosylation of proteins increases their stability, but it can also be a source of heterogeneity, which might be a problem during crystallization. To make the future X-ray study of the enzyme easier, recombinant non-glycosylated enzyme needed to be prepared. For these purposes, a synthetic gene optimized for protein expression in *Escherichia coli* was designed and synthetized. The first non-glycosylated acetylesterase obtained by the expression of its synthetic gene in *E. coli* cells was mostly insoluble or aggregated. Conditions of cell cultivation, induction of gene expression and cells disruption were necessary to optimize. Presently, after optimization of all mentioned steps, the non-glycosylated recombinant CE16 acetylesterase was prepared in the soluble and active form, ready for further downstream procedures, involving protein purification and crystallization.

Key words: acetylesterase CE16, *Hypocrea jecorina*, *Trichoderma reesei,* synthetic gene, gene expression, *Escherichia coli*

1. Introduction

Filamentous fungus *Hypocrea jecorina* (anamorph: *Trichoderma reesei)* is a representative organism today for commercial scale production of different cellulases and hemicellulases, which are currently used for production of biofuels, textile materials, detergents, food, feed and have negligible use in brewing industry. Genus *Hypocrea jecorina* has become a subject of intense studies from 1980 until now. With the increasing need to replace fossil fuels by cheaper and greener sources of energy, such as constantly forming plant biomass, scientific community has drawn attention to the enzymes produced by this fungus during its growth on cellulose and lactose, to the so called secretome (ADAV *et al*., 2011). Plant cell wall has a complex structure and

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composition, and therefore its decomposition requires a complex interaction of a range of enzymes cleaving specific types of chemical bonds (CAFFALL and MOHNEN, 2009). Xylan, a constituent of the cell walls, is a polysaccharide made from xylose units which are usually decorated by side substituents, mostly arabinose, D-glucuronic acids or acetyl groups which influences its properties and protects plant cell walls towards hydrolysis by glycoside hydrolases, i.e. acetylation slows down its subsequent digesting (CARPITA and GIBEAU, 1993). The degree of acetylation influences the enzymatic hydrolysis, since esterified polysaccharides are less soluble and less accessible to the enzymatic hydrolysis. One of the enzymes that are able to deacetylate oligosaccharides in the plant cell wall is acetylesterase (*Tr*CE16). Acetylesterase (*Tr*CE16) was identified for the first time in 1985 as a part of the cellulolytic enzyme cocktail produced by *H. jecorina* during its growth on cellulose (BIELY *et al*., 1985). According its substrate specificity, the enzyme was classified as an acetic-ester acetyl hydrolase (EC 3.1.1.6) (POUTANEN and SUNDBERG, 1988). Mentioned acetylesterase is regioselective enzyme. Moreover, it is unique by its ability to deacetylate terminally acetylated xylooligosaccharides, predominantly at position 3 and 4, from their non-reducing ends and less effectively at position 2 (KREMNICKÝ and BIELY, 2005; BIELY *et al*., 2011, BIELY *et al*., 2014). Further studies showed that acetylesterase efficiently catalyzes transacetylation in aqueous medium saturated with vinyl acetate to the position 3 of non-reducing residues of xylooligosaccharides; and it is a crucial enzyme for complete deacetylation of naturally acetylated xylans enabling their saccharification by xylanases (KREMNICKÝ and MASTIHUBA, 2004; KREMNICKÝ and BIELY, 2005). Because of the need of large quantity of acetylesterase, an acetylesterase gene from wild-type *Trichoderma reesei* QM 6a was cloned and transformed into hypercellulolytic mutant *Trichoderma reesei* Rut C-30 as a production strain. Based on amino acid sequence of recombinant acetylesterase (Aes1), a new family of carbohydrate esterases, family CE16 (CAZy classification, LOMBARD *et al.,* 2014), was established in 2008 (LI *et al*., 2008). Up to date (10 November 2015), the family CE16 comprises 190 members. Unfortunately, there is no tertiary structure information about any member yet. Moreover, only three members are biochemically characterized: acetylesterases from *T. reesei* (LI *et al*., 2008), *Myceliopthora thermophila* (KOUTANIEMI *et al*., 2013) and from *Podospora anserina* (PUCHART *et al*., 2015). Experimentally determined molecular mass of the purified recombinant acetylesterase (Aes1) was 45 kDa (LI *et al*., 2008), what matched with the molecular mass of purified non- recombinant acetylesterase *Tr*CE16 (POUTANEN and SUNDBERG, 1988), but was lower than molecular mass calculated from amino acid sequence. This difference is caused by the enzyme glycosylation. The fact that natural and recombinant acetylesterases can be *N*-glycosylated was confirmed by enzymatic cleavage by endo-β-*N*-acetylglucosaminidase, resulting in the production of lower-mass polypeptide. Glycosylation of proteins increases their stability, but it can be also a source of the heterogeneity, which might cause problems during protein crystallization. Besides this, the transformed strain of *T. reesei* produces several other acetylesterases similar to our target enzyme, which may cause problems at purification of our target acetylesterase.

The problems could be minimized by the production of non-glycosylated recombinant Aes1 protein in *Escherichia coli* cells. For these purposes, the synthetic

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gene encoding acetylesterase was designed (URBÁNIKOVÁ, unpublished data) and prepared (GenScript, Piscataway, USA). Considering further studies on the relationship between structure and function of acetylesterase, it would seem necessary to prepare a non-glycosylated recombinant enzyme, making easier its crystallization and tertiary structure determination. The advantages of the *E. coli* expression system are as following: e.g. high overexpression, relatively easy production of proteins and ability to produce proteins with His6-tag sequence, which is helpful in protein purification by IMAC (Immobilized Metal Affinity Column), and, finally, the fact that *E. coli* does not possess the glycosylation machinery. Heterologous expression is not easy to be performed. High yield and solubility of final protein are influenced by the properties of given enzyme (BASHIRI *et al*., 2015).

In this paper, the expression conditions of the synthetic gene are optimized, with the aim to reach the maximal amount of a soluble active protein.

2. Materials and methods

2.1 Bacterial strains and plasmid

Synthetic gene (GenScript, Piscataway, USA) was prepared pursuant to gene *aes1* encoding CE16 acetylesterase (Aes1), but without the part coding signal peptide. Codon usage of the synthetic gene was optimized for *E. coli* cells using the program JCat (GROTE *et al*., 2005). Optimized synthetic gene *tae1* was inserted into pET21a (Novagen, USA) expression vector. The NdeI and XhoI restriction enzymes were used to digest the sequence of the synthetic gene and pET21a vector and the recombinant pET21a-*tae1* molecule was prepared by subsequent ligation. The pET21a-*tae1* plasmid was transformed by heat-shock method to *E. coli* competent cells (FROGER and HALL, 2007). Four types of competent cells were used for testing of the gene expression: strain BL21(DE3), NiCo21(DE3), ArcticExpress and BL21-Gold(DE3) (Agilent Technologies, USA).

2.2 Media and culture conditions

For cells cultivation, LB-Miller medium (MANIATIS *et al*., 1982) was used, containing 1 % (w/v) bacto-tryptone (Serva, Germany), 0.5 % (w/v), yeast extract (Difco Laboratories, USA), and 1 % (w/v) NaCl, pH 7.5. In order to enhance the production of soluble recombinant protein LB medium containing 1 M NaCl was prepared (NAGATA *et al.,* 2005, SASAKI *et al*., 2006, SASAKI *et al*., 2012). All cultivation media were supplemented with appropriate antibiotics. The transformed cells were grown at 37 °C in 500 ml shake flasks on the shaker at 250 rpm until the OD_{600} = 0.6-1.0 was reached (about 2.5 h). Then, the temperature in the shaker was lowered to 20° C for 30 minutes and the gene expression was induced by the addition of IPTG (isopropyl–thio–β–D–1–galactopyranoside) to the final concentration of 1 mM (Serva, Germany). Afterwards, several different incubation temperatures and length of cultivation were set and tested: (i) 16 °C for 24 h, (ii) 30 °C for 6 h, and (iii) 11 °C for 24 h when ArcticExpress competent cells were used. As a control of the

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level of protein expression, samples with no IPTG added were prepared at all conditions. In order to improve the production of soluble and well folded recombinant protein, a heat shock was tested, i. e. bacterial culture was exposed to 47 °C for 30 min followed by cooling to 20° C before the IPTG addition (OGANESYAN *et al*., 2007., BURGESS and MURRAY, 2009). The protein expression was analyzed in the induced cultures and non-induced controls in the adjusted time intervals (6, 20, 24 h during cultivation) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12 % SDS-PAGE, LAEMMLI, 1970). Proteins in the gel were stained with Coomassie Brilliant Blue (Serva, Germany). After incubation, the cells were harvested by centrifugation at 10,600 *g* for 15 min at 4 °C and stored at -20 °C for further procedures.

2.3 Cell disruption

The cells were disrupted by two methods. At first, (i) chemical lysis by BugBuster Master Mix (5 ml / 1 g of pelleted cells) (Merck Millipore, USA) was performed. However, this type of cell disruption led to formation of soluble aggregates not able to bind to IMAC column. Therefore, (ii) mechanical method of disruption cell walls by sonicator (Soniprep 150, United Kingdom) was used for complete cell lysis. The cells were thawed on ice and then resuspended in a sonication buffer composed of 20 mM sodium phosphate buffer, pH 7.5, supplemented with NaCl and glucose to a final concentration of 200 mM and 25 mM. For the protein solubility improvement various detergents and additives in sonication buffer were tested. As a result, a detergent cocamidopropyl betaine, (Tego Betain F-50, Evonik Goldschmidt GmbH, Germany) and reducing agent TCEP (tris (2-carboxyethyl) phosphine) (Sigma-Aldrich, Germany) were added to the sonication buffer to the final concentration of 1 % and 10 mM, respectively. The resuspended pellet was sonicated using 15 cycles of 30 s pulses at amplitude 14 with 1 min breaks and kept on ice during the whole sonication to avoid protein denaturation. Optimal conditions of sonication were determined by time course analysis of sonication by sampling. The samples for analysis were removed during the sonication and then centrifuged for 5 min at 10,600 *g* at 4 °C. Protein concentration was determined in supernatant by spectrophotometric analysis at the wavelength of 280 nm (Nanodrop 2000c, Thermo Scientific, USA). After sonication, the lysate was clarified by centrifugation at 10,600 *g* for 15 min at 4 °C. The samples taken from both, soluble and insoluble fractions, were analyzed by 12 % SDS-PAGE (LAEMMLI, 1970) with SPECTRA^{TM} MULTICOLOR BROAD PROTEIN LADDER (10-260 kDa) (SM1841, Thermo Scientific, USA) used as a protein molecular weight control. The proteins bands were visualized by Coomassie Brilliant Blue staining protocol.

3. Results and discussion

In this work, a synthetic gene encoding *Trichoderma reesei* acetylesterase CE16 (*tae1*) was prepared, according to the sequence of *Aes1* (GenBank DQ866149), without a part encoding the signal peptide (Fig. 2). Codon usage was optimized for the

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expression in *E. coli* cells in order to overcome problems originating from the differences between the eukaryotic gene and the prokaryotic host organism (JONASSON *et al*, 2002). The prepared gene was inserted into the pET21a vector with C-terminal His6-tag sequence and a gene responsible for ampicillin resistance (Fig. 1). In general, this vector enables the expression under the control of a strong T7 promotor and also provides multi cloning site (MCS) with restriction enzymes NdeI and XhoI used for the insertion of the synthetic gene *tae1* (Fig. 1).

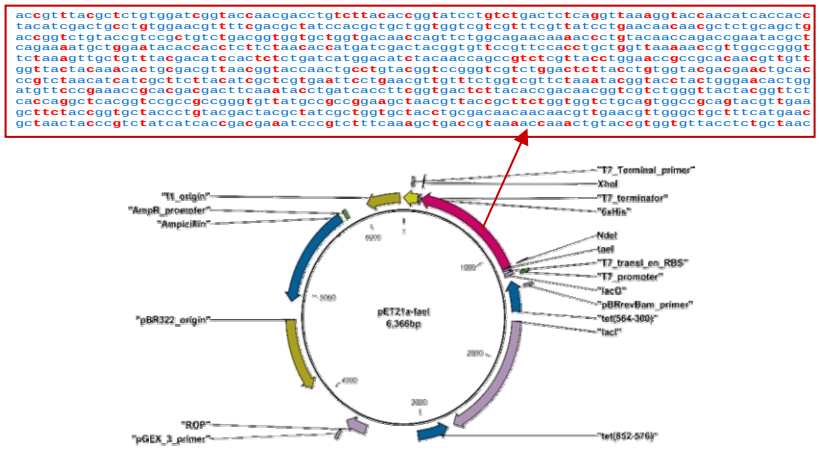

Fig. 1. The construct of a pET21a expression vector with inserted synthetic gene *tae1*. Inserted synthetic gene is marked by red color. Nucleotide sequence of the synthetic gene *tae1* with modified nucleotides in red is placed in the red box.

```
Tae1      ------------------MFPKPHDDFKYLITFGDSYTDNGRLGYYGSHQAHGPPPGVMP
Aes1      MRSILVIPSFVAVLNAFSLFPKPHDDFKYLITFGDSYTDNGRLGYYGSHQAHGPPPGVMP
                           :****************************************

Tae1      PEANVTASGGLQWPQYVEASTGATLYDYAIAGATCDNNNVERWAAFMNANYPSIITDEIP
Aes1      PEANVTASGGLQWPQYVEASTGATLYDYAIAGATCDNNNVERWAAFMNANYPSIITDEIP
          ************************************************************

Tae1      SFKADRKTKLYRGVTSANTVYALWIGTNDLSYTGILSDSQVKGTNITTYIDCLWNVFDAI
Aes1      SFKADRKTKLYRGVTSANTVYALWIGTNDLSYTGILSDSQVKGTNITTYIDCLWNVFDAI
          ************************************************************

Tae1      HAAGGRRFVILNNNALQLTGLYRPLSDGGAGDNQFWQNKTLYNQTEYAQKMLEYTTSSNT
Aes1      HAAGGRRFVILNNNALQLTGLYRPLSDGGAGDNQFWQNKTLYNQTEYAQKMLEYTTSSNT
          ************************************************************

Tae1      MIDYGVPFHLLVKNRWPGSKVAVYDIHSLIMDIYNQPSRYLEPPHNVVGYYKHCDVNGTN
Aes1      MIDYGVPFHLLVKNRWPGSKVAVYDIHSLIMDIYNQPSRYLEPPHNVVGYYKHCDVNGTN
          ************************************************************

Tae1      CLYGPGRLDSYLWYDELHPSNIIASYIAREFLNVVSGRSKYGTYWEHWLEHHHHHH
Aes1      CLYGPGRLDSYLWYDELHPSNIIASYIAREFLNVVSGRSKYGTYWEHW--------
          ***********************************************
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Fig. 2. Alignment of amino acid sequences of the two recombinant acetylesterases CE16. Tae1 = non-glycosylated acetylesterase produced by *E.coli,* Aes1 = glycosylated acetylesterase produced by *Trichoderma reesei* RutC-30, for the alignment Clustal Omega online tool was used (SIEVERS and HIGGINS, 2011). In the Tae1 sequence, amino acid residues added due to the cloning strategy are in red and His6-tag is in green color. In Aes1 sequence, amino acid residues corresponding to the signal peptide are in blue. N-glycosylation sites are in light blue color in both sequences.

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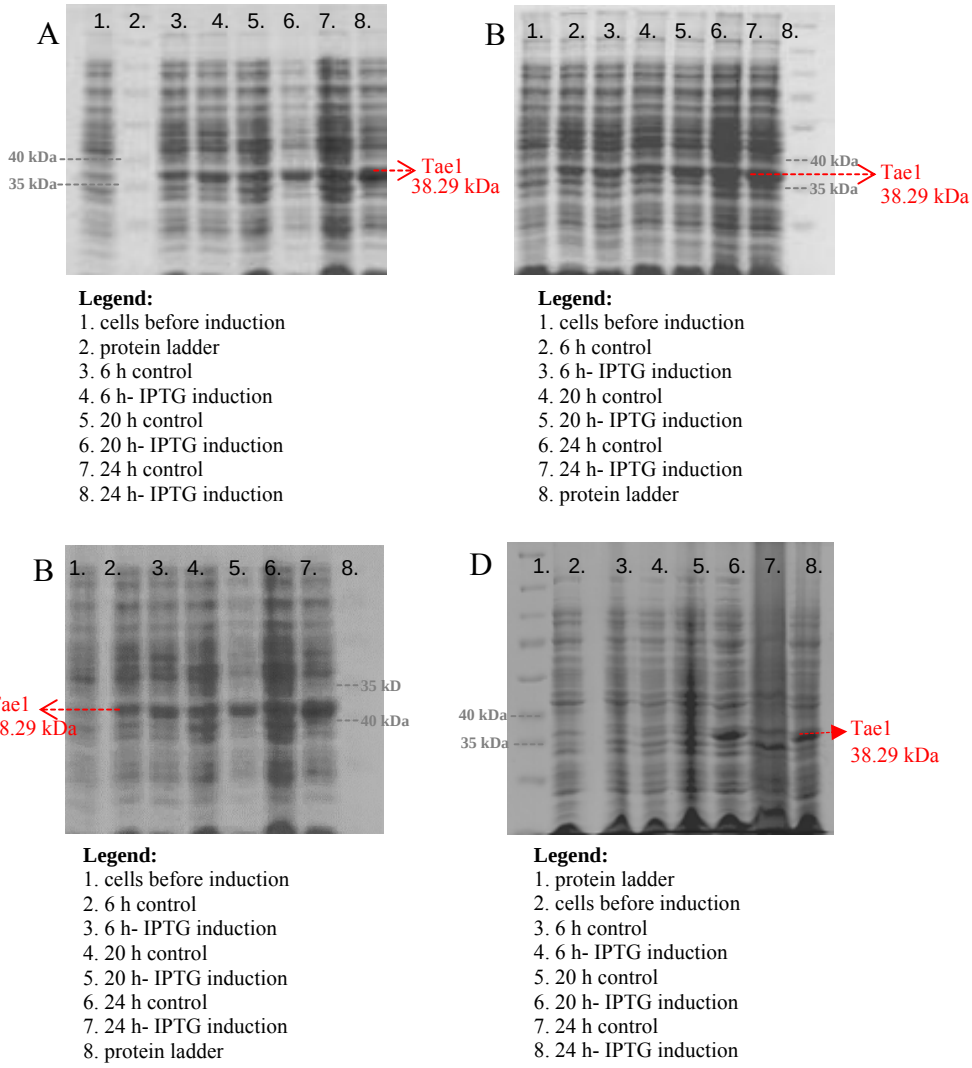

Legend:
1. cells before induction
2. protein ladder
3. 6 h control
4. 6 h- IPTG induction
5. 20 h control
6. 20 h- IPTG induction
7. 24 h control
8. 24 h- IPTG induction

Legend:
1. cells before induction
2. 6 h control
3. 6 h- IPTG induction
4. 20 h control
5. 20 h- IPTG induction
6. 24 h control
7. 24 h- IPTG induction
8. protein ladder

Legend:
1. cells before induction
2. 6 h control
3. 6 h- IPTG induction
4. 20 h control
5. 20 h- IPTG induction
6. 24 h control
7. 24 h- IPTG induction
8. protein ladder

Legend:
1. protein ladder
2. cells before induction
3. 6 h control
4. 6 h- IPTG induction
5. 20 h control
6. 20 h- IPTG induction
7. 24 h control
8. 24 h- IPTG induction

Fig. 3. Analysis of protein production by different *E.coli* strains. SDS - PAGE with samples taken at different time of the protein production in selected *E. coli* cells: **A** - BL21(DE3)-pET21a-*tae1*; **B** - NiCo21(DE3)-pET21a-*tae1*, **C** - BL21-Gold-(DE3)-pET21a-*tae1;* **D** - ArcticExpress cells-pET21a-*tae1*. Ladder: SPECTRA™ MULTICOLOR BROAD PROTEIN LADDER (10-260 kDa) (Thermo Scientific, USA). Control = cultivation without induction.

In the first experiments with a production of the non-glycosylated acetylesterase obtained by the expression of its synthetic gene in *E. coli* cells, the problems with protein solubility were found. Production of deserved protein was satisfying, but after cell homogenization, the protein was mostly insoluble or aggregated and not able to bind to IMAC column. These problems suggested incorrect folding of the protein

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(HANDRICK and HARTL, 1995). With the aim to improve the recombinant protein folding and solubility, at first, different *E. coli* expression strains were tested, and the choice of given strains for the gene expression was closely tied to the properties of the selected expression vector. Different types of expression cells were used to study the gene expression level. BL21(DE3) and NiCo21(DE3) were used as standard types of competent cells. ArcticExpress competent cells were used due to their adjustment of low-temperature cultivation in order to increase the recovery of the soluble protein (SCHEIN, 1989). BL21-Gold(DE3) competent cells are designed for easy induction and high-level protein expression. The pET21a-*tae1* plasmid was transformed into *E. coli* cells followed by the cultivation in a standard manner by described method. Gene expression was monitored by SDS-PAGE analysis. The obtained results did not show any significant differences in the synthetic gene expression among the tested BL21(DE3) (Fig.3; A), NiCo21(DE3) (Fig.3, B) and BL21-Gold(DE3) (Fig.3; C) expression cells. To emphasize, in each step of protein production and protein extraction from the cells, the total protein content was indirectly confirmed spectrophotometrically by measuring the absorbance at 280 nm. The cells were disrupted by BugBuster, but the recombinant protein was found either in the insoluble part or formed soluble aggregates and was not able to bind to IMAC column. For this reason, mechanical disruption by sonication was tested, but the majority of protein also stood in the insoluble part. The total amount of protein produced by ArcticExpress competent *E.coli* cells (Fig.3, D) was lower than in other three cases; and after homogenization, the protein remained mostly in the insoluble fraction. Therefore, these competent cells were not used in further procedures.

Legend:
1. cells before induction
2. 6 h-control
3. 6 h- IPTG induction
4. 20 h control
5. 20 h- IPTG induction
6. 24 h control
7. 24 h- IPTG induction
8. protein ladder

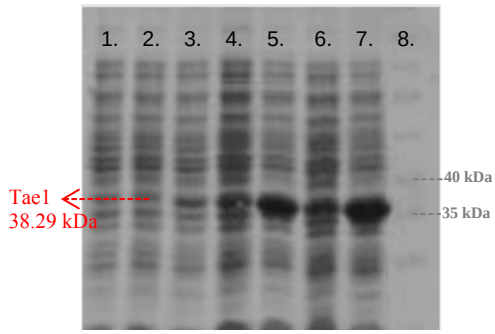

Fig. 4. SDS - PAGE with samples taken in different time of the protein production in BL21-Gold-(DE3) in LB medium supplemented by 1 M NaCl and with a heat-shock before induction. Ladder: SPECTRA^{TM} MULTICOLOR BROAD PROTEIN LADDER (10-260 kDa) (Thermo Scientific, USA). Control = cultivation without induction.

In order to improve protein solubility and reach a high level of protein production, conditions of cultivation and cell lysis were optimized. Many recombinant proteins cannot be expressed well and in a correct and soluble form in *E. coli* cells because of the absence of the proper folding environment or the absence of the post translational modification (SORENSEN *et al*; 2005). Presently, various defense mechanisms of bacteria are known to protect proteins from aggregation and incorrect folding by

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osmotic or heat stress. Defense mechanisms include intracellular accumulation of osmolytes or synthesis of heat shock proteins in the cells (KEMPF *et al*; 1998; BUKAU *et al*; 1998). Therefore, several cultivation conditions using the high concentration of NaCl (1 M) in LB medium and a heat shock (47 °C, 30 min) before induction were applied. In the view of increased temperature during heat shock and the length of cultivation, ampicillin was replaced by its stable equivalent carbenicillin. From the protein solubility point of view, the most soluble protein was obtained after 20 h cultivation at 16 °C in salted LB medium with heat shock prior to induction (Fig. 4).

The conditions for improved protein solubility were adjusted by adding Tego Betain F50 and TCEP, both before sonication. TCEP is a reducing agent that selectively reduces disulphide bonds and that is nonreactive toward other functional groups commonly present in proteins (KIRLEY *et al;* 1989). Moreover TCEP is compatible with IMAC column containing Ni^{2+} ions. Tego Betain F50 is a zwitterionic detergent containing a quaternary ammonium cation and a carboxylate groups, shown aid in protein stability and folding (NITSCH *et al*; 2005). The disadvantage of Tego Betain is high foaming, which may cause protein denaturation. To avoid denaturation and obtain the highest possible yield of the soluble protein, the concentration of Tego Betain and TCEP in sonication buffer, as well as a volume of sonication buffer per gram of cells were optimized. The number of sonication cycles (Fig. 5) was also optimized in all sonicated samples to avoid the protein denaturation; the temperature during the whole period of sonication was controlled to prevent well-known damages caused by ultrasound radiation in biological system (WOOD and LOOMIS, 1927).

The best result of both, well-expressed and most soluble protein was achieved by protein production using *E. coli* BL21-Gold(DE3) cells as a host, mechanical cell disruption by sonication using the buffer supplemented with Tego Betain F-50 and TCEP to the final concentration of 1 % and 10 mM, respectively (Fig. 6) and subjecting the cells to 15 cycles of sonication (15 x 15 s) with one minute breaks for better cooling of the sample. Above-mentioned conditions were evaluated as the best conditions for acetylesterase CE16 production in terms of the protein yield and solubility.

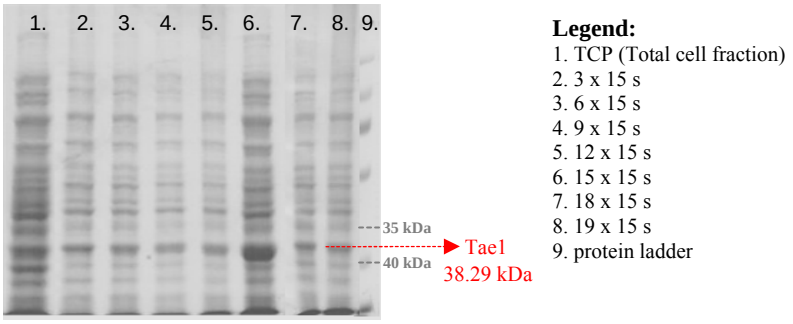

Fig. 5. Determination of optimal conditions of cell disruption by sonication. Samples were collected after each three cycles of sonication. Ladder: SPECTRA™ MULTICOLOR BROAD PROTEIN LADDER (10-260 kDa) (Thermo Scientific, USA).

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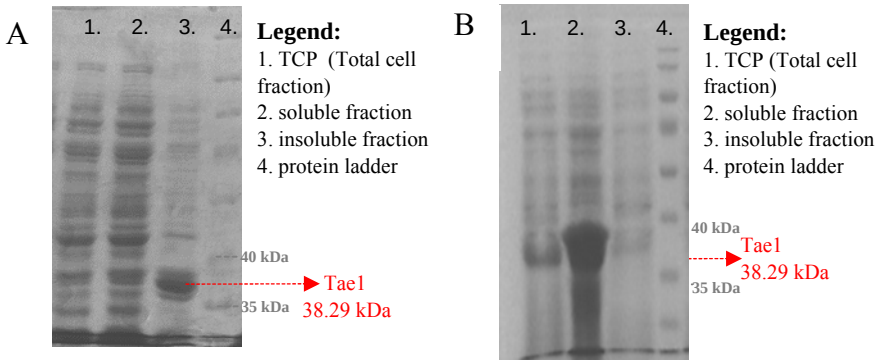

Fig. 6. Influence of sonication buffer composition on the yield of soluble protein. The comparison of the sonication results analyzed on SDS-PAGE. **A** - sonication buffer without TCEP and Tego Betain; **B** - sonication buffer containing TCEP and Tego Betain. Ladder: SPECTRA^{TM} MULTICOLOR BROAD PROTEIN LADDER (10-260 kDa) (Thermo Scientific, USA).

4. Conclusions

In the present work, conditions of recombinant molecule pET21-*tae1* expression and soluble recombinant acetylesterase production, i.e. conditions of cell cultivation, induction of protein expression and sonication of cell pellet were optimized.

E. coli BL21GOLD(DE3) strain was chosen for its property to maximally express the synthetic gene encoding acetylesterase Tae1 and produce the protein in soluble form. The cultivation of *E. coli* cells BL21GOLD(DE3) containing recombinant pET21a-tae1 construct proceeded after heat shock, until exponential phase was reached. Then the expression of the gene encoding CE16 acetylesterase was inducted by adding IPTG to the final concentration of 1 mM, in LB medium containing 1 M NaCl for 20 h at 16 °C. Collected *E. coli* cells (after storing at -20 °C) were sonicated; and solubilized protein was obtained by sonication in buffer supplemented with TCEP reducing agent and Tego Betain F50, zwitterionic detergent. The best results were achieved after 15 cycles of 30 s pulses at amplitude 14. At this stage, a non-glycosylated recombinant CE16 acetylesterase was prepared as a soluble protein fraction after sonication under specific conditions, and is stored for further downstream procedures, involving especially protein isolation by Immobilized Metal Affinity Column (IMAC), purification by size exclusion chromatography using FPLC system and crystallization for the tertiary structure determination.

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